

# Proteasome Involvement in the Repair of DNA Double-Strand Breaks

## Short Article

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### Summary

Affinity purification of the yeast 19S proteasome revealed the presence of Sem1 as a subunit. Its human homolog, DSS1, was found likewise to copurify with the human 19S proteasome. DSS1 is known to associate with the tumor suppressor protein BRCA2 involved in repair of DNA double-strand breaks (DSBs). We demonstrate that Sem1 is required for efficient repair of an HO-generated yeast DSB using both homologous recombination (HR) and nonhomologous end joining (NHEJ) pathways. Deletion of *SEM1* or genes encoding other nonessential 19S or 20S proteasome subunits also results in synthetic growth defects and hypersensitivity to genotoxins when combined with mutations in well-established DNA DSB repair genes. Chromatin immunoprecipitation showed that Sem1 is recruited along with the 19S and 20S proteasomes to a DSB in vivo, and this recruitment is dependent on components of both the HR and NHEJ repair pathways, suggesting a direct role of the proteasome in DSB repair.

### Introduction

DNA double-strand breaks are formed occasionally during DNA replication and more frequently when cells are exposed to ionizing radiation or certain other DNA damaging agents. DSBs also play a role in immunoglobulin V(D)J recombination, crossing over during mitosis and meiosis, and yeast mating-type switching (Paques and Haber, 1999). Eukaryotic cells repair DSBs primarily by two genetically separable pathways, nonhomologous end joining (NHEJ) and homologous recombination (HR) (Paques and Haber, 1999). In *S. cerevisiae*, HR utilizes

extensive homology to faithfully restore the sequence at a break site by processes that involve components of the Rad52 epistasis group, including Rad51 (the yeast homolog of bacterial RecA), Rad52, and Rad54 (Paques and Haber, 1999). In contrast, NHEJ directly rejoins the two ends of the DNA molecule at the site of the DNA DSB without any requirement for homologous DNA sequences and without necessarily restoring the sequence around the DSB. The yeast NHEJ pathway involves the Yku70-Yku80, Rad50-Mre11-Xrs2, and Dnl4-Lif1 protein complexes (Paques and Haber, 1999; Wilson and Lieber, 1999).

The 26S proteasome, which consists of a 19S regulatory cap and 20S catalytic core, degrades polyubiquitinated proteins in eukaryotic cells (Voges et al., 1999). In addition to directing ubiquitin-dependent proteolysis, the proteasome has been shown to have nonproteolytic roles in recent studies (Voges et al., 1999). Although no direct link between the proteasome and the repair of DNA DSBs has been reported, recent work has suggested a role for the 19S proteasome in nucleotide excision repair (NER) mediated by the repair protein Rad23 (Russell et al., 1999a; Schaubert et al., 1998). Russell et al. (1999a) further demonstrated that the 19S proteasome, but not the 20S core, functions in NER and that this process is independent of proteolysis. Furthermore, the 19S proteasome has been shown to have not only a stimulatory role in NER, but a negative role as well which is mediated by Rad23 (Gillette et al., 2001).

Here, we report the identification of Sem1 as a conserved subunit of the 19S proteasome and, moreover, that the 19S proteasome, as well as the 20S proteasome, is involved in the repair of DSBs in yeast.

### Results and Discussion

We purified the 19S regulatory cap of the 26S proteasome of *S. cerevisiae* by utilizing strains containing C-terminal tandem affinity purification (TAP) tags (calmodulin binding peptide plus protein A) (Rigaut et al., 1999) on 11 of its known subunits. Following affinity purification on IgG and calmodulin columns, we identified components of the resulting highly purified protein complexes by two methods: (1) direct analysis of the purified material by trypsin digestion followed by shotgun sequencing using high-performance capillary-scale liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Cagney and Emili, 2002) and (2) excision of silver-stained gel bands after SDS-PAGE followed by trypsin digestion and MALDI-TOF mass spectrometry (Krogan et al., 2002). All 19 of the previously known subunits of the 19S proteasome (Rpn1–3 and 5–13, Rpt1–6, and Ubp6) (Voges et al., 1999) were identified with high statistical confidence by both methods (Figure 1A), but we also identified Sem1 as an 19S-interacting protein (YDR363W-A) in each case. Sem1 is sufficiently small (89 amino acids; predicted  $M_r$  of 10,386 Da) that it ran off our SDS gels, but it was identified with high confidence in our gel-free analysis by LC-MS/MS.

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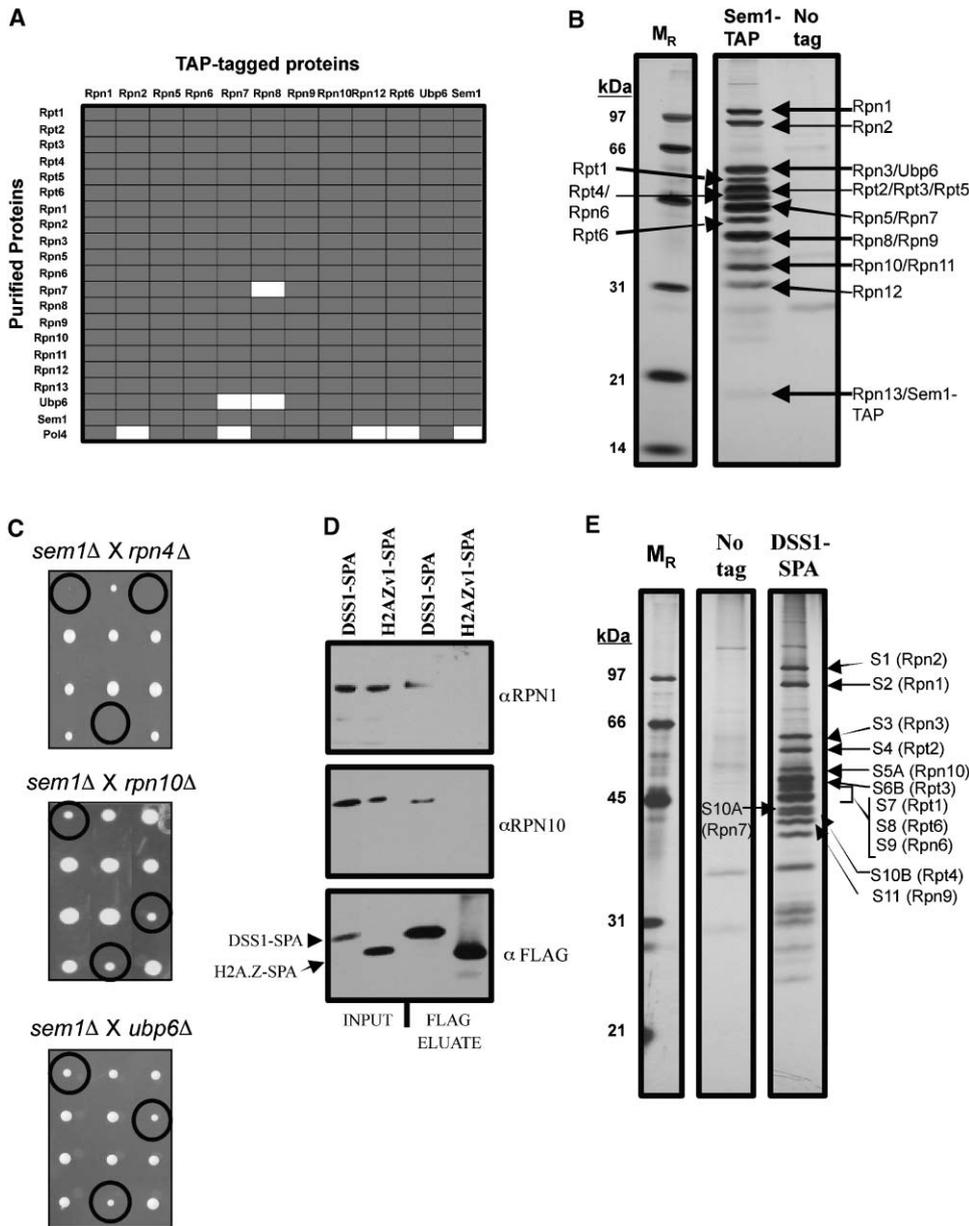


Figure 1. Sem1/DSS1 Is a Subunit of the Yeast and Human 19S Proteasomes

(A) TAP-tag purifications of 12 19S subunits. A gray box represents a protein identified in the purification by either MALDI-TOF MS or LC-MS/MS. (B) Silver-stained SDS polyacrylamide gel of purified Sem1-TAP. Arrows indicate proteins identified by gel band excision and MALDI-TOF mass spectrometry.

(C) Tetrad analysis of the growth on YPD or synthetic complete medium of 19S proteasome/Sem1 double deletion mutants. Heterozygous double mutants were sporulated, and the resulting tetrads were dissected. Circled are the colonies germinated from double mutant spores arising from tetrapype tetrads.

(D) Whole-cell extracts were prepared from HeLa S3 pTet-On cell lines stably transformed with vectors expressing either DSS1-SPA or H2A.Zv1-SPA and induced for 12 hr with doxycycline (1 μg/ml). The extracts were then incubated with anti-FLAG resin, and proteins eluted from the resin with FLAG peptide were analyzed by Western blotting either with antibodies against human 19S proteasome components or with anti-FLAG.

(E) SDS-PAGE and silver stain analysis of protein complexes purified by anti-FLAG and calmodulin affinity chromatography from  $\sim 2 \times 10^8$  stable HeLa S3 pTet-On cells expressing either DSS1-SPA or no tagged protein. The arrows indicate polypeptides identified by gel band excision and MALDI-TOF MS.

To confirm its presence in the 19S proteasome, Sem1 was similarly TAP-tagged and purified, and it was also found to copurify with all the known subunits of the 19S proteasome (Figure 1B). The nonessential *SEM1* gene was originally identified as a multicopy suppressor of

mutations affecting the secretory pathway (Jantti et al., 1999). However, Sem1 has been shown to localize exclusively to the nucleus (Huh et al., 2003; Reinman et al., 2003), implying that its role in exocytosis is almost certainly indirect. Virtually all of the other subunits of the

19S proteasome localize to the nucleus in yeast (Russell et al., 1999b), consistent with our observation that Sem1 is a stably associated component of the 19S proteasome.

We also took advantage of the fact that the 19S proteasome contains several other nonessential components (e.g., Rpn4, Rpn10, and Ubp6) to test whether *SEM1* behaves genetically as a proteasome component. Simultaneous deletion of two nonessential components of an essential protein complex might be expected to cause synthetic growth defects. Accordingly, following sporulation and tetrad dissection of heterozygous double mutant diploid strains, we found that *sem1* $\Delta$  causes a strong synthetic growth defect when combined with a deletion of *RPN4*, a transcriptional regulator of proteasome gene expression, and lesser growth defects when combined with *rpn10* $\Delta$  and *ubp6* $\Delta$  (Figure 1C), further implicating Sem1 in the normal functioning of the 19S proteasome in vivo. Additional biochemical evidence that Sem1 is a 19S proteasome subunit was published while this manuscript was under review (Sone et al., 2004).

Like other proteasome subunits (Voges et al., 1999), Sem1 is highly conserved in eukaryotes. Its human homolog, DSS1, was identified originally as a candidate gene for the split hand/split foot disorder (Crackower et al., 1996). DSS1 associates physically with human BRCA2 (Marston et al., 1999), a protein required for the efficient, error-free repair of DSBs via HR (Moynahan et al., 2001). BRCA2 has also been shown to interact with RAD51 and is thought to promote RAD51-dependent functions in HR (Marmorstein et al., 1998). Mutations in BRCA2 result in the preferential repair of DSBs by error-prone mechanisms, such as NHEJ or single-strand annealing, leading to more frequent chromosomal aberrations and often a highly penetrant, autosomal predisposition to breast and ovarian cancer (Tutt and Ashworth, 2002). More recently, BRCA2 has also been linked to the stabilization and repair of collapsed replication forks (Lomonosov et al., 2003). A high-resolution structure of DSS1 bound to a DNA-interacting domain of BRCA2 indicated that the residues in DSS1 involved in binding BRCA2 are highly conserved, while residues in BRCA2 that interact with DSS1 are mutated in certain cancers (Yang et al., 2002). Hence, the role of DSS1 appears to be functionally linked to that of BRCA2.

To ascertain whether DSS1 is associated with the human 19S proteasome, we created a stable human HeLa cell line expressing DSS1 with a C-terminal SPA tag (Zeghouf et al., 2004) (calmodulin binding peptide plus three FLAG tags) under the control of a tetracycline-inducible promoter. Following induction with doxycycline and preparation of soluble cell extracts, we immunoprecipitated DSS1-SPA with anti-FLAG antibodies. Western blotting (Figure 1D) revealed that the human 19S proteasome components RPN1 and RPN10 coprecipitated with DSS1-SPA, but not with an unrelated tagged protein, H2A.Zv1-SPA, used as a control. Sequential purification of DSS1-SPA by affinity chromatography on anti-FLAG and calmodulin columns resulted in the isolation to near homogeneity of the human 19S proteasome (Figure 1E), confirming that the Sem1/DSS1-proteasome interaction is conserved from yeast

to humans. This result suggested that the proteasome could be involved in DNA DSB repair.

To examine whether there could be a link between the 19S proteasome/Sem1 and the repair of DSBs in *S. cerevisiae*, we created and sporulated heterozygous diploid strains containing deletions of either *SEM1* or genes encoding other nonessential subunits of the 19S proteasome combined with genes known to be involved in both types of DSB repair. Tetrad dissection showed that deletions of *SEM1*, *RPN4*, and *RPN10* cause synthetic growth defects when combined with deletions of genes in the *RAD52* epistasis group (*RAD51*, *RAD52*, *RAD54*, and *RAD55*) involved exclusively in DSB repair via HR, as well as with deletions of genes encoding proteins involved in both HR and NHEJ (*RAD50* and *XRS2*) (Figures 2A and 2B). These results implied either that the proteasome participates in a DNA repair pathway other than HR or else participates in multiple repair pathways including HR.

Since the 19S proteasome interacted genetically with genes involved in both pathways, it seemed possible that Sem1 and the proteasome might act in DSB repair by both HR and NHEJ. Interestingly, 7 (out of 12) of the yeast 19S proteasome subunits that were tagged copurified with small amounts of DNA polymerase IV (Pol4) as judged by LC-MS/MS (Figure 1A). Moreover, this interaction is highly specific, because we have not identified Pol4 in purifications of more than 4000 other TAP-tagged proteins from *S. cerevisiae* (N.J.K., G.C., A.E., and J.F.G., unpublished data). Pol4 has been linked to DSB repair via NHEJ through its interaction with the Dnl4-Lif1 complex (Tseng and Tomkinson, 2002; Wilson and Lieber, 1999). Although BRCA2 is linked to DSB repair via HR, an interaction of the 19S proteasome with yeast Pol4 again suggested that the proteasome could potentially be linked to both types of DNA DSB repair. In this context, the human homolog (USP14) of the yeast 19S proteasome subunit Ubp6 has been found to interact physically with the Fanconi anemia complex, which may be involved in the repair of DNA damage and is important for chromosome stability (Reuter et al., 2003). The Brh2 protein of the fungus *Ustilago maydis* (Um) contains a BRC repeat and a DNA binding domain similar to those found in BRCA2. The *Ustilago* Sem1/DSS1 homolog umDss1 interacts with Brh2, while umDss1 null mutant cells are hypersensitive to UV radiation, are defective in HR, and display genomic instability (Kojic et al., 2003).

To examine further whether the 19S proteasome might be involved in repairing DNA DSBs, we examined the sensitivity of yeast deletion strains to camptothecin, a topoisomerase inhibitor that induces DSBs by causing replication forks to stall (Hsiang et al., 1989), hydroxyurea, an inhibitor of dNTP synthesis causing DNA replication fork collapse (Rittberg and Wright, 1989), and bleomycin, which generates free radicals which induce DNA lesions similar to those caused by ionizing radiation (Aouida et al., 2004). Deletions of genes involved in either DNA DSB repair pathway are known to result in hypersensitivity to camptothecin, bleomycin, and hydroxyurea (Aouida et al., 2004; Chang et al., 2002; Parsons et al., 2004). Although strains containing single *sem1* $\Delta$ , *rpn4* $\Delta$ , *rpn10* $\Delta$ , or *ubp6* $\Delta$  deletions displayed little or no added sensitivity to these genotoxins, double mutants combin-

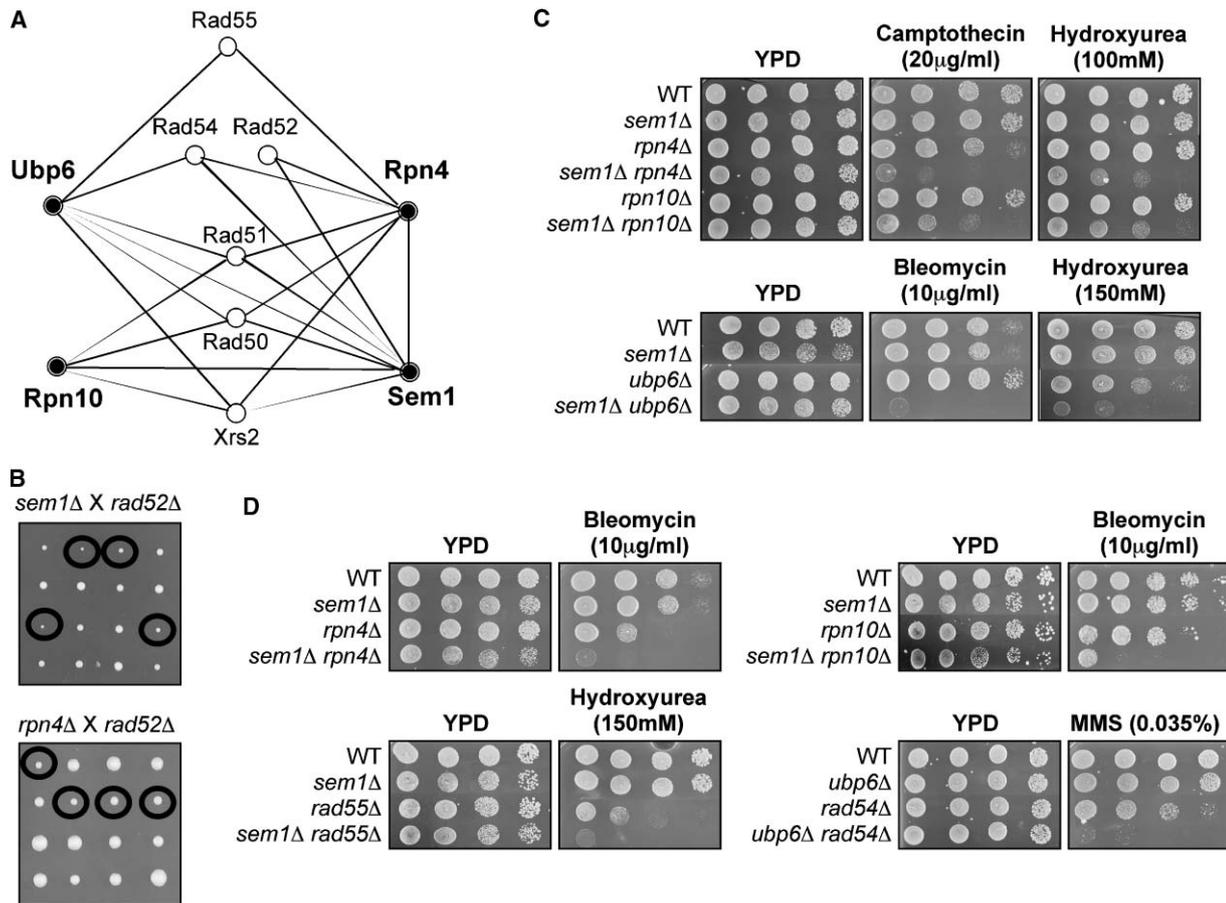


Figure 2. Involvement of the Yeast 19S Proteasome in DNA DSB Repair

(A) Mutations in Sem1 and various nonessential 19S proteasome subunits show synthetic growth defects when combined with mutations in genes involved in homologous recombination and nonhomologous end joining. A line connecting two genes indicates a synthetic growth defect in the double mutant.

(B) Tetrad analysis of 19S proteasome/Rad52 double deletion mutants. Heterozygous double mutants were sporulated and the resulting tetrads were dissected. Circled are the colonies germinated from double mutant spores arising from tetratype tetrads.

(C and D) Double mutants are hypersensitive to chemicals which cause DSBs. Five-fold serial dilutions of the indicated double mutant strains were spotted onto YPD medium lacking or containing the DNA damaging agents camptothecin (20  $\mu$ g/ml), hydroxyurea (100 or 150 mM), MMS (0.035%), or bleomycin (10  $\mu$ g/ml).

ing *sem1* $\Delta$  with either *rpn4* $\Delta$ , *rpn10* $\Delta$ , or *ubp6* $\Delta$  exhibited marked hypersensitivity to all three agents (Figures 2B and 2C; data not shown). Moreover, the double mutants were hypersensitive to the DNA alkylating agent methylmethane sulfonate (MMS; Figures 2B and 2C), which is also known to cause the accumulation of DSBs (Chang et al., 2002). Similarly, combining proteasome deletion mutations with *RAD54* or *RAD55* deletions in the HR pathway caused hypersensitivity to HU and MMS (Figure 2D). These observations further implicated the 19S proteasome in repairing DNA DSBs, either in a pathway other than HR or in multiple pathways including HR.

To provide more direct evidence of proteasome involvement in repairing DNA DSBs, we examined whether Sem1 is recruited to the site of a specific DSB by *in vivo* crosslinking and chromatin immunoprecipitation (ChIP). A single DSB was selectively created on chromosome III by using galactose to induce the expression of the site-specific homothallic (HO) endonuclease (Paques and Haber, 1999) from a galactose-regulated promoter

in a strain harboring a TAP-tagged version of Sem1 but lacking a homologous DNA sequence for repair by HR (Sugawara et al., 2003). Protein-DNA complexes were crosslinked with formaldehyde at various time points after addition of galactose (0, 60, 120, and 180 min), and then DNA fragments that coimmunoprecipitated with Sem1-TAP were amplified using seven pairs of primers, three of which anneal to DNA on the proximal side and four on the distal side of the HO break (Figure 3A). A TAP-tagged allele of Yku80 served as a positive control in these experiments since it is required for NHEJ and is recruited to DSBs (Martin et al., 1999). Yku80 bound selectively to DNA near the HO break after 60 min of induction with galactose (Figures 3B and 3E), and the signal increased at 120 and 180 min, displaying similar kinetics to that reported for Rad51 (Sugawara et al., 2003; Wolner et al., 2003). Importantly, Sem1 was also recruited to the DSB at each time point and spread in both directions away from the lesion (Figures 3D and 3E). In other experiments, we found that another 19S

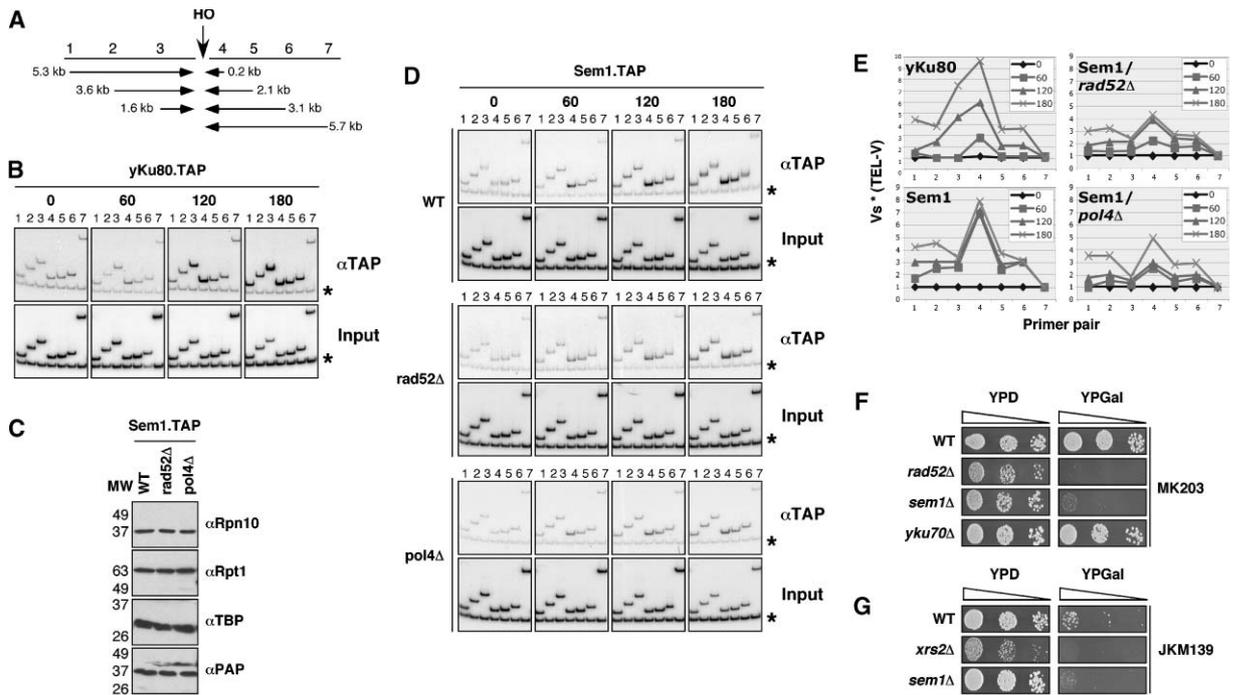


Figure 3. Sem1 Is Recruited to a DSB In Vivo and Is Involved in HR and NHEJ Repair

(A) Locations relative to the HO cleavage site of the seven sets of primers used in chromatin immunoprecipitation (ChIP) assays.  
 (B) Kinetics of recruitment of Yku80-TAP to locations near the HO site after various times of galactose induction of HO (0, 60, 120, and 180 min) in the donorless strain JKM139. The asterisk indicates a DNA band on chromosome V that was coamplified as a negative control for background precipitation in each ChIP assay (Krogan et al., 2002, 2003).  
 (C) Western analyses demonstrating that the protein levels of Sem1-TAP (as well as additional proteasome subunits Rpn10 and Rpt1 and a loading control, TBP) are unaffected in *rad52Δ* or *pol4Δ* backgrounds. PAP, peroxidase anti-peroxidase to recognize Sem1-TAP.  
 (D) Kinetics of recruitment of Sem1-TAP to regions flanking the HO site after 0, 60, 120, and 180 min of galactose induction in *rad52Δ*, *pol4Δ*, and wild-type backgrounds.  
 (E) Quantitation of the ChIP results from (B) and (D).  
 (F) HR-dependent repair of an HO endonuclease-induced DSB. In the parental strain (MK203), the HO recognition site is contained within the *URA3* gene on chromosome V, with homology on chromosome II, and the HO endonuclease is under the control of a galactose-inducible promoter.  
 (G) NHEJ-dependent repair of an HO endonuclease-induced DSB at the *MAT* locus. In the parental strain (JKM139), the *HMR* and *HML* loci are deleted and the HO endonuclease is under the control of a galactose-inducible promoter. In (F) and (G), 5-fold serial dilutions of the strains were plated on YPA with glucose or galactose.

subunit, Rpt6, was recruited with similar kinetics (data not shown). Interestingly, recruitment of Sem1 was reduced approximately 2- to 3-fold when either Rad52, which is critical for HR repair, or Pol4, a DNA polymerase linked to NHEJ repair, is deleted (Figures 3D and 3E). Since the expression of Sem1 is not affected by Rad52 or Pol4 (Figure 3C), we concluded that Sem1 is recruited by both HR and NHEJ pathways.

To test directly whether the proteasome is important for DNA DSB repair by both HR and NHEJ, we took advantage of galactose-inducible HO endonuclease systems that generate a single DNA DSB at a defined locus. In the strain JKM139, which contains deletions of the *HML* and *HMR* loci, an HO-induced DSB at the *MAT* locus can only be repaired by the relatively inefficient NHEJ pathway. Deletion of *SEM1* in this background impaired growth on galactose medium as compared to wild-type control cells (Figure 3G). Deletion of *XRS2*, a component of the NHEJ repair pathway, served as a positive control. In a complementary experiment, we analyzed the effect of the *sem1Δ* mutation on the repair of an HO-induced DSB by HR. To this end, we

took advantage of MK203, a strain in which the HO recognition site is inserted into the *URA3* gene on chromosome V and 1.2 kb of homologous sequence on chromosome II serves as a template for repair by HR. Deletion of *SEM1* caused displayed marked sensitivity to galactose (Figure 3F), comparable to that of a deletion of *RAD52*, a major player in the HR pathway. In contrast, deletion of *YKU70*, whose protein product is involved only in repair by NHEJ, caused no sensitivity to galactose. Therefore, the proteasome is important for DSB repair by both HR and NHEJ.

If the proteasome is recruited to a DSB for the purpose of proteolyzing one or more substrate proteins yet to be identified, the 20S catalytic component of the proteasome should also be present at a DSB. Therefore, we also analyzed the recruitment of a subunit of the 20S proteasome, Pre2, to the HO-induced DSB on chromosome III. As shown in Figure 4A, Pre2 was recruited to the DSB with kinetics that were similar to those observed for Sem1. Since deletion of *SEM1* did not affect the recruitment of Pre2 (data not shown), the role of Sem1 in DSB repair is likely in an aspect of proteasome function

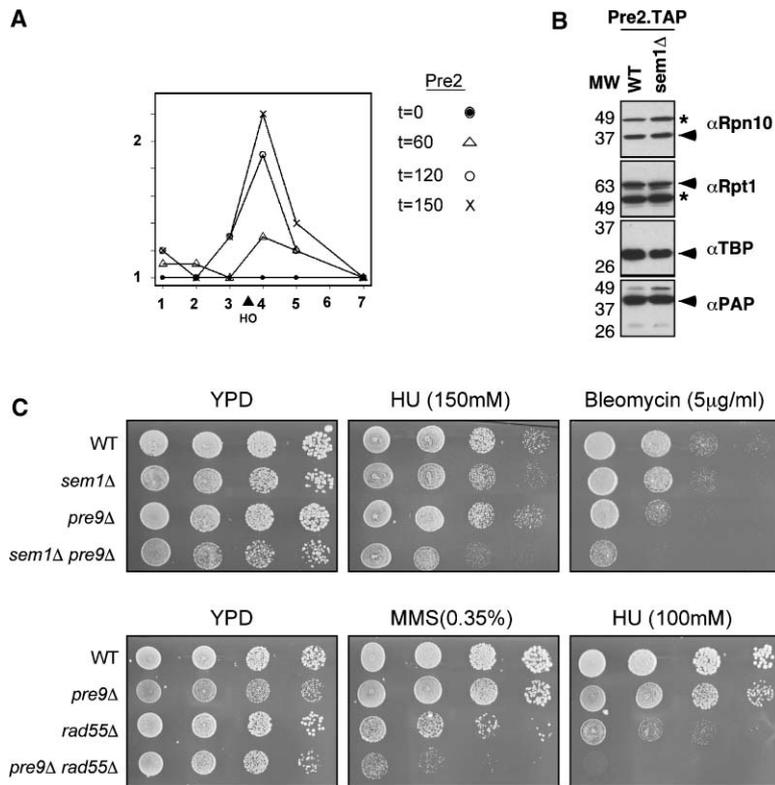


Figure 4. The Catalytic Core of the Proteasome Is Implicated in DSB Repair

(A) The 20S proteasome is recruited to a DSB in vivo. Kinetics of recruitment of Pre2-TAP to locations near the HO site after various times of HO induction (0, 60, 120, and 150 min). (B) Western analysis demonstrating that the protein levels of components of either the 19S or 20S proteasome are unaffected in a *sem1Δ* background. PAP, peroxidase anti-peroxidase to recognize Pre2-TAP. Arrows indicate a product of the expected size within each panel. Asterisks indicate a presumed second layer (goat anti-rabbit \*HRP) crossreaction with highly expressed Pre2-TAP.

(C) 20S proteasome double mutants are hypersensitive to chemicals which cause DSBs. Five-fold serial dilutions of the double mutant strains were spotted onto YPD medium lacking or containing the DNA damaging agents hydroxyurea (150 mM), MMS (0.035%), or bleomycin (5 μg/ml).

other than recruitment. Indeed, Sone et al. (2004) recently showed that deletion of *SEM1* reduces proteolytic activity of the proteasome. A slow growth phenotype is observed in a *sem1Δ pre9Δ* strain (data not shown), which becomes more pronounced in the presence of the DNA damaging agents, hydroxyurea and bleomycin (Figure 4C). A deletion of *PRE9* also results in a slow growth phenotype when combined with a deletion of *RAD55*, and the double mutation is hypersensitive to hydroxyurea and MMS (Figure 4C). Since Pre9 and Sem1 have roles in the proteolytic activity of the 20S proteasome (Sone et al., 2004; Velichutina et al., 2004), these genetic interactions and genotoxin sensitivities and the requirement of Sem1 for DSB repair by HR and NHEJ suggest that proteolysis by the proteasome is involved in DSB repair.

It is tempting to speculate that the role of the proteasome at DSBs is to degrade one or more components of the DSB repair machinery following the completion of repair. It will therefore be of considerable interest to determine whether the proteasome is required for removal of particular DSB repair proteins from the DNA following repair of the lesion. It will also be of interest to determine whether the proteasome is required along with BRCA2 for the repair of DSBs in human cells, and whether this repair function becomes perturbed in cancer. Protein degradation following the repair of DSBs may require polyubiquitination of one or more target proteins, as is perhaps consistent with the discovery that the BRCA1/BARD1 complex, important for DSB repair by HR in humans, is an E3 ubiquitination ligase (Starita and Parvin, 2003).

#### Experimental Procedures

##### Purification of the Yeast and Human 19S Proteasomes

TAP-tagged components of the yeast 19S proteasome were purified as previously described (Krogan et al., 2002) on IgG and calmodulin columns from extracts of cells (3 liters) grown in YPD medium to an  $OD_{600}$  of 1.0–1.5. We also established a stable HeLa S3 Tet-On cell line expressing tetracycline-inducible DSS1-SPA as described in the Supplemental Materials at <http://www.molecule.org/cgi/content/full/16/6/1027/DC1/>. Following purification on anti-FLAG and calmodulin columns as previously described (Zeghouf et al., 2004), the purified material was analyzed by SDS-PAGE, MALDI-TOF MS, and tandem MS essentially as previously described (Krogan et al., 2002).

##### Chromatin Immunoprecipitation

CHIP assays using TAP-tagged strains were performed as described (Krogan et al., 2002) using the donorless strain JKM139 (Sugawara et al., 2003). Cultures were grown in rich media containing 2% raffinose and 0.05% glucose until  $OD_{600} \approx 0.4$ , and then expression of HO endonuclease was induced by galactose (0, 60, 120, 150, or 180 min). Cultures were then crosslinked for 20 min and chromatin extracts isolated. IgG agarose for the precipitation of TAP-tagged proteins was from Sigma. DNA isolated from the immunoprecipitates was used in PCR reactions containing primers that have been previously described (Krogan et al., 2003; Martin et al., 1999) (see Supplemental Table S1). Quantification of the ChIP data was performed as described in detail in Supplemental Materials.

##### Yeast Genetic Methods

Strains JKM139 and MK203 have been previously described (Inbar et al., 2000; Moore and Haber, 1996). Heterozygous diploid strains containing two deletion mutations were generated using SGA technology as previously described (Tong et al., 2001), then subjected to sporulation and tetrad dissection. To determine sensitivity to MMS, hydroxyurea, camptothecin, and bleomycin, yeast strains were grown to  $OD_{600} \approx 0.5$  before being plated at 5-fold serial dilutions on yeast peptone dextrose (YPD) medium with or without the

DNA damaging agents. The plates were incubated for 2–3 days at 30°C. For galactose-inducible HO sensitivity experiments, cells were grown to OD<sub>600</sub> = 0.2, and 5-fold serial dilutions were plated on YPA plates containing either glucose or galactose. Plates were incubated at 30°C for 3–4 days.

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