The Tup1-Ssn6 complex is an important corepressor in *Saccharomyces cerevisiae* that inhibits transcription through interactions with the basal transcription machinery and by remodeling chromatin. In a two-hybrid screen for factors that interact with the *Schizosaccharomyces pombe* Tup1 ortholog, Tup11, we isolated the *pet1* eDNA. The *pet1* gene encodes an mRNA 5′-triphosphatase, which catalyzes the first step of mRNA capping reactions. *Pct1* did not interact with the *S. pombe* Ssn6 ortholog. *In vitro* glutathione S-transferase pull-down experiments revealed that *Pct1* binds to the WD repeat regions of Tup1 and the Tup1-Ssn6 complexes co-purify with the Cet1 protein, indicating that *in vivo* interactions also occur between these proteins. Over-expression of *CET1* compromised repression of an *MFA2-lacZ* reporter gene that is subject to Tup1-Ssn6 repression. These genetic and biochemical interactions between Tup1-Ssn6 and Cet1 indicate that the capping enzyme associated with RNA polymerase II is a target of the corepressor complex.

The Tup1 corepressor in budding yeast *Saccharomyces cerevisiae* was one of the first corepressor activities to be described (1). Tup1 is required for repression of genes regulated by cell type, glucose, oxygen, DNA damage, osmotic stress, and other signals (2, 3). Tup1 forms a complex in *vivo* with Ssn6 (4–6). This complex, composed of one Ssn6 and four Tup1 subunits (6), is recruited to different target gene promoters through interaction with a variety of pathway-specific repressor proteins, e.g. α2 for mating-type control (7, 8), Mig1, Nrg1, and Ste11 for glucose repression (9–11), Rox1 for oxygen repression (12, 13), Crt1 for DNA damage (14), and Sko1 for osmotic stress (15). Ssn6 may serve as an adapter between Tup1 and these DNA-binding proteins (1, 16).

Two mechanisms of repression appear operative for the Ssn6-Tup1 complex (3, 17). Genetic analyses indicate that a number of factors necessary for Ssn6-Tup1 repression are found in the RNA polymerase II holoenzyme, including Med3 (Hrs1/Pgd1), Srb7, Srb8 (Ssn5/Gig1), Srb10 (Ssn3/Gig2/Arel/Um65), Srb11 (Ssn8/Gig3), Sin4 (Ssn4/Tsfl3), Rox3 (Ssn7), and Rgr1 (18–26). Med3 and Srb7 associate with Tup1 in *vivo* and in *vivo* (18, 19). These factors provide candidates for direct targets of Ssn6-Tup1 repression.

Tup1 also mediates repression through the organization of chromatin. Ssn6-Tup1 interacts physically with class I histone deacetylases (HDACs), and simultaneous mutation of the three HDAC genes, *RPD3, HOS1, and HOS2*, abolishes repression by Ssn6-Tup1 (27). The Ssn6-Tup1 complex interacts with at least two of these HDACs, Rpd3 and Hos2 (27). Hda1 has also been reported to interact with Tup1 in *vivo* and is required for deacetylation of histones H3 and H2B at the stress-responsive *ENAI* promoter, whereas Rpd3 deacetylates the coding region of this gene (28). Microarrays reveal that expression of one Tup1-regulated genes is increased somewhat in the absence of Hda1, again suggesting that this HDAC plays a role in Tup1 functions (29). Tup1 interacts preferentially with underacetylated isoforms of histones H3 and H4 in *vivo*, genes repressed by Tup1 associate with underacetylated histones in *vivo*, and mutations in H3 and H4 synergistically reduce repression (30–33). Altogether these observations indicate that Ssn6-Tup1, when tethered to promoter regions, recruit HDACs to deacetylate adjacent nucleosomes, which in turn promotes Tup1-histone interactions and stable recruitment of the corepressor (32).

Recently, a new role for Ssn6-Tup1 as a coactivator has been described (34). The ATF/CREB (activating transcription factor/cAMP-response element-binding protein) repressor Sko1 recruits Ssn6-Tup1 to target promoters and represses transcription of the *GRE2, AHP1, HAL1* genes, etc. in the absence of osmotic stress. However, in the presence of osmotic stress, the Hog1 kinase phosphorylates Sko1 and converts the Sko1-Ssn6-Tup1 repressor into an activator that recruits the SAGA (Spt-Ada-Gcn5-acetyltransferase) histone acetylase and SWI/SNF nucleosome remodeling complexes to osmotic-inducible promoters. At the *GAL1* promoter, the Ctf6 protein links SAGA with Ssn6-Tup1 to overcome Tup1-mediated repression, even though the corepressor remains bound to the promoter in this case (35).

*In vitro* protein binding experiments and two-hybrid analyses have defined at least three protein-protein interaction domains in the 713-amino acid Tup1 protein. The N-terminal 72 amino acids of Tup1 are required for interaction with Ssn6 and for self-multimerization (36). The histone-binding domain comprises amino acids 73–385 (30). WD repeats (amino acids 333–706) in the C-terminal region of Tup1 likely form a seven-bladed β-propeller structure (37) that interacts with DNA-binding repressors including α2 (7).

† The abbreviations used are: HDAC, histone deacetylase; GST, glutathione S-transferase; HA, hemagglutinin; GAD, activation domain of the *Saccharomyces cerevisiae* Gal4 protein.

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Tup1-lion-like proteins are found in other yeasts and higher eukaryotes, e.g. Grouch in flies (38) and TLE (transducin-like enhancer of split) in humans (39). Interestingly, the fission yeast Schizosaccharomyces pombe has two functionally redundant Tup1 genes, tup11 and tup12 (40). These Tup1 orthologs have the ability to bind to HDACs and histones H3 and H4 (40–42). The tetratricopeptide repeat protein Ssn6 is also highly conserved from yeasts to humans (40), indicating that Ssn6-Tup1 corepressor complexes are commonly used for transcriptional repression in eukaryotes.

To further understand Tup1 functions in S. pombe, we performed a two-hybrid screen to isolate interacting proteins. We report here that Pct1 specifically interacts with S. pombe Tup1. Pct1 is an mRNA 5′-triposphatase, which catalyzes the first step of mRNA capping reactions. In vitro and in vivo interactions between Tup1 and the S. cerevisiae mRNA 5′-triposphatase, Cet1, were also observed. Over-expression of Cet1 compromises Ssn6-Tup1 repression. These biochemical and genetic interaction data suggest that the capping enzyme associated with RNA polymerase II is a target of the corepressor complex.

EXPERIMENTAL PROCEDURES

Yeast Strains and Genetic Methods—Strain L40 (MATa his3 Δ200 trp1–901 leu2–3,112 ade2 lys2–801am LYS2::lexAop4-HIS3 URA3::(lexAop8-locZ) was used for our two-hybrid screen. Strain JFY11 expressing hemagglutinin (HA)–tagged Tup1 from the native TUP1 promoter has been described previously (32). Strains FY250 (MATa ura3–52 leu2–31,211 trp1–Δ1 his3 Δ200) and FY250Aup1 (MATa ura3–52 leu2–Δ1 trp1–Δ1 his3 Δ200 tup1::Kan) were used for over-expression of the GST-Cet1 fusion protein. Strain MFA2-1a, containing the MFA2-locZ reporter gene, was described previously (31). Standard conditions of S. cerevisiae culture and transformation were used (44). β-Galactosidase activities from the MFA2-locZ reporter gene were measured by standard methods (44).

Plasmids—Plasmid pBTM-tup11 (40) was used to express the LexA-Tup1 fusion protein in S. cerevisiae. Plasmid pBTM-STE4 for expression of LexA-Ste4 (30–423) was constructed by cloning a 1.7-kbp PstI fragment into pBTM116. pGEX-6P-1 plasmid vector (Amersham Biosciences) was used for production of the glutathione S-transferase (GST) fusion protein in Escherichia coli. Plasmid pGEX-tup11N (amino acid positions 1–298 (40)) was used for production of GST-Tup1N. pGEX-tup11C for production of GST-Tup1C (amino acid positions 297–614) was constructed by cloning an 0.95-kbp BamHI-SalI PCR fragment into pGEX-6P-1. pGEX-tup12N for expressing GST-Tup12N (amino acid positions 627–671) was constructed by cloning an 1.1-kbp BamHI-SalI PCR fragment into pGEX-6P-1. Plasmid pGEX-tup12C for expressing GST-Tup12C (amino acid positions 272–586) was constructed by cloning an 0.95-kbp BamHI-SalI PCR fragment into pGEX-6P-1. pGEX-TUP1 for production of GST-Tup1N (amino acid positions 7–253) was constructed by cloning an 0.8-kbp NspV-BamHI fragment into pGEX-2T, in which the NspV site was converted into the BamHI site after in vitro ligation of the BamHI linker. pGEX-TUP1C for production of GST-Tup1C (amino acid positions 253–713) was constructed by cloning a 1.6-kbp BamHI-HindIII fragment into pGEX-2T. S. pombe Pct1 and S. cerevisiae Cet1 proteins were produced from pCITE2a plasmids (Novagen) and labeled with 18S-methylamine using Tnt1 Quick Coupled Transcription/Translation System (Promega). Plasmid pCITE-pct1 was constructed by cloning a 1.4-kbp EcoRI fragment containing the whole pct1′ coding region into pCITE-2a. pCITE-CET1 was constructed by cloning a 1.65-kbp BamHI-XhoI PCR fragment into pCITE-2a. Plasmids pCITE-CET1Δ204N and pCITE-CET1Δ204N were made by cloning the Pfu-HindIII and BglII-XhoI fragments, respectively, into pCITE-2a. Plasmid pCITE-CET1Δ284C was made by deleting a BglII fragment from pCITE-CET1.

pCYGST-CET1U for over-expression of GST-Cet1 was made by cloning a 1.65-kbp BamHI-XhoI prepared from pCITE-CET1 into pRD56 (45). pCYGST-CET1W for over-expression of GST-Cet1 using a different selectable marker (TRP1) for yeast transformation was made by cloning a 1.58-kbp BamHI fragment prepared from pCITE-CET1U into pRD56. pCGST-MED3U for over-expression of GST-Med3 was cloned by making a 1.3-kbp BglII-XhoI PCR fragment into pRD56.

Two-hybrid Screening—The S. cerevisiae strain L40, harboring lexAop-locZ and lexAop-HIS3 reporters, was first transformed with plasmid pBTM-tup11 to express a chimera of Tup11 and the DNA-binding domain of the E. coli LexA protein. Expression of LexA-Tup1 fusion protein in this transformant was confirmed by immunoblot using anti-LexA antibody (Clontech). Strain L40 containing pBTM-tup11 was further transformed with a library of S. pombe cDNA fragments fused to the activation domain (GAD) of the S. cerevisiae Gal4 protein. From about 6 million independent transformants, we isolated 134 histidine prototrophic (His+) clones, grown on plates lacking leucine, tryptophan, and histidine, based on the activation of the HIS3 reporter gene. Among these His+ clones, 90 clones developed blue color on 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside (X-gal) plates due to the expression of the lacZ reporter gene. Positive clones were grown on nonselective plates and then Leu Ter clones, which contained the pGAD library plasmid but not the pBTM-tup11 plasmid, were isolated. Plasmid DNAs were recovered from white, Leu Ter His− colonies and were transformed into E. coli MC1066. These pGAD library plasmids were used for retransformation of strain L40 with pBTM-tup11 to confirm the two-hybrid interactions. The nucleotide sequences of the insert cDNAs were determined by the dye-terminator method with the ABI PRISM 310 sequencing system using a primer from the GAL4 region.

GST Pull-down Assay—To express GST fusion proteins in yeast, transformants with pRD56 derivatives were cultivated in raffinose-containing medium to an A500 of 0.5, followed by a 6-h galactose induction. Cell extracts were made by glass bead breaking (20 min, 4 °C) in GST binding buffer (20 mm Tris-HCl, pH 8.0, 150 mm NaCl, 1 mm EDTA, 0.1% Triton-X, 1 mm glutamate, 1 mm dithiothreitol) containing protease inhibitors (1 mm phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, and 1 μg/ml pepstatin A) and clarified by microcentrifugation (12,000 rpm, 15 min at 4 °C).

GST fusion protein libraries were expressed in E. coli strain DH5α and purified using glutathione-Sepharose 4B beads (Amersham Biosciences) following the manufacturer’s protocol. In vitro labeled proteins (see above) or yeast extracts were incubated with comparable amounts (as determined by SDS-PAGE analysis) of the different GST fusion proteins bound to beads in GST binding buffer at room temperature for 1 h or at 4 °C overnight. Beads then were collected by centrifugation in a microcentrifuge at 500 × g for 2 min. The beads were washed three times with GST binding buffer. The washed beads were resuspended directly in 1× SDS-PAGE sample buffer. All samples were separated by SDS-PAGE and autoradiographed for detection of the 32S-labeled protein or blotted for Western analysis.

Tc7-Cet1-His6 proteins were purified using two affinity columns, His-Bind and T7-Bind columns (Novagen). GST-Tup1C and GST alone were purified using glutathione-Sepharose column (Amersham Biosciences). Tc7-Cet1-His6 proteins (10 μg) were incubated with 10 μg of GST-Tup1C or GST at 30 °C for 1 h. Glutathione-Sepharose beads (20 μl) were added and incubated at room temperature for 1 h. After the beads were washed, proteins bound to the beads were separated by SDS-PAGE and visualized by Coomassie Brilliant Blue stain. Immunoblots were performed using anti-T7 antibody to detect Tc7-Cet1.

RESULTS

S. pombe Pct1 Interacts with Tup11—Using LexA-Tup11 as bait, we screened a library of S. pombe cDNAs fused to the activation domain of the S. cerevisiae Gal4 protein (see “Experimental Procedures”). Those colonies showing histidine prototrophy and β-galactosidase activity were recovered and sequenced. One of the positive clones had a complete cDNA region identical to SPAC644.04 on chromosome 1 of S. pombe. Recently, SPAC644.04 was shown to encode an mRNA 5′-triposphatase, which removes the 5′-terminal phosphate of mRNA in the first step of mRNA capping reactions and is referred to as pct1′ (47). The nucleotide sequence of the genomic pct1′ DNA predicts that this gene has one intron. The intron sequence of pct1′ DNA was removed in our pct1′ cDNA sequence, as expected.

Tup1 contains a C-terminal WD repeat domain. This domain is found in a variety of proteins with differing functions and are thought to mediate protein-protein interactions. To exclude the possibility that Pct1 binds to WD repeat proteins in general, we examined the interactions of Pct1 with the WD repeat protein Ste4. In a two-hybrid assay, LexA-Ste4 did not interact with Gal4-Pct1 (data not shown), indicating that Pct1 does not generally bind all WD repeat proteins.
In *S. cerevisiae*, Tup1 forms a complex with the Ssn6 protein (4–6). Some sequence-specific DNA-binding proteins associate with both Tup1 and Ssn6 (7, 8, 14). Therefore, we asked whether Pct1 also interacts with the *S. pombe* Ssn6 ortholog (SPBC23E6.09) predicted by the *S. pombe* sequencing project. Pct1 did not bind to the *S. pombe* Ssn6 ortholog in the two-hybrid assay (data not shown). This *S. pombe* Ssn6 two-hybrid construct is functional and interacts with the LexA-Tup11 construct used in the above experiments. These results suggest that *S. pombe* Tup11, but not Ssn6, interacts with the mRNA 5’-triphosphatase Pct1.

*S. pombe* Tup11 and Tup12 Interact with Pct1 through Their WD Repeat Domains—The *S. cerevisiae* Tup1 and the *S. pombe* Tup11 proteins have at least three protein-binding domains, i.e. an N-terminal domain that interacts with Ssn6 (36), a middle domain that interacts with histones H3 and H4 (30), and the C-terminal WD repeat domain mentioned above (7). To define the domain of Tup11 that interacts specifically with Pct1, two Tup11 deletion derivatives were tested for *in vitro* interactions with Pct1 (Fig. 1). The N-terminal half containing the Ssn6- and histone-binding domains (amino acid positions 1–298) and the C-terminal half containing the WD repeats domain (amino acids 298–614) were fused to glutathione S-transferase (named GST-Tup11N and GST-Tup11C, respectively; Fig. 1A) and expressed in *E. coli* as GST fusions (Fig. 1A). Consistent with our results above, GST pull-down experiments indicated that *in vitro* translated Pct1 bound to GST-Tup11C (Fig. 1B, lane 3) but not to GST-Tup11N (lane 2) or GST alone (lane 6). This *in vitro* binding experiment indicates that the Pct1 protein associates with the Tup11 protein through its WD repeat domain.

Because *S. pombe* has two redundant TUP1 orthologs, *tup11`* and *tup12`* (40), we examined the ability of Tup12 to bind to Pct1 (Fig. 1). Tup12 was also separated into two regions, an N-terminal half (Tup12N, amino acid positions 67–271) and a C-terminal half containing the WD repeats (Tup12C, amino acids 272–586), that were expressed in *E. coli* as GST fusions (Fig. 1A). Consistent with our results above, GST pull-down experiments indicated that *in vitro* translated Pct1 bound to GST-Tup12C (Fig. 1B, lane 5) but not to GST-Tup12N (lane 4). We conclude that Pct1 associates with both Tup11 and Tup12 through their WD repeat regions.

*S. cerevisiae* Tup1 Interacts with Cet1—To determine whether the corepressor Tup1 interacts with mRNA 5’-triphosphatase encoded by the CET1 gene in the budding yeast *S. cerevisiae*, we tested for interaction between these two *S. cerevisiae* proteins. We were unable to detect any two-hybrid interactions between *S. cerevisiae* Tup1 and Cet1 when using either full-length or deletion constructs (data not shown). However, interactions were detected *in vitro* and *in vivo* by other means. First, GST-Tup11N (amino acid positions 7–253), GST-Tup11C (amino acid positions 253–713), or GST alone was purified from bacterial extracts and incubated with *in vitro* incubated with *in vitro* labeled Pct1. Pct1 bound to GST-Tup11C (Fig. 1B, lane 3) but not to GST-Tup11N (lane 2) or GST alone (lane 6). This *in vitro* binding experiment indicates that the Pct1 protein associates with the Tup11 protein through its WD repeat domain.

Figure 1. *In vitro* interaction of *S. pombe* Tup11 and Tup12 with Pct1. A, deletion derivatives of Tup11 and Tup12 used for *in vitro* interaction assays. The amino acid positions of the proteins are indicated. B, *in vitro* 35S-labeled Pct1 was incubated with beads bound to GST-Tup11N (lane 2), GST-Tup11C (lane 3), GST-Tup12N (lane 4), GST-Tup12C (lane 5), or GST alone (lane 6). After the beads were washed, proteins bound to the beads were analyzed by SDS-PAGE. Shown are autoradiograms detecting the labeled proteins. Input (lane 1) represents 10% of the labeled Pct1 proteins used in the binding reaction.

Figure 2. *In vitro* interaction of *S. cerevisiae* Tup1 with Cet1. A, *in vitro* 35S-labeled Cet1 proteins were incubated with beads bound to GST-Tup1N (lanes 2, 6, 10, and 14), GST-Tup1C (lanes 3, 7, 11, and 15), or GST alone (lanes 4, 8, 12, and 16). After the beads were washed, proteins bound to the beads were analyzed by SDS-PAGE. Shown are autoradiograms detecting the labeled proteins. Input (In, lanes 1, 5, 9, and 13) represents 10% of the labeled Cet1 proteins used in the binding reaction. B, deletion derivatives of Cet1 used for *in vitro* interaction assays. Full-length and truncated Cet1 are indicated schematically along with their ability to interact with Tup1. The amino acid positions of the proteins are indicated.

Figure 3. A, *in vivo* interaction of Tup1 with Pct1. Tup1 and Pct1 were epitope-tagged at their N-termini and transiently expressed in *S. pombe*. After induction, cells were transferred to fresh medium and harvested for protein extraction. Western blot analysis was conducted using antibodies against Myc (top) or Flag (bottom) and the blot was developed with ECL. B, *in vivo* interaction of Tup1 with Cet1. Tup1 and Cet1 were epitope-tagged at their N-termini and transiently expressed in *S. cerevisiae*. After induction, cells were harvested for protein extraction. Western blot analysis was conducted using antibodies against Myc and Flag and the blot was developed with ECL.
labeled Cet1 as described above (Fig. 2). GST-Tup1C interacted well with full-length Cet1 (Fig. 2A, lane 3), whereas GST-Tup1N bound only slightly to Cet1 (lane 2). No interaction of GST alone with Cet1 was observed (Fig. 2A, lane 4). These results reveal that the WD repeat domain of S. cerevisiae Tup1 can also associate with mRNA 5'-triphosphatase as was observed for S. pombe Tup1 and Cet1.

To determine the domain of Cet1 that interacts with Tup1, we constructed three deletion derivatives, Cet1Δ204N (retaining amino acids 205–549), Cet1Δ264N (retaining amino acids 265–549), and Cet1Δ284C (retaining amino acids 1–265) (Fig. 2B). These mutated Cet1 proteins were labeled with [35S]methionine and used for GST pull-down analysis with GST-Tup1N, GST-Tup1C, and GST alone (Fig. 2A). Cet1Δ204N, in which the N-terminal 204 amino acids were truncated, bound strongly to the C-terminal WD repeats region of Tup1 (Fig. 2A, lane 7) and more weakly to the N terminus of Tup1 (lane 6). A similar binding pattern was found in Cet1Δ264N, which lacks another 60 amino acids, although the intensity of the signal was weaker (Fig. 2A, lanes 13–16). On the other hand, we did not detect any interaction of Cet1Δ284C with GST-Tup1N, GST-Tup1C, or GST alone (Fig. 2A, lanes 10–12). We conclude that the Tup1 associates with the Cet1 C-terminal region. This region of Cet1 comprises a triphosphate tunnel and is highly conserved (48).

To determine whether Tup1 and Cet1 interact directly, we purified recombinant forms of these proteins expressed in E. coli. The full-length Cet1 protein was tagged with T7 (11 amino acids) in the N terminus and six copies of His in the C terminus and was purified using two affinity chromatography columns (Fig. 3A, lane 1). GST-Tup1C and GST alone were purified by glutathione-Sepharose affinity chromatography (Fig. 3A, lanes 2 and 3). T7-Cet1-His6 was mixed with GST-Tup1C or GST and pulled down with glutathione-Sepharose beads. Comparable amounts of GST-Tup1C and GST were detected by Coomassie Brilliant Blue stain (Fig. 3A, lanes 4–8). Immunoblotting of the precipitates using an anti-T7 antibody showed that T7-Cet1-His6 bound to GST-Tup1C (Fig. 3A, lane 2) but not to GST alone (lane 3). These results clearly indicate that Tup1 and Cet1 can interact directly.

To determine whether Tup1 associates with Cet1 in vivo, we expressed a GST-Cet1 hybrid protein or GST alone in the S. cerevisiae wild type or tup1 mutant cells. Protein extracts were prepared from these cells, and we analyzed proteins that copurified with GST-Cet1 or with GST alone using glutathione-Sepharose beads. Immunoblotting of the purified proteins using a polyclonal anti-Tup1 antibody revealed that whereas GST alone did not pull down Tup1 in either strain (Fig. 4, lanes 5 and 6), GST-Cet1 co-purified with Tup1 in the wild type strain (lane 5) but not in the tup1 strain (lane 7).

To further confirm the interaction of Tup1 with Cet1 in vivo, we transformed the S. cerevisiae strain JDY11, which expresses an influenza hemagglutinin epitope-tagged Tup1 (Tup1-HA) protein from the native TUP1 locus with plasmids
encoding the GST-Cet1 hybrid protein or GST alone. As described above, protein extracts were prepared from these cells, and proteins that co-purified with GST-Cet1 or with GST alone were analyzed by immunoblotting using a monoclonal anti-HA antibody (Fig. 5). Equal levels of the Tup1-HA protein were expressed in the cells containing GST-Cet1 or GST alone (Fig. 5, lanes 1 and 2). Tup1-HA co-purified with GST-Cet1 (Fig. 5, lane 3) but not with GST alone (lane 4).

Because Tup1 is in complex with Ssn6 in vivo (5, 6), we predicted that the Ssn6 protein would also co-purify with GST-Cet1. To test this hypothesis, we examined proteins pulled down in the above experiments with a polyclonal antiserum raised against Ssn6 (Fig. 6). As predicted, Ssn6 co-purified with GST-Cet1 but not with GST alone (lanes 3 and 4), consistent with the above observations. Therefore we conclude that Tup1-Ssn6 corepressor complex interacts with mRNA 5'-triphosphatase Cet1 in S. cerevisiae.

**Genetic Interaction of Corepressor Tup1 and mRNA 5'-Triphosphatase Cet1**—To determine whether over-expression of Cet1 might affect Tup1-mediated repression, we over-expressed the GST-Cet1 fusion protein in wild type strains containing an MFA2-lacZ reporter gene (Table I). Transcription of the MFA2-lacZ gene is normally repressed in haploid a cells through recruitment of Tup1-Ssn6 by the a2-Mcm1 repressor complex. Because a2 is not expressed in haploid a cells, the MFA2-lacZ gene is not repressed in these cells. We found that repression of the reporter gene was partially lost in a cells upon over-expression of the GST-Cet1 protein (MFA2.1a with pCyGST-CET1U). Although the reporter gene was not fully derepressed compared with expression levels observed in a cells (MFA2.1a with pRD56), the loss of repression in a cells was similar to that observed upon mutation of histones or mutation of RNA polymerase II mediator components (19, 22, 24, 26, 30). Cet1 over-expression did not affect the expression level of the corepressors Tup1 and Ssn6, as the amounts of Tup1 and Ssn6 are fairly equal in cells over-expressing GST-

**TABLE I**

| Plasmid          | GST Protein | β-Galactosidase activity | Fold derepression |
|------------------|-------------|-------------------------|-------------------|
| a cells          |             |                         |                   |
| pCyGST-CET1U     | GST-Cet1    | 1.00 ± 0.06             | 8.3               |
| pRD56            | GST         | 0.12 ± 0.02             | 1.0               |
| a cells          |             |                         |                   |
| pCyGST-CET1U     | GST-Cet1    | 69.31 ± 1.92            | 578               |
| pRD56            | GST         | 65.66 ± 0.70            | 547               |

*β-Galactosidase activities were determined by standard methods. Values are the average of triplicate determinations ± standard deviations.

**Fig. 5. In vivo interaction of S. cerevisiae HA-tagged Tup1 with Cet1.** Proteins were extracted from transformants of FY250α expressing GST-Cet1 (C, lanes 1, 3, and 5) or GST (G, lanes 2, 4, and 6) and incubated with glutathione-Sepharose beads. Bound proteins were analyzed by SDS-PAGE and stained with Coomassie Brilliant Blue (CBB, lanes 5 and 6). Immunoblots were performed using anti-HA antibody (lanes 1–4). Input (lanes 1 and 2) represents 10% of the protein extracts used in the binding reactions.

**Fig. 6. Co-purification of S. cerevisiae Ssn6 with Cet1.** Proteins were extracted from transformants of FY250α expressing GST-Cet1 (C, lanes 1 and 3) or GST (G, lanes 2 and 4) and incubated with glutathione-Sepharose beads as described in the legend for Fig. 4. Immunoblots were performed using anti-Ssn6 antibody. Input (lanes 1 and 2) represents 10% of protein extracts used in the binding reactions.
Cet1 or GST alone (Figs. 4–6, lanes 1 and 2). In contrast, over-expression of GST-Cet1 did not further increase expression of the MFA2-lacZ gene in a cells. These data indicate that Cet1 levels influence the function of the Tup1-Ssn6 corepressor. Derepression of the reporter gene in the Cet1-over-expressing strain might be because of sequestration of Tup1 from the repression complex. Alternatively, the increased amount of Cet1 might bypass normal sequestration of Cet1 by Tup1. In either case, these genetic data strongly suggest a functional in vivo interaction between Tup1 and Cet1.

Hrs1/Pgd1 (hereafter referred to as Med3), a component of the RNA polymerase II transcriptional mediator complex (49, 50), also interacts with Tup1 and Ssn6 (18). Med3 over-expression also derepressed transcription of the genes repressed by Tup1 and Ssn6 (18), indicating that the mediator complex is another target of the repressor complex. Because these two targets represent different steps in gene expression, it is possible that interference with both of these pathways simultaneously would lead to a greater effect on repression than does interference with either one alone. Therefore we over-expressed both Cet1 and Med3 in a cells and measured MFA2-lacZ expression (Fig. 7). Simultaneous over-expression of GST-Cet1 and GST-Med3 resulted in an additive loss of repression. A 6-fold higher β-galactosidase activity was observed in the cells over-expressing both proteins compared with cells containing empty vectors, whereas over-expression of GST-Cet1 caused a 3.5-fold and over-expression of GST-Med3 a 2.5-fold loss of repression. This additive effect of Cet1 and Med3 over-expression suggests that Tup1-Ssn6 independently targets these proteins. These findings are also consistent with observations that Tup1 interacts with Cet1 and Med3 through separate domains (18).

We tried to determine whether cet1 mutant cells exhibited loss of Tup1-Ssn6-mediated repression. Because the CET1 gene is essential for cell growth (51),2 we constructed a temperature-sensitive cet1 allele using the improved degron method (52). After shifting cet1-td cells containing the MFA2-lacZ reporter gene to the nonpermissive temperature, the HA-tagged Cet1 protein was rapidly degraded within 30 min (data not shown). However, no derepression of the MFA2-lacZ gene was observed 30, 60, or 120 min after shifting to 37°C. Another group has reported that conditional mutations in Ceg1, a partner of Cet1 in RNA capping, lead to destabilization of mRNAs (53). Thus, it is possible that any increased transcription of lacZ that occurred in our experiments upon Cet1 loss would be mitigated by increased mRNA turnover, precluding our ability to analyze directly the effects of Cet1 loss on Tup1-mediated repression.

**DISCUSSION**

*S. cerevisiae* Tup1 and its orthologs appear to function through conserved mechanisms of transcriptional repression. In this study, we have discovered a new aspect of these functions, mediated through interactions of the *S. pombe* Tup1 corepressor with Pct1, an mRNA 5′-triphosphatase that catalyzes the first step of mRNA capping reactions. Pct1 binds to Tup11 in vivo and in vitro. Moreover, Pct1 binds to the WD repeats of the Tup12 protein, which is redundant in function with Tup11. *S. cerevisiae* Tup1 also interacts with *S. cerevisiae* Cet1 in vitro and in vivo, indicating that interaction between Tup1 corepressor and mRNA 5′-triphosphatase is conserved in yeasts. Over-expression of Cet1 compromises Ssn6-Tup1 repression, indicating that the interactions between Tup1 and Cet1 impact Tup1 functions.

Both Tup1 and Ssn6 are associated with GST-Cet1 in vivo. Our in vitro binding experiments indicate that Tup1 binds directly to Cet1 and Tup11 and Tup12 bind, probably directly, to Pct1. However, *S. pombe* Ssn6 did not interact with Pct1 in the two-hybrid assay, and the interaction between Ssn6 and Cet1 in vivo may not be direct. Interestingly, Tup1 binds to the histones H3 and H4, but Ssn6 does not (30). Together, these observations are consistent with the idea that Tup1 plays the main role in repression by the Ssn6-Tup1 complex (1). However, Med3 interacts with both Tup1 and Ssn6, supporting additional roles for Ssn6 in Ssn6-Tup1 corepressor functions (18).

Genetic screens have identified a number of genes that are required for Tup1 repression, including multiple components of RNA polymerase II holoenzyme: Med3, Srb7, Srb8, Srb10, Srb11, Sin4, Rox3, and Rgr1 (18–26). These findings strongly suggest that subunits of RNA polymerase II holoenzyme are targets of Tup1 repression. Med3 also binds the Tup1 protein (18), and when over-expressed, Med3 compromises Ssn6-Tup1 repression just as Cet1 does. Srb7 also associates with Tup1, and disruption of the Tup1-Srb7 interaction causes derepression of a target gene (19). The mRNA capping enzyme in *S. cerevisiae* is composed of Cet1 and Ceg1 (mRNA guanylyltransferase) and is recruited to the phosphorylated C-terminal domain of the largest subunit of RNA polymerase II (54, 55). Therefore, at least three proteins associated with RNA polymerase II appear to be targeted by Tup1. Med3 and Cet1 seem to be independent targets of Tup1, because the effects of over-expression of Med3 and Cet1 are additive. The reason the corepressor targets multiple proteins is not known, nor is it clear whether Tup1 targets all of these polymerase II-associated proteins at every promoter or whether it interacts with different targets at different promoters. Different tetra-tripeptide repeats in Ssn6 are required for repression of different genes, and differential changes in H3 and H4 acetylation associated with Tup1-regulated genes have been observed (27, 33). Thus, different repressive architectures may exist at different genes regulated by this corepressor.

mRNA capping is considered a co-transcriptional event rather than a post-transcriptional event because it occurs when the transcript is only 25–30 nucleotides long (56, 57), coincident with the time of C-terminal domain phosphorylation and recruitment of the capping enzyme (54, 55). Interestingly, the capping enzymes are also bound to and up-regulated by the elongation factor Spt5 and HIV (human immunodeficiency virus) Tat protein (58, 59). The association of Tup1 with the mRNA capping enzyme might inhibit capping functions. However, the purified recombinant version of the C-terminal WD repeat region of Tup1, which binds to Cet1, did not inhibit the RNA 5′-triphosphatase activity of Cet1.2 Recently Cet1 was identified as a general repressor of RNA polymerase II transcription that functions at the reinitiation step of the transcription cycle (60). It is possible that Tup1 utilizes this repressor activity of Cet1 to down-regulate transcription of target genes.

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