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Fub1p, a novel protein isolated by boundary screening, binds the proteasome complex

Akira Hatanaka[1], Bo Chen[1], Jing-Qian Sun[1], Yasunobu Mano[1], Minoru Funakoshi[2], Hideki Kobayashi[2], Yunfeng Ju[3], Tetsuya Mizutani[3,4], Kaori Shinmyozu[5], Jun-ichi Nakayama[6], Kaoru Miyamoto[3,4], Hiroyuki Uchida[1] and Masaya Oki[1,4,7]*

[1]*Department of Applied Chemistry & Biotechnology, Graduate School of Engineering, University of Fukui, 3-9-1 Bunkyo, Fukui 910-8507, Japan*
[2]*Center for Faculty Development, Okayama University, Tsushima-naka 2-1-1, Kita-ku, Okayama 700-8530, Japan*
[3]*Department of Biochemistry, Faculty of Medical Science, University of Fukui, Fukui 910-1193, Japan*
[4]*Research and Education Program for Life Science, University of Fukui*
[5]*Proteomics support unit and* [6]*Laboratory for Chromatin Dynamics, Center for Developmental Biology, RIKEN, Kobe 650-0047, Japan*
[7]*PRESTO, Japan Science and Technology Agency (JST), 4-1-8 Honcho Kawaguchi, Saitama 332-0012, Japan*

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Silenced chromatin domains are restricted to specific regions. Eukaryotic chromosomes are organized into discrete domains delimited by domain boundaries. From approximately 6,000 genes in *Saccharomyces cerevisiae*, we previously isolated 55 boundary genes. In this study, we focus on the molecular function of one of boundary genes, *YCR076C/FUB1* (function of boundary), whose function has not been clearly defined *in vivo*. Biochemical analysis of Fub1p revealed that it interacted with multiple subunits of the 20S proteasome core particle (20S CP). To further clarify the functional link between Fub1p and proteasome, several proteasome mutants were analyzed. Although only 20S CP subunits were isolated as Fub1p interactors, a genetic interaction was also observed for component of 19S regulatory particle (19S RP) suggesting involvement of Fub1p with the whole proteasome. We also analyzed the mechanism of boundary establishment by using proteasome composition factor-deficient strains. Deletion of *pre9* and *ump1*, whose products have effects on the 20S CP, resulted in a decrease in boundary function. Domain analyses of Fub1p identified a minimum functional domain in the C terminus that was essential for boundary establishment and showed a limited sequence homology to the human PSMF1, which is known to inhibit proteasome activity. Finally, boundary assay showed that human PSMF1 also exhibited boundary establishment activity in yeast. Our results defined the functional correlation between Fub1p and PSMF1.

Key words: boundary, euchromatin and heterochromatin, gene silencing, proteasome, *Saccharomyces cerevisiae*

INTRODUCTION

Eukaryotic chromosomes are comprised of accessible euchromatic domains contained in loose chromatin structures and inaccessible heterochromatic domains within condensed structures that restrict the expression of internal genes. In *S. cerevisiae*, heterochromatin-like regions have been found at *HM* silent mating-type loci (*HMR* and *HML*), the telomeres, and in *rRNA*-encoding DNA. This transcriptional silencing is achieved and spread through propagation of the SIR (silent information regulator) protein complex, composed of Sir2p, Sir3p, and Sir4p. In the Sir complex, Sir2p has histone deacetylation activity (Imai et al., 2000) and moves along the DNA by deacetylating the N-terminal of histone to allow the other Sir pro-

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* Corresponding author. E-mail: ma4sa6ya@u-fukui.ac.jp

teins to bind to the nucleosomes (Rusche et al., 2003; Sun et al., 2011). While Sir2p, Sir3p, and Sir4p are all required for silencing at the *HMR* region, *HML* region and the telomeres, silencing of genes at the *rDNA* region requires only Sir2p (Rusche et al., 2003).

To regulate transcriptional silencing, boundaries are necessary to prevent uncontrolled gene silencing. Thus far, two models have been proposed to explain how silencing might be limited; DNA sequence-dependent and DNA sequence-independent. In the former model, STAR (subtelomeric anti-silencing regions) sequences near the telomeres (Fourel et al., 1999) and *tDNA* sequence at the right side of the *HMR* domain regulate the spread of silencing (Dhillon et al., 2009; Donze et al., 1999; Donze and Kamakaka, 2001, 2002; Oki and Kamakaka, 2005). The DNA sequence-independent model, on the other hand, relies on competition between histone deacetylation by Sir2p and histone acetylation by Sas2p, a subunit of the SAS-I complex, to define the silencing boundaries (Kimura and Horikoshi, 2004; Kimura et al., 2002; Suka et al., 2002).

We developed a genome-wide screening system to investigate whether the Gal4p DNA-binding domain (GBD) fused protein maintains boundary function during the silencing process. We isolated 55 boundary related genes from approximately 6,000 genes in *S. cerevisiae* (Oki et al., 2004). The function of one of these boundary proteins (Fub1p/Ycr076cp) was still unknown. In the past syudies, Fub1p had been shown to associate to more than 20 proteins, for example, proteasome associated proteins, histone modification proteins, transcriptional regulator proteins, small G-protein regulator proteins, cell cycle check point proteins, ER and/or Golgi related proteins, mitochondria related proteins and transport factor proteins with many kinds of comprehensive screening (Collins et al., 2007; Costanzo et al., 2010; Krogan et al., 2006). Interestingly, Fub1p has a PI31 proteasome regulator domain with higher eukaryote protein, and it is known to inhibit proteasome activity (Kirk et al., 2008; Mccutchen-Maloney et al., 2000; Zaiss et al., 1999, 2002). The proteasome is a proteolytic enzyme comprised of multiple component particles that degrades unneeded or damaged proteins with a polyubiquitinated targeting sequence (Hochstrasser, 1996). The 26S proteasome is comprised of more than 33 different subunits and is the most common form of proteasome. It contains one 20S proteasome core particle (20S CP) and two 19S regulatory particles (19S RPs) with multiple ubiquitin-binding sites that allow polyubiquitinated proteins to enter into the catalytic core (Baumeister et al., 1998; Coux et al., 1996; Voges et al., 1999). The 20S CP consists of four sevenfold symmetric rings with two outer α rings and two inner β rings (Groll et al., 1997). The 19S RP consists of two subassemblies: a lid that associates deubiquitination, and a base that binds to the 20S CP directly and maintains six ATPases that support the rapid degradation of unfolded proteins (Glickman et al., 1998). However, new attention is being paid to the non-proteolytic functions of the proteasome that have recently been reported, such as regulation of transcription (Szutorisz et al., 2006).

Thus far, a relationship between the proteasome and transcriptional silencing has not been reported. This study focuses on analyzing the relationship between Fub1p, which plays a role in the establishment of transcriptional silencing boundaries, and the proteasome complex, which interacts directly with Fub1p.

MATERIALS AND METHODS

Strains and plasmids The yeast strains and plasmids used in this experiment are listed in Tables 1 and 2. All domain analysis plasmids were generated by PCR-mediated sequence deletion to obtain plasmids in which specific amino acids were deleted. Using a pair of phosphorylated primers designed against sequences just outside the target domain, the remainder of the plasmid was amplified and ligated, effectively deleting the target amino acids.

A human cDNA library was prepared by RT-PCR using mRNA from mesenchymal stem cells (MSCs) as a template. An *Eco*RI/*Not*I PSMF1 fragment was amplified by PCR using the primers 5'- aattagaattcatggcgggcctgga - 3' and 5'- ttaaagcggccgctcacaggtaca - 3' (underlined areas indicate restriction sites) and cloned into a pGBK-RC plasmid (Ito et al., 2000) to generate pFOM279.

Construction of gene disruption strains To construct the gene disruption strains, the genes were replaced by the *KanMX* gene (Yeast Knock Out Strain Collection, Open Biosystems). PCR products amplified from genomic DNA containing *geneΔ::KanMX* were individually transformed into yeast cells. They were confirmed by PCR.

Patch mating assays Patch mating assays were performed as described previously (Donze et al., 1999). Cells that were transformed by GBD or GBD-fused genes were grown on selective medium (YMD) lacking tryptophan at 30°C for 3 days. Single colonies were spread on YMD plates containing mating tester lawns JRY19a with an OD_{600} of 2 and leucine and adenine for diploid colonies. The plates were incubated at 30°C for 3 days, and were photographed daily.

Protein affinity purification Fub1p-3xFLAG purification was performed as described previously (Iida and Araki, 2004), with some modifications. The cells were grown in YPD medium to an OD_{600} of 2, washed once with PBS buffer and resuspended in 0.8 ml lysis buffer [50 mM HEPES-KOH (pH 7.5), 300 mM KCl, 0.05% Tween-20, 0.005% NP-

Table 1. Yeast strains

Strain	Genotype	Source
FUY31	*MATa ADE2 lys2Δ his3-11 leu2-5,112 trp1-1 ura3-1*	*W-303 wild type*
FUY95	*FUY31 FUB1-3×FLAG::KanMX*	This study
ROY2041	*MATα ADE2 lys2Δ his3-11 leu2-5,112 trp1-1 hmrΔ::bgl-bclΔ ura3-1::HMRΔI::URA3*	(Oki et al., 2004)
ROY2042	*MATα ADE2 lys2Δ his3-11 leu2-5,112 trp1-1 hmrΔ::bgl-bclΔ ura3-1::HMRΔI+4×Gal4 bs at mat a2 (ECONI)::URA3*	(Oki et al., 2004)
FUY165	*ROY2041 fub1Δ::KanMX*	This study
FUY166	*ROY2042 fub1Δ::KanMX*	This study
JRY19a	*MATa ADE2 LYS2 his4-519 leu2-3,112 trp1 ura3-52*	(Donze et al., 1999)
FUY309	*ROY2042 hsm3Δ::KanMX*	This study
FUY310	*ROY2042 rpn10Δ::KanMX*	This study
FUY311	*ROY2042 rpn13Δ::KanMX*	This study
FUY312	*ROY2042 ump1Δ::KanMX*	This study
FUY375	*ROY2042 blm10Δ::KanMX*	This study
FUY563	*ROY2042 pre9Δ::KanMX*	This study
YPH499	*MATa ade2-101 lys2-801 his3Δ-200 leu2Δ1 trp1Δ63 ura3-52*	(Sikorski and Hieter, 1989)
MHY4464	*YPH499 rpt6(cim3-1)*	(Ghislain et al., 1993)
MHY4466	*YPH499 rpt1(cim5-1)*	(Ghislain et al., 1993)
des3	*YPH499 pre2-75*	(Funakoshi et al., 2002)
des5	*YPH499 rpt4-G106D*	(Funakoshi et al., 2009)
des16	*YPH499 rpn1-821*	(Funakoshi et al., 2002)
FUY567	*YPH499 fub1Δ::KanMX*	This study
FUY568	*YPH499 fub1Δ::KanMX rpt6(cim3-1)*	This study
FUY569	*YPH499 fub1Δ::KanMX rpt1(cim5-1)*	This study
FUY570	*YPH499 fub1Δ::KanMX pre2-75*	This study
FUY571	*YPH499 fub1Δ::KanMX rpt4-G106D*	This study
FUY572	*YPH499 fub1Δ::KanMX rpn1-821*	This study

40, 10% glycerol, 0.2 μg/ml pepstatin A, 0.2 μg/ml leupeptin, 1 mM PMSF, 2 mM β-glycerophosphate, 2 mM NaF, 0.4 mM Na_3VO_4, 0.5 mM Na-pyrophosphate]. Cells were disrupted by glass beads with vortex mixer. Cell lysates were cleared by centrifugation for 20 min at 4°C. Protein extracts were adsorbed onto 0.5 ml of sepharose 4B Fast Flow (GE Healthcare) for 1 hour at 4°C. Then, the beads were pelleted and the supernatant was recovered and mixed with 50 μl anti-Flag-agarose (M2) beads (Sigma) for 3 hours at 4°C. The beads were washed six times with 1 ml cold lysis buffer containing 0.1 mg/ml BSA, and then washed five times with 1 ml cold lysis buffer. The complex was eluted by incubating the beads with lysis buffer containing 100 μg/ml 3X FLAG peptide (Sigma).

Mass spectrometry Affinity-purified proteins were resolved by 15% SDS-PAGE. After silver staining with *SilverQuest™ Silver Staining Kit* (Invitrogen), the peptide bands were excised from the gel and subjected to in-gel reduction with 10 mM DTT, alkylation with 55 mM iodoacetamide, and digestion with 10 μg/ml modified trypsin (Promega) at 37°C for 16 hours. After in-gel digestion, the collected peptides were subjected to mass spectrometry analysis as described previously (Sadaie et al., 2008).

RESULTS

Fub1p binds the 20S CP structure A total 55 genes related to silencing region control were isolated from approximately 6,000 genes in *S. cerevisiae* by genome-wide screening (Oki et al., 2004). We focused on *YCR076C/FUB1* whose biological function was still unknown.

To investigate the function of Fub1p, we used a FLAG purification system to isolate Fub1p binding proteins. Because tag-fused proteins sometimes lose activity, we assessed the activity of a Fub1p-3xFLAG fusion protein by the same method used for genome-wide screening (Oki et al., 2004). Our results showed that Fub1p retained function even when fused to a 3xFLAG at the C terminus (data not shown). Therefore, we used this construct to

Table 2. Plasmid

Plasmid		Comment
p3FLAG-KanMX		(Gelbart et al., 2001)
pFOM151	p3FLAG-KanMX plus FUB1	This study
p426GAL	pRS426 plus GAL promoter	(Funk et al., 2002)
p426GAL-FUB1	pRS426 plus GAL promoter-FUB1	This study
pGBK-RC		(Ito et al., 2000)
pFOM51	pGBK-RC plus GBD-Sas2p (open reading frame [ORF])	(Oki et al., 2004)
pFOM57	pGBK-RC plus GBD-Fub1p (open reading frame [ORF])	(Oki et al., 2004)
pFOM380	pGBK-RC plus GBD- Fub1p (124–250aa.)	This study
pFOM381	pGBK-RC plus GBD- Fub1p (1–123aa.)	This study
pFOM382	pGBK-RC plus GBD- Fub1p (61–250aa.)	This study
pFOM383	pGBK-RC plus GBD- Fub1p (⊿61–164aa.)	This study
pFOM384	pGBK-RC plus GBD- Fub1p (1–164aa.)	This study
pFOM385	pGBK-RC plus GBD- Fub1p (1–60aa.)	This study
pFOM386	pGBK-RC plus GBD- Fub1p (165–250aa.)	This study
pFOM387	pGBK-RC plus GBD- Fub1p (⊿165–229aa.)	This study
pFOM388	pGBK-RC plus GBD- Fub1p (⊿161–207aa.)	This study
pFOM389	pGBK-RC plus GBD- Fub1p (⊿165–195aa.)	This study
pFOM390	pGBK-RC plus GBD- Fub1p (⊿196–229aa.)	This study
pFOM391	pGBK-RC plus GBD- Fub1p (196–250aa.)	This study
pFOM392	pGBK-RC plus GBD- Fub1p (165–195aa.)	This study
pFOM393	pGBK-RC plus GBD- Fub1p (196–213aa.)	This study
pFOM394	pGBK-RC plus GBD- Fub1p (196–224aa.)	This study
pFOM395	pGBK-RC plus GBD- Fub1p (196–237aa.)	This study
pFOM279	pGBK-RC plus GBD- PSMF1	This study

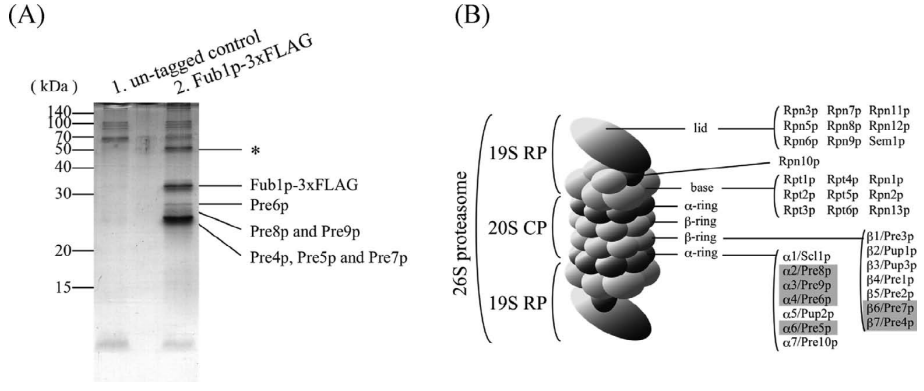

Fig. 1. Purification of Fub1p-interacting proteins and identification of 20S CP components. (A) Silver-stained gels show purification of Fub1p-3xFLAG and an untagged control strain. The approximate position of the Fub1p complex components identified by mass spectrometry is indicated. All proteins belong to the 20S CP of the proteasome. The asterisk indicates an immunoglobulin. Line 1 is the untagged control (FUY31). Line 2 is Fub1p-3xFLAG (FUY95). (B) Molecular structure of the proteasome. The 26S proteasome is composed of more than 33 different subunits, containing one 20S CP structure and two 19S RPs. The shadow box shows active subunits isolated from Fub1p-3xFLAG.

generate the yeast strain, FUY95. The Fub1p-3xFLAG protein was purified using anti-FLAG antibodies and eluted using FLAG peptides. Following SDS-PAGE and silver staining, bands were excised for LC-MS/MS analysis to identify proteins bound to Fub1p. The results identified a number of 20S CP core-associated factors, including β7/Pre4p, α6/Pre5p, α4/Pre6p, β4/Pre7p, α2/Pre8p and α3/Pre9p (Fig. 1A), all of which belong to the α or β subunit of the 20S CP (Fig. 1B).

***FUB1* has genetic interaction with proteasome particles** Because Fub1p was shown to bind to proteasome particles (Fig. 1A), a genetic approach was used to clarify the mechanism of interaction. To examine the genetic interaction, we utilized target gene overexpression and deletion analysis. Most proteasome components are essential genes, so we chose well-characterized temperature sensitive (ts) mutants. Some components of the 20S CP were isolated by LC-MS/MS, but these components were not proteolytically active. Among the 20S CP components, β1/Pre3p, β2/Pup1p and β5/Pre2p are known to possess proteolytic activities, thus, we chose the *pre2-75* temperature sensitive mutant. On the other hand, the 19S RP was not isolated by our assay. Therefore, we tested the genetic interaction in *rpt1 (cim5-1), rpt6 (cim3-1), rpt4-G106D and rpn1-821* temperature sensitive mutants. First, we observed the effects of overexpression of Fub1p in both wild-type (WT) and proteasome mutants. When Fub1p was overexpressed in WT, the cells exhibited obvious growth defects. Furthermore, Fub1p overexpression in *pre2-75* (20S CP) mutant cells and *rpn1-821, rpt1 (cim5-1)* (19S RP) mutants resulted in much more serious growth defects than were seen in WT. However, there was no obvious effect of Fub1p overexpression in the *rpt4-G106D, rpt6 (cim3-1)* (19S RP) mutant cells (Fig. 2A).

Next, we analyzed the effects of *fub1* deletion because no phenotype had been reported between *fub1Δ* and proteasome components. A double mutant of *fub1Δ* and *rpt1 (cim5-1)*, a temperature sensitive mutant of the 19S RP, exhibited a synthetic sick growth phenotype. However, genetic interaction with Fub1p was not observed in *pre2-75, rpt4-G106D, rpt6 (cim3-1)* or *rpn1-821* mutants (Fig. 2B). Based on these results, Fub1p interacts not only with the 20S CP but also with the 19S RP.

The relationship between the proteasome and the boundary function of Fub1p To determine whether Fub1p could maintain its boundary function in proteasome mutant strains, analysis was performed using the same method as the patch mating assay with genome-wide boundary screening (Oki et al., 2004). First, a plasmid (pFOM57), Gal4-binding domain (GBD) fused to full-length Fub1p, was introduced into wild-type (WT) and proteasome component deleted strains that had a Gal4-binding sequence adjacent to the silencing domain at *HMR*. If the Fub1p maintained boundary function, expression of the reporter gene *a1* would be de-repressed and the cells would be unable to form diploid colonies on selective plates. Conversely, if cells did form diploid colonies on selective plates, this would indicate that the reporter *a1* gene was repressed and that Fub1p had lost boundary function. Of the proteins isolated by LC-MS/MS, only Pre9p (a component of the 20S CP) was a non-

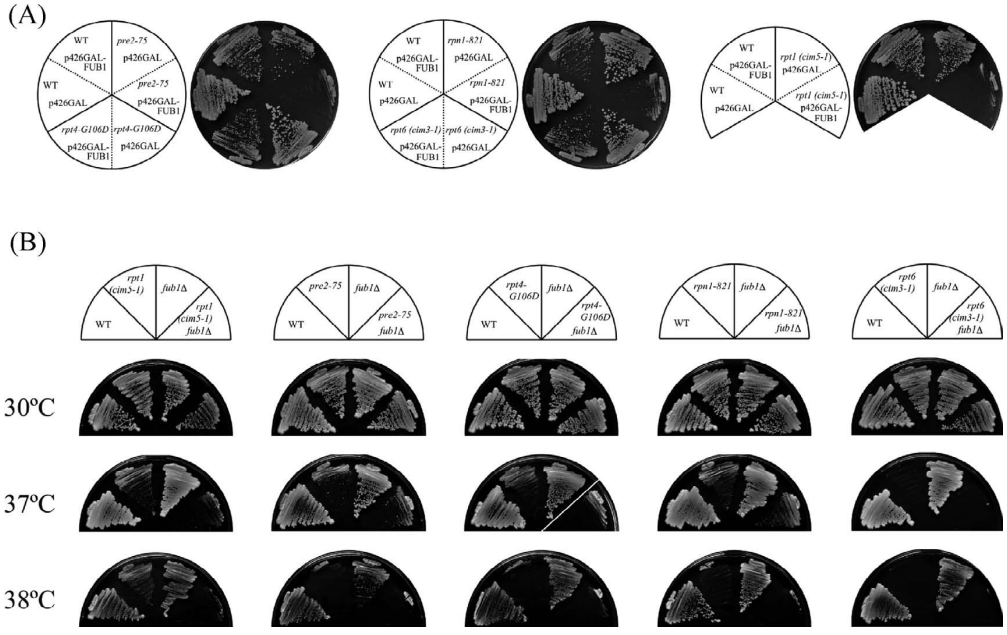

Fig. 2. Characterization of the genetic interactions between Fub1p and the proteasome complex components. (A) Effect of Fub1p overexpression on yeast growth. Control (p426GAL) and Fub1 overexpression plasmids (p426GAL-FUB1) were introduced in wild-type (WT, YPH499) and the proteasome temperature sensitive (ts) mutant strains, *pre2-75* (des3), *rpt4-G106D* (des5), *rpn1-821* (des16), *rpt6 (cim3-1)* (MHY4464) and *rpt1 (cim5-1)* (MHY4466). All plates were YM+galactose plates lacking uracil. Yeast were grown for 3 days at 30°C. (B) The *fub1Δ rpt1 (cim5-1)* double mutant showed a synthetic sick growth phenotype. WT (YPH499), proteasome ts mutants (*rpt1 (cim5-1)*, *pre2-75, rpt4-G106D, rpn1-821* and *rpt6 (cim3-1)*, *fub1Δ* (FUY567), and a double mutant of *fub1Δ* and proteasome ts mutants were streaked on YPD plates and grown for 3 days at 30°C, 37°C and 38°C.

essential gene (Hochstrasser, 1996), and Fub1p boundary function was lost in the *pre9* mutant strain (Fig. 3). This result suggested that the 20S CP is important for Fub1p boundary function. Next, we tested whether the *ump1* mutant strain would affect the 20S CP since Ump1p plays a role in assembly of the 20S CP (Bochtler et al., 1999; Collins et al., 2007; Maurizi, 1998; Ramos et al., 1998). As shown in Fig. 3, the *ump1* mutant also lost boundary function of Fub1p. As a control, Sas2p, the histone acetyltransferase (HAT) catalytic subunit of the SAS-I complex, maintained its boundary function in all mutant strains as well as in the WT strain. These results suggested that Fub1p cooperated with Pre9p and Ump1p to regulate transcriptional silencing. Meanwhile, mutants of the 19S RP components *rpn10* and *rpn13*, whose products recognize and bind to polyubiquitin chains (Husnjak et al., 2008), showed no change in the barrier activity of Fub1p. We also tested the *hsm3* mutant, which is defective in base assembly, and observed the same result (Funakoshi et al., 2009; Roelofs et al., 2009; Saeki et al., 2009). Based on these results, we hypothesized that the 19S RP was unnecessary for the boundary function of Fub1p. We also observed loss of Blm10p because Blm10p is a multifunctional protein similar to mammalian PA200, which functions as an ATP-independent 20S CP activator associated with 20S CP assembly (Doherty et al., 2004; Fehlker et al., 2003; Schmidt et al., 2005; Stadtmueller and Hill, 2011). However, our results revealed no effect on the boundary function of Fub1p.

To determine whether other boundary proteins isolated by genome-wide screening (Oki et al., 2004) were involved

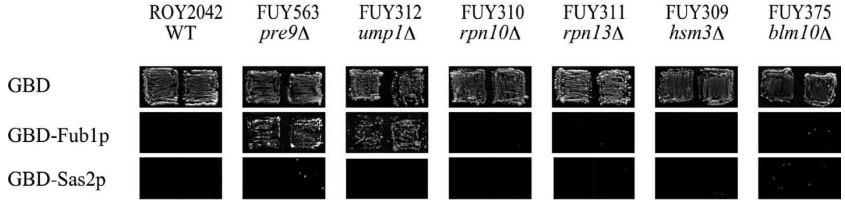

Fig. 3. Boundary activity in mutant strains. Patch mating assay is shown with WT (ROY2042), pre9*Δ* (FUY563), *ump1Δ* (FUY312), *rpn10Δ* (FUY310), *rpn13Δ* (FUY311), *hsm3Δ* (FUY309) and *blm10Δ* (FUY375) strains to detect genes necessary for the boundary function of Fub1p or Sas2p. GBD is used as boundary formational negative control and GBD-Sas2p is used as positive control.

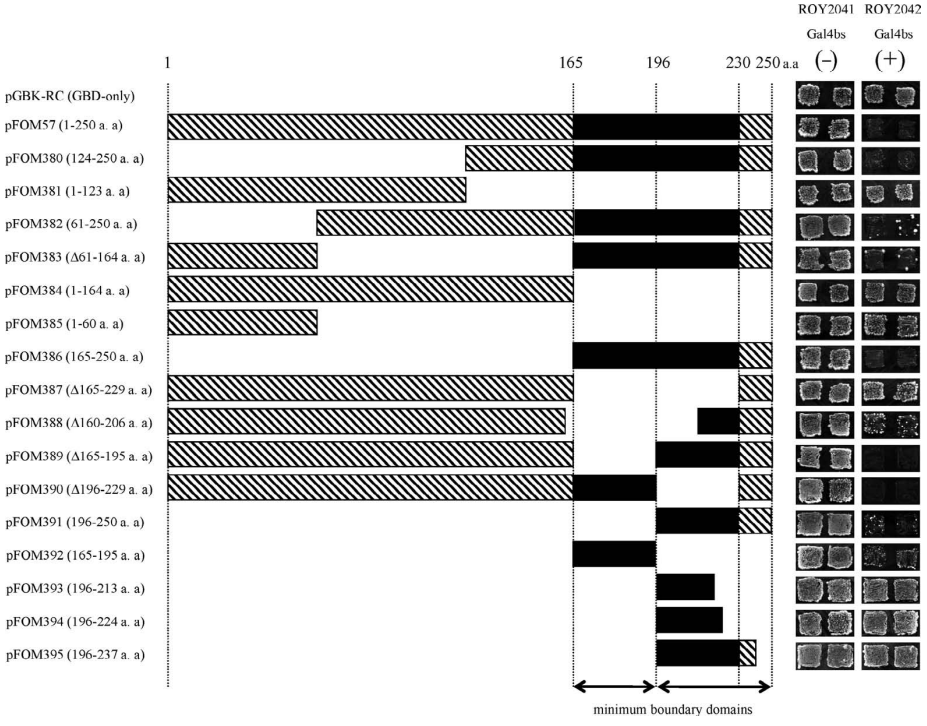

Fig. 4. Domain analysis of Fub1p boundary activity. Schematic presentation of Fub1p domain analyses that were tested with patch mating assay. (–) and (+) indicate constructs without and with the Gal4p binding sequence, respectively. The full length of Fub1p is shown as 1–250 aa and the black box (165–229 aa) indicates the PI31 proteasome regulator domain.

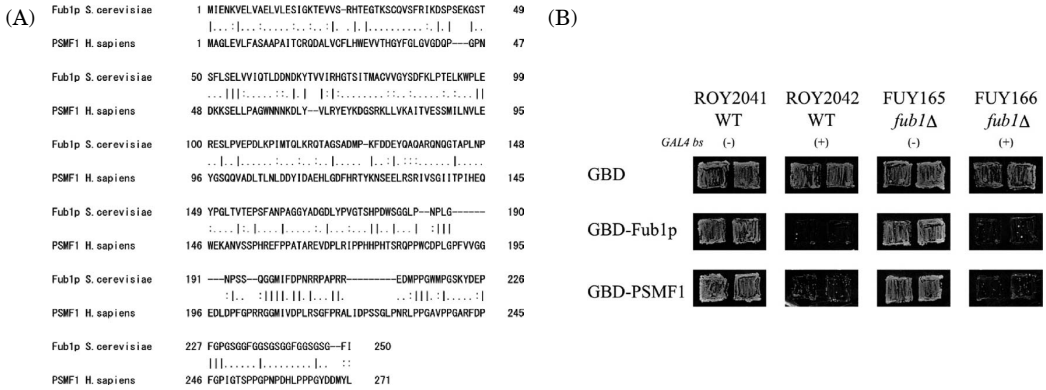

Fig. 5. Boundary activity of the human PSMF1. (A) Sequence alignment of *S. cerevisiae* Fub1p and *Homo sapiens* PSMF1/PI31. Stretcher (Version 6.3.1; http://www.ebi.ac.uk/Tools/psa/emboss_stretcher/) was used for alignments. A line (|) at the bottom of the column of aligned amino acids indicates 100% identity, two dots (:) indicate similar amino acids. (B) *H. sapiens* PSMF1 also had boundary function. Wild-type (ROY2041 and ROY2042) and *fub1Δ* mutant strains (FUY165 and FUY166) were transformed with GBD, GBD-Fub1p or GBD-PSMF1 plasmids, and patch mating assays were performed as described previously. (−) and (+) indicate constructs without and with Gal4p-binding sites, respectively.

in boundary establishment by Fub1p, the boundary activities of some of these proteins were analyzed in a *fub1*-deleted strain. The proteins selected for analysis were related to the histone modification complex (Sas2p, Sas5p, Dot1p, Ada1p and Ada2p), TFIID complex (Taf47p), chromatin remodeling complex (Snf6p), mediator (Med2p), and cell cycle (Clb1p), as well as other factors such as Gic1p and Gds1p. The results showed that they did not appear to have any effect on boundary function of Fub1p (data not shown). Based on this finding, it appears that Fub1p functions independently, rather than cooperatively with the other boundary factors.

Minimum domain analysis of Fub1p To analyze which domain of Fub1p is crucial for boundary function, a minimum domain analysis was performed (Fig. 4). The domain containing amino acids 165–229 of Fub1p is reported to be the PI31 proteasome regulator domain conserved in higher eukaryotes (Zaiss et al., 2002). *In vitro*, PSMF1, a human protein containing the PI31 proteasome regulator domain, behaves as a proteasome inhibitor (Mccutchen-Maloney et al., 2000; Zaiss et al., 1999, 2002). Patch mating assay results showed that the Fub1p domain containing amino acids 165–250 retained Fub1p boundary function (pFOM386, 165–250 aa). When the PI31 proteasome regulator domain was divided into two fragments (pFOM391, 196–250 aa; pFOM392, 165–195 aa), both maintained boundary function, but pFOM392 was not as strong as that of pFOM391. However, boundary function was lost when the C terminal domain (pFOM395, 196–237 aa), which has low homology to other species, was deleted.

Human homolog of Fub1p, PSMF1, has boundary function in *S. cerevisiae* The PI31 proteasome regulator domain in Fub1p where the boundary function is maintained is widely conserved from yeast to mammals (Fig. 5A, (Zaiss et al., 2002), http://www.ebi.ac.uk/interpro/IEntry?ac=IPR013886). Fub1p and PSMF1 are 19.6% homologous (similarity 32.2%) at the full length and 30.8% homologous (similarity 46.2%) in the PI31 proteasome regulator domain. Because there was high homology between Fub1p and PSMF1, we tested whether the boundary function of Fub1p was conserved in PSMF1. To test boundary function by patch mating assay, we cloned PSMF1 from a human cDNA library and constructed a GBD-PSMF1 fusion plasmid. To prevent false positive results due to the formation of heterodimers between GBD-PSMF1 and endogenous yeast Fub1p, GBD-fused plasmids (GBD, GBD-Fub1p and GBD-PSMF1) were introduced in both wild-type (WT) and *fub1Δ* mutant strains. As shown in Fig. 5B, PSMF1 maintained the boundary function at approximately the same level as Fub1p in both wild-type yeast and the *fub1Δ* mutant strains.

DISCUSSION

To investigate the *in vivo* role of Fub1p in the control of transcriptional silencing, we performed LC-MS/MS. Although Fub1p was previously reported to interact with the proteasome composition factors Pre2p and Pup3p (Collins et al., 2007; Krogan et al., 2006), this association had yet to be analyzed in depth. To determine whether Fub1p interacted with the 20S CP, the 26S proteasome consisting of both 19S RP and 20S CP, or just Pre2p and Pup3p, we purified the interacting proteins using an

Fub1p-3xFLAG strain. The results demonstrated that Fub1p bound to subunits of the 20S CP; however, subunits of the 19S RP were not detected. Based on *in vitro* analysis with the PI31 proteasome regulator domain of PSMF1, which is highly homologous to the Fub1p C terminal region, the PI31 proteasome regulator domain appeared to bind to the α ring of the 20S CP and inhibit its activity (Chu-Ping et al., 1992; Mccutchen-Maloney et al., 2000). To analyze the genetic interaction of Fub1p with the proteasome, growth defects were assessed following overexpression of Fub1p. These defects are thought to be caused by inhibition of the enzymatic activity of the 20S CP or by deregulation of the ubiquitin-proteasome system. The slow growth of the *pre2-75* mutant strain was likely due to reduced enzymatic activity of the 20S CP, while the more severe growth defects seen in the *rpn1-821* and *rpt1 (cim5-1)* mutant strains were probably caused by a decreased unfolding ability of the subunits of the 19S RP, which reduced the efficiency of translocation into the central channel of the 20S CP. On the other hand, the 19S RP mutant strains *rpt4-G106D* and *rpt6 (cim3-1)* did not exhibit an obvious growth defect phenotype. We believe the reason for this is that another 20S CP activator, such as Blm10p/PA200 (Yeast/Human) or PA26/PA28 (Yeast/Human), was able to compensate for 19S RP activity in these strains (Stadtmueller and Hill, 2011). We isolated the 20S CP factor that interacted with the Fub1p by affinity purification. Furthermore, in genetic interaction, both the 20S CP factor and the 19S RP factor were detected. Although this appears to contradict our earlier results, it is possible that the 19S was not detectable under the conditions of the affinity purification experiment. Direct interaction between the 19S RP and Fub1p might be observed by changing the experimental conditions.

When the boundary function in our focused silencing domain was examined, Fub1p lost boundary function in cells in which *pre9* was deleted, the only single factor that can be disrupted among all the subunits of the 20S CP. Fub1p boundary function was also lost in cells lacking *ump1*, a chaperone of the 20S CP. These results suggested that the entire 20S CP plays an important role in boundary formation for controlling the spread of transcriptional silencing. On the other hand, when all the factors of the 20S CP α ring were fused with the GBD, no boundary function was observed using the same methods as in Fig. 3 and Fig. 4 (data not shown). Since the GBD was fused to the amino terminus of the protein in our system, it is possible that the GBD-20S CP did not maintain normal function. Conversely, there was no loss of Fub1p boundary function in the 19S RP mutant strains, indicating that the 19S RP might not be necessary for direct boundary function, or that 19S RP function could be compensated for by other 20S CP activators, such as Blm10p/PA200 and PA26/PA28 (Stadtmueller and Hill, 2011).

It was very important to clarify the native barrier function of Fub1p. Therefore, we generated a Fub1p-3xFLAG strain and investigated whether Fub1p was present at the native boundary region using ChIP assay with anti-FLAG antibody. In budding yeast, a native barrier is located in the telomeric region of each chromosome, *HML*, *HMR* and an *rDNA* coding region, and we examined the left side boundary region at *HMR*. However, no Fub1p was observed in this region (data not shown). To determine whether the silencing region would extend to the left side in a *fub1Δ* mutant strain considering the loss of the left side boundary at *HMR*, we used ChIP assay with anti-Sir3p antibody, and no extension was found (data not shown). It was also possible that Fub1p participated in the barrier function at other silencing regions, such as the telomeres, *HML*, and the *rDNA* coding region. As other possibility, no obvious change was observed in the boundary function or extension of the silencing domain by removing either of the two elements, the cis-element and HAT (a histone acetylation enzyme complex), that participated in the function of the *HMR* right side boundary (Oki and Kamakaka, 2005).

Many reports examine the relationship between chromatin and the proteasome. For example, it is reported that the proteasome interacts with chromatin directly (Sikder et al., 2006), the composition factor of the proteasome is required for extension in RNA transcription (Bhaumik and Malik, 2008; Ferdous et al., 2001), proteasome works on the chromatin (Szutorisz et al., 2006). In addition, ts mutations in both the 19S RP and 20S CP proteasome resulted in the loss of silencing activity (Ezhkova and Tansey, 2004). Furthermore, double mutation of *sem1* and *ubp6*, a composition factor of the 19S RP and deubiquitinating protein, respectively, weakened the effect of Sir proteins at the telomere silencing domain (Qin et al., 2009). Similarly, a similar result was observed at the telomere domain following loss of *rpn10* (Hang and Smith, 2011). These data also supported that the proteasome might affect the boundary function. From the above findings, we hypothesize that Fub1p interacts with the proteasome on chromatin and forms a boundary either by making use of the activity of the 20S CP or by weakening silencing through inhibiting proteasome function.

The PI31 proteasome regulator domain lies in the C terminus of Fub1p. Fub1p possesses two boundary formation domains, as shown by the results of the minimum domain analysis (Fig. 4). Both of these domains retained boundary function, even in the absence of the full PI31 proteasome regulator domain, although pFOM392 was not as strong as either pFOM391 or the full Fub1 domain. Based on these findings, it is plausible that both or one of these domains binds the 20S CP. In fact, interaction between the PI31 proteasome regulator domain of PSMF1 and the 20S CP has already been

reported (Mccutchen-Maloney et al., 2000; Zaiss et al., 1999, 2002). Therefore, it will be interesting to ascertain whether the other minimum domain can also bind the 20S CP or other factors, and to identify any additional factors that interact with the N terminus of Fub1p.

It is thought that human PSMF1 may be an ortholog of Fub1p based on conservation of the PI31 proteasome regulator domain at the C terminus of Fub1p. In addition, as the proteasome is present in species from bacteria to the eukaryotes, it is tempting to speculate that Fub1p may have a similar boundary function in other species. However, because the mechanism by which PSMF1 might participate in the process boundary formation to restrict the spread of silencing in human cells is still unknown, future studies are needed.

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REFERENCES

Baumeister, W., Walz, J., Zuhl, F., and Seemuller, E. (1998) The proteasome: paradigm of a self-compartmentalizing protease. Cell **92**, 367–380.

Bhaumik, S. R., and Malik, S. (2008) Diverse regulatory mechanisms of eukaryotic transcriptional activation by the proteasome complex. Crit. Rev. Biochem. Mol. Biol. **43**, 419–433.

Bochtler, M., Ditzel, L., Groll, M., Hartmann, C., and Huber, R. (1999) The proteasome. Annu. Rev. Biophys. Biomol. Struct. **28**, 295–317.

Chu-Ping, M., Slaughter, C. A., and Demartino, G. N. (1992) Purification and characterization of a protein inhibitor of the 20S proteasome (macropain). Biochim. Biophys. Acta **1119**, 303–311.

Collins, S. R., Kemmeren, P., Zhao, X. C., Greenblatt, J. F., Spencer, F., Holstege, F. C., Weissman, J. S., and Krogan, N. J. (2007) Toward a comprehensive atlas of the physical interactome of *Saccharomyces cerevisiae*. Mol. Cell. Proteomics **6**, 439–450.

Costanzo, M., Baryshnikova, A., Bellay, J., Kim, Y., Spear, E. D., Sevier, C. S., Ding, H., Koh, J. L., Toufighi, K., Mostafavi, S., et al. (2010) The genetic landscape of a cell. Science **327**, 425–431.

Coux, O., Tanaka, K., and Goldberg, A. L. (1996) Structure and functions of the 20S and 26S proteasomes. Annu. Rev. Biochem. **65**, 801–847.

Dhillon, N., Raab, J., Guzzo, J., Szyjka, S. J., Gangadharan, S., Aparicio, O. M., Andrews, B., and Kamakaka, R. T. (2009) DNA polymerase epsilon, acetylases and remodellers cooperate to form a specialized chromatin structure at a tRNA insulator. EMBO J. **28**, 2583–2600.

Doherty, K., Pramanik, A., Pride, L., Lukose, J., and Moore, C. W. (2004) Expression of the expanded *YFL007w* ORF and assignment of the gene name *BLM10*. Yeast **21**, 1021–1023.

Donze, D., and Kamakaka, R. T. (2001) RNA polymerase III and RNA polymerase II promoter complexes are heterochromatin barriers in *Saccharomyces cerevisiae*. EMBO J. **20**, 520–531.

Donze, D., and Kamakaka, R. T. (2002) Braking the silence: how heterochromatic gene repression is stopped in its tracks. Bioessays **24**, 344–349.

Donze, D., Adams, C. R., Rine, J., and Kamakaka, R. T. (1999) The boundaries of the silenced *HMR* domain in *Saccharomyces cerevisiae*. Genes Dev. **13**, 698–708.

Ezhkova, E., and Tansey, W. P. (2004) Proteasomal ATPases link ubiquitylation of histone H2B to methylation of histone H3. Mol. Cell **13**, 435–442.

Fehlker, M., Wendler, P., Lehmann, A., and Enenkel, C. (2003) Blm3 is part of nascent proteasomes and is involved in a late stage of nuclear proteasome assembly. EMBO Rep. **4**, 959–963.

Ferdous, A., Gonzalez, F., Sun, L., Kodadek, T., and Johnston, S. A. (2001) The 19S regulatory particle of the proteasome is required for efficient transcription elongation by RNA polymerase II. Mol. Cell **7**, 981–991.

Fourel, G., Revardel, E., Koering, C. E., and Gilson, E. (1999) Cohabitation of insulators and silencing elements in yeast subtelomeric regions. EMBO J. **18**, 2522–2537.

Funakoshi, M., Sasaki, T., Nishimoto, T., and Kobayashi, H. (2002) Budding yeast Dsk2p is a polyubiquitin-binding protein that can interact with the proteasome. Proc. Natl. Acad. Sci. USA **99**, 745–750.

Funakoshi, M., Tomko, R. J., Jr., Kobayashi, H., and Hochstrasser, M. (2009) Multiple assembly chaperones govern biogenesis of the proteasome regulatory particle base. Cell **137**, 887–899.

Funk, M., Niedenthal, R., Mumberg, D., Brinkmann, K., Ronicke, V., and Henkel, T. (2002) Vector systems for heterologous expression of proteins in *Saccharomyces cerevisiae*. Methods Enzymol. **350**, 248–257.

Gelbart, M. E., Rechsteiner, T., Richmond, T. J., and Tsukiyama, T. (2001) Interactions of Isw2 chromatin remodeling complex with nucleosomal arrays: analyses using recombinant yeast histones and immobilized templates. Mol. Cell. Biol. **21**, 2098–2106.

Ghislain, M., Udvardy, A., and Mann, C. (1993) *S. cerevisiae* 26S protease mutants arrest cell division in G2/metaphase. Nature **366**, 358–362.

Glickman, M. H., Rubin, D. M., Fried, V. A., and Finley, D. (1998) The regulatory particle of the *Saccharomyces cerevisiae* proteasome. Mol. Cell. Biol. **18**, 3149–3162.

Groll, M., Ditzel, L., Lowe, J., Stock, D., Bochtler, M., Bartunik, H. D., and Huber, R. (1997) Structure of 20S proteasome from yeast at 2.4 A resolution. Nature **386**, 463–471.

Hang, M., and Smith, M. M. (2011) Genetic analysis implicates the Set3/Hos2 histone deacetylase in the deposition and remodeling of nucleosomes containing H2A.Z. Genetics **187**, 1053–1066.

Hochstrasser, M. (1996) Ubiquitin-dependent protein degradation. Annu. Rev. Genet. **30**, 405–439.

Husnjak, K., Elsasser, S., Zhang, N., Chen, X., Randles, L., Shi, Y., Hofmann, K., Walters, K. J., Finley, D., and Dikic, I. (2008) Proteasome subunit Rpn13 is a novel ubiquitin receptor. Nature **453**, 481–488.

Iida, T., and Araki, H. (2004) Noncompetitive counteractions of DNA polymerase epsilon and ISW2/yCHRAC for epigenetic inheritance of telomere position effect in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **24**, 217–227.

Imai, S., Armstrong, C. M., Kaeberlein, M., and Guarente, L. (2000) Transcriptional silencing and longevity protein Sir2 is an NAD-dependent histone deacetylase. Nature **403**, 795–800.

Ito, T., Tashiro, K., Muta, S., Ozawa, R., Chiba, T., Nishizawa,

M., Yamamoto, K., Kuhara, S., and Sakaki, Y. (2000) Toward a protein-protein interaction map of the budding yeast: A comprehensive system to examine two-hybrid interactions in all possible combinations between the yeast proteins. Proc. Natl. Acad. Sci. USA **97**, 1143–1147.

Kimura, A., and Horikoshi, M. (2004) Partition of distinct chromosomal regions: negotiable border and fixed border. Genes Cells **9**, 499–508.

Kimura, A., Umehara, T., and Horikoshi, M. (2002) Chromosomal gradient of histone acetylation established by Sas2p and Sir2p functions as a shield against gene silencing. Nat. Genet. **32**, 370–377.

Kirk, R., Laman, H., Knowles, P. P., Murray-Rust, J., Lomonosov, M., Meziane El, K., and Mcdonald, N. Q. (2008) Structure of a conserved dimerization domain within the F-box protein Fbxo7 and the PI31 proteasome inhibitor. J. Biol. Chem. **283**, 22325–22335.

Krogan, N. J., Cagney, G., Yu, H., Zhong, G., Guo, X., Ignatchenko, A., Li, J., Pu, S., Datta, N., Tikuisis, A. P., et al. (2006) Global landscape of protein complexes in the yeast *Saccharomyces cerevisiae*. Nature **440**, 637–643.

Maurizi, M. R. (1998) Proteasome assembly: biting the hand. Curr. Biol. **8**, 453–456.

Mccutchen-Maloney, S. L., Matsuda, K., Shimbara, N., Binns, D. D., Tanaka, K., Slaughter, C. A., and Demartino, G. N. (2000) cDNA cloning, expression, and functional characterization of PI31, a proline-rich inhibitor of the proteasome. J. Biol. Chem. **275**, 18557–18565.

Oki, M., and Kamakaka, R. T. (2005) Barrier function at *HMR*. Mol. Cell **19**, 707–716.

Oki, M., Valenzuela, L., Chiba, T., Ito, T., and Kamakaka, R. T. (2004) Barrier proteins remodel and modify chromatin to restrict silenced domains. Mol. Cell. Biol. **24**, 1956–1967.

Qin, S., Wang, Q., Ray, A., Wani, G., Zhao, Q., Bhaumik, S. R., and Wani, A. A. (2009) Sem1p and Ubp6p orchestrate telomeric silencing by modulating histone H2B ubiquitination and H3 acetylation. Nucleic Acids Res. **37**, 1843–1853.

Ramos, P. C., Hockendorff, J., Johnson, E. S., Varshavsky, A., and Dohmen, R. J. (1998) Ump1p is required for proper maturation of the 20S proteasome and becomes its substrate upon completion of the assembly. Cell **92**, 489–499.

Roelofs, J., Park, S., Haas, W., Tian, G., Mcallister, F. E., Huo, Y., Lee, B. H., Zhang, F., Shi, Y., Gygi, S. P., et al. (2009) Chaperone-mediated pathway of proteasome regulatory particle assembly. Nature **459**, 861–865.

Rusche, L. N., Kirchmaier, A. L., and Rine, J. (2003) The establishment, inheritance, and function of silenced chromatin in *Saccharomyces cerevisiae*. Annu. Rev. Biochem. **72**, 481–516.

Sadaie, M., Shinmyozu, K., and Nakayama, J. (2008) A conserved SET domain methyltransferase, Set11, modifies ribosomal protein Rpl12 in fission yeast. J. Biol. Chem. **283**, 7185–7195.

Saeki, Y., Toh, E. A., Kudo, T., Kawamura, H., and Tanaka, K. (2009) Multiple proteasome-interacting proteins assist the assembly of the yeast 19S regulatory particle. Cell **137**, 900–913.

Schmidt, M., Haas, W., Crosas, B., Santamaria, P. G., Gygi, S. P., Walz, T., and Finley, D. (2005) The HEAT repeat protein Blm10 regulates the yeast proteasome by capping the core particle. Nat. Struct. Mol. Biol. **12**, 294–303.

Sikder, D., Johnston, S. A., and Kodadek, T. (2006) Widespread, but non-identical, association of proteasomal 19 and 20 S proteins with yeast chromatin. J. Biol. Chem. **281**, 27346–27355.

Sikorski, R. S., and Hieter, P. (1989) A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. Genetics **122**, 19–27.

Stadtmueller, B. M., and Hill, C. P. (2011) Proteasome activators. Mol. Cell **41**, 8–19.

Suka, N., Luo, K., and Grunstein, M. (2002) Sir2p and Sas2p opposingly regulate acetylation of yeast histone H4 lysine16 and spreading of heterochromatin. Nat. Genet. **32**, 378–383.

Sun, J. Q., Hatanaka, A., and Oki, M. (2011) Boundaries of transcriptionally silent chromatin in *Saccharomyces cerevisiae*. Genes Genet. Syst. **86**, 73–81.

Szutorisz, H., Georgiou, A., Tora, L., and Dillon, N. (2006) The proteasome restricts permissive transcription at tissue-specific gene loci in embryonic stem cells. Cell **127**, 1375–1388.

Voges, D., Zwickl, P., and Baumeister, W. (1999) The 26S proteasome: a molecular machine designed for controlled proteolysis. Annu. Rev. Biochem. **68**, 1015–1068.

Zaiss, D. M., Standera, S., Holzhutter, H., Kloetzel, P., and Sijts, A. J. (1999) The proteasome inhibitor PI31 competes with PA28 for binding to 20S proteasomes. FEBS Lett. **457**, 333–338.

Zaiss, D. M., Standera, S., Kloetzel, P. M., and Sijts, A. J. (2002) PI31 is a modulator of proteasome formation and antigen processing. Proc. Natl. Acad. Sci. USA **99**, 14344–14349.