

Mutants of the Paf1 Complex Alter Phenotypic Expression of the Yeast Prion [PSI+]

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The yeast [PSI+] prion is an epigenetic modifier of translation termination fidelity that causes nonsense suppression. The prion [PSI+] forms when the translation termination factor Sup35p adopts a self-propagating conformation. The presence of the [PSI+] prion modulates survivability in a variety of growth conditions. Nonsense suppression is essential for many [PSI+]–mediated phenotypes, but many do not appear to be due to read-through of a single stop codon, but instead are multigenic traits. We hypothesized that other global mechanisms act in concert with [PSI+] to influence [PSI+]–mediated phenotypes. We have identified one such global regulator, the Paf1 complex (Paf1C). Paf1C is conserved in eukaryotes and has been implicated in several aspects of transcriptional and posttranscriptional regulation. Mutations in Ctr9p and other Paf1C components reduced [PSI+]–mediated nonsense suppression. The *CTR9* deletion also alters nonsense suppression afforded by other genetic mutations but not always to the same extent as the effects on [PSI+]–mediated read-through. Our data suggest that the Paf1 complex influences mRNA translatability but not solely through changes in transcript stability or abundance. Finally, we demonstrate that the *CTR9* deletion alters several [PSI+]–dependent phenotypes. This provides one example of how [PSI+] and genetic modifiers can interact to uncover and regulate phenotypic variability.

INTRODUCTION

Termination of translation is signaled when a stop codon in the mRNA reaches the A site of the ribosome. In eukaryotes, the conserved proteins eRF1 and eRF3 recognize the stop codon and catalyze polypeptide release from the ribosome to terminate translation (Frolova *et al.*, 1994; Stansfield *et al.*, 1995b; Zhouravleva *et al.*, 1995). Termination is a highly accurate process, and several factors that contribute to this fidelity have been identified (reviewed in von der Haar and Tuite, 2007). However, the molecular mechanism of how many of these factors contribute to faithful translation termination has not been fully elucidated. Mutant screens in *Saccharomyces cerevisiae* have identified genetic factors such as suppressor tRNAs, 18S rRNA, and ribosomal proteins that contribute to the accuracy of translation termination (reviewed in Beier and Grimm, 2001; Bertram *et al.*, 2001; Valente and Kinzy, 2003). Factors that regulate polyA tail length and mRNA stability, such as Upf1p, Upf2p, Upf3p, Dcp1p, Pab1p, Pop2p, and Tpa1p, also influence faithful termination (Weng *et al.*, 1996; Czaplinski *et al.*, 1998; Cosson *et al.*, 2002; Keeling *et al.*, 2006; Kofuji *et al.*, 2006). Overexpression of Mtt1p/Ecm32p, Itt1p, Sso1p, Stu2p, eEF1B α ,

snR18, and Ssb1p, specific mutations in actin, up-regulation of tRNAs, and loss of *SAL6/PPQ1* or *DBP5* have all been shown to modify the efficiency of translation termination (Song and Liebman, 1987; Czaplinski *et al.*, 2000; Urakov *et al.*, 2001; Kandl *et al.*, 2002; Kwapisz *et al.*, 2002; Namy *et al.*, 2002; Gross *et al.*, 2007; Hatin *et al.*, 2007); however, the mechanisms by which many of these factors affect termination have not been elucidated. Epigenetic factors that influence translation termination such as [PSI+] and [ISP+] have also been identified (Cox, 1965; Volkov *et al.*, 2002).

In *S. cerevisiae* and other yeasts, eRF3 is encoded by *SUP35* (Stansfield *et al.*, 1995b). Interestingly, eRF3/Sup35p exists in two different states in yeast (Patino *et al.*, 1996; Paushkin *et al.*, 1996). In the soluble state, Sup35p functions with eRF1/Sup45p to terminate translation (Stansfield *et al.*, 1995b). However, Sup35p can fold into an alternative conformation that is self-propagating, referred to as the prion [PSI+] (Patino *et al.*, 1996; Paushkin *et al.*, 1996). In the [PSI+] state, Sup35p is aggregated and the protein aggregates are heritable through mitosis and meiosis. [PSI+] cells exhibit increased nonsense suppression (or read-through of stop codons), presumably because aggregated Sup35p cannot participate effectively in translation termination. Multiple strain variants of the [PSI+] prion, differing in their level of nonsense suppression, can exist within different cells of the same genetic background (Derkatch *et al.*, 1996). The level of nonsense suppression exhibited by strains of [PSI+] inversely correlates with the amount of soluble Sup35p (Zhou *et al.*, 1999; Kochneva-Pervukhova *et al.*, 2001; Uptain *et al.*, 2001). Strong [PSI+] strain variants have high nonsense suppression, show little soluble Sup35p, and generally have high mitotic and meiotic stability (Derkatch *et al.*, 1996; Zhou *et al.*, 1999; Kochneva-Pervukhova *et al.*, 2001; Uptain *et al.*, 2001). In contrast, [PSI+] strain variants with lower nonsense suppression have more soluble Sup35p as well as

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Abbreviations used: Δ , null; GdnHCl, guanidine hydrochloride; GFP, green fluorescent protein; NM, prion-forming domain of Sup35p; Paf1C, Paf1 complex; SD, synthetic dropout; s[PSI+], strong [PSI+]; SDD-AGE, semi-denaturing detergent agarose gel electrophoresis; w[PSI+], weak [PSI+]; WT, wild-type; YPD, yeast extract, peptone, and dextrose.

lower mitotic and meiotic stability and are called weak $[PSI^+]$. $[PSI^+]$ strain variants rarely interconvert and primarily arise by spontaneous or induced conversion from $[psi^-]$. Prion strain variants are not due to genetic differences; rather they are due to different self-replicating Sup35p conformations (Derkatch *et al.*, 1996; Krishnan and Lindquist, 2005; Tanaka *et al.*, 2005; Toyama *et al.*, 2007).

The domains of Sup35p that function in prion propagation and translation termination are separable (Ter-Avanesyan *et al.*, 1993, 1994). The N-terminal region alone is capable of forming transmissible prion aggregates (Ter-Avanesyan *et al.*, 1994; Li and Lindquist, 2000). Mutations in, or deletion of, the Sup35p N-terminal domain result in an inability to propagate $[PSI^+]$ but allow faithful translation termination (Doel *et al.*, 1994; Ter-Avanesyan *et al.*, 1994; DePace *et al.*, 1998). Mutations in the essential C-terminal domain that reduce function result in less efficient translation termination and cause nonsense suppression (Ter-Avanesyan *et al.*, 1993; Bradley *et al.*, 2003). However, there is cross-talk between the domains. The N-terminal domain influences nonsense suppression in Sup35 C-terminal domain mutants (Volkov *et al.*, 2007). Nonetheless, the N-terminal region is both necessary and sufficient for $[PSI^+]$ prion propagation, and the C-terminal domain alone is necessary and sufficient for translation termination.

Interestingly, we and others have previously shown that completely isogenic $[PSI^+]$ and $[psi^-]$ yeast strains differ in many different growth phenotypes, including changes in colony morphology (Eaglestone *et al.*, 1999; True and Lindquist, 2000; Namy *et al.*, 2008). Furthermore, the specific phenotypic effects were dependent on the genetic background of the strains used. $[PSI^+]$ permitted better growth in some conditions tested, whereas $[psi^-]$ enhanced growth in other conditions. In further studies, we demonstrated that many of the phenotypic changes could be attributed to $[PSI^+]$ -mediated nonsense suppression and not other properties of $[PSI^+]$, such as protein aggregation (True *et al.*, 2004). Nonsense suppression can occur at stop codons at the 3' ends of normal mRNAs or at premature stop codons. As a result, $[PSI^+]$ -mediated nonsense suppression has the potential to produce polypeptides with C-terminal extensions, to merge contiguous open reading frames into a single polypeptide, and to facilitate the translation of pseudogenes and other disabled open reading frames (Harrison *et al.*, 2002; Namy *et al.*, 2002, 2003, 2008; Giacomelli *et al.*, 2007). The hidden variation revealed by $[PSI^+]$ in these normally untranslated regions has led to the proposal that $[PSI^+]$ may generate adaptive phenotypic variants and increase the rate of evolvability (True and Lindquist, 2000; Masel and Bergman, 2003; Masel, 2006). In addition, $[PSI^+]$ -mediated read-through affects mRNA stability both by stabilizing mRNAs normally subjected to nonsense mediated decay and by triggering nonstop decay (Wilson *et al.*, 2005). These may be additional sources of $[PSI^+]$ -mediated genetic variation (True *et al.*, 2004; Wilson *et al.*, 2005). Interestingly, although read-through was essential for the phenotypic effects, many did not appear to be due to nonsense suppression of a single transcript but instead were complex, polygenic traits. This suggests that many modifiers, and perhaps many other cellular pathways, can contribute to the effects of $[PSI^+]$ on phenotypic diversity. Furthermore, because many of the phenotypes are multigenic, modifiers may exist that broadly affect the level of read-through and have the potential to influence many phenotypes. In this study, we identify a genetic modifier of $[PSI^+]$ -mediated traits and show that $[PSI^+]$ and genetic modifiers can interact to regulate phenotypic variability.

MATERIALS AND METHODS

Yeast Strains and Plasmids

Yeast cells were cultured and manipulated using standard techniques. Yeast strains were grown in rich media (YPD: 1% yeast extract, 2% peptone, 2% dextrose) or synthetic dropout media (SD) lacking one or more components as needed to select for plasmids or assay for nonsense suppression. Solid media contained 2% agar. Additions of G418, guanidine hydrochloride (GdnHCl), hygromycin B, neomycin sulfate, and cycloheximide were at the final concentrations noted in the figure legends. Colony morphology was assayed on KOAc media (1% yeast extract, 2% peptone, 3% potassium acetate, 2% agar). Suppression of the *lys1-1* allele in 10B-H49 was assayed on SD medium lacking lysine with 3% glycerol, as the use of glycerol as the carbon source enhances nonsense suppression in 10B-H49. Diploids were sporulated on 0.98% potassium acetate, 0.05% glucose, 0.1% yeast extract, 1.5% agar, 0.08% CSM powder (BIO-101, Carlsbad, CA) for ~10 d before micromanipulation of asci.

All yeast strains used in this study except for those used in Figure 9 were derived from 74-D694 (*MATa ade1-14 trp1-289 his3-Δ200 ura3-52 leu2-3112*). 74-D694 containing strong $[PSI^+]$ was a gift of Y. Chernoff (Georgia Institute of Technology, Atlanta, GA) (Chernoff *et al.*, 1995), as was the 74-D694 strain OT69 containing the *sup45* mutation. The weak $[PSI^+]$ (Derkatch *et al.*, 1996) and $[psi^-]$ *sup35-Y351C* (Bradley *et al.*, 2003) strains were provided by S. Liebman (University of Illinois at Chicago, Chicago, IL). The $[psi^-]$ strain was generated in our laboratory by multiple passages of the strong $[PSI^+]$ strain on YPD supplemented with 3 mM GdnHCl. Strains used in Figure 9 were derivatives of 5V-H19 (*MATa ade2-1 can1-100 leu2-3112 ura3-52 SUQ5*; Ter-Avanesyan *et al.*, 1993) or 10B-H49 (*MATa ade2-1 SUQ5 lys1-1 his3-11,15 leu1 kar1-1*; Ter-Avanesyan *et al.*, 1994) and were obtained from M. Ter-Avanesyan (Institute of Experimental Cardiology, Moscow, Russia). In these strains the nonsense suppression depends on both $[PSI^+]$ and *SUQ5*. The coding regions of *CTR9*, *PAF1*, *CDC73*, *LEO1*, and *RTF1* were replaced with *HIS3MX6* or *loxP-LEUMX-loxP* using the method of Baudin *et al.* (1993) and pFA6a-*HIS3MX6* (Wach *et al.*, 1997) or pUG73 (Gueldener *et al.*, 2002) as template. All knockouts were confirmed by colony PCR.

Plasmids were constructed using standard molecular biology techniques and maintained in DH5α. The shuttle vectors pRS315 (*LEU2 CEN6 ARSH4*) and pRS316 (*URA3 CEN6 ARSH4*) were described previously (Sikorski and Hieter, 1989). pRS316kanMX4 contains *kanMX4* in the f1 origin of pRS316 and can be selected in yeast via *URA3* or *kanMX4*. This plasmid was constructed by removing *kanMX4* from pFA6a-*kanMX4* (Wach *et al.*, 1994) with BglII and SacI, making the fragment blunt with Klenow, and ligating it into the NgoMIV site (made blunt with Klenow) of pRS316 (Sikorski and Hieter, 1989). Wild-type *CTR9* from 74-D694a and mutant *ctr9* from SV16 were cloned into pRS316kanMX4 by PCR amplifying the gene from each strain with oligos (5'-CTACGCGCGCTTTTGTGAATTACACTATCCATC-3' and 5'-CTACTC-GAGAGGACACGAAAAGGTGGATG-3'), digesting the resulting PCR products with EagI and XhoI and ligating them into the same sites of pRS316kanMX4.

Screen for Factors that Reduce Nonsense Suppression

Strain 74-D694a containing strong $[PSI^+]$ was mutagenized with ethylmethane sulfonate as previously described (Lawrence, 1991) to 17% viability. Cells were plated onto YPD plates at ~500–700 colonies per plate and incubated at 30°C for 6–7 d before visually screening for colonies that were darker pink than the original, unmutagenized light pink parental strain. Approximately 133,000 colonies were screened. Solid, darker pink colonies were restreaked to YPD at least two additional times to assess the maintenance of the phenotype. Thirty-two mutants that consistently and uniformly produced solid, darker pink colonies were kept for further analysis.

Cloning SV16 by Complementation

The gene that is responsible for the reduced nonsense suppression in mutant SV16 was cloned by complementation of the poor growth phenotypes on SD-ade and YPD containing 3 mM GdnHCl. SV16 was independently transformed with two different libraries. *S. cerevisiae* genomic libraries in either p366 (*LEU2 CEN*; American Type Culture Collection, Manassas, VA; number 77162, deposited by Philip Hieter) or pRS316kanMX4 (*URA3 kanMX4 CEN*) made in our laboratory (unpublished data) were used. Approximately 108,000 and 17,400 transformants from the *LEU2 CEN* and *URA3 kanMX4 CEN* libraries, respectively, were screened. Library plasmids that complemented both poor growth phenotypes were rescued from transformants and retested. On retesting, eight isolates were identified that complemented both phenotypes. To evaluate specificity, each of the plasmids was tested in strong $[PSI^+]$, weak $[PSI^+]$, and $[psi^-]$ strains for an effect on nonsense suppression by color or growth on SD-ade. Six of the plasmids passed this secondary test. Both ends of the inserts in the complementing plasmids were sequenced (Protein Nucleic Acid Chemistry Laboratory, Washington University School of Medicine). All six library plasmids contained overlapping regions of the same genomic region. *CTR9* was the only gene common to all six plasmids.

To compare the sequences of *CTR9* from SV16 and wild-type, the sequence of the *CTR9* locus from SV16 or 74-D694a was gap-repaired into plasmid A186

(isolated from the *LEU2 CEN* genomic library) digested with *SwaI* and *MluI* to remove most of the *CTR9* coding region. *CTR9* in the two gap-repaired plasmids was sequenced using ABI Big Dye v3.1 chemistry and the sequences from wild-type 74-D694 and SV16 were compared.

Growth Assays

[PSI+]-mediated phenotypes were assayed by growing cultures to mid-log phase in YPD or dropout media to select for a plasmid if necessary. All cultures within a single experiment were normalized to the same density by A_{600} before making fivefold serial dilutions. All phenotypes were assayed multiple times from independent cultures with identical results.

Biochemical Analysis

Sup35p aggregates were analyzed by semidenaturing detergent agarose gel electrophoresis (SDD-AGE) as previously described (Bagriantsev and Liebman, 2004) with the modifications of Tank *et al.* (2007). Unless indicated differently in the figure legend or text, samples were incubated for 7 min at room temperature before being loaded on the gel.

To examine Sup35p and Ctr9p expression levels, protein lysates were prepared as for SDD-AGE except denaturing loading buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 0.1% bromophenol blue, 10% glycerol, and 100 mM dithiothreitol) was added to cleared lysates, and samples were incubated at 95°C for 5 min. Equal amounts of protein, as determined by Bradford Assay, were loaded on polyacrylamide gels for SDS-PAGE. The protein was subsequently transferred to PVDF for Western blotting. Membranes were probed with antibodies recognizing Sup35p (Patino *et al.*, 1996; diluted 1:2000), Ctr9p yN-18 (Santa Cruz Biotechnology, Santa Cruz, CA; diluted 1:400), or Pgk1p (Molecular Probes, Eugene, OR; diluted 1:10,000) followed by horseradish peroxidase-conjugated goat anti-rabbit IgG (Sigma-Aldrich, St. Louis, MO; diluted 1:10,000), rabbit anti-goat IgG (Jackson ImmunoResearch Laboratories, West Grove, PA; diluted 1:4000), or rabbit anti-mouse IgG (Sigma-Aldrich; diluted 1:10,000), respectively. Immunoblots were developed using enhanced chemiluminescence.

Fluorescence Microscopy

Yeast strains were transformed with the copper-inducible plasmid mCNMG containing a fusion between sequences coding for the Sup35p prion domain (NM) and green fluorescent protein (GFP; Patino *et al.*, 1996). Expression of the fusion protein was induced with a final concentration of 60 μ M copper sulfate for 6 h before visualization. Visualization was on an Olympus IX70 fluorescence microscope (Melville, NY) equipped with an enhanced GFP filter cube and a Photometrics Coolsnap HQ camera and QED in vivo software (Tucson, AZ).

Read-through Assay

β -Galactosidase reporter plasmids pUKC817, pUKC818, and pUKC819 (Stansfield *et al.*, 1995a) were provided by M. Tuite (University of Kent, Canterbury, United Kingdom). Transformants were grown to log-phase in liquid culture, harvested by centrifugation, lysed with glass beads, and assayed by the Galacto-Light Chemiluminescent Reporter Gene Assay System (Applied Biosystems, Bedford, MA). Detection was on a Sirius Single Tube Luminometer (Berthold Detection Systems, Pforzheim, Germany) with a delay time of 2.0 s and read time of 5.0 s. For each strain, read-through was calculated from three independent cultures as the average relative light units per microgram of protein assayed as determined by Bradford Assay. Read-through for the [*psi*-] culture was set to a value of 1.0 and read-through for each of the other strains was expressed as the fold-change from [*psi*-]. This method of normalization was used because our data suggest that each individual transcript may be influenced differently by the loss of Ctr9.

Northern Blot

Total RNA was isolated as described (Schmitt *et al.*, 1990). Briefly, total RNA was extracted using three organic extractions: once in hot, acidic phenol-chloroform and twice in cold, acidic phenol-chloroform. The RNA was precipitated overnight with 3 M sodium acetate, pH 5.2, and 100% ethanol at -20°C. The RNA pellet was washed once in 70% ethanol and resuspended in diethylpyrocarbonate (DEPC)-treated water. Equal amounts (20 μ g) of RNA was loaded onto a 0.8% denaturing formaldehyde agarose gel in 1 \times MOPS buffer. RNA was transferred onto a nylon membrane (Amersham, Piscataway, NJ; Hybond-N) by capillary action overnight. The membrane was stained with methylene blue to visualize the 18S and 25S rRNA. The RNA was UV-cross-linked onto the membrane. The membrane was prehybridized in Amersham Rapid-hyb Buffer at 55°C for 1 h. Radioactive probes for the Northern analysis were prepared by random primed labeling of *ADE1* PCR product (Roche, Indianapolis, IN). The oligos used to amplify *ADE1* product were 5' ATGTCAATTACGAAGACT and 5' TTAGTGAGACCATTGACCC. Hybridization was performed at 55°C for 2 h. The membrane was washed three times: once for 15 min at room temperature, followed by a 30 min wash at 60°C (1 \times SSPE, 0.1% SDS), and final wash for 45 min at 60°C (0.5 \times SSPE, 0.25% SDS). The membrane was exposed to film for visualization of bands.

3' RACE and Quantitative RT-PCR Analysis

Total RNA was isolated as described above. Total RNA, 5 μ g, was reverse-transcribed using Superscript III (Invitrogen, Carlsbad, CA) and 50 μ M of an oligo adaptor-dT₁₉: 5' GTTCCAGTCAGATCTTTTTTTTTTTTTTTTTTTT. The RNA was reverse transcribed at 50°C for 1 h, and the reverse transcriptase was inactivated at 70°C for 15 min. The cDNA was subsequently treated with RNase H at 37°C for 20 min. The cDNA was diluted 20-fold into a 50- μ l PCR reaction. The oligos used in the PCR amplification were 5' TACGGATCCTATGAAACATTGACAGGGTC and 5' GTTCCAGTCAGATCT. The 3' RACE reactions were resolved on a 1.5% agarose gel. Quantitative RT-PCR (qRT-PCR) was performed using Sybr-Green (Bio-Rad, Richmond, CA) according to the manufacturer's instructions. The PCR products for *LacZ* and *ACT1* were generated using the following primer pairs: *LacZ*, 5' GTTGCTGATTCCGAGCGGTTAAC and 5' GGCTTCATCCACCACATACAG and *ACT1*, 5' CTACGTTCCATCCAAGCCG and 5' CCACGTTCACTCAAGATCTC.

Invasive Growth Assay

Invasive growth was assayed as described previously (Roberts and Fink, 1994).

RESULTS

Identification of Mutants with Weakened Nonsense Suppression

Read-through of stop codons during translation (nonsense suppression) to produce elongated polypeptide chains is one mechanism that can give rise to phenotypic variability (True and Lindquist, 2000; True *et al.*, 2004; Wilson *et al.*, 2005). The [PSI+] prion reduces the fidelity of translation termination in a slightly metastable, yet heritable manner, and has been shown to influence the phenotype of numerous traits in yeast (True and Lindquist, 2000). Further, many of these traits are complex in nature (True *et al.*, 2004). Although many of the phenotypes require nonsense suppression, other gene products appear to interact and contribute. With the goal of understanding how [PSI+] interacts with other cellular components to contribute to genetic variation and phenotypic diversity, we screened for factors that weakened the nonsense suppression of a [PSI+] yeast strain.

Nonsense suppression is commonly monitored using auxotrophic alleles of biosynthetic pathway genes that contain premature stop codons (Chernoff *et al.*, 2002). For example, the *ade1-14* allele contains a premature stop codon in the *ADE1* gene that encodes a component of the adenine biosynthetic pathway. Because cells with the *ade1-14* allele do not make enough functional Ade1 protein, they require adenine in the media to grow. Although they grow well on rich medium, [*psi*-] *ade1-14* cells accumulate an intermediate in the adenine biosynthetic pathway that causes the colonies to be red in color. In [PSI+] cells, however, translation proceeds through the stop codon to produce some full-length Ade1 protein and thereby complete the adenine biosynthetic pathway. Thus, [PSI+] *ade1-14* cells do not require adenine in the media, and the colonies are white or pale pink in color.

We utilized the *ade1-14* allele in the 74-D694 yeast strain to screen for factors that weaken the nonsense suppression of a strong [PSI+] variant. Cells with weakened nonsense suppression should make less Ade1 protein and produce darker pink colonies than strong [PSI+]. Wild-type pale pink [PSI+] *ade1-14* cells were mutagenized with ethylmethane sulfonate to 17% viability, and the resulting colonies were screened visually for a darker pink color. Of 133,000 colonies screened, 32 consistently produced darker pink colonies than wild-type strong [PSI+] after multiple restreaks.

ctr9 Weakens Nonsense Suppression

One mutant (SV16) will be described here. The SV16 mutant strain produces medium pink colonies on rich media (YPD) and grows slowly on media lacking adenine (SD-ade) com-

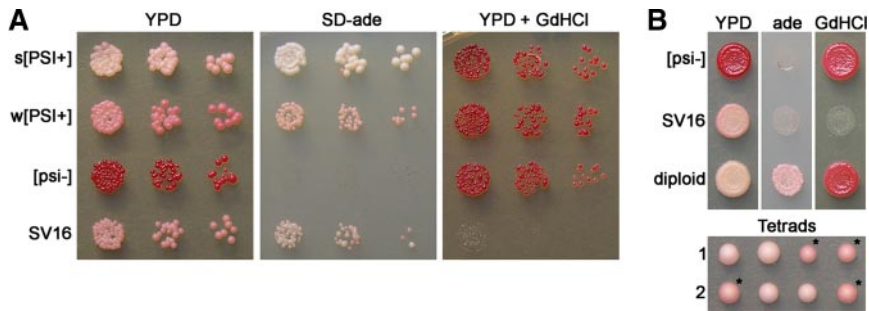


Figure 1. Mutant strain SV16 contains a recessive, Mendelian mutation that weakens the nonsense suppression of $[PSI^+]$. (A) Fivefold serial dilutions of indicated yeast cultures at equal cell density were spotted onto YPD, SD-ade, and YPD containing 3 mM GdnHCl. $s[PSI^+]$ and $w[PSI^+]$, strong and weak $[PSI^+]$ strains, respectively. (B) Spotting of $[psi^-]$, SV16, and the wild-type $[psi^-] \times$ SV16 diploid on YPD, SD-ade, and YPD containing 3 mM GdnHCl (top). Two representative tetrads from sporulation of the wild-type $[psi^-] \times$ SV16 diploid are shown (bottom). Asterisks denote spores that inherited the mutant allele.

pared with unmutagenized strong $[PSI^+]$ cells (Figure 1A). The $[PSI^+]$ prion can be cured by transient growth on media containing millimolar amounts of GdnHCl (Tuite *et al.*, 1981). Interestingly, SV16 cells were inviable in the presence of 3 mM GdnHCl (Figure 1A). However, the SV16 mutant was viable on lower concentrations of GdnHCl and was able to be cured of the $[PSI^+]$ prion (data not shown).

The SV16 mutant was backcrossed to a wild-type 74-D694 $[psi^-]$ strain, and the resulting diploid was analyzed. The diploid produced pale pink colonies on rich media and grew well on SD-ade (Figure 1B), indicating that the SV16 mutation was recessive and that the mutant still contained the $[PSI^+]$ prion. When the meiotic progeny were analyzed, two haploids were pale pink and two were medium pink in each tetrad. The 2:2 segregation pattern suggested that a single mutation was contributing to the phenotype. Furthermore, the recovery of strong $[PSI^+]$ haploids in each tetrad indicated that the mutation had to be present to obtain the reduced nonsense suppression and that the mutation had not permanently altered the structure to create a new prion strain variant.

To identify the mutation in SV16, we complemented the weak nonsense suppression phenotype with two yeast genomic libraries. Complementation was assayed by robust growth on media lacking adenine and by resistance to 3 mM GdnHCl. Six plasmids were identified that complemented both phenotypes and passed all secondary tests (data not

shown). The ends of each rescuing library plasmid were sequenced. All six plasmids contained overlapping genomic fragments. Only one gene, *CTR9*, was common to all six plasmids. *Ctr9p* is a component of the Paf1 protein complex (Paf1C) implicated in the transcription and transcriptional processing of some mRNAs (Shi *et al.*, 1996; Koch *et al.*, 1999; Krogan *et al.*, 2002; Mueller and Jaehning, 2002; Pokholok *et al.*, 2002; Squazzo *et al.*, 2002; Mueller *et al.*, 2004; Rondon *et al.*, 2004; Penheiter *et al.*, 2005). To test whether the increase in nonsense suppression conferred by the library plasmids to SV16 was specific to the SV16 mutant or due to a general increase in nonsense suppression, we transformed a representative library plasmid containing *CTR9* into a wild-type $[psi^-]$ strain as well as strong and weak variants of $[PSI^+]$. The library plasmid did not significantly affect the color of the wild-type $[PSI^+]$ or $[psi^-]$ strains on rich media or their growth on media lacking adenine (Figure 2A).

Because *CTR9* was the only gene in common to all six library plasmids, we tested whether *CTR9* was responsible for the complementation of SV16. *CTR9* was cloned and tested for complementation of the reduced growth of SV16 on SD-ade and for rescue of other phenotypes associated with SV16. During our analysis we found that the SV16 mutant grows slower than wild-type, is slightly temperature sensitive at 37°C, and is hypersensitive to hygromycin B, neomycin sulfate, and hydroxyurea (Figure 2B and data not shown). The introduction of wild-type *CTR9* on a plasmid

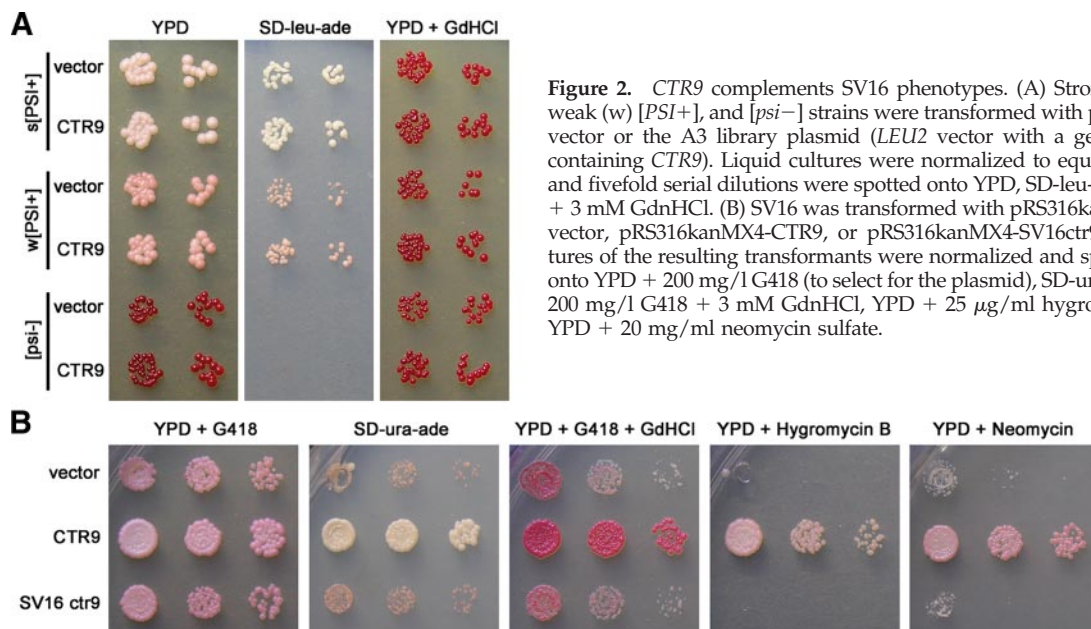


Figure 2. *CTR9* complements SV16 phenotypes. (A) Strong (s) $[PSI^+]$, weak (w) $[PSI^+]$, and $[psi^-]$ strains were transformed with pRS315 empty vector or the A3 library plasmid (*LEU2* vector with a genomic region containing *CTR9*). Liquid cultures were normalized to equal cell density and fivefold serial dilutions were spotted onto YPD, SD-leu-ade, and YPD + 3 mM GdnHCl. (B) SV16 was transformed with pRS316kanMX4 empty vector, pRS316kanMX4-*CTR9*, or pRS316kanMX4-SV16ctr9. Liquid cultures of the resulting transformants were normalized and spotted as in A onto YPD + 200 mg/l G418 (to select for the plasmid), SD-ura-ade, YPD + 200 mg/l G418 + 3 mM GdnHCl, YPD + 25 μ g/ml hygromycin B, and YPD + 20 mg/ml neomycin sulfate.

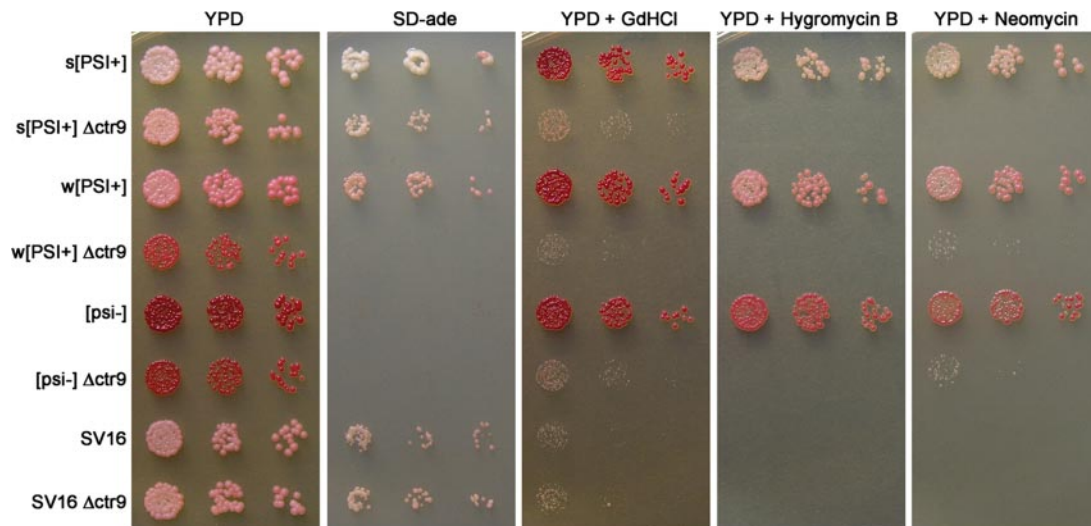


Figure 3. *Δctr9* reduces nonsense suppression of strong and weak [PSI+] and exhibits phenotypes similar to SV16. *CTR9* was deleted in strong (s) [PSI+], weak (w) [PSI+], [psi-], and SV16 strains. Liquid cultures were normalized to equal cell density, and fivefold serial dilutions were spotted onto the indicated media. Drug concentrations in the media are the same as those listed in the legend of Figure 2.

permitted the rapid growth of SV16 on SD-ade compared with the empty vector (Figure 2B). In addition, *CTR9* complemented the slow-growth phenotype of SV16 on rich media as well as the sensitivities to GdnHCl, hygromycin B, and neomycin sulfate (Figure 2B). Moreover, when we cloned the *ctr9* allele out of the SV16 mutant into the same vector and tested it for complementation, we found that it failed to rescue any of the SV16 mutant phenotypes (Figure 2B).

Because *CTR9* rescued the weakened nonsense suppression phenotype of SV16, we hypothesized that a mutation in *CTR9* may be responsible for the weakened nonsense suppression in the SV16 mutant. We sequenced *CTR9* from SV16 and the isogenic wild-type strain and found one difference that resulted in a change in amino acid (C227Y) in the SV16 mutant compared with wild-type.

If the C227Y mutation in *ctr9* was in fact causing the recessive phenotypes of the SV16 mutant, then we predicted that the complete deletion of the nonessential *CTR9* gene would also weaken nonsense suppression. The entire *CTR9* open reading frame was deleted in strong [PSI+], weak [PSI+], [psi-], and SV16 strains and the resulting strains were assessed phenotypically. Deletion of *CTR9* did reduce the [PSI+]-mediated nonsense suppression of the *ade1-14* allele (Figure 3). The null mutant produced darker pink colonies on YPD in the strong [PSI+] strain. In addition, *Δctr9* in a strong [PSI+] strain grew significantly slower on SD-ade than the wild-type strong [PSI+] strain. When *ctr9* was deleted in a weak [PSI+] strain, the colonies became red in color and did not grow on media lacking adenine. Because these colonies appeared phenotypically like [psi-], we wanted to determine if [PSI+] was still present. Deletion of *CTR9* could cure the weak [PSI+] prion. Alternatively, the [PSI+] prion could still be present but phenotypically masked by more faithful translation termination at the premature *ade1-14* stop codon in the *CTR9* deletion. To genetically test for the presence of [PSI+], we crossed *Δctr9* made in the weak [PSI+] strain to a [psi-] strain and examined the color of the resulting diploids on YPD. If [PSI+] was present but masked in the *Δctr9* strains, then the diploid would appear pink because of the rescued nonsense suppression by one wild-type copy of *CTR9* in the diploid. However, if

[PSI+] had been cured by the deletion of *CTR9*, then the diploid would be red. The diploid was pink in color, indicating deletion of *CTR9* did not cure weak [PSI+] (data not shown). We also tested for the presence of aggregated Sup35p in [PSI+] *Δctr9*. Sup35 protein aggregates were still detected by SDD-AGE (data not shown). Collectively, this data suggests that the [PSI+] prion was still present and that deletion of *CTR9* reduced the nonsense suppression phenotype of [PSI+]. In addition, *Δctr9* in strong [PSI+], weak [PSI+], and [psi-] strains were all temperature sensitive at 37°C and produced similar sensitivities to GdnHCl, hygromycin B, neomycin sulfate, and hydroxyurea as the SV16 point mutant (Figure 3 and data not shown).

We also knocked out *ctr9* from the SV16 mutant strain. Interestingly, we noted that the complete deletion of *ctr9* in the SV16 strain was phenotypically similar to the SV16 point mutant (Figure 3), suggesting that the Ctr9-C227Y protein could be produced at a lower level, unstable, or severely impaired functionally. To test if the Ctr9-C227Y protein was present at a steady-state level similar to wild-type, lysates were prepared from wild-type and *ctr9*-C227Y [PSI+] strains and equal protein quantities were analyzed by SDS-PAGE and immunoblotting with antibodies to Ctr9p. The Ctr9-C227Y protein was barely detectable compared with wild-type Ctr9p (data not shown), suggesting less mutant protein is made or that it is very unstable.

Loss of Select Paf1C Members Also Reduces Nonsense Suppression

Because Ctr9p is part of a protein complex that contains Paf1p, Cdc73p, Leo1p, and Rtf1p (Koch *et al.*, 1999; Krogan *et al.*, 2002; Mueller and Jaehning, 2002; Squazzo *et al.*, 2002), we tested whether other members of the complex also affected [PSI+]-mediated nonsense suppression. Deletions of *CTR9* and *PAF1* have been reported to have many phenotypes in common, whereas deletion of other members of Paf1C often produces fewer and weaker phenotypes (Betz *et al.*, 2002; Mueller and Jaehning, 2002). Because *CTR9* influences nonsense suppression, we predicted that the deletion of other Paf1C members would also affect the [PSI+] phenotype. We individually deleted the genes encoding members of Paf1C in strong [PSI+], weak [PSI+], [psi-], and

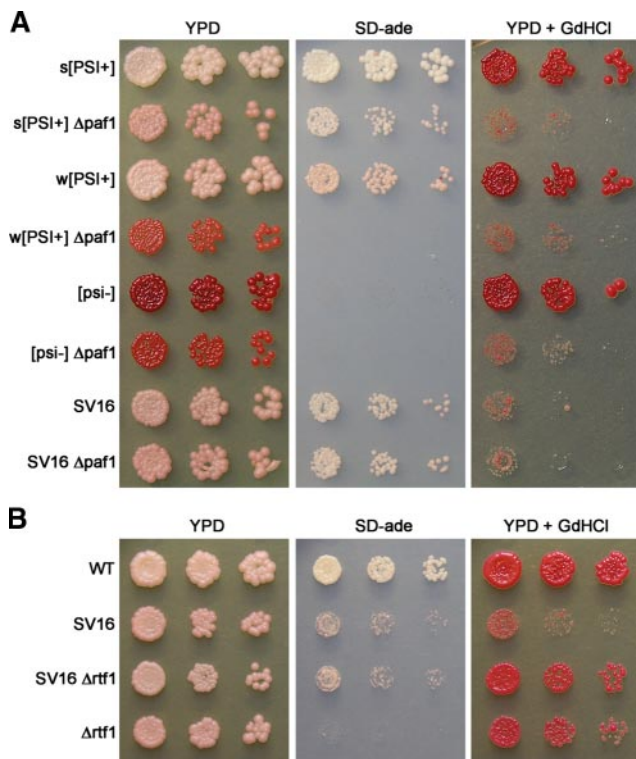


Figure 4. Some components of Paf1C affect nonsense suppression of $[PSI^+]$. (A) Deletion of *PAF1* weakens $[PSI^+]$ -mediated nonsense suppression. Deletions of *PAF1* were made in the following strains: strong (s) $[PSI^+]$, weak (w) $[PSI^+]$, $[psi^-]$, and SV16. Each deletion mutant and parental wild-type control strain was spotted as described in the legend of Figure 3. (B) Deletion of *RTF1* suppresses the GdnHCl sensitivity of SV16 but not the reduction in nonsense suppression of *ade1-14*. A wild-type (wt) strong $[PSI^+]$ strain, SV16, SV16 $\Delta rtf1$, and strong $[PSI^+]$ $\Delta rtf1$ strain were spotted as described in the legend of Figure 3.

SV16 strains. The deletion of *PAF1* weakened nonsense suppression in both strong and weak $[PSI^+]$ strains and rendered the cells sensitive to GdnHCl (Figure 4A).

The individual deletion of *RTF1* also weakened nonsense suppression in both strong and weak $[PSI^+]$ strains (Supplemental Figure S1). However, $\Delta rtf1$ strains were only mildly sensitive to GdnHCl (data not shown). Interestingly, the deletion of *CDC73* resulted in an intermediate phenotype. Weakened nonsense suppression of *ade1-14* by $\Delta cdc73$ was readily detected in a weak $[PSI^+]$ strain but not in a strong $[PSI^+]$ strain (Supplemental Figure S1). It is possible that $\Delta cdc73$ does weaken nonsense suppression in a strong $[PSI^+]$ strain but that a more sensitive assay is required to detect it. Deletion of *LEO1* had no detectable effect on nonsense suppression of *ade1-14* in either strong or weak $[PSI^+]$ strains by this assay (Supplemental Figure S1). Moreover, $\Delta cdc73$ and $\Delta leo1$ strains were not sensitive to GdnHCl (data not shown).

Deletion of *RTF1* Suppresses Some of the SV16 Phenotypes

Loss of Leo1p or Rtf1p has been reported to suppress many of the phenotypes of $\Delta ctr9$ and $\Delta paf1$ mutants (Mueller and Jaehning, 2002). To test whether the loss of other components of Paf1C can suppress the *ctr9-C227Y* mutant, we assayed deletions of *PAF1*, *CDC73*, *LEO1*, and *RTF1* in the SV16 mutant strain for rescue of the *ctr9-C227Y* phenotypes. The deletions of *PAF1*, *CDC73*, and *LEO1* had no significant

effect on the nonsense suppression of the *ade1-14* allele in the $[PSI^+]$ SV16 strain (Figures 4A and Supplemental Figure S1). Interestingly, $\Delta rtf1$ suppressed some of the phenotypes associated with *ctr9-C227Y* in the SV16 mutant strain. Loss of Rtf1p suppressed the sensitivity of SV16 to 3 mM GdnHCl and 0.15 M hydroxyurea (Figure 4B and data not shown). However, it did not alter the level of nonsense suppression of *ade1-14* (Figures 4B and Supplemental Figure S1).

To determine whether another member of Paf1C can functionally replace Ctr9p in translation termination, we assayed whether the overexpression of *PAF1*, *CDC73*, *LEO1*, or *RTF1* could suppress the phenotypes of the SV16 mutant. None of the other Paf1C components expressed from a 2- μ m plasmid restored the nonsense suppression of *ade1-14* or the GdnHCl resistance in the $[PSI^+]$ SV16 mutant strain (data not shown).

Loss of *CTR9* Alters *Sup35p* Aggregates

In $[PSI^+]$ cells, Sup35p is aggregated in a heritable, self-propagating conformation (Patino *et al.*, 1996; Paushkin *et al.*, 1996). One way mutants of *CTR9* could affect nonsense suppression in $[PSI^+]$ cells is by altering the expression or aggregation of Sup35p. To determine if the level of Sup35 protein was altered by *ctr9-C227Y* or $\Delta ctr9$, Sup35p levels in the SV16 and $\Delta ctr9$ strains were compared with a wild-type $[PSI^+]$ strain by Western blot. No change in the steady-state Sup35p level was detected between the wild-type and mutant strains (Figure 5A), indicating that the weakened nonsense suppression in the *CTR9* mutants cannot be explained simply by a difference in the amount of total Sup35p.

Another mechanism by which *ctr9* could weaken nonsense suppression in $[PSI^+]$ cells is by altering the aggregation of the Sup35p. Mutants of *CTR9* could alter the conformation of the aggregates, resulting in a change in the $[PSI^+]$ strain variant being propagated by the cells. In addition, the stability of the aggregates could be altered such that a smaller percentage of the Sup35p is aggregated, thereby generating a larger pool of soluble Sup35p for translation termination activity. We examined these possibilities by several complementary methods. First, we tested whether the Sup35p was still aggregated in the SV16 mutant by ectopically expressing the prion forming domain of Sup35 (NM) fused to GFP and visualizing the cells by direct fluorescence microscopy. NM-GFP gets incorporated into the preexisting Sup35p aggregates in $[PSI^+]$ cells, thereby acting as an indicator of the $[PSI]$ state (Patino *et al.*, 1996; Lindquist *et al.*, 2001). One to three large, bright foci were typically observed in cells when NM-GFP was expressed in strong and weak $[PSI^+]$ strains (Figure 5B). In contrast, no foci were present in $[psi^-]$ cells, and NM-GFP was diffuse in the cytoplasm. When NM-GFP was expressed in the SV16 mutant, foci were observed, indicating that Sup35p was still aggregated (Figure 5B). In some cells, the amount of diffuse NM-GFP in the cytoplasm appeared to be increased in the SV16 mutant strain as compared with the wild-type strong and weak $[PSI^+]$ strains. However, this was not uniform among all cells examined (data not shown).

Second, we determined if there was a gross change in the size or conformation of the Sup35p aggregates by SDD-AGE. The difference in size of the aggregates between prion strain variants can be detected as a change in electrophoretic mobility by SDD-AGE (Kryndushkin *et al.*, 2003). As seen previously (Kryndushkin *et al.*, 2003), Sup35p from a strong $[PSI^+]$ strain migrated slightly farther into the gel than Sup35p from a weak $[PSI^+]$ strain (Figure 5C). In the SV16 and $\Delta ctr9$ $[PSI^+]$ strains, the smear of Sup35p aggregates migrated even farther into the gel than strong $[PSI^+]$, and

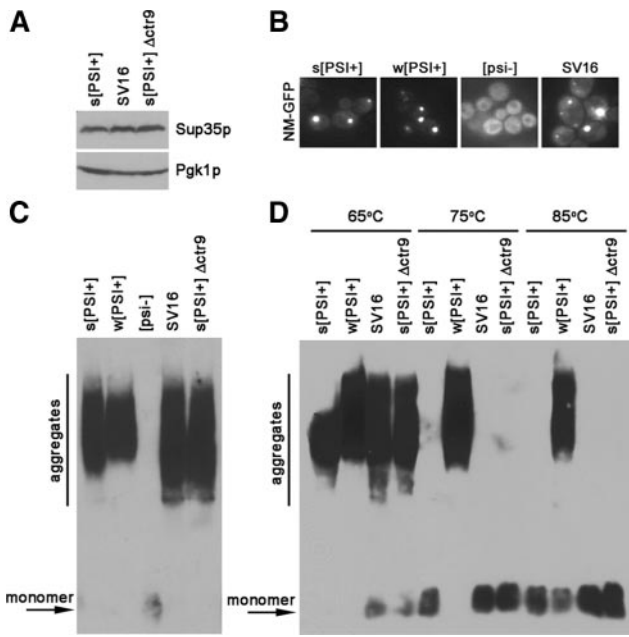


Figure 5. Sup35p aggregates in SV16 and $\Delta ctr9$ are similar to those of strong [PSI+]. (A) Mutations in *CTR9* do not alter the steady-state expression level of Sup35p. Equal quantities of protein were loaded on a SDS-polyacrylamide gel, transferred to PVDF, and immunoblotted with antibodies recognizing Sup35p or Pgf1p. Pgf1p was used as a control for equal loading. (B) NM-GFP aggregates in the SV16 strain. NM-GFP was expressed in the indicated strains and visualized by direct fluorescence microscopy after a 6-h induction with 60 μ M copper sulfate. (C) Sup35p aggregates in SV16 and $\Delta ctr9$ strains migrate as a broader smear compared with those of strong [PSI+]. Equal amounts of protein were analyzed by SDD-AGE and immunoblotting with antibodies recognizing Sup35p. The positions of monomeric and aggregated Sup35p are indicated to the left of the blot. (D) Sup35p aggregates in SV16 and $\Delta ctr9$ strains exhibit thermal stability similar to those from a wild-type strong [PSI+] strain. SDD-AGE was performed as in C except the lysates were heated at 65, 75, or 85°C before loading on the gel. (A–D) s[PSI+] and w[PSI+], strong and weak [PSI+] strains, respectively.

the smear covers a slightly broader range (Figure 5C). In addition, a small amount of monomeric Sup35p was sometimes detected in the SV16 and $\Delta ctr9$ mutants but not in wild-type [PSI+] (data not shown). To further investigate a possible structural change in the Sup35p aggregates in the mutants, we examined their thermal stability. Protein lysates from strong [PSI+], weak [PSI+], SV16, and $\Delta ctr9$ were heated at various temperatures before SDD-AGE (Figure 5D). At 65°C, the Sup35p aggregates appeared similar to those of the standard assay temperature of \sim 25°C, but some change in size or stability was beginning to be apparent for strong [PSI+], SV16, and $\Delta ctr9$ (compare 65°C in Figure 5D with standard assay in Figure 5C). At 75°C, aggregates from strong [PSI+], but not weak [PSI+], were broken down to monomer. By 85°C, aggregates from weak [PSI+] also began to break down to show some monomeric protein. In the SV16 and $\Delta ctr9$ mutant strains, the Sup35p aggregates broke down to monomer at 75°C, similar to the strong [PSI+] strain. Taken together with our genetic data, this suggests that although there may be a small effect on the size of the Sup35p aggregates in the population within the SV16 and $\Delta ctr9$ mutants, the heritable structure of the aggregates is not grossly changed from those of the original strong [PSI+] parental strain.

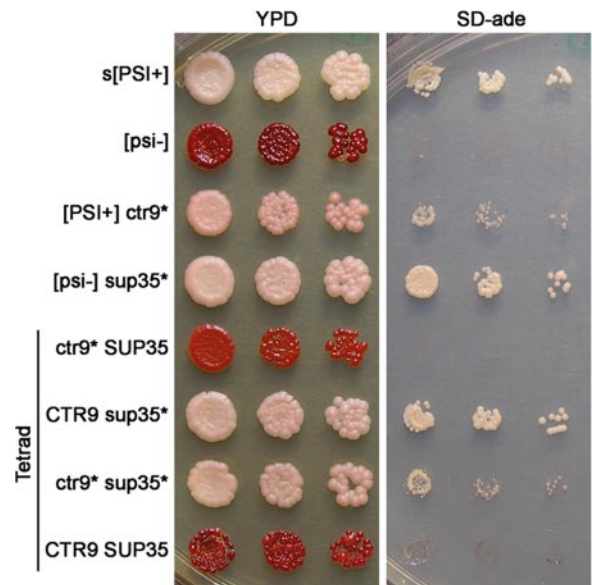


Figure 6. *ctr9-C227Y* reduces nonsense suppression of *ade1-14* in the absence of Sup35p aggregates. SV16 [PSI+] was crossed to [psi-] *sup35-Y351C*, and the resulting diploid was cured of [PSI+] by growth on media containing 3 mM GdnHCl before sporulation. Fivefold serial dilutions of yeast cultures at equal cell density were spotted on YPD and SD-ade. The top four rows show control strong (s) [PSI+] and [psi-] strains and the [PSI+] SV16 ([PSI+] *ctr9**) and [psi-] *sup35-Y351C* ([psi-] *sup35**) parental strains used to generate the diploid. The bottom four rows show a representative tetrad with genotype indicated on the left. Presence of the *ctr9-C227Y* and *sup35-Y351C* alleles is designated with an asterisk adjacent to the gene name. Wild-type alleles are designated in capital letters. All progeny are [psi-].

$\Delta ctr9$ Alters Both the Aggregation of Sup35p and Nonsense Suppression

Finally, we used genetic analyses to distinguish the effects of *CTR9* on the Sup35p aggregates in [PSI+] cells from those on translation termination and nonsense suppression. In [psi-] cells, mutations in the *SUP35* C-terminal domain that reduce function create a nonsense suppression phenotype that mimics that of [PSI+] cells (Wickner *et al.*, 1995). Furthermore, these Mendelian mutations cause inviability in combination with [PSI+] due to high levels of nonsense suppression (Cox, 1977; Liebman and All-Robyn, 1984; Zhou *et al.*, 1999). We crossed one of these mutants, [psi-] 74-D694 *sup35-Y351C*, to SV16, sporulated the diploid, and analyzed the tetrads. Only two haploids were recovered from each tetrad (data not shown), further supporting the cell biological and biochemical results that the SV16 mutant still contains the [PSI+] prion. When we cured the diploid cells of [PSI+] by passing them on media containing GdnHCl before sporulation, all four meiotic progeny were viable. Using these [psi-] progeny, we then asked whether the *ctr9-C227Y* mutant altered the nonsense suppression of a *sup35* genetic mutant. As shown by a representative tetrad in Figure 6, the *ctr9-C227Y sup35-Y351C* double mutant exhibited less nonsense suppression of the *ade1-14* allele than the *sup35-Y351C* mutant alone, suggesting that one way the *ctr9* mutant is promoting more efficient termination is by genetically modifying nonsense suppression. However, the effect of *ctr9-C227Y* on [PSI+] appeared to be stronger than on *sup35-Y351C*, because the difference in color between [PSI+] *ctr9-C227Y* and the parental [PSI+] strain is slightly greater than it is between the *ctr9-C227Y sup35-Y351C* and *sup35-*

Y351C strains (Figure 6, YPD). This is also consistent with our result that *ctr9* has some effect on the size of the Sup35p aggregates in *[PSI+]* (Figure 5C). This suggests that the loss of Ctr9p may affect *[PSI+]*-mediated phenotypes in multiple ways (see below). We next tested whether the effects of Ctr9 on nonsense suppression were specific to Sup35. The nonsense suppression phenotype resulting from mutations in *sup45* is also reduced in $\Delta ctr9$ strains (Supplemental Figure S2). The extent to which the loss of Ctr9 alters nonsense suppression of *sup45* mutants was not as striking as the effect on *[PSI+]*-mediated nonsense suppression.

Ctr9 Affects Transcript Processing and Translatability of mRNA

The *[PSI+]* prion causes suppression of all three stop codons in yeast (Tuite *et al.*, 1983; Firoozan *et al.*, 1991). We have shown that $\Delta ctr9$ weakens nonsense suppression of the *ade1-14* allele, which contains a premature UGA stop codon. To investigate whether the effect of *ctr9* on nonsense suppression is specific to UGA and the *ade1-14* allele, we expressed reporter constructs with each one of the stop codons fused in front of *lacZ* (Stansfield *et al.*, 1995a). If the stop codon is read through in these reporters, then β -galactosidase is produced. Each reporter was expressed in wild-type *[PSI+]*, *[psi-]*, and $\Delta ctr9$ *[PSI+]* strains. As expected, the strong *[PSI+]* strain exhibited a higher level of read-through of each stop codon than the isogenic *[psi-]* strain (Figure 7A). Furthermore, the weak *[PSI+]* strain produced a level of read-through that was intermediate to the strong *[PSI+]* and *[psi-]* strains. Deletion of *CTR9* in strong *[PSI+]* caused a reduction in *[PSI+]*-mediated nonsense suppression of all three stop codons (Figure 7A), further supporting the idea that $\Delta ctr9$ weakens nonsense suppression in general.

To investigate whether this reduction in read-through by $\Delta ctr9$ was specific to the prion, we introduced the same reporters into a *[psi-]* *sup35-Y351C* $\Delta ctr9$ double mutant strain along with each single mutant and the wild-type control. The effect on read-through was not entirely specific to the *[PSI+]* prion for two reasons. First, the *[psi-]* $\Delta ctr9$ mutant alone had significantly lower read-through than the *[psi-]* wild-type strain (Figure 7B; unpaired *t* test, $p < 0.0003$ for all stop codons), indicating that the loss of Ctr9p reduces basal read-through of UAA, UAG, and UGA stop codons. Second, the *sup35-Y351C* $\Delta ctr9$ double mutant also had significantly lower read-through of all three stop codons than the *sup35-Y351C* mutant alone (Figure 7B; unpaired *t* test, $p < 0.0024$ for all stop codons). Interestingly, $\Delta ctr9$ had a greater effect on *[PSI+]*-mediated read-through than on *sup35-Y351C*-mediated read-through for each of the stop codons (compare fold change in Figure 7, A and B). The effect is more striking on UAG and UGA than on UAA. This difference, along with the slight effect of $\Delta ctr9$ on the size of the Sup35p aggregates (Figure 5C), supports the idea that $\Delta ctr9$ affects *[PSI+]*-mediated nonsense suppression in multiple ways. Collectively, these results indicate that nonsense suppression of all three stop codons is reduced in the absence of Ctr9p.

Interestingly, we also noted a twofold reduction in the level of specific activity of β -galactosidase with the deletion of *ctr9* using the nonstop control *lacZ* constructs. This suggests that Ctr9 and the Paf1 complex effects transcript stability or translatability of the mRNA. Indeed, qRT-PCR confirmed a reduction in the steady-state level of nonstop *lacZ* mRNA (eightfold), and the reduction was verified by Northern blot (data not shown).

Next, we wanted to test if the level of *ade1-14* transcript was reduced, hence accounting for the decreased amount of

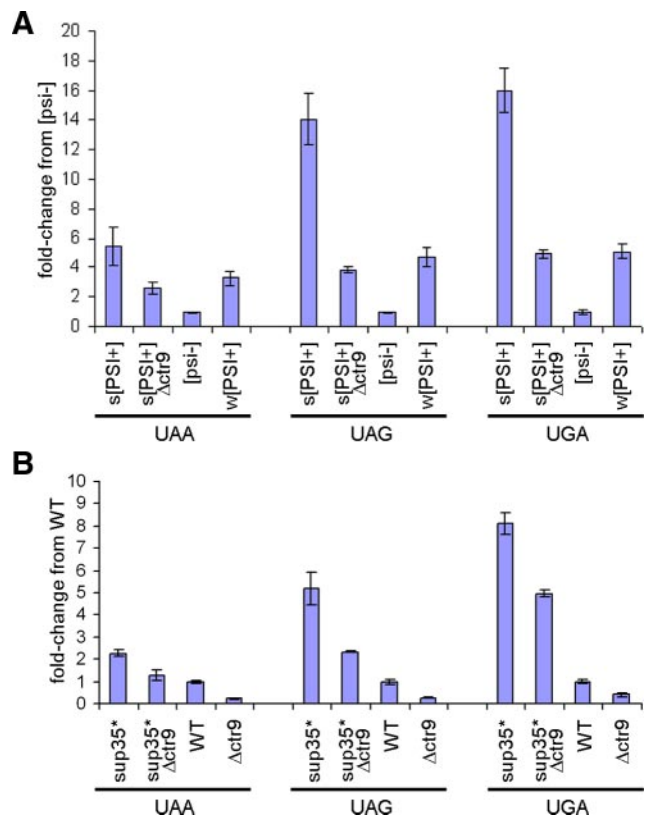


Figure 7. Deletion of *CTR9* reduces nonsense suppression of all three stop codons. Read-through of reporter constructs containing the indicated stop codon was assayed and expressed as the fold change of the *[psi-]* strain. Each bar in the graphs represents the mean and SD of three independent cultures assayed on the same day. Whereas the absolute fold change varied between days, the overall trend remained the same. (A) $\Delta ctr9$ reduces nonsense suppression by *[PSI+]* at each of the stop codons. s[*PSI+*] and w[*PSI+*], strong and weak *[PSI+]* strains, respectively. (B) $\Delta ctr9$ also reduces nonsense suppression by *sup35-Y351C* (designated sup35*). All strains are *[psi-]*. WT, wild-type.

Ade1 product and change in phenotype in $\Delta ctr9$. Curiously, we did not find a change in the steady-state levels of *ade1-14* transcript by Northern blot analysis (Figure 8A, top). Loading of RNA was assessed by methylene blue staining of the 18S and 25S rRNA (Figure 8A, bottom). Because there was no change in the levels of *ade1-14* transcript, another possibility that could account for the reduced amount of Ade1p in $\Delta ctr9$ would be a change in the translatability of the *ade1-14* mRNA. Supporting this hypothesis, a recent genome-wide analysis revealed that *ADE1* transcript was predicted to have two or more poly(A) addition sites (Nagalakshmi *et al.*, 2008). Because the Paf1C has been shown to play a role in posttranscriptional 3' end processing (Penheiter *et al.*, 2005; Nordick *et al.*, 2008), we wanted to test if the loss of *CTR9* could affect the 3' end processing of the *ade1-14* transcript and consequently affect its translatability. Using 3' rapid amplification of cDNA ends (RACE) analysis, we found that the shorter product of the *ade1-14* 3' end was the predominant isoform in $\Delta ctr9$ mutant as compared with a longer product in the wild-type strain (Figure 8B).

Phenotypic Effects of *ctr9* Are Not Limited to *ade1-14*

Isogenic *[PSI+]* and *[psi-]* yeast strains differ in numerous different growth phenotypes, many of which can be attrib-

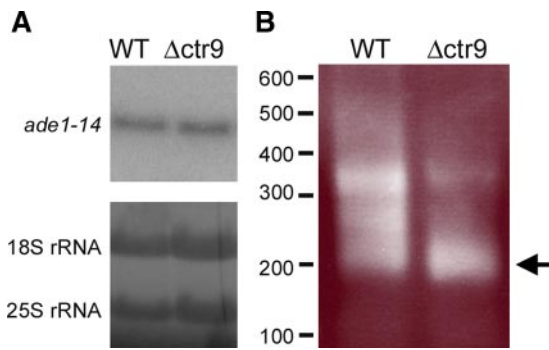


Figure 8. $\Delta ctr9$ alters the 3' processing of the *ade1-14* transcript. (A) Northern analysis of *ade1-14* transcript levels are shown (top). Equal amounts of total RNA was loaded on the gel, and this was visualized by methylene blue staining of 18S and 25S rRNA (bottom). One representative blot is shown. (B) 3' RACE analysis of total RNA isolated from wild-type (WT) and $\Delta ctr9$ strains. The *ade1-14* transcript has multiple isoforms in the WT and $\Delta ctr9$ strain. Arrow indicates the predominant isoform that accumulates in the $\Delta ctr9$ strain. The molecular-weight marker in base pairs is indicated on the left side of the gel.

uted to [PSI+]-mediated nonsense suppression (True and Lindquist, 2000; True *et al.*, 2004). We wanted to determine if the loss of Ctr9p affected any of these [PSI+]-dependent traits. Few strong [PSI+]-dependent phenotypes were previously reported in the 74-D694 strain background (True and Lindquist, 2000). However, many [PSI+]-dependent phenotypes were identified in the 5V-H19 and 10B-H49 yeast strain backgrounds (True and Lindquist, 2000). Therefore, we made deletions of *CTR9* in [PSI+] isolates of 5V-H19 and 10B-H49, cured the prior to obtain isogenic [psi-] $\Delta ctr9$ strains, and examined the strain pairs for the effect of $\Delta ctr9$ on some of the previously reported [PSI+]-dependent traits (True and Lindquist, 2000). Because $\Delta ctr9$ alone is sensitive to many different growth conditions and compounds (Betz *et al.*, 2002 and this study), we were limited in the number and range of phenotypes we could test.

Both 5V-H19 and 10B-H49 contain a premature UAA stop codon in *ADE2* (the *ade2-1* allele) that is suppressible by [PSI+]. Similar to our results with *ade1-14* in 74-D694, $\Delta ctr9$ weakened [PSI+]-mediated nonsense suppression of *ade2-1* in both 5V-H19 and 10B-H49 (data not shown). 10B-H49 also contains a [PSI+]-suppressible allele of *LYS1* (*lys1-1*), a component of the lysine biosynthetic pathway. 10B-H49 [psi-] cells grow poorly on media lacking lysine because they are unable to make enough full-length Lys1p. However, 10B-H49 [PSI+] cells make more Lys1p and can grow in the absence of lysine. When we deleted *CTR9* in the 10B-H49 [PSI+] strain, the colonies grew slower than the wild-type 10B-H49 [PSI+] strain on media lacking lysine (Figure 9A), suggesting $\Delta ctr9$ weakened the nonsense suppression of *lys1-1*. Another [PSI+]-dependent phenotype in 10B-H49 is resistance to cycloheximide. However, the gene target(s) of [PSI+] that contribute to this phenotype are not yet known. 10B-H49 [PSI+] cells grow better than isogenic [psi-] cells in the presence of 0.2 μ g/ml cycloheximide (True and Lindquist, 2000 and Figure 8A). Surprisingly, the [psi-] $\Delta ctr9$ strain was more resistant to cycloheximide than the wild-type [psi-] strain (Figure 9A).

Invasive growth and flocculation are two related [PSI+]-dependent phenotypes in 5V-H19 (True and Lindquist, 2000 and Figure 8B). [PSI+] cells invade the agar during growth on solid media and flocculate in liquid media, whereas [psi-] cells do not invade the agar and do not flocculate. When we deleted *CTR9* from 5V-H19 [PSI+] cells, the cells invaded the agar much less than the wild-type [PSI+] strain on solid media and no longer flocculated in liquid media (Figure 9B and data not shown). Another [PSI+]-dependent trait in 5V-H19 that may be related to the flocculation and invasive growth phenotypes is a change in colony morphology. [PSI+] cells produce large colonies with a puckered and dimpled surface when grown on media containing 3% potassium acetate as the sole carbon source (True and Lindquist, 2000). In contrast, [psi-] cells produce smaller colonies with a smooth, rounded surface. The genes influenced by [PSI+] that are associated with this phenotype are unknown. However, the phenotype can be at least partially

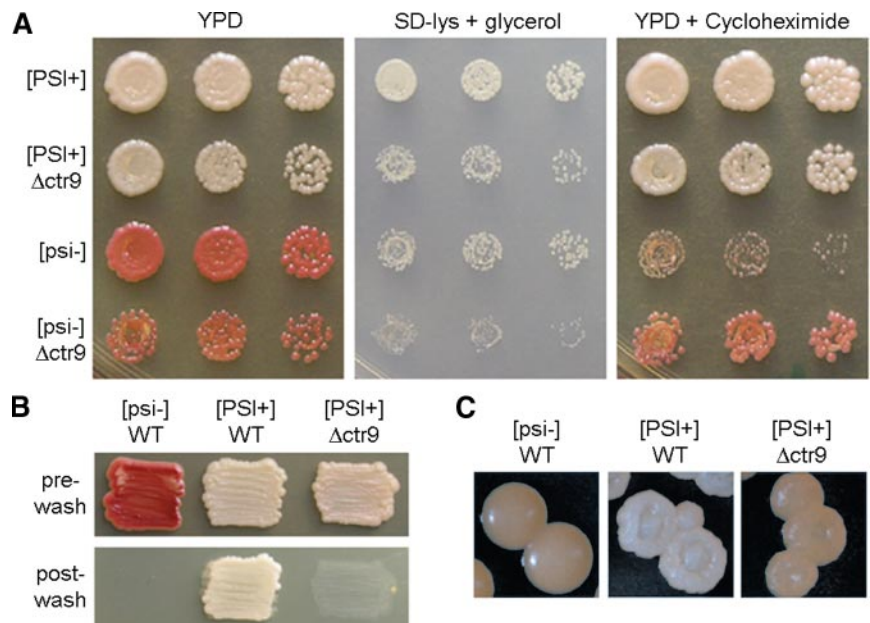


Figure 9. $\Delta ctr9$ modifies [PSI+]-dependent phenotypes. (A) *CTR9* was deleted in [PSI+] and [psi-] derivatives of 10B-H49 and assayed for nonsense suppression of the *lys1-1* allele on SD-lys + glycerol and for the alteration of [PSI+]-dependent cycloheximide resistance (YPD + 0.2 μ g/ml cycloheximide). (B) Wild-type (WT) [psi-], wild-type [PSI+], and [PSI+] $\Delta ctr9$ derivatives of 5V-H19 were grown on YPD (pre-wash) and assayed for invasive growth by washing the plate with distilled water (post-wash). (C) Colony morphology of the same strains as B grown on media containing potassium acetate as the sole carbon source. The puckered colony morphology was reduced in strong [PSI+] $\Delta ctr9$ but not completely lost.

attributed to nonsense suppression (H. L. True, unpublished data). Loss of Ctr9p in the [PSI⁺] strain caused the cells to revert to a more [psi⁻]-like appearance (Figure 9C). Collectively, these results show that $\Delta ctr9$ modifies multiple [PSI⁺]-dependent phenotypes.

DISCUSSION

We identified Ctr9p, a component of Paf1C, in a screen for factors that weaken [PSI⁺]-mediated nonsense suppression. Furthermore, we show that other members of the complex also affect [PSI⁺]-mediated nonsense suppression. In addition to Ctr9p and Paf1p, the complex is composed of Cdc73p, Leo1p, and Rtf1p (Koch *et al.*, 1999; Krogan *et al.*, 2002; Mueller and Jaehning, 2002; Squazzo *et al.*, 2002). Paf1C is conserved from yeast to humans (Rozenblatt-Rosen *et al.*, 2005; Zhu *et al.*, 2005). However, the complex in humans does not appear to contain Rtf1p even though a homolog exists. We show that the loss of Paf1C weakens nonsense suppression and is a genetic modifier of [PSI⁺]-dependent phenotypes. How might Paf1C affect nonsense suppression? One possibility is that Paf1C is affecting transcript levels via initiation or elongation by RNA polymerase II. Paf1C is associated with RNA polymerase II from the promoters to the polyadenylation (polyA) sites of actively transcribed genes and is involved in the transcription and posttranscriptional processing of some mRNAs (Shi *et al.*, 1996; Wade *et al.*, 1996; Krogan *et al.*, 2002; Pokholok *et al.*, 2002; Squazzo *et al.*, 2002; Mueller *et al.*, 2004; Rondon *et al.*, 2004; Penheiter *et al.*, 2005; Nordick *et al.*, 2008). Paf1C has been reported to influence the efficiency of transcription initiation and elongation (Pokholok *et al.*, 2002; Squazzo *et al.*, 2002; Rondon *et al.*, 2004). However, it is unclear whether Paf1C directly affects these processes (Mueller *et al.*, 2004; Penheiter *et al.*, 2005). Paf1C is required for methylation of histone H3 (Krogan *et al.*, 2003a,b; Ng *et al.*, 2003), monoubiquitylation of histone H2B (Xiao *et al.*, 2005), 3' end formation of snoRNAs (Sheldon *et al.*, 2005), and 3' end formation of mRNAs in determining polyA site usage and polyA tail length (Penheiter *et al.*, 2005). Recent work suggests that the influence of Paf1C on some histone modifications is likely to be an indirect consequence of reduced methyltransferase recruitment to RNA polymerase II in Paf1C mutants (Nordick *et al.*, 2008).

The function of Paf1C in 3' end formation of mRNAs is just beginning to be unraveled. Loss of Paf1C components leads to altered transcripts due to the change in usage of more distal polyA sites (Penheiter *et al.*, 2005; Nordick *et al.*, 2008). This effect on 3' end formation has been characterized for specific transcripts (Penheiter *et al.*, 2005; Nordick *et al.*, 2008), but it is likely that more targets exist (Mozdy *et al.*, 2008). Links between Paf1C and other factors that affect posttranscriptional 3' end formation have recently been identified. Paf1C is required for the recruitment of cleavage and polyadenylation factors Cft1p and Pcf11p to RNA polymerase II (Mueller *et al.*, 2004; Nordick *et al.*, 2008). It was also shown that Paf1C directly binds Cft1p (Nordick *et al.*, 2008).

One explanation for the influence of Paf1C on the phenotypic effects of [PSI⁺]-mediated nonsense suppression is via its effects on posttranscriptional 3' end formation. Loss of Paf1C produces transcripts that have an altered 3' end (Penheiter *et al.*, 2005; Nordick *et al.*, 2008). The 3'-extended MAK21 transcripts in $\Delta paf1$ cells were targeted for degradation by the nonsense-mediated decay pathway, causing a reduction in the steady-state level of MAK21 transcript (Penheiter *et al.*, 2005). This is likely to be true of additional

targets of Paf1C; transcripts altered in 3' end position and polyadenylation can be targeted for rapid degradation by the nonsense mediated decay pathway (Muhlrad and Parker, 1999). If the transcripts subjected to [PSI⁺]-mediated read-through are also targets of Paf1C-mediated processing, then we would expect that the loss of Paf1C components could lead to 3'-extended transcripts that are unstable. A smaller pool of transcript should result in less overall substrate for translation and translational read-through in [PSI⁺] cells that would thereby alter [PSI⁺]-mediated phenotypes. However, a change in the 3' end processing may not alter transcript stability but may change translatability of the message. Indeed, the 3' untranslated region has been shown to control polyadenylation of individual transcripts and translational efficiency (Beilharz and Preiss, 2007). A recent genome-wide study mapping transcribed regions in *S. cerevisiae* identified 540 genes that utilize more than one polyA site (Nagalakshmi *et al.*, 2008). Interestingly, two sites of polyA tail addition were identified for ADE1. Our results demonstrate that individual deletions of CTR9, PAF1, RTF1, and CDC73 weaken [PSI⁺]-mediated nonsense suppression of the *ade1-14* allele. This may be a consequence of a change in the utilization of the two polyA tail sites in ADE1. In support of this, we detected a significant and reproducible change in the ratio of the most abundant species of *ade1-14* transcript in $\Delta ctr9$ compared with the wild-type strain by 3'RACE. Interestingly, this change did not correspond to a reduced steady-state level of *ade1-14* mRNA in cells deleted for *ctr9*. This suggests that there may be multiple ways by which the Paf1 complex influences mRNA translatability.

We identified four other [PSI⁺]-dependent phenotypes (cycloheximide resistance, flocculation, invasive growth, and colony morphology) that $\Delta ctr9$ modifies. Because the loss of Ctr9p results in many drug sensitivities (Betz *et al.*, 2002 and this study), we speculate that more slight alterations in the regulation of Paf1C might affect many other [PSI⁺]-dependent phenotypes. The genes involved in many [PSI⁺]-mediated phenotypes have not yet been identified. The cycloheximide-resistance phenotype is particularly interesting because [PSI⁺] grows better than [psi⁻], yet $\Delta ctr9$ causes the [psi⁻] cells to become more resistant. This suggests that producing less of a specific transcript increases resistance to cycloheximide. If the ribosome reads through the normal stop codon in [PSI⁺] cells and reaches the 3' end of the transcript without encountering another stop codon, this would trigger nonstop decay. In [psi⁻] cells, the transcript would be stable and translated, resulting in cycloheximide sensitivity. Loss of CTR9 in [psi⁻] cells could provide a different mechanism to destabilize the transcript and make cells more resistant to cycloheximide. Precedence exists for changes in nonstop decay modifying [PSI⁺]-dependent phenotypes (Wilson *et al.*, 2005). Moreover, Paf1C itself may affect nonstop decay. The human PAF1C coimmunoprecipitates with the human SKI complex that mediates nonstop decay (Zhu *et al.*, 2005). Furthermore, human PAF1C is required for the recruitment of the SKI complex to transcriptionally active genes (Zhu *et al.*, 2005). Thus, different mechanisms to alter transcript stability could result in similar phenotypic consequences.

The strain 5V-H19 shows a [PSI⁺]-dependent increase in flocculation and invasive growth. Flocculation and invasive growth in yeast are regulated by the transcription factor FLO8 (Kobayashi *et al.*, 1996, 1999; Liu *et al.*, 1996). FLO8 is inactivated in many laboratory strains by a premature stop codon (Liu *et al.*, 1996). However, FLO8 is not mutated in the 5V-H19 strain background (H. L. True, unpublished data) and is, therefore, not a likely target of read-through in 5V-

H19 to produce the [PSI+]-dependent flocculation and invasive growth phenotypes. Loss of Paf1C reduces the effect of [PSI+] on flocculation and invasive growth. Interestingly, Paf1C affects the modification of histones at *FLO8*. Trimethylation of lysine 36 on histone 3 at *FLO8* is reduced in $\Delta paf1$ and $\Delta ctr9$ mutants and acetylation of histones 3 and 4 on the 3' end of *FLO8* is increased in $\Delta paf1$ and $\Delta ctr9$ mutants (Chu *et al.*, 2007). Although the genes involved in the [PSI+]-mediated changes in flocculation and invasive growth are unknown, it is plausible to speculate that [PSI+] and $\Delta ctr9$ affect different genes in the flocculation and invasive growth pathways. However, for other phenotypes, both [PSI+] and Paf1C may affect the translation of the same transcript.

Transcriptional read-through of proximal polyA sites presumably occurs at a low rate. However, the use of alternative transcription termination sites may have biological functions, including the regulation of viral gene expression (Bousse *et al.*, 2002; Poenisch *et al.*, 2008). We describe another scenario, one in which changes in posttranscriptional processing modifies several [PSI+]-dependent phenotypes in yeast. The effect of Paf1C and 3' end formation on translational nonsense suppression adds to the growing list of posttranscriptional processes that genetically modify [PSI+]-dependent traits. These links between transcriptional and translational control highlight the potential continuum for phenotypic diversity. If [PSI+]-mediated read-through provides an advantage to the organism whereby a phenotype can be altered in response to environmental changes, the ability to fine-tune the traits during transitions may enhance the organism's ability to adapt and survive. We have previously shown that it is possible to fix traits and become [PSI+]-independent (True *et al.*, 2004). However, for an organism that must thrive in a fluctuating environment, fixation may not always be beneficial. Given that [PSI+]-mediated phenotypes are largely multifactorial, we postulate that there may be other global mechanisms for modulating the spectrum of phenotypes. One such mechanism may involve posttranscriptional processing through Paf1C.

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