Sem1 is a functional component of the nuclear pore complex–associated messenger RNA export machinery

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The evolutionarily conserved protein Sem1/Dss1 is a subunit of the regulatory particle (RP) of the proteasome and, in mammalian cells, binds the tumor suppressor protein BRCA2. Here, we describe a new function for yeast Sem1. We show that sem1 mutants are impaired in messenger RNA (mRNA) export and transcription elongation, and induce strong transcription-associated hyper-recombination phenotypes. Importantly, Sem1, independent of the RP, is functionally linked to the mRNA export pathway. Biochemical analyses revealed that, in addition to the RP, Sem1 coenriches with components of two other multisubunit complexes: the nuclear pore complex (NPC)-associated TREX-2 complex that is required for transcription-coupled mRNA export, and the COP9 signalosome, which is involved in deneddylation. Notably, targeting of Thp1, a TREX-2 component, to the NPC is perturbed in a sem1 mutant. These findings reveal an unexpected nonproteasomal function of Sem1 in mRNA export and in prevention of transcription-associated genome instability. Thus, Sem1 is a versatile protein that might stabilize multiple protein complexes involved in diverse pathways.

Introduction

In eukaryotes, RNA polymerase II (RNAPII)-driven production of mRNAs in the nucleus initiates gene expression. The nascent pre-mRNAs are processed and matured into mRNAs by several protein complexes that are loaded onto transcription sites via their interaction with the C-terminal domain of RNAPII (for reviews see Reed and Cheng, 2005; Sommer and Nehrbass, 2005; Cole and Scarcelli, 2006). Processing and maturation events include the 5′ capping and splicing, and 3′ polyadenylation of the pre-mRNAs. Further, these steps are coupled to the dynamic interaction of mRNAs with several proteins, including export factors that facilitate their transport through the nuclear pore complex (NPC) into the cytoplasm. Studies over the past years have revealed that all steps during gene expression, starting from gene activation to the nuclear export of mRNAs, are tightly coupled (Suntharalingam and Wente, 2003; Reed and Cheng, 2005; Sommer and Nehrbass, 2005; Köhler and Hurt, 2007).

In budding yeast, the THO–transcription export (TREX) complex and the Sac3–Thp1–Sus1–Cdc31 (TREX-2; also called THSC) complex are involved in transcription-coupled mRNA export (Köhler and Hurt, 2007). The THO–TREX complex is thought to be recruited to the elongating RNAPII via the THO subunits (Hpr1, Tho2, Mft1, and Thp2), which function in transcription and biogenesis of mRNA protein complexes (mRNPs). The additional TREX factors Sub2 and Yra1 are involved in recruiting the Mex67-Mtr2 export receptor to the mRNP, thereby coupling mRNP biogenesis with their nuclear export (for reviews see Aguilera, 2005; Reed and Cheng, 2005; Köhler and Hurt, 2007). TREX-2
potentially coordinates Spt7-Ada2-Gcn5 acetyltransferase (SAGA)-mediated transcription of a subset of genes at the nucleoplasmic face of the NPC (Rodríguez-Navarro et al., 2004). An integral component of TREX-2 is Sac3, a multidomain protein that serves as a binding platform for other members of the complex. The N-terminal and middle domain (N+M) of Sac3 binds Tif1 and Mex67-Mtr2, whereas the C-terminal domain mediates its NPC targeting (Fischer et al., 2002) and recruits the centrin Cdc31 as well as Sus1 (Fischer et al., 2004). Recent works from several groups have demonstrated a requirement of TREX-2 in the dynamic repositioning of a subset of gene loci from the nuclear interior to the nuclear periphery upon their activation (Brickner and Walter, 2004; Casolari et al., 2004; Cabal et al., 2006; Taddei et al., 2006; Kurshakova et al., 2007).

In addition to their direct function in mRNA export, THO–TREX and TREX-2 play an important role in preventing transcription-associated genomic instability. Impaired components of both complexes induce transcription elongation defects, in particular for long and GC-rich DNA sequences (for review see Aguilera, 2005) and repeat-containing genes (Voynov et al., 2006). THO and TREX-2 mutants show defects that lead to hyper-recombination phenotypes via the cotranscriptional formation of RNA/DNA hybrids (R loops) between the emerging RNA and the transcribed single-stranded DNA (ssDNA; Huertas and Aguilera, 2003). R loops are likely to become obstacles for subsequent elongating RNAPIIIs, thus impairing transcription elongation or generating mRNA-RNAPII-DNA tertiary structures that can obstruct replication, leading to genome instability (Aguilera and Gómez-González, 2008).

Sem1 is a small acidic protein that is highly conserved among all eukaryotic species. SEM1 was originally isolated as a multicopy suppressor of exocyst mutants in budding yeast (Jäntti et al., 1999). Mutations in Sem1 lead to several pleiotropic phenotypes, such as defects in exocytosis, pseudohyphal growth, and defects in the cell cycle (Jäntti et al., 1999; Marston et al., 1999). Genetic screens and proteomic approaches identified Sem1 as a component of the lid subcomplex of the 19S regulatory particle (Krogan et al., 2004; Sone et al., 2004). THO and TREX-2 mutants show defects that lead to hyper-recombination phenotypes via the cotranscriptional formation of RNA/DNA hybrids (R loops) between the emerging RNA and the transcribed single-stranded DNA (ssDNA; Huertas and Aguilera, 2003). R loops are likely to become obstacles for subsequent elongating RNAPIIIs, thus impairing transcription elongation or generating mRNA-RNAPII-DNA tertiary structures that can obstruct replication, leading to genome instability (Aguilera and Gómez-González, 2008).

Sem1 but not the RP is required for nuclear poly(A)+ RNA export

We initiated our analysis by asking whether a budding yeast strain deficient for Sem1 (sem1Δ) exhibits nuclear accumulation of poly(A)+ RNA. We assessed this phenotype by FISH using Cy3-labeled oligo (dT) probes. Nearly all the sem1Δ cells, when grown at 30°C, showed a nuclear accumulation of poly(A)+ RNA (Fig. 1 A). This phenotype was also observed at 25°C and 37°C (not depicted). Furthermore, the nuclear accumulation of poly(A)+ RNA was found to be comparable to TREX-2 deletion mutants (i.e., thp1Δ and sac3Δ; Fischer et al., 2002) but less severe than in the mex67-5 strain at 37°C (Fig. 1 A).

Because Sem1 was previously identified as a nonessential, bona fide subunit of the RP, we tested whether deletion of other nonessential components also induces defects in poly(A)+ RNA export. However, strains deficient in PRE9, RPN10, RPN4, UBP6, and RPN9 were not found to accumulate poly(A)+ RNA inside the nucleus (Figs. 1 B and S1 A). Using temperature-sensitive (ts) mutants (cin3-1 and cin5-1), we tested whether impairment of essential components of the RP induced nuclear accumulation of poly(A)+ RNA. Neither cin3-1 nor cin5-1 were found to show this phenotype at the restrictive temperature (Fig. 1 B). Finally, the treatment of the drug-permeable yeast strain erg6Δ (Lee and Goldberg, 1998) with the proteasomal inhibitor MG132 did not induce a nuclear accumulation of poly(A)+ RNA (Fig. S1 B). Thus, Sem1 is the unique component of the RP that is required for proper nuclear export of poly(A)+ RNA.

Sem1 interacts genetically with mRNA export factors

Next, we asked whether Sem1 is genetically linked to factors involved in mRNA export. We found that sem1Δ is indeed synthetic lethal (sl) or synthetic enhanced (se) in combination with
Figure 1. Poly(A)+ RNA export is inhibited in sem1Δ but is not impaired in mutants of the RP.
[A] The depicted yeast strains were grown in YPD at 30°C; the localization of poly(A)+ RNA in the
strains was assessed by in situ hybridization using Cy3-oligo (dT)30, and DNA was stained with
DAPI. The mex67-5 strain was grown at 25°C and subsequently shifted to 37°C for 1 h. [B] The
cim3-1 and cim5-1 were grown to logarithmic phase at 25°C and subsequently shifted to 37°C
for 4 h. Other details were as in A.
impaired alleles of known mRNA export factors such as Mex67, Mtr2, Yra1, and Sub2 (Fig. 2 A). In contrast, other nonessential components of the RP (rpn9Δ, pre9Δ, rpn4Δ, rpn10Δ, and ubp6Δ), when combined with mex67 and mtr2 mutants, were not found to be sl or se (Figs. 2 B and S2, and not depicted). Notably, we found that overexpression of Sub2, a component of TREX, strongly inhibited growth of sem1Δ, but not of the RP mutants (Fig. 2 C and not depicted). Similar effects of increased Sub2 levels have been described previously in sac3Δ, thp1Δ and sus1Δ mutants (Gallardo, 2003; Gonzalez-Aguilera et al., 2008).
protease (TEV) eluates were subjected to SDS-PAGE and Western analysis using antibodies directed against Sem1 and Rpt6, a core component of the RP. Rpn1-TAP and a yeast lysate containing no TAP-tagged bait protein served as positive and negative controls, respectively. These biochemical analyses show that Sem1 specifically coenriches with Thp1-TAP and protein A – Sac3 (Fig. 4, A and B). However, Sem1 is not found in any other protein complex involved in mRNA export such as THO – TREX (Mft1-TAP, Thp2-TAP) and the exosome (Rrp46-TAP; Fig. 4 A and not depicted). Importantly, Rpt6 was only detected in the Rpn1-TAP and not in any of the other purifications (Fig. 4 A). Thus, Sem1 coenriches with TREX-2 independently of the RP.

Previously, the N+M domain of Sac3 has been reported to interact with Thp1 and the mRNA export adaptor Mex67-Mtr2, whereas the NPC-targeting C domain of Sac3 interacts with Sus1 and Cdc31 (Fischer et al., 2002; Fischer et al., 2004). We performed purifications from yeast lysates containing protein A–Sac3 (Fig. 4 A and not depicted). Importantly, Rpt6 was only detected in the Rpn1-TAP and not in any of the other purifications (Fig. 4 A). Thus, Sem1 coenriches with TREX-2 independently of the RP.

Previously, the N4M domain of Sac3 has been reported to interact with Thp1 and the mRNA export adaptor Mex67-Mtr2, whereas the NPC-targeting C domain of Sac3 interacts with Sus1 and Cdc31 (Fischer et al., 2002; Fischer et al., 2004). We performed purifications from yeast lysates containing protein A–Sac3, protein A–N4M–Sac3, and protein A–C–Sac3. We found that the N4M domain but not the C domain of Sac3 is required for coenrichment of Sem1 (Fig. 4 A and not depicted). Together, we conclude that Sem1 is non-functional in the mRNA export pathway.

Hence, to investigate whether Sem1 associates with TREX-2, we directly purified this complex using Thp1-TAP and protein A–Sac3 as bait proteins. The tobacco etch viral protease (TEV) eluates were subjected to SDS-PAGE and Western analysis using antibodies directed against Sem1 and Rpt6, a core component of the RP. Rpn1-TAP and a yeast lysate containing no TAP-tagged bait protein served as positive and negative controls, respectively. These biochemical analyses show that Sem1 specifically coenriches with Thp1-TAP and protein A–Sac3 (Fig. 4, A and B). However, Sem1 is not found in any other protein complex involved in mRNA export such as THO – TREX (Mft1-TAP, Thp2-TAP) and the exosome (Rrp46-TAP; Fig. 4 A and not depicted). Importantly, Rpt6 was only detected in the Rpn1-TAP and not in any of the other purifications (Fig. 4 A). Thus, Sem1 coenriches with TREX-2 independently of the RP.

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Figure 4. **Sem1 is a bona fide subunit of TREX-2.** (A and B) The indicated TAP- or protein A–tagged bait proteins were isolated from yeast lysates by affinity purification using an IgG-Sepharose. TEV eluates of purified bait proteins were separated on a 4–12% SDS–polyacrylamide gradient gel and subjected to silver staining. Western blot analysis of the purified protein complexes was performed with indicated antibodies against CBP, Sem1, and Rpt6. Asterisks indicate the positions of bait proteins. (C) In order to show that Sem1 is a stoichiometric component of TREX-2, sequential affinity purifications were performed. In the first step, protein A–Sac3 was purified from lysates derived from strains expressing Thp1-GFP and Thp1 (negative control) to isolate TREX-2. The bound material was released by TEV cleavage, and a part of the eluates was separated on a 4–12% SDS–polyacrylamide gradient gel, then analyzed by Western blotting using antibodies against GFP and Sem1 (top). In the second step, the rest of the TEV eluates were incubated with Sepharose coupled to anti-GFP antibodies. The bound material was eluted and separated on a 4–12% SDS–polyacrylamide gradient gel and analyzed by Western blotting using antibodies against GFP and Sem1 [IP anti-GFP, bottom]. (D) Protein A–Sac3 and Thp1-TAP were isolated from yeast lysates from thp1Δ and sac3Δ strains by affinity purification using an IgG-Sepharose. Lysates derived from wildtype strains were used as controls. The bound material was released by TEV cleavage. TEV eluates of purified bait proteins were separated on a 4–12% SDS–polyacrylamide gradient gel and subjected to silver staining. Western blot analysis of the purified protein complexes was performed with indicated antibodies against CBP and Sem1. (E) Thp1-TAP was isolated from yeast lysates in which Sac3 was tagged with GFP by affinity purification using an IgG-Sepharose. The bound material was incubated with and without 20 μg/ml RNase A for 1 h. The IgG-Sepharose resin with the bound material was washed with 5 bed volumes of wash buffer. The bound TREX-2 was released by TEV cleavage, and the TEV eluates were separated on a 4–12% SDS–polyacrylamide gradient gel; Western analyses were performed using antibodies against GFP, CBP, and Sem1 to detect components of TREX-2.
purifications (Fig. 4 D). These biochemical data suggest that the was probed by Western analysis. Sem1 failed to coenrich in both purifications (Fig. 4 D). These biochemical data suggest that the enrichment of Sem1 with TREX-2 requires the presence of both Sac3 and Thp1. Importantly, the enrichment of Sem1 with TREX-2 was found to be insensitive to RNase A treatment (Fig. 4 E). Thus, Sem1 association with TREX-2 requires both Sac3 and Thp1, and is independent of its association with RNA. All together, these biochemical data strongly suggest that, like in the RP, Sem1 is a bona fide component of TREX-2.

Sem1 coenriches with Sac3-germinal center-associated nuclear protein (GANP) and PCI-associated module (PAM) domain-containing protein complexes

Previously, sequence analysis of individual subunits of the RP and TREX-2 had revealed one common feature: two subunits of each complex contain a Sac3-GANP domain and a PAM domain, respectively. The Sac3-GANP domains are present in Rpn12 (RP) and Sac3 (TREX-2), whereas Rpn3 (RP) and Thp1 (TREX-2) contain the PAM domains (Fig. 5; Ciccarelli et al., 2003; Scheel and Hofmann, 2005). Both domains are predicted to be α-helical and have been proposed to mediate protein–protein interactions (Ciccarelli et al., 2003). Notably, Csn12, a subunit of the CSN, has also been reported to contain a PAM domain (Fig. 5; Ciccarelli et al., 2003). Large-scale proteomic approaches had revealed a coenrichment of the Sac3-GANP domain-containing protein Ypr045c in the Csn12-TAP purification (Krogan et al., 2006). Therefore, we asked whether Sem1 also coenriches with these components. Western analysis of Csn12-TAP (Fig. 4 A) and Ypr045c-TAP (not depicted) preparations show that this is indeed the case. Because the PAM domain is often found upstream of prota-some-COP9-initiation factor (PCI) domains, we investigated the ability of Sem1 to bind to PCI domains. We were unable to detect Sem1 in a Nip1 purification (Fig. 4 A), which coenriches eIF3, a PCI domain containing translation initiation factor. Therefore, we conclude that Sem1 specifically coenriches with at least three distinct protein complexes that contain Sac3-GANP and PAM domains.

Sem1 is required for proper targeting of Thp1 to the NPC in vivo

Previous studies have shown that the C-terminal domain of Sac3 is required for its anchorage to the NPC, whereas the N+M domain of Sac3 was shown to provide a docking platform for Thp1 (Fischer et al., 2002). We asked whether deletion of Sem1 affects the NPC localization of Sac3 and Thp1. For this, we tagged both Sac3 and Thp1 at their C termini with GFP in sem1Δ strains at their genomic loci. As described previously, Thp1–GFP and Sac3–GFP have a punctate distribution at the nuclear periphery in wild-type cells (Fischer et al., 2002). In sem1Δ cells, the association of Thp1-GFP with the NPC is severely perturbed (Fig. 6 A). Furthermore, we observed an increase in cytoplasmic staining of Thp1-GFP in sem1Δ cells. However, the punctate staining of Sac3-GFP is only slightly affected in sem1Δ cells. Mislocalization of Thp1-GFP in sem1Δ cells is specific because we did not observe this phenotype in other deletion mutants of the RP (Fig. S3 A). Localization of the structural nucleoporin Nic96-GFP remained unaltered in a sem1Δ strain, which strongly suggests that NPCs remained intact (Fig. S3 B). Moreover, Western analysis of whole cell lysates showed no change in the levels of Thp1 in wild type and sem1Δ (Fig. 6 B). Finally, Thp1-TAP purification from a sem1Δ strain reproducibly revealed a reduced enrichment of Sac3-GFP as compared with the control strain that contained SEM1 (Fig. 6 C). Together, we conclude that Sem1 is required for efficient association of Thp1 with the NPC.

sem1Δ shows transcription-dependent hyper-recombination and transcriptional defects similar to thp1Δ, sac3Δ, and sus1Δ mutants

The data so far show that Sem1 interacts both functionally and physically with TREX-2. Hence, we wondered whether sem1Δ could induce similar transcription-associated recombination
Figure 6. Thp1 is inefficiently recruited to the NPC in sem1Δ cells. (A) The depicted yeast strains were grown to logarithmic phase at 30°C. The localization of Thp1-GFP and Sac3-GFP in sem1Δ cells and the isogenic wild-type strain was visualized by fluorescence microscopy. (B) Whole cell extracts were prepared from Thp1-GFP in wild-type and sem1Δ strains. The extracts were analyzed by SDS-PAGE and subjected to Western blot analysis using anti-GFP antibodies. Ribosomal protein L1 was used as loading control. (C) Thp1-TAP purification was performed from SEM1 and sem1Δ strains in which Sac3 was tagged with GFP. TEV eluates were separated on a 4–12% SDS-polyacrylamide gradient gel, and Western blot analysis was performed with indicated antibodies against GFP (Sac3-GFP), CBP (Thp1-CBP), and Sem1.

(TAR) and transcription deficiency phenotypes that have been observed in thp1Δ, sac3Δ, and sus1Δ mutants (Aguilera and Gómez-González, 2008). Transcription through long and GC-rich genes like LacZ was found to be impaired in thp1Δ and sac3Δ mutants (Gallardo, 2003). To test whether this is also the case for sem1Δ, we analyzed gene expression in the pLAUR system that contains a 4.15-kb lacZ-URA3 translational fusion under the control of the tet promoter (Jimeno et al., 2002). sem1Δ cells carrying the pLAUR system were unable to form colonies on synthetic medium lacking uracil (SC-Ura-Trp) and did not show β-galactosidase activity (Fig. 7 A), which indicates that they did not properly express the lacZ-URA3 fusion. Northern analysis of sem1Δ showed decreased mRNA levels compared with the wild type (Fig. 7 A). This reduction was not caused by an impairment of transcription initiation at the tet promoter, as the expression of the pCM189-LEU2 system (short, GC-poor) was not affected in the sem1Δ mutant (Fig. 7 B). We asked whether Sem1-TAP, which still interacts with the RP but is defective in mRNA export, is able to properly express the lacZ-URA3 fusion. As expected, Sem1-TAP was found to be defective in expressing the lacZ-URA3 fusion (Fig. 7 C). Importantly, mutants of the RP (rpm103Δ and pre9Δ) carrying the pLAUR system were still able to support growth on synthetic medium lacking uracil (SC-Ura-Trp) and showed β-galactosidase activity (Fig. 7 A). Thus, Sem1, not as part of the RP, is required for proper expression of long GC-rich genes. Importantly, this phenotype was found to be very similar to that observed in thp1Δ and sac3Δ mutants.

For the analysis of TAR, we transformed the LNA and LNAT systems (see Fig. S4 for a scheme of the various recombinant systems used), based on 0.6-kb leu2 repeats into the sac3Δ, thp1Δ sem1Δ, Sem1-TAP, pre9Δ, and rpm103Δ mutant strains. The frequencies of recombination observed in the above mentioned strains were obtained by quantifying the number of revertants that grew on media lacking leucine. sem1Δ and Sem1-TAP strains exhibit a vast increase in recombination above wild-type levels (Fig. 8 A). To see whether this increase in recombination is dependent on transcription, we used the LNAT system, identical to LNA but with a transcriptional terminator located downstream of the leu2Δ3′ fragment so that the intervening region is not transcribed. In this construct, recombination levels are found to resemble those seen in the wild type (Fig. 8 A), which suggests that the hyper-recombination phenotype of sem1Δ and Sem1-TAP is transcription dependent. To check further whether the phenotypes observed in sem1Δ mutants were independent of its RP function, we addressed whether mutants of the RP (pre9Δ and rpm103Δ) show similar recombination or transcription phenotypes. For the analysis of TAR, we used systems similar to those described above, based on 0.6-kb leu2 repeats: LYΔNS in which transcription has to proceed through a long and GC-rich intervening sequence between the leu2 repeats, and L that is identical to LYΔNS but without an intervening sequence between the repeats. Mutants of the RP did not show an increase in recombination in either system, whereas an increase in recombination was specifically observed in the LYΔNS system for Sem1-TAP and in mutants of TREX-2 (thp1Δ and sac3Δ; Fig. 8 B). These results are consistent with the fact that the Sem1-TAP strain is not able to properly transcribe the LacZ-URA3 fusion of pLAUR and only reaches 37% of mRNA levels observed in the wild-type strain (Fig. 7 C), whereas the mutants of the RP did not show gene expression defects in the pLAUR system (Fig. 7 A). Altogether, these data strongly suggest that the observed hyper-recombination phenotypes in sem1Δ and Sem1-TAP are due to its association with TREX-2 and not linked to the role of Sem1 as part of the RP.

To further demonstrate this, we determined the effect of sem1Δ on recombination events in the L-lacZ and GL-lacZ
The accumulation of poly(A)⁺ RNA, defects in expression of long GC-rich genes, and the observed TAR phenotypes of sem1Δ and Sem1-TAP strains suggest that transcription elongation may be impaired. To address this question, we analyzed RNAPII elongation in vivo. RNAPII recruitment was assayed by chromatin immunoprecipitation (ChIP) along the 8 kb – long YLR454w gene fused to the GAL1 promoter (Mason and Struhl, 2005). RNAPII occupancy was determined at the 5‘/H11032, middle, and 3‘/H11032 region of YLR454w in wild-type and sem1Δ cells. The presence of RNAPII at the middle and 3‘ end of the gene was found to be reduced with respect to the 5‘ end to 50% compared with wild-type levels (Fig. 8 D). This result confirms that transcription elongation is indeed impaired in sem1Δ mutants in vivo. Altogether, and consistent with the genetic links, these data demonstrate that like other members systems, based on the same 0.6-kb leu2 direct repeats as before but flanking the lacZ open reading frame, in which the leu2Δ3′ repeat was under the control of either its endogenous promoter or the inducible GAL1 promoter. Studies were performed under conditions of low (GAL1 promoter in 2% glucose), medium (LEU2 promoter), and high levels of transcription (GAL1 promoter in 2% galactose). The increase in recombination under low transcription conditions was less pronounced (sixfold above wild type) compared with medium transcription conditions (260-fold above wild type) and most pronounced (414-fold above wild type) at high transcription levels (Fig. 8 C). We therefore conclude that hyper-recombination in sem1Δ and Sem1-TAP strains strongly depends on transcription and resembles the phenotypes seen in other mutants defective in mRNP formation and export (Jimeno et al., 2002; Huertas and Aguilera, 2003).
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recombination in yeast THO and thp1 Δ mutants (Gómez-
González and Aguilera, 2007; Gonzalez-Aguilera et al., 2008).
This increase is consistent with the formation of R loops in
THO mutants, where the nontranscribed strand is exposed as
ssDNA (Huertas and Aguilera, 2003) and thereby rendered
of TREX-2, Sem1 likewise functions in transcription elonga-
tion and maintenance of genomic integrity.

\textit{sem1Δ} favors the action of ssDNA-
specific human activation-induced cytidine
deaminase (AID)

AID is a B cell–specific enzyme essential for Ig somatic hyper-
mutation and class switching. One of its in vivo targets is the
S region of Ig genes, in which R loops are formed (Yu et al.,
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Figure 8. \textit{sem1Δ} and Sem1-TAP, but not mutants of the RP, induce TAR. (A and B) Recombination frequency of \textit{sem1Δ}, \textit{sac3Δ}, \textit{thp1Δ}, Sem1-TAP, and RP mutants (\textit{rpn10Δ} and \textit{pre9Δ}), and an isogenic wild-type strain (BY4741) in the plasmid-borne recombination systems. A small diagram of the systems
(not drawn to scale) is shown. Repeats are shown as gray boxes, and gray arrows indicate relevant transcripts produced from the construct. Recombinants
were selected as \textit{leu}+. Median and SD of three independent experiments are shown. (C) Recombination analysis of \textit{sem1Δ} and isogenic wild type in the
plasmid-borne systems \textit{L:lacZ} and \textit{GL: lacZ}. Recombination frequencies, plotted as a function of the transcription levels, are shown. "Low" transcription refers
to the \textit{GL: lacZ} system, in which the first \textit{leu2} repeat is under the control of the \textit{GAL1} promoter, in strains cultured in 2% glucose; "medium" refers to \textit{L: lacZ},
in which the \textit{leu2} repeat is under its own promoter, in 2% glucose; and "high" refers to \textit{GL: lacZ} in 2% galactose. Other details were as in A. (D) RNAPII
occupancy at the \textit{GAL1pr-YLR454w} gene in \textit{sem1Δ} mutant. ChIP analyses (using N20 or 8WG16 anti-RNAPII antibodies) in wild-type (W303-1A) and
\textit{sem1Δ} isogenic strains carrying the \textit{GAL1pr::YLR454w} fusion construct located at the endogenous \textit{YLR454w} chromosomal locus are shown. The scheme of
the gene and the PCR-amplified fragments are shown. The DNA ratios in the \textit{S1} (1), middle (2), and \textit{S3} (3) regions, were calculated from the DNA amount
of these regions relative to the intergenic region. The recruitment data shown are first referred to the value of the \textit{S1} region taken as 100% and then relative
to the wild-type levels for each region. Median and SD of three independent experiments are shown. WT, isogenic wild-type strain.
susceptible to mutation by AID. We wondered, therefore, whether sem1Δ also stimulates accumulation of mutations caused by AID activity as an indirect way to assess the formation of R loops in this mutant. We used the pLAUR system in which caused by AID activity as an indirect way to assess the formation of R loops in this mutant. We used the pLAUR system in which

![Figure 9. Spontaneous and AID-induced mutation frequencies in sem1Δ.](image)

Analysis of the genetic instability (mutation and recombination) phenotype in W303-1A and sem1Δ isogenic strains, using the tetpr::lacZ-URA3 fusion construct. Ura− mutants were selected on 5-FOA. The human AID gene, present in the p413-GALAID plasmid, was either overexpressed in 2% galactose (+AID) or repressed in 2% glucose (−AID). Median and SD of three independent experiments are shown.

What could be the role of Sem1 in the CSN? The CSN is composed of eight distinct subunits and is highly homologous to the lid subcomplex of the RP. Deletions of members of the CSN complex have been shown to accumulate neddylated culin Cdc53 (Maytal-Kivity et al., 2002). Functional analysis of the PAM domain containing Csn12 showed that it does not participate in the deneddylation of Cdc53 (Maytal-Kivity et al., 2002). Furthermore, we did not observe any alterations in Cdc53 deneddylation in sem1Δ and ypr045cΔ strains (Fig. S5). Moreover, both csn12Δ and ypr045cΔ strains are not defective in mRNA export (Fig. S1 A), which suggests that Sem1 has an additional role in the CSN that is independent of its function in the RP and TREX-2. While this work was under revision, Wilmes et al. (2008) showed that Sem1 coenriches with distinct complexes (RP, TREX-2, and CSN). Furthermore, they showed that the Csn12-Ypr045c-Sem1 subcomplex associates with the splicing machinery and that the csn12Δ and ypr045cΔ strains were defective in pre-mRNA splicing. Thus, we speculate that the Csn12–Ypr045c–Sem1 subcomplex is likely to affect a yet undetermined activity of the CSN.

### Discussion

Previous studies in budding and fission yeasts had identified Sem1 as a bona fide subunit of the RP of the proteasome (Funakoshi et al., 2004; Krogan et al., 2004; Sone et al., 2004). Here, we provide several lines of evidence that in addition to its proteasomal role, Sem1 functions in mRNA export as part of TREX-2. First, we show that Sem1 is the unique nonessential subunit of the RP that is impaired in mRNA export and functionally linked to the mRNA export machinery. Second, like sac3Δ and thr1Δ, sem1Δ mutants are defective in transcription of long GC-rich genes, which leads to transcription-dependent hyper-recombination phenotypes and susceptibility to the mutagenic action of AID (Gallardo and Aguilera, 2001; Tous and Aguilera, 2007; González-Aguilera et al., 2008). Finally, we show that Sem1 specifically coenriches with TREX-2, independently of the RP. The reason why association of Sem1 with TREX-2 was not previously observed appears to be due to the use of Sem1-TAP alleles. The functionality of Sem1-TAP was not previously tested (e.g., in an sl relationship). Sem1-TAP is nonfunctional in the mRNA export pathway, as the Sem1-TAP strain showed a defect in mRNA export and, when combined with mex67 and mtr2 mutant alleles, was found to be sl. Thus, only a combined approach of genetic and biochemical assays was able to unravel the function of Sem1 in mRNA export.

With what could be the role of Sem1 in the CSN? The CSN is composed of eight distinct subunits and is highly homologous to the lid subcomplex of the RP. Deletions of members of the CSN complex have been shown to accumulate neddylated culin Cdc53 (Maytal-Kivity et al., 2002). Functional analysis of the PAM domain containing Csn12 showed that it does not participate in the deneddylation of Cdc53 (Maytal-Kivity et al., 2002). Furthermore, we did not observe any alterations in Cdc53 deneddylation in sem1Δ and ypr045cΔ strains (Fig. S5). Moreover, both csn12Δ and ypr045cΔ strains are not defective in mRNA export (Fig. S1 A), which suggests that Sem1 has an additional role in the CSN that is independent of its function in the RP and TREX-2. While this work was under revision, Wilmes et al. (2008) showed that Sem1 coenriches with distinct complexes (RP, TREX-2, and CSN). Furthermore, they showed that the Csn12–Ypr045c–Sem1 subcomplex associates with the splicing machinery and that the csn12Δ and ypr045cΔ strains were defective in pre-mRNA splicing. Thus, we speculate that the Csn12–Ypr045c–Sem1 subcomplex is likely to affect a yet undetermined activity of the CSN.

What could be the role of Sem1 in these three protein complexes? The common feature of the RP, TREX-2, and CSN is the presence of Sac3-GANP and PAM domain–containing subunits (Fig. 9; Hofmann and Bucher, 1998; Ciccarelli et al., 2003; Scheel and Hofmann, 2005). Importantly, Sem1 specifically coenriches with the N+M–Sac3 domain that contains a Sac3-GANP domain and also binds the PAM domain–containing Thp1. Recently, a specific acidic motif in Sem1 has been shown to directly interact with Rpn3 in vitro (Wei et al., 2008). Furthermore, a combination of chemical cross-linking followed by mass spectrometry and yeast two-hybrid analyses of the RP suggests that Sem1 lies in the near vicinity of Rpn3 (PAM) and Rpn12 (Sac3-GANP; Sharon et al., 2006; Gudmundsdottir et al., 2007). These findings have led us to speculate that Sem1 might facilitate interactions between the Sac3-GANP-containing subunit and its PAM domain counterpart. Consistent with this notion, we have observed that the tethering of Thp1 to the NPC is severely affected in sem1Δ. Previously, an RP purified from sem1Δ cells was shown to be considerably destabilized and defective in ubiquitin-mediated proteasomal degradation (Funakoshi et al., 2004; Sone et al., 2004). Whether Sem1 “glues” the Sac3-GANP and PAM domains together still remains to be determined. Another possibility is that Sem1 stabilizes the PAM domain, which then would facilitate a robust interaction with the Sac3-GANP-containing subunit or vice versa. Previously, Dss1, the human homologue of Sem1, has been implicated in maintaining the correct conformation of BRCA2, as BRCA2 is largely insoluble in absence of Dss1 (Yang et al., 2002; Kojic et al., 2003). Further biochemical work could provide insights into how Sem1 might facilitate complex formation between Sac3-GANP and PAM-containing proteins.
Can Sem1 interact with other protein complexes? Here, the structure of Dss1 in complex with a part of BRCA2 can provide possible insights. Dss1 was shown to bind the helix turn helix motif (helical domain) and an oligonucleotide-binding domain (OB) of BRCA2 in an extended conformation. Dss1 appears to make contacts with numerous residues on the helical domain and the OB surface that is rich in basic, aromatic, and hydrophobic residues (Yang et al., 2002). The Sac3-GANP and PAM domains contain periodic hydrophobic patches followed by conserved positive residues typically found in α-helical structures (Hofmann and Bucher, 1998; Scheel and Hofmann, 2005). Interestingly, Sac3-GANP and PAM domains have neither structural nor sequence homology with the region of BRCA2 that interacts with Sem1. Thus, it is very likely that Sem1 will target multiple folds present in several unrelated protein complexes. Interestingly, alleles of Mex67 and Mtr2 (mex67-kraa and mtr2-33), which are specifically defective in 60S export (Bassler et al., 2001; Yao et al., 2007), in combination with sem1Δ were also found to be sl (Fig. 2), which indicates a functional link of Sem1 to export of the large ribosomal subunit. Purification of the complexes associated with human Dss1 has identified the Integrator complex (involved in snRNA processing) in addition to proteasome and the metazoan-specific BRCA2 (Baillat et al., 2005).

A large body of work in mammalian cells has implicated mutations in the tumor suppressor protein BRCA2 in genomic instability. BRCA2 functions in the repair of DSBs by homologous recombination (Gudmundsdottir et al., 2004, 2007; Li et al., 2006). Dss1 has been shown to stabilize BRCA2, and consistently, depletion of either BRCA2 or Dss1 was shown to induce similar phenotypes of genomic instability (Li et al., 2006). Although yeast lacks an obvious homologue of BRCA2, Sem1, as part of the RP, was shown to be important for the repair of DSBs by both homologous recombination and nonhomologous end-joining pathways (Krogan et al., 2004). Our work has uncovered a nonproteosomal role of Sem1 in preventing transcription-associated genomic instability by regulating the integrity of TREX-2. Mutations in genes encoding components of TREX-2, which functions at the interface between transcription and mRNA export, induce transcription-associated hyper-recombination phenotypes that are very similar to sem1Δ. We show that, like thrp1Δ, the sem1Δ mutation facilitates the mutagenic action of AID on transcribed DNA. As AID preferentially acts on ssDNA, this supports the idea that in sem1Δ mutants, ssDNA regions accumulate during transcription, which is consistent with the possibility of R-loop formation, as observed in THO complex mutants (Gómez-González and Aguilera, 2007). Recently GANP, the human homologue of Sac3, has been shown to suppress hyper-recombination and AID activity in mammalian cells (Yoshida et al., 2007).

Interestingly, several components that directly participate in mRNA export show moonlighting functions. The TREX-2 component Cdc31 performs an additional role in spindle pole body duplication (Fischer et al., 2004). Similarly, the heterodimeric mRNA export factor Mex6-Mtr2 has been shown to be required for proper nuclear export of the large ribosomal subunit (Yao et al., 2007). Recently, the NPC-associated factors Gle1 and Dpb5 have been shown to function in transcription (Bolger et al., 2008). Thus, this study adds Sem1 to the list of versatile proteins that function in multiple pathways, in addition to their direct involvement in mRNA export.

Materials and methods

Yeast strains and microbiological methods

Microbiological techniques such as plasmid transformation, mating, sporation of diploids, and tetrad analysis were done according to standard procedures. Genomic disruptions were performed as described previously (Longtine et al., 1998; Janke et al., 2004). The yeast strains used in this study are given in Table S1.

Affinity purifications

TAP purification of various bait proteins was performed as described previously (Rigaut et al., 1999). The TEV eluates from calmodulin-Sepharose were trichloroacetic acid precipitated, resuspended in SDS sample buffer, and analyzed on an SDS 4–12% gradient polyacrylamide gel (Invitrogen). Antibodies used for Western blotting were monoclonal anti-CBP (1:3,000; Open Biosystems), polyclonal rabbit anti-Sem1 (1:2,000; gifts from the Kobayashi and Jäntti laboratories), and monoclonal anti-RP6 (1:5,000; Bio-Concept). For Western blotting, enhanced chemiluminescence detection was performed (GE Healthcare).

Recombination and mutation analysis

Transcription efficiency, recombination, and mutation frequencies were performed as described previously (Gallardo and Aguilera, 2001; Gómez-González and Aguilera, 2007). Mutation frequencies were derived from the mean of three to four median frequencies from two different transformants each and for each genotype tested. Median frequencies were obtained as described previously (Santos-Rosa and Aguilera, 1994) from six independent colonies per transformant.

Miscellaneous

Detection of nuclear accumulation of poly(A)⁺ RNA by FISH was performed as described previously (Grosshans et al., 2000). Both fixed cells (FISH analyses) and live cells (resuspended in water) were examined by microscopy using a fluorescence microscope (Imager Z1) equipped with a 63× NA 1.4 Plan-Apochromat oil immersion lens, using DICIII, HE-GFP, DAPI, or HECy3 filters (all from Carl Zeiss, Inc.). Images were acquired with an AxioCam MRm camera and AxioVision 4.3 software (Carl Zeiss, Inc.) at 25°C. Northern analyses were performed according to standard procedures with ³²P-radiolabeled probes as described previously (Chávez et al., 2000). RNA levels in arbitrary units (A.U.) were obtained in an FLA 3000 (Fujifilm) and were normalized with respect to rRNA levels of each sample.

CHIP analysis

For ChIP experiments, strains were grown in synthetic complete medium (SC), 2% glucose and 2% lactate to an OD₆₆₀ of 0.5. The culture was split into two; one half was supplemented with 2% glucose (repressed transcription) and the other half with 2% galactose (activated transcription). Samples were taken after 4 h, and ChIP assays were performed as described previously (Mason and Struhl, 2005). Monoclonal anti-Rpb1-CTD antibodies BWG16 and N20 (Covance), and protein A–Sepharose were used for RNAPII immunoprecipitation. The GFX purification system (GE Healthcare) was used for the last DNA purification step. We used the PCR of the intergenic region at positions 9,716–9,863 of chromosome V as a negative control. Real-time quantitative PCR and calculation of the relative abundance of each DNA fragment were performed as described previously (Huertas et al., 2006). For the evaluation of RNAPII occupancy, data are expressed as the result of two steps of normalization. First, for each strain, we consider the value of the occupancy in the 5' region as 100%, and the values of the middle and the 3' region are normalized to the value of the corresponding position in the wild-type strain (the percentage that is obtained for the occupancy of RNAPII in each region of the wild type when it is compared with its 5' region, taken as 100%).

Online supplemental material

Fig. S1 contains additional FISH data showing the absence of poly(A)⁺ RNA accumulation in deletions of nonessential proteasome components. Fig. S2 reveals that RP components are not genetically linked to mRNA.
export. Fig. S3 demonstrates that NPCs remain intact in sem1Δ and that targeting of Thp1-GFP is unperturbed in mutants of the RP. Fig. S4 illustrates that NPCs remain intact in mutants of the RP. Fig. S5 demonstrates the various recombination systems used. Fig. S5 contains the Western analysis showing that the Csn12–Sem1–Ypr045c subcomplex is not involved in deneddylation of the cullin Cdc53. Table S1 lists all yeast strains used in this study. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200810059/DC1.

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