Two Routes to Genetic Suppression of RNA Trimethylguanosine Cap Deficiency via C-Terminal Truncation of U1 snRNP Subunit Snp1 or Overexpression of RNA Polymerase Subunit Rpo26

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ABSTRACT The trimethylguanosine (TMG) caps of small nuclear (sn) RNAs are synthesized by the enzyme Tgs1 via sequential methyl additions to the N2 atom of the m7G cap. Whereas TMG caps are inessential for Saccharomyces cerevisiae vegetative growth at 25°C to 37°C, tgs1Δ cells that lack TMG caps fail to thrive at 18°C. The cold-sensitive defect correlates with ectopic stoichiometric association of nuclear cap-binding complex (CBC) with the residual m7G cap of the U1 snRNA and is suppressed fully by Cbc2 mutations that weaken cap binding. Here, we show that normal growth of tgs1Δ cells at 18°C is also restored by a C-terminal deletion of 77 amino acids from the Snp1 subunit of yeast U1 snRNP. These results underscore the U1 snRNP as a focal point for TMG cap function in vivo. Casting a broader net, we conducted a dosage suppressor screen for genes that allowed survival of tgs1Δ cells at 18°C. We thereby recovered RPO26 (encoding a shared subunit of all three nuclear RNA polymerases) and RPO31 (encoding the largest subunit of RNA polymerase III) as moderate and weak suppressors of tgs1Δ cold sensitivity, respectively. A structure-guided mutagenesis of Rpo26, using rpo26Δ complementation and tgs1Δ suppression as activity readouts, defined Rpo26-(78-155) as a minimized functional domain. Alanine scanning identified Glu89, Glu124, Arg135, and Arg136 as essential for rpo26Δ complementation. The E124A and R135A alleles retained tgs1Δ suppressor activity, thereby establishing a separation-of-function. These results illuminate the structure activity profile of an essential RNA polymerase component.

KEYWORDS trimethylguanosine synthase U1 snRNP RNA polymerase subunit Rpo26

Trimethylguanosine (TMG) cap structures are characteristic of small nuclear (sn) RNAs, small nucleolar (sno) RNAs, and telomerase RNA. TMG is formed post-transcriptionally by the enzyme Tgs1, which catalyzes two successive methyl additions to the N2 atom of the m7G cap (Mouaikel et al. 2002; Hausmann et al. 2007). tgs1Δ yeast cells display apparently normal steady-state snRNA levels (Mouaikel et al. 2002; Hausmann et al. 2007) and no overt aberrations in the RNA or protein contents of their spliceosomal snRNPs, except for the acquisition of the nuclear cap-binding complex (CBC) as a stoichiometric component of the U1 snRNP (Schwer et al. 2011). S. cerevisiae can grow in the absence of Tgs1 because the effects of ablating the TMG cap of the spliceosomal U snRNAs are genetically buffered, either by spliceosome assembly factors that are themselves inessential for vegetative growth (Hausmann et al. 2008; Wilmes et al. 2008; Chang et al. 2010) or by otherwise dispensable domains of the essential branchpoint binding protein Ma5 (Chang et al. 2010).
Nonetheless, there are two situations in which the lack of TMG caps per se elicits a profound phenotype. First, *S. cerevisiae* tgs1Δ diploids are unable to properly execute meiosis and sporulation because they are defective in splicing certain meiotic pre-mRNAs (Qiu et al. 2011). Second, although *S. cerevisiae* haploid tgs1Δ cells grow normally at 30°–37°, they are unable to grow at 18°–20° (Mouaiel et al. 2002; Hausmann et al. 2008), signifying that one or more essential cellular transactions becomes reliant on TMG caps at low temperatures.

To gain insights to the basis for tgs1Δ cold sensitivity, we sought to identify genetic suppressors that restore the growth of tgs1Δ cells at restrictive temperature. Based on our findings that the residual m7G cap of the U1 snRNP in tgs1Δ cells is accessible to and occupied by nuclear CBC, we queried whether mutating the cap-binding site of CBC (in the Cbc2 subunit of yeast CBC) might suppress tgs1Δ cs growth. We thereby identified a series of hypomorphic mutations of Cbc2 predicted to weaken cap-binding (Mazza et al. 2002; Calero et al. 2002), which had no effect on vegetative growth in TGS1 cells yet restored the growth of tgs1Δ cells at 18°–20° (Schwer et al. 2011; Qiu et al. 2012). We inferred from these results that the cs phenotype is, at least in part, by the ectopic association of nuclear CBP with the m7G cap of U1 snRNA.

This focused our attention on the U1 snRNP as a potential source of additional tgs1Δ suppressors. Yeast U1 snRNP consists of a 568-nt U1 snRNA, a 7-subunit Sm protein ring, and 10 U1-specific snRNP subunits: Prp39, Prp40, Snu71, Snu56, Luc7, Prp42, Nam8, Snp1, Mud1, and Yhc1 (Gottschalk et al. 1998; Schwer et al. 2011). The Nam8 and Mud1 subunits are inessential for vegetative growth. Rather than suppressing tgs1Δ, we learned early on that nam8Δ and mud1Δ deletions were synthetic lethal with tgs1Δ (Hausmann et al. 2008). The essential Yhc1 subunit (the yeast homolog of human U1-C) interacts directly with the conserved U1 snRNA 5’ leader sequence m2,2-7Gppp1AUCAU1UCCU that base-pairs to the complementary sequence of the consensus yeast 5’ splice site (GUAGU) (Kondo et al. 2015). Extensive mutagenesis of Yhc1, by C-terminal truncation and structure-guided alanine scanning, has yielded a collection of hypomorphic YHC1 alleles that have no effect per se on vegetative growth, but have strong negative genetic interactions with Mud1, Nam8, and Mud2 (Schwer and Shuman 2014, 2015). Testing the YHC1 mutant collection for interactions with tgs1Δ uncovered many instances of synthetic lethality and sickness, but no case in which the tgs1Δ cs phenotype was suppressed. The same was true of a series of truncation and alanine mutants of the SmD3 and SmB subunits of the yeast Sm ring (Schwer and Shuman 2015).

Despite these discouraging results, we continued the search for tgs1Δ suppressors along two lines, one U1-centric, and one unbiased. As we report here, both approaches bore fruit. In the first instance, we generated a series of viable truncation mutants of U1 subunit Snp1 and screened them for genetic interactions. We found two C-terminal truncations of Snp1 that restored the growth of tgs1Δ cell at low temperature.

In parallel, we screened a 2-μm plasmid-based yeast genomic library for candidate dosage suppressors of the tgs1Δ cs defect. The rationale was that bypass by overexpression might identify specific gene products or cellular transactions that are limiting when TMG caps are absent. We report the results of the screen, which identified the RNA polymerase subunit Rpo26 as capable of reviving the growth of tgs1Δ cells at restrictive temperature when expressed from plasmid vectors. Rpo26, a 155-aa polypeptide, is an essential constituent of all three nuclear RNA polymerases (Pol I, Pol II, and Pol III) (Archambault et al. 1990; Woychik et al. 1990). Rpo26 plays key roles in nuclear RNA polymerase assembly and function (Nouraini et al. 1996a; Tan et al. 2003). It makes direct atomic contacts to the catalytic Rpo21 subunit of yeast Pol II (Cramer et al. 2001), and hypomorphic mutations of Rpo26 are synthetic lethal in combination with an rpo21Δ allele (Archambault et al. 1990; Nouraini et al. 1996a). Rpo26 interacts similarly with the catalytic Rpo190 subunit in the crystal structure of yeast Pol I (Fernández-Tornero et al. 2013) and is presumed to do so with the catalytic Rpo31 subunit of Pol III. Our screen also identified Rpo31 as a weaker tgs1Δ suppressor.

We proceeded to conduct a structure-guided mutational analysis of Rpo26 and thereby delineated a minimal functional domain, Rpo26-(78–155), capable of rpo26Δ complementation and tgs1Δ cs suppression. We identified separation-of-function mutations within this domain that abolished rpo26Δ complementation without affecting tgs1Δ suppression.

**MATERIALS AND METHODS**

**Snp1 C-terminal truncations and tests of function**

A 1.59-kbp DNA segment bearing the SNP1 gene (nucleotides 400 to +1190) was amplified from *S. cerevisiae* genomic DNA by PCR using primers that introduced restriction sites for inserting the gene into the yeast expression plasmids pRS316 (CEN URA3) and pRS413 (CEN HIS3). The resulting plasmids p316-SNP1 and p413-SNP1 were constructed to introduce a 5’ BamHI site and a 3’ SpeI site immediately flanking the open reading frame. C-terminal truncation alleles SNP1-(1–223), SNP1-(1–208), and SNP1-(1–193) were generated by PCR amplification with reverse primers that introduced a STOP codon in lieu of codons for Thr224, Ser209, or Phe194 and a flanking SpeI site. The mutated PCR fragments were digested and inserted into p413-SNP1 in lieu of the wild-type SNP1 gene. The plasmidborne genes were sequenced completely to confirm that no unwanted changes were introduced during PCR and cloning.

To assess the effects of SNP1 mutations, we first generated a haploid snp1Δ [p316-SNP1] strain by sporulating and dissecting heterozygous SNP1 snp1Δ:kanMX diploids (Open Biosystems) that had been transfected with p316-SNP1. SNP1 [p316-SNP1] cells were resistant to G418 and unable to grow on medium containing 5-Fluoroorotic acid (FOA). To assess the function of SNP1 alleles, SNP1-Δ [p316-SNP1] cells were transfected with p413-SNP1 (HIS3 CEN) plasmids. His+ transformants were selected and streaked on agar medium containing FOA. The plates were incubated at 20°, 30°, and 37°, and mutants that failed to form colonies at any temperature after 8 d were deemed lethal. Individual FOA-resistant colonies of viable SNP1 alleles were grown to mid-log phase in YPD broth and adjusted to A600 of 0.1. Aliquots (3 μl) of serial 10-fold dilutions were spotted to YPD agar plates, which were then incubated at temperatures ranging from 18° to 37°. We also developed plasmid shuffle assays to test mutational effects on SNP1 function in tgs1Δ, nam8Δ, mud1Δ, mud2Δ, and CBC2-Y244A cells using standard genetic manipulations of mating, sporulation, and dissection.

**Screen for dosage suppressors of tgs1Δ cold sensitivity**

tgs1Δ cells were transfected with a yeast genomic DNA library in vector YEp24 (2 μm, URA3). Approximately 41,000 Ura+ transformants were plated on medium lacking uracil at 18°. The 2 μm plasmid was isolated from 20 colonies that grew at 18° and then transformed into *Escherichia coli*. Plasmids were prepared from cultures of individual ampicillin-resistant transformants. The 20 candidate suppressor plasmids were re-tested by transformation into the tgs1Δ strain; 16 of them rescued growth of tgs1Δ cells at 18°. Primers flanking the cloning site were used to sequence the ends of the genomic DNA inserts in 1362 | Z. R. Qiu, B. Schwer, and S. Shuman
these 16 plasmids and thereby identify the genes contained in each clone. Eight of the clones contained the TGS1 gene. Six plasmids contained a yeast genomic DNA locus, provisionally named DTS1 (DTS = deletion of TGS1 suppressor). Two plasmids contained a different yeast genomic locus, provisionally named DTS2.

**Yeast expression plasmids**

The following DNA fragments with flanking BamHI sites at both 5’ and 3’ ends were amplified by PCR using DTS1 or DTS2 plasmids as templates: (i) the RPO26 ORF and intron (544 bp) plus 419 bp and 239 bp of 5’ and 3’ flanking genomic DNA; (ii) the ML2C ORF (492 bp) plus 443 bp and 240 bp of 5’ and 3’ flanking genomic DNA; (iii) the PZF1 ORF (1.3 kbp) plus 455 bp and 244 bp of 5’ and 3’ flanking genomic DNA; (iv) the SK13 ORF (3.0 kbp) plus 442 bp and 231 bp of 5’ and 3’ flanking genomic DNA; (v) the AZF1 ORF (2.7 kbp) plus 441 bp and 303 bp of 5’ and 3’ flanking genomic DNA; (vi) the TRS33 ORF plus 463 bp and 276 bp of 5’ and 3’ flanking genomic DNA; and (vii) the YOR114w ORF plus 475 bp and 232 bp of 5’ and 3’ flanking genomic DNA. A DNA fragment containing the RPO31 ORF (4.4 kbp) plus 426 bp of upstream (5’) and 230 bp of downstream (3’) chromosomal DNA was amplified by PCR from the DTS2 plasmid using primers that introduced SaII sites at both the 5’ and 3’ ends. The RPO26 intron was trimmed cleanly from its genomic fragment via two-stage overlap extension PCR to generate the cDNA (RPO26+) with its genomic DNA flanks and BamHI terminal restriction sites. The PCR products were then restricted at the terminal sites and inserted into yeast expression vector YEp24 (2 μ URA3). The restricted fragments of RPO26, RPO26+, and RPO26-1 were also inserted into yeast expression vector pRS415 (CEN LEU2). RPO26 was inserted into BamHI-cut pRS316 (CEN URA3) to yield pRS316-RPO26 for use in the plasmid shuffle assays described below. The plasmid inserts were sequenced completely to exclude the acquisition of unwanted changes during PCR amplification and cloning. Plasmids p360-TGS1 (CEN URA3 TGS1) and pUN100-TGS1 (CEN LEU2 TGS1) used as positive controls were described previously (Hausmann et al. 2008).

**Rpo26 mutants**

An intron-less RPO26 ORF was PCR-amplified with sense-strand primers designed to introduce a BamHI restriction site immediately upstream of the translation start codon and an antisense primer that introduced an Xhol site downstream of the stop codon. The PCR product was digested with BamHI and Xhol and then inserted into a yeast expression vector pRS415-TPI (2 μ LEU2) to yield pRS415-TPI-RPO26, in which expression of RPO26 is driven by the yeast TPI1 promoter, contained in a 2.2-kb PvuII fragment from pYX1132 (Novagen).

Truncated RPO26 alleles were constructed by PCR amplification with: (i) sense strand primers that introduced a new start codon at the positions specified plus a flanking BamHI site and/or (ii) antisense strand primers that introduced a new stop codon after the positions specified plus a flanking Xhol site. Single-alanine mutations R79A, E89A, R97A, E124A, R135A, R136A, D145A, and E150A were introduced into RPO26 (78-155) by two-stage PCR overlap extension with mutagenic primer oligonucleotides. The PCR products containing the mutated RPO26 ORFs were digested with BamHI and Xhol and inserted into BamHI/Xhol-cut pRS415-TPI-RPO26 in lieu of the wild-type RPO26 gene. The inserts of all plasmid clones were sequenced to exclude the acquisition of unwanted mutations during amplification and cloning.

**Plasmid shuffle assay for rpo26Δ complementation**

A heterozygous diploid S. cerevisiae RPO26 rpo26Δ::kanMX heterozygous strain (purchased from Open Biosystems) was transformed with pRS316-RPO26. The resulting Ura+ diploid was sporulated and tetrad were dissected. We thereby recovered viable rpo26Δ::kanMX haploids that were resistant to G418 and unable to grow on medium containing 0.75 mg/ml FOA (5-fluoroorotic acid), a drug that selects against the URA3 RPO26 plasmid. The rpo26Δ strain was used to test plasmid-borne alleles of RPO26 for rpo26Δ complementation by plasmid shuffle as follows. rpo26Δ::kanMX-RPO26 cells were transfected with pRS425-TPI-RPO26 plasmids containing wild-type or mutant RPO26 alleles. Individual transformants were selected and patched on SD-Leu agar medium. Cells from each isolate were streaked on agar medium containing 1.0 mg/ml FOA at 30°C. In cases where the RPO26-containing plasmid supported growth on FOA, two isolates of each mutant amplified from single FOA-resistant colonies were tested for growth by spotting 3-μl aliquots of serial 10-fold dilutions of cells (from liquid cultures grown in SD-Leu medium to mid-log phase at 30°C and adjusted to A600 of 0.1) to YPD agar and incubating the plates at 18°C for 7 d, 25°C for 4 d, 30°C for 3 d, or 37°C for 2 d.

**RESULTS**

**Synthetic genetics of Snpl truncation mutants**

Snpl, a 300-aa polypeptide, is the yeast homolog of human U1-70K (437-aa). Alignment of their primary structures highlights 98 positions of side-chain identity/similarity over the N-terminal 207-aa segment of Snpl (Figure 1A). In the 3.3 Å crystal structure of the core human U1 snRNP (Kondo et al. 2015), the N-terminal 60-aa segment of U1-70K is a highly extended polypeptide that drapes across the surface of the U1 particle, making contacts to U1-C/Ytc1 near the U1 snRNA 5’ terminus, and to the 5 m loop subunits, and to the U1 snRNA 3’ of the 5 m site. The segment of U1-70K from aa 61-202 (underlined in Figure 1A), comprising a long α helix and an RRM domain, binds to the conserved stem-loop 1 (SL1) of the U1 snRNA (Kondo et al. 2015). The C-terminal domains of Snpl and U1-70K differ in length and amino acid sequence and are expected to be poorly structured based on their amino acid composition. The conserved N-terminal 21-aa peptide of Snpl that interacts with U1-C/Ytc1 and SmD3 could be deleted without effect on yeast vegetative growth at any temperature (Schwer and Shuman 2015). A SNP1-(22-300) tgs1Δ double-mutant displayed the same cs growth defect as tgs1Δ (Schwer and Shuman 2015).

Here, we constructed three C-terminal truncation mutants of Snpl with distal margins indicated by the reverse arrowheads in Figure 1A. The wild-type and truncated alleles were placed on CEN HIS3 plasmids under the control of the native SNP1 promoter and tested by plasmid shuffle for complementation of a snplΔ [CEN URA3 SNP1] strain. The resulting SNP1-(1-223), SNP1-(1-208), and SNP1-(1-193) strains were viable after FOA selection and grew as well as wild-type YHC1 cells on YPD agar (Figure 1B).

We surveyed genetic interactions of the benign Snpl C-terminal truncations with mud2Δ, nam8Δ, and mud1Δ. The results (Figure 1B) disclosed an informative hierarchy of synthetic mutational effects. SNP1-(1-223), SNP1-(1-208), and SNP1-(1-193) were lethal at all temperatures in the absence of Mud2, indicating that the essential contributions of the Snpl segment downstream of the RRM module to early spliceosome assembly/stability are buffered by the cross-intron bridging interactions of Mud2 (engaged with Mud5 at the branchpoint) with U1 snRNP at the 5’ splice site.
SNP1-(1-223) and SNP1-(1-208) were barely viable in the nam8Δ genetic background and SNP1-(1-193) was synthetically lethal with nam8Δ. By contrast, SNP1-(1-223) and SNP1-(1-208) supported normal growth of mud1Δ cells at 20°–34° and slightly slowed growth at 37° (Figure 1B). The salient finding was that the more truncated SNP1-(1-193) allele was synthetically lethal in the mud1Δ strain, signifying that the Snp1 peptide 184FKPRRLGGGLGGRGY208 is critical for U1 snRNP function in vivo in the absence of Mud1. The corresponding peptide in U1-70K makes direct contacts to the SL1 loop (Kondo et al. 2015).

SNP1-(1-223) and SNP1-(1-208) suppress tgs1Δ

The standout finding was that the SNP1-(1-223) and SNP1-(1-208) truncation alleles restored the growth of tgs1Δ cells at 25° and 20° (Figure 1B) and at 18° (not shown). Colony size of the SNP1-(1-223) tgs1Δ and SNP1-(1-208) tgs1Δ strains on YPD medium at cold
temperatures was indistinguishable from the SNP1, TGS1 wild-type strain (Figure 1B). Thus, deletion of the Snp1 segment from aa 224-300, downstream of the RRM domain, elicited a gain-of-function for the U1 snRNP that lacks a TMG cap. This positive genetic interaction was severed when the C-terminal truncation was extended into the RRM domain, i.e., the SNP1-(1-193) tgs1Δ reverted to cs growth (à la tgs1Δ) and acquired a new ts phenotype (Figure 1B).

**Mutational synergy of SNP1-(1-223) and SNP1-(1-208) with CBC2-Y24A**

The Y24A mutation in the m7G-binding pocket of Cbc2 suppresses the tgs1Δ cs growth defect (Qi et al. 2012), as do C-terminal truncations 1-223 and 1-208 of the U1 snRNP subunit Snp1. To query potential connections between Cbc2 and Snp1, we tested by plasmid shuffle the effects of the SNP1-(1-223) and SNP1-(1-208) alleles in CBC2 snp1Δ and CBC2-Y24A snp1Δ strain backgrounds. We also tested in parallel the N-terminal truncation allele SNP1-(22-300), which eliminates a conserved peptide segment of Snp1/U1-70K that was severed when the C-terminal truncation was extended into the CBC2-Y24A background, both SNP1-(22-300) and SNP1-(1-208) elicited a severe cold-sensitive defect in CBC2-Y24A cells (Figure 2), one that recapitulates the cold-sensitive growth defect of a cbc2Δ null strain (Qi et al. 2012).

**RPO26 and RPO31 are dosage suppressors of tgs1Δ cs growth**

The dosage suppressor screen entailed transformation of S. cerevisiae tgs1Δ cells with a 2-μ URA3 plasmid-based wild-type genomic DNA library and selection for Ura+ colonies that grew at 18°C. Plasmid DNA was recovered from individual yeast colonies grown at 18°C and then transformed into E. coli. Candidate suppressors were retransformed into the original tgs1Δ strain and tested for growth at 18°C. Sequencing the insert junctions of the plasmids that restated faithfully revealed that the rescueing clones contained either TGS1 (as was to be expected) or one of two distinct extragenic suppressor loci, which we provisionally named DTS1 and DTS2, respectively (DTS = deletion of TGS1 suppressor). Note that whereas the 2-μ DTS1 or 2-μ DTS2 plasmids restored growth at 18°C, compared to tgs1Δ cells carrying the empty 2-μ vector, neither 2-μ DTS1 nor 2-μ DTS2 was as effective as a TGS1 plasmid, as gauged by colony size (Figure 3).

**Defining the suppressor loci within the 2μ DTS1 and DTS2 plasmids**

DTS1 spans a segment of chromosome XVI that includes four complete genes—PZF1, RPO26, MLC2, and SKI3—plus a 3’ fragment of the RPC82 gene (Figure 3, top panel). To map the suppressor, we constructed a series of 2-μ vectors containing the individual PZF1, RPO26, MLC2, and SKI3 open reading frames and ~200–400 bp of 5’ and 3’ flanking genomic DNA. tgs1Δ cells transformed with these plasmids were tested for growth at 18°C, thereby revealing that the suppressor activity was inherent to RPO26 (Figure 3, top panel), the yeast gene encoding a shared 155-amino acid subunit of nuclear RNA polymerases I, II, and III.

DTS2 comprises a region of chromosome XV that embraces the complete AZF1, YOR114w, TRS33, and RPO31 genes, plus a 3’ fragment of the CEX1 gene (Figure 3, bottom panel). When 2-μ vectors containing the individual AZF1, YOR114w, TRS33, and RPO31 open reading frames and ~200–400 bp of 5’ and 3’ flanking genomic DNA were transformed into tgs1Δ cells, only RPO31 revived growth at 18°C compared to the vector control (Figure 3, bottom panel). Rpo31 encodes the 1460-amino acid largest subunit of nuclear RNA polymerase III. Side-by-side comparison of the growth of tgs1Δ cells bearing 2-μ RPO26 or RPO31 plasmids revealed that RPO26 was a better suppressor of the cold-sensitive defect, as gauged by colony size (Figure 3, bottom panel).

**RPO26 and RPO31 suppress tgs1Δ at low gene dosage**

The identification of two RNA polymerase subunits as dosage suppressors of tgs1Δ suggested a novel connection between TMG caps and transcription. The connection via Rpo31 to RNA polymerase III, which is responsible for the synthesis of many essential noncoding RNAs (5S rRNA, U6 snRNA, tRNAs), was particularly puzzling insofar as none of the known Pol III transcripts have 5’ TMG (or m7G) caps. One scenario that might explain the genetic suppressor results is that the loss of TMG caps affects nucleolar architecture and function (Colau et al. 2004) such that the assembly or activity of Pol III is compromised at cold temperature, and this defect can be overcome, in part, by overexpressing either RPO31 or RPO26. If this is the case, then we might expect that simultaneously overexpressing RPO31 and RPO26 would afford better growth of tgs1Δ cells at 18°C than increasing the gene dosage of either gene alone. We tested this by introducing RPO31 and RPO26 on the same 2-μ plasmid, but observed no better rescue of tgs1Δ growth in the cold than that afforded by 2-μ RPO26 (data not shown). Another prediction of the above scenario is that tgs1Δ suppression should require high gene dosage. To address this issue, we placed the RPO31 and RPO26 genes on CEN plasmids and transformed them into tgs1Δ cells. The striking finding was that provision of RPO26 or RPO31 on a CEN plasmid was just as effective as the 2-μ RPO26 or RPO31 plasmids in restoring tgs1Δ growth at restrictive temperature (Figure 4).

**Figure 2**

Mutational synergy of SNP1-(1-223) and SNP1-(1-208) with CBC2-Y24A. The wild-type and truncated SNP1 alleles were tested for activity by plasmid shuffle in CBC2 snp1Δ and CBC2-Y24A snp1Δ strains. FOA-resistant isolates were spot-tested for growth on YPD agar at the temperatures specified. Synthetic growth defects are denoted by *.
Figure 3 Rpo26 and Rpo31 are dosage suppressors of tgs1Δ cold sensitivity. DTS1 (8.6 kb) and DTS2 (12.4 kb) are the two genomic inserts in the 2-μ URA3 plasmids that were isolated in the dosage suppressor screen for reversal of tgs1Δ growth. Individual genes with complete ORFs within the DTS1 and DTS2 inserts including PZF1, RPO26, MLC2, SK13, AZF1, RPO31, TRS33, and YOR114w were cloned into a 2-μ URA3 vector. These plasmids, an empty CEN vector (negative control), a CEN URA3 plasmid containing wild-type TGS1 (positive control), and the plasmids containing DTS1 or DTS2 were transformed into tgs1Δ cells. Ura+ transformants were selected and grown at 30°C in liquid medium lacking uracil. The cultures were adjusted to an A600 of 0.1 and aliquots of serial 10-fold dilutions were spotted on agar medium lacking uracil. The plates were photographed after incubation for 7 d at 18°C.

to us the more plausible target of such an effect, because: (i) TMG caps are certainly implicated genetically in pre-mRNA splicing; (ii) the Rpo26 gene contains an intron, whereas Rpo31 does not; and (iii) prior studies had shown that a 60% reduction in the level of mature Rpo26 mRNA (caused by a mutation in the Rpo26 promoter) resulted in a cold-sensitive growth defect (Nouraini et al. 1996b). We initially considered a scenario in which adequate Rpo26 expression might somehow require the presence of an intron in the pre-mRNA, akin to what has been described for the yeast Sus1 and the intron-containing SUS1 pre-mRNA (Cuencabono et al. 2011; Hossain et al. 2011). In that case, we would expect that an intron-less cDNA version of Rpo26 would not be able to suppress tgs1Δ. However, we found that the Rpo26 cDNA (designated Rpo26* in Figure 3) was just as effective as the native Rpo26 gene in promoting tgs1Δ growth at 18°C, whether delivered on a 2-μ vector or a CEN vector (Figure 4).

N- and C-terminal truncations of Rpo26 delineate a minimal functional domain

The crystal structure of yeast RNA polymerase II (Cramer et al. 2001) revealed the fold of the C-terminal segment of Rpo26 from amino acids 72 to 155, which comprises two α-helices and a β-hairpin (Figure 5). The N-terminal 71-amino-acid segment was disordered in the Pol II structure. In the recent crystal structure of yeast Pol I, the N-terminal 54-amino-acid segment of Rpo26 was disordered and the segment from amino acids 55 to 71 comprised an α-helix (Fernández-Tornero et al. 2013). A previous study had shown that deleting 42 amino acids from the N-terminus of Rpo26 did not affect the viability of yeast cells when the truncated Rpo26Δ42 allele was driven by the strong GAL10 promoter in galactose-containing medium; however, deletion of 84 amino acids from the Rpo26 N-terminus was lethal (Nouraini et al. 1996a).

Here, we tested the effects of finer incremental N- and C-terminal truncations on the in vivo activity of Rpo26, using two genetic readouts of function: (i) dosage suppression of tgs1Δ and (ii) complementation of rpo26Δ. The truncated Rpo26 alleles were placed on 2-μ plasmids under the control of the yeast TPI1 promoter. The N-terminal deletion alleles Rpo26-(40-155), Rpo26-(70-155), and Rpo26-(78-155) were as effective as Rpo26 in supporting tgs1Δ growth at 18°C, whereas Rpo26-(1-80), a truncated version encoding just the disordered N-terminal segment of Rpo26, had no salutary effect (Figure 5). The Rpo26-(78-155) allele complemented rpo26Δ in a plasmid shuffle assay. Rpo26-(78-155) cells grew as well as wild-type Rpo26 yeast at

Figure 4 Rpo26 and Rpo31, at low gene dosage, are capable of restoring growth of tgs1Δ at 18°C. (Left) Yeast tgs1Δ cells were transformed with a CEN URA3 plasmid bearing wild-type TGS1 (positive control), an empty 2-μ URA3 vector (negative control), and 2-μ URA3 plasmids expressing wild-type Rpo26, intron-less Rpo26 cDNA (Rpo26*), or Rpo31. Ura+ transformants were selected at 30°C and then tested for growth at 18°C by spotting serial 10-fold dilutions of liquid cultures (grown at 30°C in SD-Ura medium) on Ura– agar plates. The plates were photographed after incubation for 7 d at 18°C. (Right) Yeast tgs1Δ cells were transformed with a CEN LEU2 plasmid bearing wild-type TGS1 (positive control), an empty CEN LEU2 vector (negative control), and CEN LEU2 plasmids expressing wild-type Rpo26, Rpo26*, or Rpo31. Leu+ transformants were selected at 30°C and then tested for growth at 18°C by spotting serial 10-fold dilutions of liquid cultures (grown at 30°C in SD-Leu medium) on SD-Leu agar plates. The plates were photographed after incubation for 7 d at 18°C.
Figure 5 N- and C-terminal truncations of Rpo26 delineate a minimal functional domain for tgs1Δ suppression. (Left) Tertiary structure of Rpo26-(72-155), from the yeast Pol II crystal structure (pdb 1I3Q), with the N and C termini indicated. (Bottom right) The amino acid sequence of yeast Rpo26. The C-terminal segment visualized in the Pol II crystal structure is in black font; the disordered N-terminal segment is in red font. The margins of the N- and C-terminal truncations are denoted by forward and reverse arrows. For the N-terminal deletions, the arrows specify the residues that were mutated to methionine to initiate the truncated proteins. Black arrows denote the truncations that allow the mutants to restore tgs1Δ growth at 18°C, whereas the gray arrows denote the truncations that disable tgs1Δ suppression. (Top right) tgs1Δ cells were transformed with a CEN LEU2 plasmid bearing wild-type TGS1 (positive control), an empty 2-μ LEU2 TP11 vector (negative control), or 2-μ LEU2 TP11-RPO26 plasmids bearing wild-type RPO26 or the indicated truncation mutants. Leu+ transformants were selected at 30°C and then tested for growth at 18°C by spotting serial 10-fold dilutions of liquid cultures (grown at 30°C in SD-Leu medium) on SD-Leu agar plates. The plates were photographed after incubation for 7 d at 18°C.

18°C, 25°C, 30°C, and 37°C, as gauged by colony size (Figure 6B). We conclude that the N-terminal 77 amino acids are dispensable for Rpo26 function as a subunit of the three RNA polymerases and as a suppressor of tgs1Δ. By contrast, RPO26-(84-155) was a feeble suppressor of tgs1Δ at 18°C (Figure 5) and was unable to complement rpo26Δ in the plasmid shuffle (not shown), signifying that the 78QRATTP83 peptide is important for Rpo26 activity.

We then tested the effects of deleting 5 amino acids or 16 amino acids from the C-terminus of the biologically active Rpo26-(70-155) polypeptide. Whereas RPO26-(70-150) was able to support growth of tgs1Δ cells at 18°C, the RPO26-(70-140) allele was not (Figure 5). RPO26-(70-140) failed to complement rpo26Δ (not shown). By contrast, RPO26-(70-150) did complement rpo26Δ, albeit with a conditional phenotype whereby RPO26-(70-150) cells grew well at 18°C and 25°C, formed small colonies at 30°C, and failed to grow at 37°C (Figure 6B). Thus, the decapeptide segment 141GSFEDWSVEE150 is necessary for Rpo21 function at warmer temperatures. Because a previous study had shown that a nonsense mutant allele encoding Rpo26-(1-145) was unable to complement rpo26Δ (Nouriaini et al. 1996a), we can surmise that the pentapeptide 146WSVEE150 contains features essential for Rpo26 activity in vivo.

Structure-guided alanine scan identifies amino acids essential for rpo26Δ complementation

The crystal structure of Rpo26 in the context of RNA polymerase II highlights a network of intramolecular side chain contacts entailing salt bridge, hydrogen bonding, and π-cation interactions (Figure 6A). Here, we performed a structure-guided alanine scan of eight residues that comprise this network: Arg79, Glu89, Arg97, Glu124, Arg135, Arg136, Asp145, and Glu150. The alanine mutations were introduced into the biologically active RPO26-(78-155) gene on 2-μ plasmids under the control of the TP11 promoter and tested for complementation of rpo26Δ by plasmid shuffle. Four of the alanine mutations were lethal: E89A, E124A, R135A, and R136A. Three of the alanine mutants—R79A, D145A and E150A—were viable and grew as well as “wild-type” RPO26-(78-155) at 18°C, 25°C, 30°C, and 37°C (Figure 6B). R79A cells grew at 25°C and 30°C but displayed a cold-sensitive growth defect at 18°C and grew slowly at 37°C, as gauged by colony size (Figure 6B). We interpret the mutational data in light of the crystal structure, as follows.

Arg79 is located within the 78QRATTP83 hexapeptide defined as essential by our deletion analysis; Arg79 forms a salt bridge to Glu150 at 18°C, whereas the gray arrows denote the truncations that disable tgs1Δ suppression. (Top right) tgs1Δ cells were transformed with a CEN LEU2 plasmid bearing wild-type TGS1 (positive control), an empty 2-μ LEU2 TP11 vector (negative control), or 2-μ LEU2 TP11-RPO26 plasmids bearing wild-type RPO26 or the indicated truncation mutants. Leu+ transformants were selected at 30°C and then tested for growth at 18°C by spotting serial 10-fold dilutions of liquid cultures (grown at 30°C in SD-Leu medium) on SD-Leu agar plates. The plates were photographed after incubation for 7 d at 18°C.

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The TPYMT peptide loop preceding the first α-helix are necessary for Rpo26 folding and function. A conservative R136K mutation in RPO26 elicits a temperature-sensitive growth defect (Nouraini et al. 1996a).

Arg135 and Asp145 are situated on the opposite face of the β-hairpin, where they form an interstrand salt bridge (Figure 6A). It was noteworthy that whereas subtracting the Asp145 side chain had no apparent impact on cell growth, the loss of Arg135 was lethal. Thus, the Asp-Arg salt bridge is not essential for Rpo26 activity. Arg135 forms a cation-π stack on Phe143 (Figure 6A), and we suspect that this cation-π interaction accounts for the essentiality of Arg135. Consistent with this idea, replacing Arg135 with lysine, which would, in principle, preserve the cation-π interaction, had no effect on yeast growth (Nouraini et al. 1996a).

The essential Glu124 side chain participates in a network of ionic and hydrogen bond contacts involving the two α-helices and the connecting loop (Figure 6A). Glu124 (in α1) makes a bidentate salt bridge to Arg97 (in α1 and conditionally essential at 18°C) and accepts a hydrogen bond from the main-chain amide of Phe108 (in the loop). Arg97, in turn, donates hydrogen bonds to Gln100 and the main-chain carbonyl of Pro106. It was shown previously that replacing Gln110 with arginine results in cold-sensitive and temperature-sensitive growth defects (Tan et al. 2003).

Rpo26 mutations that separate rpo26Δ complementation and tgs1Δ suppression activities

As one might expect, the R79A, D145A, and E150A mutants that complemented rpo26Δ at 18°C were also active in suppressing tgs1Δ (Figure 6C). Mutations E89A and R136A that unconditionally abolished rpo26Δ complementation also eliminated tgs1Δ suppression. The salient findings were that: (i) two other mutants, R135A and E124A, that were unconditionally defective in rpo26Δ complementation retained tgs1Δ suppressor activity and (ii) mutant R79A, which was inactive in rpo26Δ complementation at 18°C, nonetheless.
complemented tgs1Δ growth at 18°C. Thus, R135A, E124A, and R97A exemplify separation of function mutations that distinguish the global role of Rpo26 in transcription by all nuclear RNA polymerases from its particular ability to act as a dosage suppressor of the cold sensitivity of tgs1Δ cells.

DISCUSSION
The present study provides new genetic insights to the impact of the lack of TMG caps in budding yeast. Prior screening for synthetic lethal and sick tgs1Δ interactions had drawn attention specifically to the U1 snRNP as a focal point for TMG cap function in vivo (Hausmann et al. 2008). This idea was fortified by the findings that the only overt change in the composition of yeast spliceosomal snRNPs in tgs1Δ cells was the gain of CBC as a stoichiometric component of the U1 snRNP, by virtue of its binding to the residual m7G cap on the U1 snRNA (Schwer et al. 2011). In TGS1 cells, CBC is loosely associated with the U1 snRNP at low sub-stoichiometric levels compared to the intrinsic U1 snRNP subunits (Schwer et al. 2011). It is thought that CBC interacts with one or more of the U1 snRNP proteins to facilitate bridging interactions between CBC bound to the pre-mRNA m7G cap and the U1 snRNP at the 5′ splice site (Lewis et al. 1996; Görnemann et al. 2005). Hypomorphic mutations in Cbc2 that weaken cap binding suppress the tgs1Δ cs growth defect (Qiu et al. 2012).

Here, we show that restoration of growth of tgs1Δ cells in the cold can also be achieved by deleting the C-terminal 77-aa segment of the essential Snp1 subunit of the U1 snRNP. Although it had been appreciated earlier that this C-terminal portion of Snp1 was dispensable for vegetative growth (Hilleren et al. 1995), the genetic interactions of the Snp1-Cα truncations were not interrogated. Underscoring the theme of redundancy in the yeast U1 snRNP, we show that otherwise benign Snp1-(1-223) and Snp1-(1-208) mutations are catastrophic in the absence of Mud2 or Nam8. Yet these same Snp1 truncation alleles elicit a gain-of-function in the tgs1Δ genetic background. Our frugal speculation is that tgs1Δ suppression by Snp1-Cα is mediated via an effect on CBC association with the residual U1 snRNA m7G cap, whereby the C-terminal segment of Snp1 is itself a point of contact of CBC with the U1 snRNP. In this scenario, weakening of the CBC-U1 snRNP interaction by Snp1 truncation would diminish CBC association with the U1 m7G cap in tgs1Δ cells and allow growth in the cold (more or less mimicking the Cbc2 cap-binding site mutants with respect to tgs1Δ suppression). In TGS1 cells that have TMG caps, the Cbc2 cap-binding site lesion Y24A and the Snp1-Cα truncations, which cause no growth defects per se, synergized when combined to mimic the severe cold sensitivity of the cbc2Δ null mutant. These findings are consistent with the idea that the Snp1 C-terminus contributes to the interaction of CBC with the U1 snRNP.

The C-terminal 77-aa segment of yeast Snp1 is rich in arginine (n = 11), serine (n = 12), and alanine (n = 11) and is predicted to be strongly hydrophilic, with the exception of one hydrophobic tract (265PLLSSATPTAAVT520). The amino acid sequence and composition are suggestive of structural disorder or a structure that is templated by the association of this polypeptide with other proteins. Because this segment is not conserved in human U1-70K and the C-terminus of U1-70K is not seen in U1 snRNP crystal structures, we cannot intuit what contacts might be made by the Snp1 C-terminus. This will be an interesting topic for future studies given the broad impact, both positive and negative, of Snp1 C-terminal deletion on yeast physiology when other components of the splicing apparatus are simultaneously perturbed.

In a separate approach entailing a genomic library screen, we identified Rpo26 as a dosage suppressor of the cold-sensitive phenotype of tgs1Δ cells. Because even a nominally single extra copy of the Rpo26 gene on a CEN plasmid revived tgs1Δ growth at 18°C, we surmise that Rpo26 is an especially vulnerable target of the effect of the tgs1Δ mutation. This vulnerability is unlikely to reflect a fastidious gene-specific requirement for TMG caps or other splicing factors in removing the Rpo26 intron, insofar as the Rpo26 5′-splice site, branchpoint, and 3′-splice site adhere perfectly to the yeast consensus sequences and the intron is situated close to the 5′ end of the Rpo26 ORF, as is the case for most yeast genes. Rather, it is the fact that even small changes in Rpo26 expression can result in an overt growth defect in the cold (Nouraini et al. 1996b) that allowed us to recover a singularly sensitive target gene in the suppressor screen.

As discussed above, we implicate ectopic binding of nuclear CBC to the m7G cap of the U1 snRNP of tgs1Δ cells as a principal factor in the cold sensitivity of tgs1Δ cells. Mutations in the cap-binding site of CBC or deletion of the Snp1 C-terminus completely restore normal growth of tgs1Δ cells at 18°C, unlike the dosage suppression by Rpo26, which promotes growth of s1Δ cells at 18°C, albeit not as well as TGS1 or the hypomorphic CBC2 and Snp1-Cα mutations. These findings fortify the inference that the cold sensitivity of tgs1Δ arises not from the lack of TMG caps, but from the effect of U1-bound CBC on yeast nuclear mRNAs, among which Rpo26 stands out.

Our studies shed new light on the structure-activity relations of Rpo26. We refine the margins of the minimal functional Rpo26 domain, identify essential side chains by alanine scanning, and interpret the mutational effects by reference to the crystal structure of Rpo26 in RNA polymerase II. Especially instructive were the mutations that separated the tgs1Δ dosage suppression activity of Rpo26 from its globally essential function in nuclear transcription. An appealing explanation for this separation is that certain Rpo26 mutations selectively impact Rpo26 function in one (or two) of the nuclear RNA polymerases while sparing its function in the other polymerase(s) (Tan et al. 2003). In that case, we infer that the Rpo26 R135A, E124A, and R97A mutants, which are lethal or conditionally lethal with respect to Rpo26Δ complementation, can provide Rpo26 function for the nuclear RNA polymerase that is most affected in tgs1Δ cells at low temperatures. That we isolated RPO31, the gene encoding the largest subunit of Pol III, in the same suppressor screen that yielded Rpo26 suggests to us that Pol III is especially sensitive to the level of Rpo26 subunit in tgs1Δ cells at low temperatures. Rpo26 is in intimate contact with the large subunits of nuclear RNA polymerases during and after assembly of the polymerases (Wild and Cramer 2012) and there are well-documented genetic interactions of Rpo26 with Rpb1, the largest Pol II subunit (Archambault et al. 1990; Nouraini et al. 1996a). We speculate that the relatively weaker suppression of tgs1Δ by increased RPO31 gene dosage (compared to Rpo26 suppression) reflects enhanced assembly of Pol III when Rpo26 levels are limiting.

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