A fungal anticodon nuclease ribotoxin exploits a secondary cleavage site to evade tRNA repair

BIRTHE MEINEKE,[1,4] ALENE KAST,[2,4] BEATE SCHWER,[3] FRIEDHELM MEINHARDT,[2] STEWART SHUMAN,[1,6] and ROLAND KLASSEN[2,5,6]

[1]Molecular Biology Program, Sloan-Kettering Institute, New York, New York 10065, USA
[2]Institut für Molekulare Mikrobiologie und Biotechnologie, Westfälische Wilhelms-Universität Münster, D-48149 Münster, Germany
[3]Department of Microbiology and Immunology, Weill Cornell Medical College, New York, New York 10065, USA

ABSTRACT

PaOrf2 and γ-toxin subunits of *Pichia acaciae* toxin (PaT) and *Kluyveromyces lactis* zymocin are tRNA anticodon nucleases. These secreted ribotoxins are assimilated by *Saccharomyces cerevisiae,* wherein they arrest growth by depleting specific tRNAs. Toxicity can be recapitulated by induced intracellular expression of PaOrf2 or γ-toxin in *S. cerevisiae*. Mutational analysis of γ-toxin has identified amino acids required for ribotoxicity in vivo and RNA transesterification in vitro. Here, we report that PaOrf2 residues Glu9 and His287 (putative counterparts of γ-toxin Glu9 and His209) are essential for toxicity. Our results suggest a similar basis for RNA transesterification by PaOrf2 and γ-toxin, despite their dissimilar primary structures and distinctive tRNA target specificities. PaOrf2 makes two sequential incisions in tRNA, the first of which occurs 3′ from the mcm^{5}s^{2}U wobble nucleoside and depends on mcm^5. A second incision two nucleotides upstream results in the net excision of a di-nucleotide. Expression of phage and plant tRNA repair systems can relieve PaOrf2 toxicity when tRNA cleavage is restricted to the secondary site in *elp3* cells that lack the mcm^5 wobble U modification. Whereas the endogenous yeast tRNA ligase Trl1 can heal tRNA halves produced by PaOrf2 cleavage in *elp3* cells, its RNA sealing activity is inadequate to complete the repair. Compatible sealing activity can be provided in *trans* by plant tRNA ligase. The damage-rescuing ability of tRNA repair systems is lost when PaOrf2 can break tRNA at both sites. These results highlight the logic of a two-incision mechanism of tRNA anticodon damage that evades productive repair by tRNA ligases.

Keywords: RNA repair; anticodon nuclease; mcm^5; tRNA; toxin

INTRODUCTION

Infliction of tRNA damage by site-specific endoribonucleases (''ribotoxins'') underlies cellular stress responses and self-nonself discrimination in a wide range of microbial taxa. tRNA ribotoxins incise the tRNA anticodon loop to leave 2′,3′-cyclic phosphate and 5′-OH ends at the break. Anticodon breakage inhibits protein synthesis via depletion of the functional pool of specific tRNA isoacceptors, which, in turn, results in growth arrest or cell death.

The secreted eukaryal tRNA ribotoxins zymocin and PaT are elaborated by cytoplasmic linear DNA plasmids resident in the fungi *Kluyveromyces lactis* and *Pichia acaciae*, respectively (Klassen et al. 2004, 2008a; Lu et al. 2005). Zymocin is a heterotrimer of α, β, and γ subunits; PaT is a heterodimer of PaOrf1 and PaOrf2 subunits (Stark and Boyd 1986; McCracken et al. 1994; Klassen et al. 2004). Zymocin and PaT arrest growth of the nonself yeast species *Saccharomyces cerevisiae* by exposure from without. The α and β subunits of zymocin and the PaOrf1 subunit of PaT interact with the target cell surface to effect the transport of the γ subunit and the PaOrf2 subunit (the anticodon endonuclease enzymes) into the cytoplasm of the target cell (Jablonowski et al. 2004; Jablonowski and Schaffrath 2007; Meinhardt and Klassen 2009). Toxicity can also be achieved from within by induced intracellular expression in *S. cerevisiae* of just the γ-toxin or PaOrf2 subunits (minus their N-terminal signal peptides) (Butler et al. 1991; Klassen et al. 2004).

γ-toxin is an anticodon nuclease that cleaves its in vivo target tRNA^{Glu} at a single phosphodiester 3′ from the modified wobble base mcm^{5}s^{2}U (5-methoxycarbonylmethyl-2-thiouridine) of the UUC anticodon, leaving 2′,3′-cyclic

[4]These authors contributed equally to this work.
[5]**Present address:** Department of Biochemistry and Molecular Biology, University of Texas Medical Branch, Galveston, TX 77555, USA
[6]**Corresponding authors**
E-mail s-shuman@ski.mskcc.org
E-mail roklasse@utmb.edu
Article published online ahead of print. Article and publication date are at http://www.rnajournal.org/cgi/doi/10.1261/rna.034132.112.

phosphate and 5′-OH ends at the break. γ-toxin can also cleave the two other yeast tRNAs ($tRNA^{Gln}$ and $tRNA^{Lys}$) that have an mcm^5s^2U wobble base, albeit less efficiently than it incises $tRNA^{Glu}$. The wobble modification is required for tRNA cleavage by γ-toxin; yeast mutants that lack any of the enzymes responsible for synthesizing the mcm^5 moiety are resistant to zymocin (Frohloff et al. 2001; Huang et al. 2005; Lu et al. 2005; Jablonowski et al. 2006; Huang et al. 2008). The latter includes the Elongator subunits (Elp1-6) and Elongator interacting proteins Kti11, Kti12, and Kti13, as well as the methylase complex Trm9-Trm112 and the phosphatase Sit4 and kinase Kti14 (Jablonowski et al. 2004; Jablonowski and Schaffrath 2007; Huang et al. 2008; Studte et al. 2008; Mehlgarten et al. 2009). Individual loss of the wobble uridine 2-thio modification of $tRNA^{Glu}$ provides only partial zymocin resistance and weak cleavage stimulation in vitro (Huang et al. 2008; Lu et al. 2005), indicating the mcm^5 side chain, but not the 2-thio group of mcm^5s^2U, to be crucial for tRNA cleavage by γ-toxin.

PaOrf2 cleaves the same three yeast mcm^5s^2U-containing tRNAs in vitro, though its toxicity in vivo is attributed to specific cleavage and depletion of $tRNA^{Gln}$ (Klassen et al. 2008a). Whereas PaOrf2 and γ-toxin are functionally analogous and have overlapping tRNase activities, PaOrf2's cytotoxicity in *S. cerevisiae* is not stringently dependent on the presence of the mcm^5-modified wobble uridine in the target tRNA (Klassen et al. 2008a). In the absence of mcm^5, PaOrf2 can cleave $tRNA^{Gln}_{(UUG)}$ at an alternative phosphodiester upstream of the wobble U34 nucleoside, a property that is not shared with γ-toxin (Klassen et al. 2008a). Interestingly, PaOrf2 is unable to cleave its substrates when the wobble uridine methylase Trm9 (part of a complex with Trm112) is lacking (Klassen et al. 2008a). Since Trm9-Trm112 catalyzes the last step of mcm^5/mcm^5s^2 biosynthesis (Kalhor and Clarke 2003; Mazauric et al. 2010; Chen et al. 2011), and *trm9* mutants accumulate ncm^5s^2 (5-carbamoylmethyl-2-thiouridine) instead of mcm^5s^2 (Chen et al. 2011), it appears that this moiety is inhibitory for PaT.

A remarkable feature of the two known fungal tRNA anticodon nucleases is that, at first glance, they have no discernible primary structure similarity to one another (via BLAST queries), to any of the bacterial tRNA ribotoxins (PrrC, colicin D, colicin E5, VapC), or to any known ribonucleases or phosphotransferases. Alignment of the protein sequences in search of a structural or evolutionary connection between PaOrf2 and γ-toxin is now aided by the identification of two other putative plasmid-encoded fungal ribotoxins (PiT of *Pichia inositovora* and DrT of *Debaryomyces robertsiae*) and by the results of an extensive mutational analysis of γ-toxin that identified an ensemble of amino acids required for toxicity in vivo and anticodon nuclease activity in vitro (Jablonowski et al. 2006; Keppetipola et al. 2009; Jain et al. 2011).

The PiT toxin subunit (PiOrf4) shows weak primary structure similarity to γ-toxin (Klassen and Meinhardt 2003) but none to PaOrf2. The DrT toxin subunit (DrOrf3) shows strong similarity to PaOrf2 but none to γ-toxin (Klassen et al. 2004). An alignment of all four fungal polypeptides revealed only eight positions of side chain identity (exclusive of the N-terminal methionine of the signal peptide) (Fig. 1). Two of these correspond to γ-toxin Glu9 and His209, which are essential for toxicity and are the imputed general acid-base catalysts of RNA transesterification at the wobble uridine (Jablonowski et al. 2006; Jain et al. 2011). Here, we used the alignment in Figure 1 to guide a mutational analysis of PaOrf2 and thereby identified five side chains that are critical for toxicity in vivo, including the conserved glutamate and histidine residues. These results hint at a common chemical mechanism of RNA incision by the fungal anticodon nucleases.

We also queried the rationale for the capacity of PaOrf2 to break its tRNA target at more than one site in the anticodon loop. A growing body of evidence highlights the existence of RNA repair systems in diverse taxa that are capable of sealing broken RNAs with 2′,3′-cyclic phosphate and 5′-OH ends (Amitsur et al. 1987; Martins and Shuman 2004, 2005; Nandakumar et al. 2008; Chan et al. 2009; Tanaka et al. 2011). In some cases, it is clear that the biological role of RNA repair is to "cure" ribotoxic tRNA damage. For example, during bacteriophage T4 infection of *Escherichia coli*, the viral enzymes Pnkp and Rnl1 repair a 2′,3′-cyclic phosphate/5′-OH "nick" in the anticodon loop of $tRNA^{Lys}$, inflicted by virus-activation of the host ribotoxin PrrC (Amitsur et al. 1987). The budding yeast *S. cerevisiae* has an RNA repair enzyme, Trl1, that is responsible for joining the 2′,3′-cyclic phosphate and 5′-OH exons (broken in the anticodon loop) during tRNA splicing. Trl1 executes RNA repair via end-healing and end-sealing steps catalyzed by its three enzymatic domains (Apostol et al. 1991; Sawaya et al. 2003). Therefore, the toxicity of the fungal tRNA anticodon nucleases to *S. cerevisiae* must rely on either (1) an innate inability of Trl1 to repair the broken tRNA ends generated by the fungal ribotoxin, or (2) the rate and extent of tRNA damage greatly exceeding those of the endogeneous tRNA repair enzymes. There is good evidence in favor of the first scenario, insofar as (1) *S. cerevisiae* cells are susceptible to γ-toxin because the sealing domain of yeast Trl1 is unable to seal a break at the modified wobble base of $tRNA^{Glu}$, and (2) the cytotoxicity of γ-toxin in *S. cerevisiae* can be ameliorated by expressing heterologous RNA repair enzymes from plant or bacteriophage T4 (Nandakumar et al. 2008). These findings underscored how the *K. lactis* ribotoxin exploits an Achilles' heel in the target cell's tRNA repair system by specifically nicking its target flanking a "lesion" (the modified wobble base) that impedes the endogenous repair system.

What about the *Pichia acaciae* ribotoxin? An appealing hypothesis is that dual cleavage of a tRNA molecule by PaOrf2 would result in the excision of a di-nucleotide,

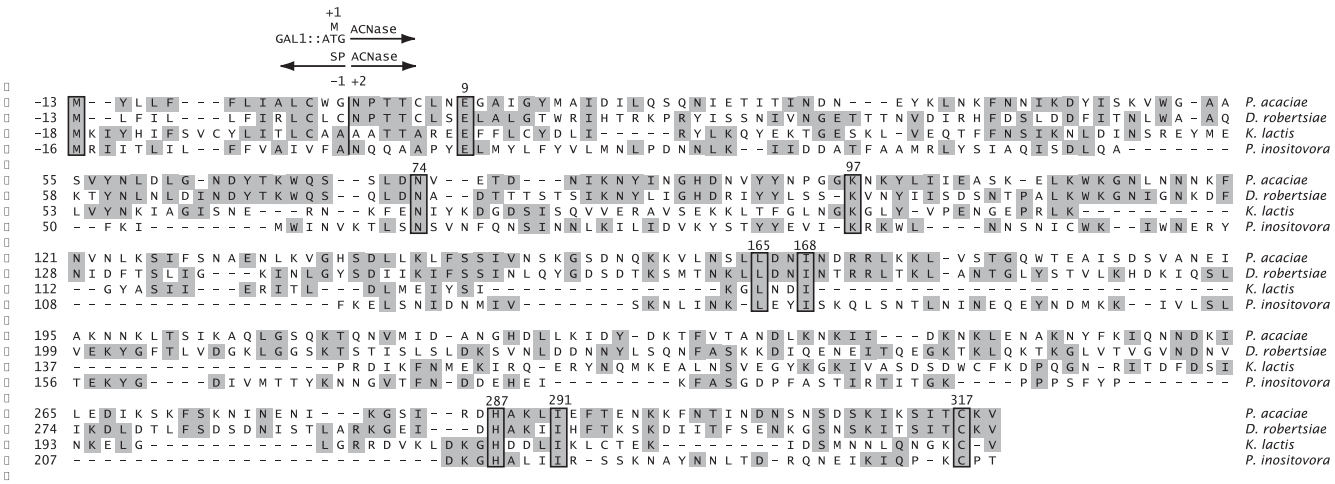

FIGURE 1. Alignment of putative ACNase subunits of killer toxins from *Pichia acaciae, Debaryomyces robertsiae, Kluyveromyces lactis,* and *Pichia inositovora*. For the multiple alignment, ClustalV (PAM250) was applied. Positions which have the same amino acid in at least two of the four proteins are shaded gray. Positions of side chain identity in all four proteins are shown in boxes. Negative positions represent the predicted signal peptide of each protein; position +1 accounts for the methionine added to achieve intracellular expression of variants lacking the signal peptide. Numbering of conserved residues refers to the intracellular version of PaOrf2-(1-319). NCBI GenBank accession numbers: *P. acaciae* pPac1-2 ORF2, CAE84960.1; *D. robertsiae* pWR1A ORF3, CAE84956.1; *K. lactis* pGKL1 ORF4, YP_001648056.1; *P. inositovora* pPin1-3 ORF4, CAD91887.1.

thereby rendering the damage functionally irreversible, because repair of such ends (if it occurred) would yield an inactive tRNA with a 2-nt deletion in the anticodon loop. Here, we flesh out this model by (1) showing that PaOrf2 does perform sequential cleavages at two sites in a tRNA and (2) testing the ability of Trl1 and plant and phage RNA repair enzymes to protect against PaOrf2 toxicity under conditions that favor nicking versus excision of the anticodon loop.

RESULTS

Phylogenetically guided mutational analysis of the PaOrf2 ribotoxin

Alignment of the γ-toxin, PaOrf2, PiOrf4, and DrOrf3 polypeptides highlighted eight positions of side chain identity, corresponding to Glu9, Asn74, Lys97, Leu165, Ile168, His287, Ile291, and Cys317 in the PaOrf2 protein (Fig. 1). To check the relevance of these eight conserved residues for PaOrf2 toxicity in vivo, we mutated them individually to alanine and conditionally expressed mutant and wild-type *PaORF2* in *S. cerevisiae* under the control of a *GAL* promoter, which is repressed by glucose and induced by galactose. Whereas cells carrying the wild-type *PaORF2* plasmid grew as well on glucose-containing medium as control cells carrying the empty vector, induced expression of wild-type *PaORF2* inhibited cell growth on galactose medium (Fig. 2). Induced expression of *PaORF2-Ala* variants revealed that the E9A, L165A, I168A, H287A, and I291A variants lost their toxicity, while N74A, K97A, and C317A remained toxic (Fig. 2). The equivalents of PaOrf2 Glu9 and His287 in γ-toxin (Glu9 and His209) are imputed to be active site residues, at which even conservative substitutions led to a loss of toxicity in vivo and ablation of anticodon RNase activity in vitro (Jain et al. 2011). Here, we substituted PaOrf2 Glu9 with glutamine and aspartate and His287 with glutamine and asparagine and checked the effect on the in vivo activity of PaOrf2. The E9Q, E9D, H287Q, and H287N mutants were nontoxic

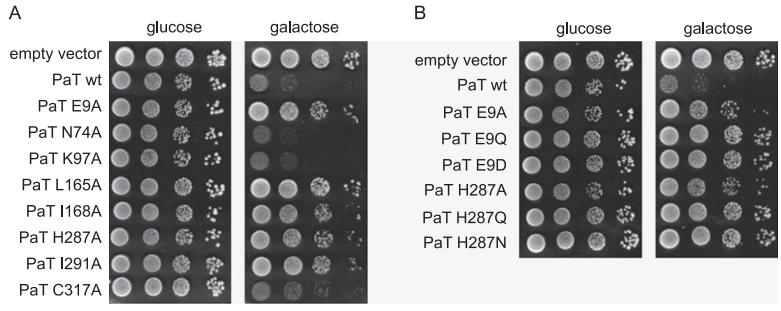

FIGURE 2. Mutational effects on PaOrf2 toxicity. (*A*) Alanine substitutions of residues conserved among putative fungal ACNases. (*B*) Conservative substitutions of Glu9 and His287 (putative counterparts of γ-toxin Glu9 and His209). Expression vectors carrying the indicated *PaORF2* alleles were transformed into *S. cerevisiae*. Serial dilutions of the plasmid-bearing yeast cells were spotted on agar medium containing 2% glucose or galactose. All mutant variants except C317A were expressed from 2μ plasmids. The C317A variant was expressed from a *CEN* plasmid. The plates were photographed after incubation at 30°C for 3 d.

(Fig. 2). Thus, five of the eight residues targeted, including those directly involved in the proposed transesterification mechanism of γ-toxin (Jain et al. 2011), were essential for PaOrf2 function, suggesting similarities in the γ-toxin and PaOrf2 active sites.

Analysis of in vitro tRNA cleavage by PaOrf2

We showed previously that PaOrf2 cleaves $tRNA^{Gln}_{(UUG)}$, $tRNA^{Glu}_{(UUC)}$, and $tRNA^{Lys}_{(UUU)}$ in vitro, even though $tRNA^{Gln}_{(UUG)}$ is the main target in vivo (Klassen et al. 2008a). Loss of the mcm^5 modification at U_{34} in an *elp3* mutant strain confers resistance to γ-toxin, which correlates with a lack of detectable tRNA cleavage in vivo in *elp3* cells expressing γ-toxin and with a ~100-fold reduction in γ-toxin-induced cleavage in vitro of tRNA lacking the mcm^5 wobble modification (Huang et al. 2005; Lu et al. 2005). In contrast, PaOrf2 is able to cleave hypomodified tRNA from *elp3* yeast in vivo and in vitro. Mapping the site of in vitro cleaved $tRNA^{Gln}$ from *elp3* cells revealed the incision to occur at a position 2 nt upstream of the wobble nucleoside (Klassen et al. 2008a). PaOrf2 cleavage of tRNA from wild-type cells yields two different 5′ cleavage products, corresponding to $N_{1–32}$ and $N_{1–34}$ of $tRNA^{Gln}$, suggesting that there are two alternative sites of incision, U_{34} and U_{32} (Klassen et al. 2008a). It was unclear if one of these sites is cleaved preferentially and whether PaT is capable of catalyzing an excision of 2 nt from the anticodon loop by double cleavage of the same tRNA molecule.

Here, we analyzed the temporal order of the incisions of the anticodon loop. Total yeast tRNA was incubated with recombinant PaOrf2 (Klassen et al. 2008a), and aliquots were removed and quenched at intervals of 30 min. $tRNA^{Gln}$ 5′ half-molecules were detected by Northern analysis using a probe that hybridizes to the 5′ end of $tRNA^{Gln}$ (Fig. 3A). (In vitro transcribed $tRNA^{Gln}$ half-molecules corresponding to nt 1–37, 1–35, and 1–33 served as standards.) After 30 min, there was a reduction in signal strength for full-length $tRNA^{Gln}$, concomitant with the appearance of signals for the $N_{1–32}$ and $N_{1–34}$ cleavage products. By 60 and 90 min, full-length $tRNA^{Gln}$ had declined further, while the $N_{1–34}$ to $N_{1–32}$ products persisted (Fig. 3A).

Northern blot analysis of the kinetics of PaOrf2 incision $tRNA^{Glu}$ in vitro revealed that the larger $N_{1–34}$ cleavage product appeared prior to the smaller $N_{1–32}$ product, indicating a preference for PaOrf2 to cleave $tRNA^{Glu}$ at the wobble U_{34} position. The appearance of the $N_{1–32}$ product was concomitant with the disappearance of the $N_{1–34}$ species, suggesting a precursor-product relationship. These results suggest that PaOrf2 incises $tRNA^{Gln}$ initially at either U_{32} or U_{34}, whereas it breaks $tRNA^{Glu}$ first at U_{34} and subsequently at U_{32}. Thus, PaOrf2 can cleave one of its target tRNAs twice, resulting in a net excision of 2 nt.

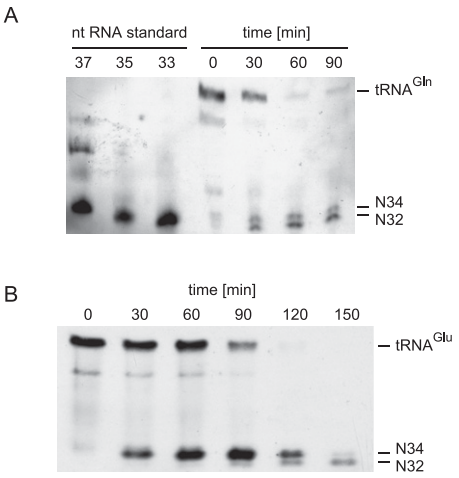

FIGURE 3. Northern blot analysis of tRNA cleavage by PaOrf2. Total yeast tRNA was incubated with $PaOrf2His_6$, and aliquots were withdrawn and quenched at indicated times. Detection of $tRNA^{Gln}$ (*A*) and $tRNA^{Glu}$ (*B*) and their 5′ half-cleavage products $N_{1–32}$ (N32) and $N_{1–34}$ (N34) was carried out by probing the membrane with DIG-labeled oligonucleotides complementary to $tRNA^{Gln}$ or $tRNA^{Glu}$. A set of in vitro transcribed $tRNA^{Gln}$ half-molecules comprising nucleotide 1–37, 1–35, and 1–33 served as standards.

Alleviation of PaOrf2 ribotoxicity by RNA repair enzymes

Growth arrest of *S. cerevisiae* by exogenous zymocin or intracellular γ-toxin can be "cured" by intracellular expression of eukaryotic and phage-encoded tRNA repair enzymes (Nandakumar et al. 2008). In Eukarya, tRNA repair is an essential component of tRNA splicing (Abelson et al. 1998). Bacteriophage T4 exploits tRNA repair to evade a ribotoxin-mediated host antiviral response (Amitsur et al. 1987). Ribotoxic tRNA damage results in 2′,3′-cyclic phosphate and 5′-OH ends, which need to be converted to 5′-PO_4 and 3′-OH ends (the "end-healing" phase of tRNA repair) before they can be sealed by a classic ATP-dependent RNA ligase.

The yeast tRNA ligase Trl1 and the orthologous plant tRNA ligase AtRNL have three discrete enzymatic activities within three modular catalytic domains (see Fig. 5, below). A carboxyl-terminal 2′,3′ cyclic phosphodiesterase (CPD) hydrolyzes the 2′,3′-cyclic phosphate end to a 2′-PO_4, 3′-OH end. A central polynucleotide kinase domain transfers the γ-phosphate from an NTP donor to the broken 5′-OH end to yield a 5′-PO_4. An amino-terminal ATP-dependent ligase domain then joins the healed ends to form a 3′-5′ phosphodiester, 2′-PO_4 repair junction (Apostol et al. 1991; Sawaya et al. 2003; Wang et al. 2006). The 2′-PO_4 is subsequently removed by the phosphotransferase Tpt1 (Spinelli et al. 1997). Bacteriophage T4 encodes a two-protein RNA repair system that performs related end-healing and end-sealing reactions. T4 polynucleotide kinase-phosphatase (Pnkp) is a bifunctional healing enzyme. The carboxyl-terminal phosphatase domain removes the 2′,3′-cyclic

phosphate to yield a 2′-OH, 3′-OH end and the amino-terminal kinase domain phosphorylates the 5′-OH end. The healed ends are then joined by T4 RNA ligase 1 (Rnl1) to generate a 3′-5′ phosphodiester repair junction (Amitsur et al. 1987).

Expression of the phage or plant tRNA repair enzymes in yeast confers γ-toxin resistance, whereas the endogenous ScTrl1 is apparently unable to carry out repair of γ-toxin-induced damage even when ScTrl1 is overexpressed (Nandakumar et al. 2008). ScTrl1 is able to heal the ends of γ-toxin cleaved $tRNA^{Glu}$ halves, but not seal them. It was suggested that the presence of the bulky mcm^5s^2U nucleobase on the 3′-OH side of the break inhibits sealing by ScTrl1 but is tolerated by AtRNL or T4 Rnl1 (Nandakumar et al. 2008).

Because PaOrf2 can cleave its substrate at two alternative positions, one of which (U_{32}) is not mcm^5-modified, we asked whether these sites are accessible to tRNA repair by plant and phage enzymes. PaOrf2 was conditionally expressed from a *CEN* plasmid under control of the *GAL* promoter in the presence or absence of plasmids constitutively expressing AtRNL or T4 Pnkp+Rnl1. In wild-type *TRL1* cells, neither plant nor phage RNA repair enzymes could alleviate the galactose-dependent toxicity of PaOrf2 (Fig. 4). However, in *trl1*Δ cells, in which the essential tRNA splicing activity was provided by AtRNL or Pnkp+Rnl1 (Schwer et al. 2004), we observed that the toxicity of PaOrf2 was ameliorated by the phage tRNA repair enzymes but not by AtRNL (Fig. 4). We surmise that (1) AtRNL is unable to repair PaOrf2-mediated tRNA damage, and (2) the capacity of T4 Pnkp and Rnl1 to repair tRNAs damaged by PaOrf2 is inhibited by the endogenous ScTrl1.

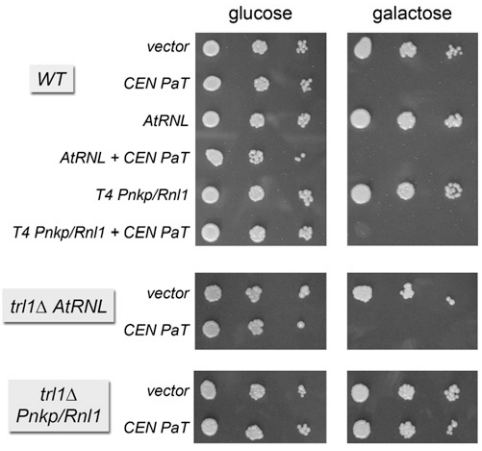

FIGURE 4. Rescue of PaOrf2 toxicity by Pnkp and Rnl1 in a *trl1*Δ strain background. Serial 10-fold dilutions of *S. cerevisiae* cells bearing a *CEN PaORF2* plasmid (PaT) or an empty vector in the presence or absence of an additional plasmid encoding AtRNL (2μ) or T4 Pnkp and Rnl1 were spotted on selective agar plates with 2% glucose or galactose. The plates were photographed after incubation at 30°C for 2 d (glucose) or 3 d (galactose).

We queried which activity of ScTrl1 is inhibitory to the repair of PaOrf2-mediated damage by individually expressing Trl1's sealing (ligase) or healing (kinase-CPD) domains in the *trl1*Δ *Pnkp Rnl1* yeast strain (Fig. 5). Whereas the Trl1 ligase domain did not diminish the protective effects of phage tRNA repair, the kinase-CPD domain rendered the *trl1*Δ *Pnkp Rnl1* strain sensitive to galactose-dependent growth arrest by PaOrf2 (Fig. 5). Thus, healing of PaOrf2-damaged tRNA by endogenous ScTrl1 prevented completion of tRNA repair by the phage pathway. Because the kinase modules of the phage and yeast end-healing enzymes are structurally similar and the reaction products are identical (i.e., a 5′-PO_4 RNA end), we ascribe the repair-defeating effect of the ScTrl1 kinase-CPD domain to the distinctive 3′-OH, 2′-PO_4 end configuration generated by yeast CPD versus the 3′-OH, 2′-OH end produced by Pnkp. In other words, the yeast CPD diverts the processing of the 3′ end of the PaOrf2-damaged tRNAs to a 3′-OH, 2′-PO_4 structure that is not sealed as effectively by T4 Rnl1 as is a 3′-OH, 2′-OH terminus. This inhibitory effect of CPD on the T4 repair system is apparently particular to PaOrf2-damaged tRNAs and is either inapplicable or relatively benign with respect to physiologically spliced yeast tRNAs, insofar as ScTrl1 kinase-CPD expression itself did not affect the growth of the *trl1*Δ *Pnkp Rnl1* yeast strain (Fig. 5).

Rescue of PaOrf2 ribotoxicity in *elp3* yeast cells by RNA repair

Ablation of the yeast *ELP3* gene eliminates the mcm^5 modification of the wobble uridine (U_{34}) of the anticodon in the tRNAs targeted by zymocin and PaT (Huang et al. 2005). Whereas *elp3* mutants are resistant to growth arrest by zymocin, PaT retains its toxicity to *elp3* cells, albeit at higher PaT concentrations compared to *ELP3* cells (Klassen et al. 2004, 2008a; Lu et al. 2005). PaOrf2's ability to cleave tRNAs isolated from *elp3* cells exclusively at the secondary site (U_{32}) (Klassen et al. 2008a) raises the question of whether tRNA restriction at a single site flanking the anticodon affects the ability of tRNA repair enzymes to protect *elp3* cells from PaOrf2 toxicity.

Galactose-induced expression of *PaORF2* from a *CEN* vector in *elp3* cells did not cause growth arrest (data not shown). However, when *PaORF2* was expressed in *elp3 TRL1* cells at high gene dosage from a 2μ plasmid, growth arrest was observed on galactose medium (Fig. 6). When phage or plant tRNA repair enzymes were expressed in *elp3 TRL1* cells, the toxicity of 2μ *PaORF2* was partially alleviated, resulting in cell growth under inducing conditions (albeit more slowly that control cells, as judged by colony size) (Fig. 6). Neither Pnkp/Rnl1 nor AtRNL could rescue 2μ *PaORF2* toxicity in wild-type *ELP3* cells (Fig. 6). We infer that AtRNL and T4 Pnkp/Rnl1 (but not the endogenous ScTrl1) were able to repair the PaOrf2 damage when the target tRNAs were broken uniquely at U_{32}.

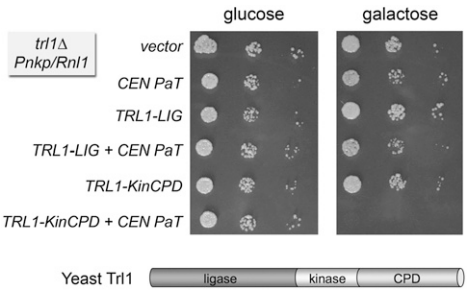

FIGURE 5. The ScTrl1 healing domain inhibits the rescue of PaOrf2 toxicity by Pnkp and Rnl1. Serial 10-fold dilutions of *S. cerevisiae* cells expressing PaOrf2 (PaT) from a *CEN* vector or an empty vector or in the presence of an additional 2μ plasmid encoding full-length ScTrl1 or its component end-healing (Kin-CPD) or end-sealing (LIG) domains were spotted on –Ura–His agar plates with 2% glucose or galactose. The plates were photographed after incubation at 30°C for 2 d (glucose) or 3 d (galactose).

In order to gauge whether the inability of ScTrl1 to rescue toxicity in the *elp3* background was due to limitations of its healing or sealing activities, we expressed ligase-dead (K152A), kinase-dead (S701A), or CPD-dead (T1001A) mutants of AtRNL (Wang et al. 2006) in *elp3 TRL1* cells bearing the 2μ *PaORF2* plasmid. The kinase-dead AtRNL S701A mutant conferred resistance to PaOrf2 comparable to that of wild-type AtRNL (Fig. 7), signifying that the endogenous ScTrl1 kinase activity was competent for repair of the PaOrf2 damage. The CPD-dead T1001A mutant also conferred rescue, albeit not as well as wild-type AtRNL, as judged from the smaller colony size on galactose medium (Fig. 7). In contrast, the ligase-dead K152A AtRNL mutant was unable to ameliorate PaOrf2 toxicity (Fig. 7). Thus, we infer that ScTrl1 could heal tRNA ends broken at position 32 in *elp3* cells but was unable to seal them. This deficiency may reflect a fastidiousness of the ScTrl1 ligase with respect to the position of the break within the tRNA anticodon loop (spliced yeast tRNAs are incised 3′ from the anticodon at position 37) that is not shared by AtRNL or Rnl1.

DISCUSSION

Two distinct eukaryotic ACNase toxins have been identified in the yeasts *K. lactis* and *P. acaciae* (Lu et al. 2005; Klassen et al. 2008a). Both toxins are encoded by cytoplasmic linear plasmids that are phylogenetically related and share a common receptor-binding and *trans*-membrane passage apparatus, consisting of chitin-binding and hydrophobic domains located on the same (in PaT) or different (in zymocin) subunits (Jablonowski et al. 2001; Klassen et al. 2004). Yet, the anticodon nuclease subunits PaOrf2 and γ-toxin are seemingly unrelated (Klassen et al. 2004). An expanded comparison to putative ribotoxins encoded by related linear plasmids from *P. inositovora* and *D. robertsiae* led us to identify a small number of conserved residues, two of which—a glutamate and a histidine—are known to be essential for γ-toxin activity (Keppetipola et al. 2009; Jain et al. 2011). We find that the corresponding Glu and His residues of PaOrf2 are essential for its toxicity in vivo. We suggest that the Glu and His side chains conserved in the primary structure of the four fungal ribotoxins are constituents of the endonuclease active site. Leu165, Ile168, and Ile291 of PaOrf2 are also conserved among all four fungal ribotoxins and are essential for the in vivo toxicity of PaOrf2. It is possible that the fungal ribotoxins adopt related tertiary structures, notwithstanding their sparse primary structure similarity.

PaOrf2 and γ-toxin have distinctive substrate specificities. Whereas γ-toxin cleaves tRNA exclusively at the mcm^5s^2-modified wobble base, PaOrf2 cleaves at the same position and at a secondary site 2 nt upstream (Lu et al. 2005; Klassen et al. 2008a). Time-resolved monitoring of PaOrf2 cleavage products revealed that the $tRNA^{Glu}$ substrate is initially cleaved at position 34, while cleavage at position 32 occurs with a delay, implying that PaOrf2 can excise a di-nucleotide in vitro. $tRNA^{Gln}$ appears to be cleaved at either position 34 or 32. At least one other anticodon nuclease toxin, *Geobacillus kaustrophilus* RloC, a homolog of the bacterial single-incision *Escherichia coli* PrrC ribotoxin (Davidov and Kaufmann 2008), also exhibits anticodon "excisase" activity. RloC breaks $tRNA^{Lys}$ at two positions, immediately 3′ from and 5′ of the wobble uridine nucleoside, resulting in net excision of the wobble mononucleotide. Thus, the advancement from an incisase to an excisase mode of ribotoxins action may have occurred

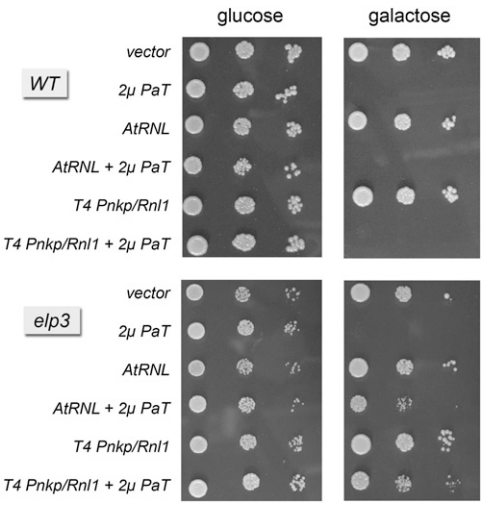

FIGURE 6. Rescue of high-copy PaOrf2 toxicity by plant T4-encoded tRNA repair in the absence of U_{34} mcm^5 modification. Serial 10-fold dilutions of *S. cerevisiae* cells bearing a 2μ *PaORF2* plasmid (PaT) or an empty vector plus an additional plasmid encoding AtRNL (2μ) or T4 Pnkp and Rnl1 were spotted on selective agar plates with 2% glucose or galactose. The plates were photographed after incubation at 30°C for 2 d (glucose) or 3 d (galactose).

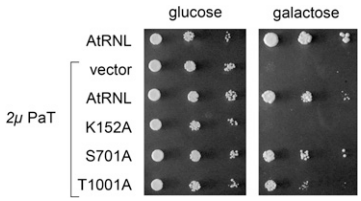

FIGURE 7. ScTrl1 can heal U_{32} breaks but requires AtRNL sealing activity for rescuing PaOrf2 toxicity in *elp3*. Serial 10-fold dilutions of *S. cerevisiae* cells bearing a 2μ *PaORF2* plasmid (PaT) or an empty vector plus 2μ plasmids encoding full-length AtRNL or ligase-dead (K152A), kinase-dead (S701A), and CPD-dead (T1001A) variants as specified were spotted on –Ura–His agar plates with 2% glucose or galactose. The plates were photographed after incubation at 30°C for 2 d (glucose) or 4 d (galactose).

repeatedly during evolution and provides an increased benefit to the ribotoxin producer. It has been suggested that evasion of RNA repair is one such benefit (Davidov and Kaufmann 2008).

Our studies of the effect of RNA repair enzymes on PaOrf2 ribotoxicity provide new insights into this issue (Fig. 8). First, heterologous plant and phage T4 RNA repair pathways, but not endogenous yeast Trl1, can counteract tRNA damage when cleavage is restricted to U_{32} in an *elp3* mutant that lacks the mcm^5 wobble uridine modification. ScTrl1 can provide the end-healing activities to counteract PaOrf2 ribotoxicity in *elp3* cells (when supplemented by the AtRNL ligase activity), but it is apparently unable to carry out the ligation step (Fig. 8A). The ScTrl1 ligase activity in vivo appears to be sensitive to the position of the break within the anticodon loop (U_{32} "rejected") and/or the presence of a modified nucleobase on the 3′-OH side of the break (mcm^5U_{34} "rejected"). In contrast, AtRNL sealing activity is not thwarted by these variations.

The ability of plant and T4 tRNA repair pathways to antagonize PaOrf2 toxicity changes in *ELP3* cells when dual incisions of tRNA are allowed. Since both pathways can operate, in general, on single U_{32} and U_{34} tRNA breaks but fail under conditions that allow PaT to access both sites, we conclude that PaT's toxicity in vivo involves excision cleavage, even though in vitro, double cleavage was restricted to $tRNA^{Glu}$. It is clear, however, that target specificities of PaT are modulated by additional, yet unknown factors in vivo, as the highly specific targeting $tRNA^{Gln}$ observed in vivo cannot be recapitulated in vitro. Results of tRNA repair accessibility strongly support our model (Fig. 8B), in which double cleavage of a toxicity-relevant target tRNA does occur in PaT-challenged wild-type cells. In this context, AtRNL is ineffective, presumably because PaOrf2 can cleave enough of its targets twice before the plant enzymes can act, so that any break sealing that occurs thereafter would be nonproductive. Pnkp and Rnl1 can rescue PaOrf2 ribotoxicity in *ELP3* cells when the endogenous Trl1 activity is ablated. The implication here is that the phage enzymes can access and repair enough of the tRNAs incised singly at U_{34} before a second incision at position 32 renders them irreparable (Fig. 8B). Our speculation that the rates of tRNA repair in vivo by plant and phage repair enzymes may differ, with the phage pathway acting more effectively than AtRNL, is in keeping with the findings that the specific activity of the AtRNL ligase domain in tRNA splicing in vitro is ~10-fold lower than that of T4 Rnl1 (Nandakumar et al. 2008). The ill effect of yeast Trl1 on the repair of PaOrf2-inflicted RNA damage by T4 Rnl1 signifies that the healed 3′-OH, 2′-PO_4 ends generated by the CPD domain of ScTrl1 are poor

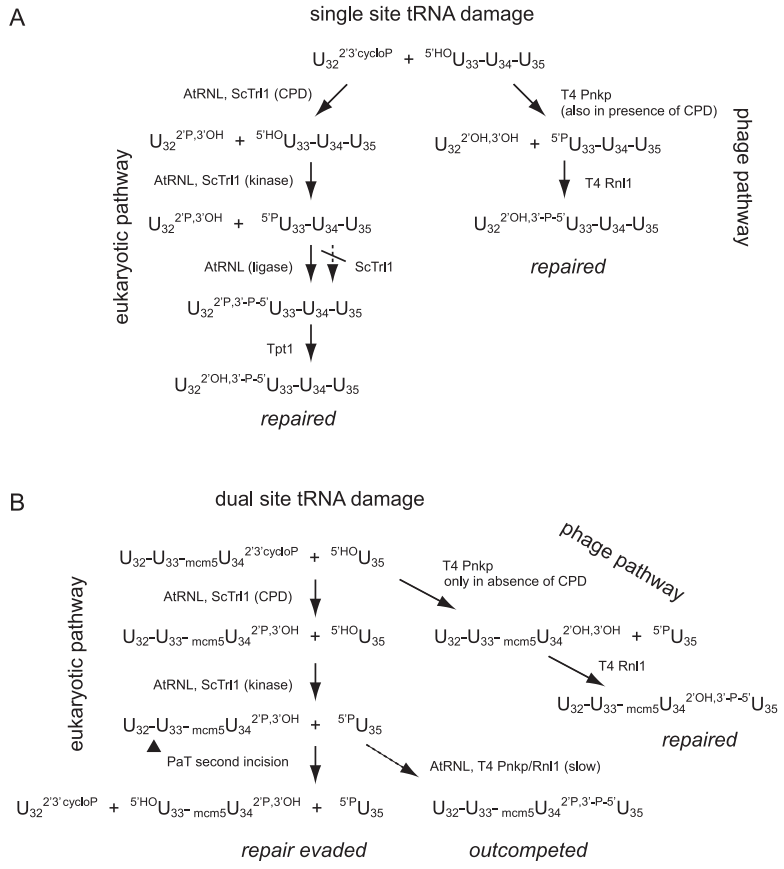

FIGURE 8. Summary model of eukaryal and phage T4 RNA repair pathways operating on PaOrf2-cleaved tRNA. (*A*) Single cleavage site action of PaOrf2 in the absence of mcm^5 wobble uridine modification in *elp3* cells. (*B*) Dual cleavage site action of PaOrf2 in *ELP3* cells. (P) Phosphate, (cycloP) cyclo-phosphate. See text for details.

substrates for Rnl1. Such 2′-PO_4 ends are also less than optimal substrates for yeast tRNA splicing by Rnl1, insofar as a *trl1Δ RNL1 TRL1(389–827)* yeast strain that relies on the combination of the phage ligase and the yeast kinase-CPD domain to perform tRNA splicing is viable at 25° and 30°C but not at 37°C (Schwer et al. 2004).

Because PaT and zymocin are functional analogs produced by related fungal plasmids, it is reasonable to think that the second tRNA incision inflicted by PaOrf2 represents an acquired advantage over the single-cutting endonuclease γ-toxin. Given that AtRNL is able to repair single-site tRNA damage flanking a modified wobble base, and that all fungi have tRNA ligases homologous to Trl1 and AtRNL, we envision that some fungal tRNA ligases will resemble the plant enzyme in their ability to repair ribotoxic tRNA incision. The excisional mode of action of a secreted anticodon nuclease toxin potentially expands the spectrum of self-nonself discrimination by overriding target cell resistance in taxa that have an AtRNL-like repair capacity. Additionally, excision cleavage may represent an adaptation of the toxin-antitoxin system encoded by the parasitizing virus-like DNAs to counteract the potential occurrence of an AtRNL-like repair capacity within the host species and prevent the establishment of autoimmunity via RNA repair.

MATERIALS AND METHODS

Expression plasmids and *S. cerevisiae* strains

PaOrf2 was expressed in haploid *S. cerevisiae* in a galactose-inducible manner. The strains used are derivatives of W303a or CEN.PK2-1c and have been described (van Dijken et al. 2000; Sawaya et al. 2003; Huang et al. 2005; Jablonowski et al. 2006; Nandakumar et al. 2008). The expression constructs (encoding for PaOrf2 lacking the N-terminal 12-aa signal peptide) pPACTOXLF (*ARS-CEN LEU2*, in pLF16), pPACBX (2μ *LEU2 URA3*), and pRS415-Gal1-PaT (*CEN LEU2*) are described (Klassen et al. 2004, 2008a; Tanaka et al. 2011). An additional construct (pPACTOXEP) containing the GAL1-promotor-*PaORF2* fusion in YEplac181 was made by subcloning via PstI-PvuII from pPACTOXLF. We found that the cloned gene in pPACTOXLF and pPACTOXEP differs at two nucleobase positions from the sequence in GenBank (NCBI accession number: CAE84960.1). The resulting coding changes N82K and K199I do not affect PaOrf2 toxicity. Missense mutations were introduced into the *PaORF2* gene in pPACTOXEP or pPACTOXLF via site-directed mutagenesis using two partially overlapping primers. The mutated genes were sequenced to verify that no unwanted coding changes were acquired. The RNA repair plasmids pRS423-TPI-AtRNL (2μ *HIS3*), pRS423-TPI-Trl1 (2μ *HIS3*), and pRS413-Pnkp/Rnl1 and plasmids for separate expression of the yeast Trl1 healing (1–388) and sealing (389–827) domains were described previously (Nandakumar et al. 2008; Meineke and Shuman 2012).

PaORF2 toxicity assay

The indicated *S. cerevisiae* strains were transformed with plasmid DNA by the lithium acetate method (Schiestl and Gietz 1989). Transformants were selected on appropriate minimal synthetic media on 2% (w/v) bacto agar plates. Cells derived from single transformants were grown at 30°C in liquid culture in selective media containing 2% glucose. The cultures were adjusted to A_{600} of 0.1 and then serially diluted in 10-fold steps in water. Aliquots (3 μL) of the dilutions were spotted in parallel on selective agar plates containing either 2% glucose or 2% galactose. The plates were photographed after incubation at 30°C for 2 d (glucose) or 3 or 4 d (galactose) as indicated.

Analysis of tRNA cleavage in vitro

Recombinant PaOrf2 protein was produced in *E. coli* as a C-terminal His_6 fusion and isolated from a soluble extract by Ni-affinity chromatography as described previously (Klassen et al. 2008a). tRNA was isolated from wild-type yeast cells (*S. cerevisiae* CEN.PK2-1c) (van Dijken et al. 2000) as described (Klassen et al. 2008b). Five μg of tRNA was incubated at 30°C in a total volume of 100 μL in 10 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 50 mM NaCl, 1 mM dithiothreitol, containing 50 ng of PaOrf2His_6. At time intervals of 30 min, 10-μL aliquots (containing 0.5 μg of tRNA) were removed, mixed with an equal volume of formamide loading buffer (95% w/v formamide, 0.025% w/v SDS, 0.025% w/v bromophenol blue, 0.025% xylene cyanol), then incubated at 65°C for 30 min and analyzed by electrophoresis through a 12% polyacrylamide gel containing 7.5 M urea in TBE. Synthetic tRNAGln half-molecules comprising nucleotides 1–37, 1–35, and 1–33 were produced by in vitro transcription as described (Klassen et al. 2008a). The gel contents were transferred to positively charged nylon membranes by semidry electroblotting. The membrane was incubated overnight at 37°–40°C in hybridization buffer (Roche, Mannheim) containing 50% formamide and 10 pmol μL^{-1} digoxygenin-labeled oligonucleotides specific for $tRNA^{Gln}_{(UUG)}$ or $tRNA^{Glu}_{(UUC)}$ (Klassen et al. 2008a). Detection of the annealed primers was carried out using phosphatase-conjugated anti-DIG antibodies and chemiluminescent alkaline phosphatase substrate CDPstar as recommended by the manufacturer (Roche, Mannheim).

ACKNOWLEDGMENTS

This research was supported by NIH grant GM42498 (S.S.). S.S. is an American Cancer Society Research Professor.

Received April 30, 2012; accepted June 14, 2012.

REFERENCES

Abelson J, Trotta CR, Li H. 1998. tRNA splicing. *J Biol Chem* **273:** 12685–12688.

Amitsur M, Levitz R, Kaufman G. 1987. Bacteriophage T4 anticodon nuclease, polynucleotide kinase, and RNA ligase reprocess the host lysine tRNA. *EMBO J* **6:** 2499–2503.

Apostol BL, Westaway SK, Abelson J, Greer CL. 1991. Deletion analysis of a multifunctional yeast tRNA ligase polypeptide: Identification of essential and dispensable functional domains. *J Biol Chem* **266:** 7445–7455.

Butler AR, Porter M, Stark MJ. 1991. Intracellular expression of *Kluyveromyces lactis* toxin γ subunit mimics treatment with

exogenous toxin and distinguishes two classes of toxin-resistant mutant. *Yeast* **7:** 617–625.

Chan CM, Zhou C, Huang R. 2009. Reconstituting bacterial RNA repair and modification in vitro. *Science* **326:** 247. doi: 10.1126/science.1179480.

Chen C, Huang B, Anderson JT, Byström AS. 2011. Unexpected accumulation of ncm^{5}U and ncm^{5}S^{2}U in a *trm9* mutant suggests an additional step in the synthesis of mcm^{5}U and mcm^{5}S^{2}U. *PLoS ONE* **6:** e20783. doi: 10.1371/journal.pone.0020783.

Davidov E, Kaufmann G. 2008. RloC: A wobble nucleotide-excising and zinc-responsive bacterial tRNase. *Mol Microbiol* **69:** 1560–1574.

Frohloff F, Fichtner L, Jablonowski D, Breunig KD, Schaffrath R. 2001. *Saccharomyces cerevisiae* Elongator mutations confer resistance to the *Kluyveromyces lactis* zymocin. *EMBO J* **20:** 1993–2003.

Huang B, Johansson MJO, Byström AS. 2005. An early step in wobble tRNA modification requires the Elongator complex. *RNA* **11:** 424–436.

Huang B, Lu J, Byström AS. 2008. A genome-wide screen identifies genes required for formation of the wobble nucleoside 5-methoxycarbonylmethyl-2-thiouridine in *Saccharomyces cerevisiae*. *RNA* **14:** 2183–2194.

Jablonowski D, Schaffrath R. 2007. Zymocin, a composite chitinase and tRNase killer toxin from yeast. *Biochem Soc Trans* **35:** 1533–1537.

Jablonowski D, Fichtner L, Martin VJ, Klassen R, Meinhardt F, Stark MJR, Schaffrath R. 2001. *Saccharomyces cerevisiae* cell wall chitin, the potential *Kluyveromyces lactis* zymocin receptor. *Yeast* **18:** 1285–1299.

Jablonowski D, Fichtner L, Stark MJ, Schaffrath R. 2004. The yeast Elongator histone acetylase requires Sit4-dependent dephosphorylation for toxin-target capacity. *Mol Biol Cell* **15:** 1459–1469.

Jablonowski D, Zink S, Mehlgarten C, Daum G, Schaffrath R. 2006. tRNA^{Glu} wobble uridine methylation by Trm9 identifies Elongator's key role for zymocin-induced cell death in yeast. *Mol Microbiol* **59:** 677–688.

Jain R, Poulos MG, Gros J, Chakravarty AK, Shuman S. 2011. Substrate specificity and mutational analysis of *Kluyveromyces lactis* γ-toxin, a eukaryal tRNA anticodon nuclease. *RNA* **17:** 1336–1343.

Kalhor HR, Clarke S. 2003. Novel methyltransferase for modified uridine residues at the wobble position of tRNA. *Mol Cell Biol* **23:** 9283–9292.

Keppetipola N, Jain R, Meineke B, Diver M, Shuman S. 2009. Structure-activity relationships in *Kluyveromyces lactis* γ-toxin, a eukaryal tRNA anticodon nuclease. *RNA* **15:** 1036–1044.

Klassen R, Meinhardt F. 2003. Structural analysis of the killer element pPin1-3 from *Pichia inositovora*. *Mol Genet Genomics* **270:** 190–199.

Klassen R, Teichert S, Meinhardt F. 2004. Novel yeast killer toxins provoke S-phase arrest and DNA damage checkpoint activation. *Mol Microbiol* **53:** 263–273.

Klassen R, Paluszynski J, Wemhoff S, Pfeiffer A, Fricke J, Meinhardt F. 2008a. The primary target of the killer toxin from *Pichia acaciae* is tRNA^{Gln}. *Mol Microbiol* **69:** 681–697.

Klassen R, Fricke J, Pfeiffer A, Meinhardt F. 2008b. A modified DNA isolation protocol for obtaining pure RT-PCR grade RNA. *Biotechnol Lett* **30:** 1041–1044.

Lu J, Huang B, Esberg A, Johansson MJ, Byström AS. 2005. The *Kluyveromyces lactis* γ-toxin targets tRNA anticodons. *RNA* **11:** 1648–1654.

Martins A, Shuman S. 2004. Characterization of a baculovirus enzyme with RNA ligase, polynucleotide 5′ kinase and polynucleotide 3′ phosphatase activities. *J Biol Chem* **279:** 18220–18231.

Martins A, Shuman S. 2005. An end-healing enzyme from *Clostridium thermocellum* with 5′ kinase, 2′,3′ phosphatase, and adenylyltransferase activities. *RNA* **11:** 1271–1280.

Mazauric MH, Dirick L, Purushothaman SK, Bjork GR, Lapeyre B. 2010. Trm112p is a 15-kDa zinc finger protein essential for the activity of two tRNA and one protein methyltransferases in yeast. *J Biol Chem* **285:** 18505–18515.

McCracken DA, Martin VJ, Stark MJR, Bolen PL. 1994. The linear-plasmid-encoded toxin produced by the yeast *Pichia acaciae*: Characterization and comparison with the toxin of *Kluyveromyces lactis*. *Microbiology* **140:** 425–431.

Mehlgarten C, Jablonowski D, Breunig KD, Stark MJ, Schaffrath R. 2009. Elongator function depends on antagonistic regulation by casein kinase Hrr25 and protein phosphatase Sit4. *Mol Microbiol* **73:** 869–881.

Meineke B, Shuman S. 2012. Determinants of the cytotoxicity of PrrC anticodon nuclease and its amelioration by tRNA repair. *RNA* **18:** 145–154.

Meinhardt F, Klassen R. 2009. Yeast killer toxins: Fundamentals and applications. In *The Mycota* (ed. T Anke, D Weber), pp. 107–130. Springer, New York, NY.

Nandakumar J, Schwer B, Schaffrath R, Shuman S. 2008. RNA repair: An antidote to cytotoxic eukaryal RNA damage. *Mol Cell* **31:** 278–286.

Sawaya R, Schwer B, Shuman S. 2003. Genetic and biochemical analysis of the functional domains of yeast tRNA ligase. *J Biol Chem* **278:** 43928–43938.

Schiestl RH, Gietz RD. 1989. High efficiency transformation of intact yeast cells using single stranded nucleic acids as a carrier. *Curr Genet* **16:** 339–346.

Schwer B, Sawaya R, Ho CK, Shuman S. 2004. Portability and fidelity of RNA-repair systems. *Proc Natl Acad Sci* **101:** 2788–2793.

Spinelli SL, Consaul SA, Phizicky EM. 1997. A conditional lethal yeast phosphotransferase (*tpt1*) mutant accumulates tRNAs with a 2′-phosphate and an undermodified base at the splice junction. *RNA* **3:** 1388–1400.

Stark MJ, Boyd A. 1986. The killer toxin of *Kluyveromyces lactis*: Characterization of the toxin subunits and identification of the genes which encode them. *EMBO J* **5:** 1995–2002.

Studte P, Zink S, Jablonowski D, Bär C, von der Haar T, Tuite MF, Schaffrath R. 2008. tRNA and protein methylase complexes mediate zymocin toxicity in yeast. *Mol Microbiol* **69:** 1266–1277.

Tanaka N, Meineke B, Shuman S. 2011. RtcB, a novel RNA ligase, can catalyze tRNA splicing and *HAC1* mRNA splicing *in vivo*. *J Biol Chem* **286:** 30253–30257.

van Dijken JP, Bauer J, Brambilla L, Duboc P, Francois JM, Gancedo C, Giuseppin ML, Heijnen JJ, Hoare M, Lange HC, et al. 2000. An interlaboratory comparison of physiological and genetic properties of four *Saccharomyces cerevisiae* strains. *Enzyme Microb Technol* **26:** 706–714.

Wang LK, Schwer B, Englert M, Beier H, Shuman S. 2006. Structure–function analysis of the kinase-CPD domain of yeast tRNA ligase (Trl1) and requirements for complementation of tRNA splicing by a plant Trl1 homolog. *Nucleic Acids Res* **34:** 517–527.