Determinants of eukaryal cell killing by the bacterial ribotoxin PrrC

Birthe Meineke[1], Beate Schwer[2], Raffael Schaffrath[3] and Stewart Shuman[1,*]

[1]Molecular Biology Program, Sloan-Kettering Institute, [2]Department of Microbiology and Immunology, Weill Cornell Medical College, New York, NY 10065 USA and [3]Department of Genetics, University of Leicester, Leicester, LE1 7RH, UK

ABSTRACT

tRNA damage inflicted by the *Escherichia coli* anticodon nuclease PrrC (*Eco*PrrC) underlies an antiviral response to phage T4 infection. PrrC homologs are present in many bacterial proteomes, though their biological activities are uncharted. PrrCs consist of two domains: an N-terminal NTPase module related to the ABC family and a distinctive C-terminal ribonuclease module. In this article, we report that the expression of *Eco*PrrC in budding yeast is fungicidal, signifying that PrrC is toxic in a eukaryon in the absence of other bacterial or viral proteins. Whereas *Streptococcus* PrrC is also toxic in yeast, *Neisseria* and *Xanthomonas* PrrCs are not. Via analysis of the effects of 118 mutations on *Eco*PrrC toxicity in yeast, we identified 22 essential residues in the NTPase domain and 11 in the nuclease domain. Overexpressing PrrCs with mutations in the NTPase active site ameliorated the toxicity of wild-type *Eco*PrrC. Our findings support a model in which *Eco*PrrC toxicity is contingent on head-to-tail dimerization of the NTPase domains to form two composite NTP phosphohydrolase sites. Comparisons of *Eco*PrrC activity in a variety of yeast genetic backgrounds, and the rescuing effects of tRNA overexpression, implicate tRNA$^{Lys(UUU)}$ as a target of *Eco*PrrC toxicity in yeast.

INTRODUCTION

Transfer RNAs are essential components of the translation machinery; they are also vulnerable targets for bacterial and fungal endoribonuclease toxins (ribotoxins) that incise specific tRNA anticodons and arrest cell growth. Secreted tRNA ribotoxins, such as bacterial colicins D and E5, *Kluyveromyces lactis* γ-toxin and *Pichia acaciae* toxin, provide a means to discriminate self from non-self species and suppress growth of the latter (1–5). Intracellular ribotoxins are normally maintained in a latent state, but are activated in response to cellular stress or viral infection (6–8).

The *Escherichia coli* PrrC anticodon nuclease (ACNase) represents an RNA-based intracellular innate immune system of host defense against a foreign invader (6). PrrC is maintained in a latent state by association with the host DNA restriction-modification enzyme encoded by the *prrA*, *prrB* and *prrD* ORFs of the *E. coli prr* operon (9,10). The PrrC ACNase is activated by a virus-encoded protein, Stp, synthesized early during bacteriophage T4 infection (11–13). Active PrrC incises tRNA^{Lys} at a single site in the anticodon loop, 5′ of the modified wobble uridine (mnm^{5}s^{2}U), to generate a 2′,3′-cyclic phosphate and a 5′-OH at the broken ends. Unopposed depletion of tRNA^{Lys} interdicts synthesis of viral late proteins and prevents spread of the virus through the population. However, the phage thwarts the RNA-damaging host defense by encoding an RNA repair system, consisting of T4 polynucleotide kinase-phosphatase (Pnkp) and T4 RNA ligase 1 (Rnl1), that heals and seals the broken tRNA ends (14). tRNA restriction as a defense mechanism against phages is seemingly widespread in the bacterial domain, insofar as: (i) PrrC homologs are present in many diverse bacteria; and (ii) RNA repair enzymes are encoded by viruses other than T4 (15).

E. coli PrrC (*Eco*PrrC) is the only member of the large PrrC-like family for which a biological role (antiviral host defense) and a specific RNA target have been defined. *Eco*PrrC is a 396-aa polypeptide composed of two putative domains: an N-terminal nucleoside triphosphate phosphohydrolase (NTPase) module (aa 1–264) related to the ABC transporter family and a C-terminal 'nuclease' module (aa 265–396) that has no apparent similarity to any known nuclease or tRNA-binding protein. Gabriel Kaufmann's laboratory has reported that: (i) active *Eco*PrrC is a homo-oligomeric complex; (ii) *Eco*PrrC nuclease activity is triggered by GTP hydrolysis and activated allosterically by dTTP; (iii) tRNA^{Lys} is the

*To whom correspondence should be addressed. Tel: +1 212 639 7145; Fax: +1 212 717 3623; Email: s-shuman@ski.mskcc.org

specific target of *Eco*PrrC *in vivo* and (iv) *Eco*PrrC activity is influenced by base modifications in the tRNA anticodon loop (16–19).

Davidov and Kaufmann (20) recently identified *Geobacillus kaustophilus* RloC (*Gka*RloC) as the exemplar of a distinct subfamily of bacterial PrrC-like ribotoxins, in which the NTPase domain contains a large modular insert with a putative coiled-coil/ zinc-hook structure reminiscent of Rad50. *Gka*RloC incises the anticodon loop of tRNA^{Glu} and to a lesser extent tRNA^{Lys}, tRNA^{Arg} and tRNA^{Gln}, when expressed in *E. coli* cells. In contrast to *Eco*PrrC, which merely nicks the tRNA backbone, *Gka*RloC performs two nuclease reactions on either side of the wobble uridine of a tRNA^{Lys} substrate, leaving 2′,3′-cyclic phosphate and 5′-OH termini at each cleavage site. The net result is excision of the wobble nucleoside, which effectively precludes regeneration of a functional tRNA by a T4-like RNA-repair system.

Biochemical and structural studies of *Eco*PrrC have been hindered by the self-limiting capacity for expression of active PrrC in bacteria (i.e. PrrC curtails bacterial protein synthesis). Nonetheless, the Kaufmann laboratory has identified several functionally important components of the *Eco*PrrC protein, by surveying for mutations that affect PrrC toxicity in *E. coli* or assaying ACNase activity in extracts of *E. coli* expressing PrrC mutants (18,19,21). We and others have exploited budding yeast *Saccharomyces cerevisiae* as a surrogate system to study the effects of intracellular expression of tRNA anticodon nucleases, e.g. γ-toxin, colicin D, colicin E5 (22–24). Here we apply this strategy to *Eco*PrrC and PrrC homologs from other bacteria. We find that *Eco*PrrC is toxic to yeast cells, as is *Streptococcus mutans* PrrC (*Smu*PrrC). In contrast, the *Neisseria meningitidis* (*Nme*) and *Xanthomonas campestris* (*Xca*) PrrCs are nontoxic. We gained new insights to structure–function relationships in the PrrC family via an extensive mutational analysis of *Eco*PrrC. Comparisons of PrrC activity in a variety of yeast genetic backgrounds implicate tRNA^{Lys} as a relevant target of *Eco*PrrC toxicity in a eukaryon, as it is in bacteria, notwithstanding the differences in the anticodon base-modification profiles of eukaryal and bacterial tRNA^{Lys}. We discuss possible therapeutic niches for enzymatic ribotoxins in eukarya.

MATERIALS AND METHODS

PrrC expression plasmids

The *E. coli prrC* gene was inserted into yeast plasmid YCplac111 (*CEN LEU2*) under the transcriptional control of a *GAL1* promoter to yield pYC-EcoPrrC. The amino acid sequence of the plasmid-encoded 396-aa *Eco*PrrC polypeptide is shown in Figure 2A. Missense mutations were introduced in the *prrC* gene in pYC-EcoPrrC by two-stage overlap extension PCR with mutagenic primers. The *prrC* ORF was sequenced in each case to verify the intended coding change and exclude the acquisition of unwanted coding changes during amplification and cloning. EcoRI/SalI fragments containing

GAL1-prrC-Ala expression cassettes were excised from pYC-EcoPrrC-Ala and inserted into multicopy yeast plasmid pRS423 (2 μ *HIS3*).

The genes encoding PrrC homologs from *Neisseria meningitidis* (accession NP_273873), *Streptococcus mutans* (accession NP_721301) and *Xanthomonas campestris* (accession NP_635858) were amplified by PCR from genomic DNAs obtained from ATCC. The sense-strand PCR primers were designed to introduce an NdeI site at the translation start codon. The antisense primers introduced a SalI site downstream of the stop codon. The PCR products were digested with NdeI and SalI and inserted between the corresponding sites in pYC-EcoPrrC in lieu of the EcoPrrC fragment. Sequencing of the inserts in the resulting pYC-NmePrrC, pYC-SmuPrrC and pYC-XcaPrrC plasmids verified that no coding changes had been introduced during amplification and cloning.

tRNA expression plasmids

The 2 μ *URA3* plasmids bearing the yeast genes for tRNA$^{Glu(UUC)}$, tRNA$^{Lys(UUU)}$, tRNA$^{Gln(UUG)}$, tRNA$^{Arg(UCU)}$, tRNA$^{Gly(UCC)}$, tRNA$^{Leu(UAA)}$ and tRNA$^{Tyr(GUA)}$ are described (25). A 2 μ *URA3* tRNA$^{Lys(CUU)}$ plasmid was constructed by PCR amplifying a 1-kb fragment of *S. cerevisiae* chromosome III genomic DNA containing this tRNA gene and inserting it between BamHI and SalI sites in YEplac195 to generate pLysCUU. A KpnI/BglII fragment containing the tRNA$^{Lys(UUU)}$ gene was then inserted between KpnI and BamHI site of pLysCUU to generate pLysCUU/UUU bearing both tRNA^{Lys} isoacceptors. The sequences of the tRNA inserts were verified for each of the new constructs used in this study.

PrrC toxicity assays

The haploid *S. cerevisiae* strain W303 was used in all experiments unless specified otherwise. The *trm9Δ* and *tot3Δ* derivatives of W303 are described (25,26). Yeast cells were transformed with plasmid DNAs by using the lithium acetate method (27). Transformants were selected on appropriate selective minimal synthetic media on 2% (w/v) bacto agar plates.

Toxicity of the plasmid-encoded PrrC proteins was gauged as follows. 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 diluted in water in serial 5-fold decrements. Aliquots (3 μl) of the dilutions were then 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 (glucose) or 3 days (galactose).

Alternatively, the growth and viability of yeast cells bearing *CEN LEU2* PrrC plasmids were monitored in liquid cultures as follows. Cells derived from single transformants were grown overnight at 30°C in SD–Leu medium containing 2% raffinose. The cultures were adjusted to A_{600} of 0.1 by dilution into –Leu media containing either 2% glucose or 2% galactose (time 0). The

cultures were then incubated at 30°C with constant shaking and A_{600} was monitored at 3-h intervals. Viable cell counts were determined by withdrawing aliquots at 3 h intervals, diluting them 1:50 in water, and then plating 10, 100 and 250 μl of this sample on SD–Leu agar plates containing 2% glucose. Colonies were counted after incubation for 2 days at 30°C.

Dominant negative effects of PrrC-Ala mutants

Yeast cells were cotransformed with pYC-EcoPrrC (*CEN LEU2*) or the empty *CEN* vector and each of 15 pRS-EcoPrrC-Ala plasmids (2μ *HIS3*) encoding nontoxic *Eco*PrrC mutants or the empty 2μ vector. Cells derived from single transformants were grown at 30°C in liquid culture in SD–Leu–His medium containing 2% glucose. The cultures were adjusted to A_{600} of 0.1 and then diluted in water in serial 5-fold decrements. Aliquots (3 μl) of the dilutions were then spotted in parallel on –Leu–His agar plates containing either 2% glucose or 2% galactose. The plates were photographed after incubation at 30°C for 2 (glucose) or 3 days (galactose). Alternatively, the growth of yeast cells bearing pYC-EcoPrrC and pRS-EcoPrrC-Ala (or empty vector controls) was monitored in liquid cultures. Cells derived from single transformants were grown at 30°C in SD–Leu–His medium containing 2% raffinose until A_{600} reached ∼2. Aliquots of the cultures were then diluted

with SD–Leu–His medium containing 2% galactose to an A_{600} of 0.1. The cultures were incubated at 30°C with constant shaking for 18 h, at which time A_{600} was measured.

RESULTS AND DISCUSSION

Induced expression of *E. coli* PrrC in budding yeast is toxic

We installed the *E. coli prrC* gene in yeast on a *CEN* plasmid under the control of a glucose-repressed/galactose-induced *GAL1* promoter. Galactose induction of PrrC production suppressed yeast growth on agar medium (Figure 1A) and in liquid culture (Figure 1B). By analyzing yeast survival after transient galactose induction and return to glucose, we found that PrrC expression was profoundly fungicidal (Figure 1C). The number of viable cells in the yeast culture decreased by 19-fold within 3 h of *Eco*PrrC induction, by 350-fold after 9 h and by 550-fold after 12 h (Figure 1C). Control experiments verified that *CEN prrC* yeast cells grew as well as cells bearing the empty *CEN* vector on glucose-containing agar and liquid media (Figure 1A and B). These results showed that *Eco*PrrC is an effective toxin in a eukaryal cell, in the absence of any other bacterial or bacteriophage proteins, including the DNA restriction enzyme with
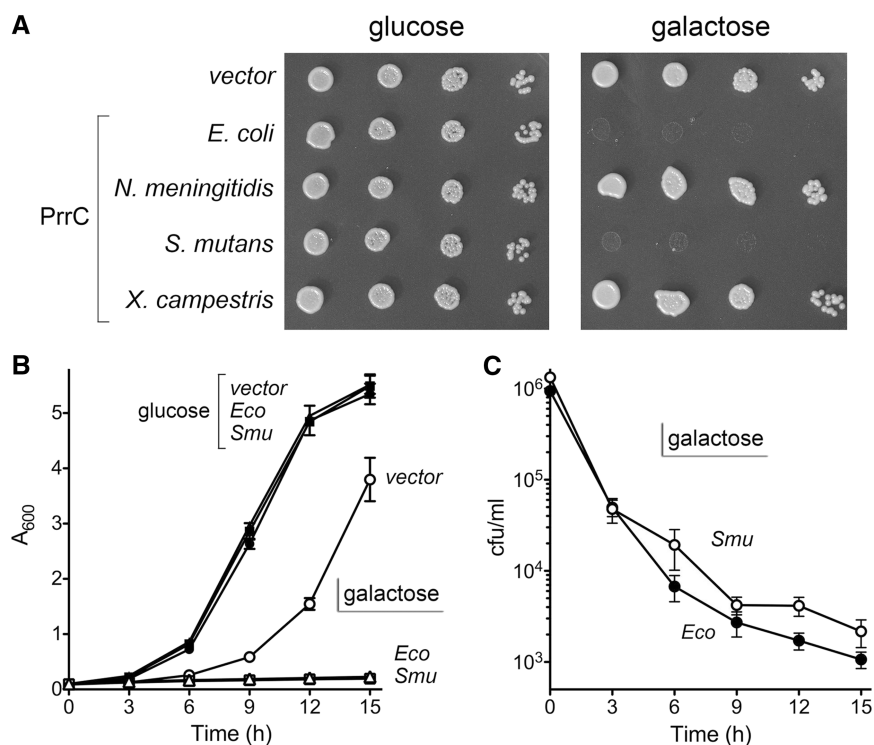

Figure 1. Induced expression of *Eco*PrrC and *Smu*PrrC is toxic to *S. cerevisiae*. (**A**) Serial 5-fold dilutions of yeast cells bearing a *CEN* plasmid encoding the indicated galactose-regulated *prrC* gene or an empty *CEN* vector were spotted on –Leu agar plates containing 2% glucose or galactose as specified. (**B**) Growth of liquid cultures of yeast cells bearing *Eco*PrrC or *Smu*PrrC plasmids or the empty vector was monitored by determining A_{600} at serial times after transfer from raffinose medium to media containing glucose or galactose. Each datum is the average of three independent growth experiments ±SEM. (**C**) Viable cell counts of liquid cultures of yeast cells bearing a *Eco*PrrC or *Smu*PrrC plasmid were determined immediately prior to (time 0) and at serial 3-h intervals after galactose induction, by plating aliquots on –Leu agar containing 2% glucose. Each datum is the average of three independent galactose-induction experiments ±SEM.

which it interacts in *E. coli* and the phage Stp protein that triggers PrrC activity during virus infection.

Remarkably, not all bacterial PrrCs are created equal in this respect. *Nme*- and *Xca*PrrC were nontoxic in yeast, while *Smu*PrrC was toxic (Figure 1A). Like *Eco*PrrC, *Smu*PrrC arrested yeast growth in liquid medium (Figure 1B) and was fungicidal after transient galactose induction and return to glucose (Figure 1C). We infer from these results that the toxic *Eco* and *Smu* PrrC proteins can incise essential target RNAs in yeast cells. The failure of *Nme*PrrC to arrest yeast growth was surprising to us, insofar as the nontoxic *Nme*PrrC protein has a significantly higher degree of amino acid identity (57%) with the *Eco*PrrC polypeptide than does *Smu*PrrC (42%). It is conceivable that: (i) *Nme*PrrC and *Xca*PrrC are nontoxic in yeast because they lack RNase activity, or (ii) *Nme*PrrC and *Xca*PrrC are *bona fide* ribotoxins, but their targets are not present in budding yeast (or are present but not essential for yeast growth). With respect to the latter issue, we tested the effects of induced expression of the four PrrCs on the growth of *E. coli* and found that the results were concordant those observed in yeast. Namely, *Eco*PrrC and *Smu*PrrC were toxic to *E. coli*, whereas *Nme*PrrC and *Xca*PrrC were not (data not shown). Thus, it is not simply a matter of the eukaryal milieu that masks an underlying ribotoxin activity of *Nme*PrrC and *Xca*PrrC. We surmise that members of the PrrC family differ with respect to their biological activity, which could reflect distinctive RNA target specificities and/or reliance on unique coactivators, e.g. if *Nme*PrrC and *Xca*PrrC require additional proteins from the cognate bacterium to manifest their RNase functions.

Structure-function analysis of *Eco*PrrC by alanine-scanning

The toxicity elicited by *Eco*PrrC expression in yeast affords a convenient assay to probe structure-activity relations. Our aim in this study was to map the amino acid functional groups of *Eco*PrrC required for cytotoxicity via alanine-scanning guided by a primary structure alignment of the *Eco*, *Nme* and *Smu* PrrC proteins (Figure 2A). We tested 53 *Eco*PrrC-Ala mutants for galactose-induced toxicity (Table 1). We thereby identified 20 nonessential residues (colored yellow in Figure 2A) and 33 essential residues (colored green in Figure 2A). In the case of nonessential residues such as His23, Lys238 and Lys325, their replacement by alanine still allowed for virtually complete growth inhibition on galactose-containing medium (Figure 2B). The *Eco*PrrC-C386A mutant also retained cytoxicity, albeit with very faint growth of the expressing yeast cells on galactose agar (Figure 2B), suggesting that C386A might be a hypomorph (see below.) Essential PrrC residues were those—like His295, Arg320, Arg349 and His356 in the C-terminal domain—at which alanine changes eliminated toxicity and permitted growth on galactose that was similar to that of the vector control (Figure 2B). Among the essential residues in the N-terminal domain were the five PrrC counterparts (Lys46, Thr47, Asp215, Asp216 and His251) of the

constituents of the conserved NTP-binding site of the ABC transporter NTPases (28). We infer that NTP binding/hydrolysis is essential for *Eco*PrrC toxicity in yeast. The collection of 33 essential PrrC residues (22 in the N-domain and 11 in the C-domain) included six histidines, five lysines, three arginines, six aspartates, five asparagines, three glutamates, two threonines, two serines and a tryptophan (Table 1).

Structure–activity relationships at essential residues in the NTPase domain

We proceeded to determine structure–activity relationships for each of the 22 essential residues in the NTPase domain by testing the effects of 43 conservative substitutions. The results are summarized in Table 1 and discussed below, wherever possible, in light of structures of the homologous motor domains of ABC-family NTPases bound to nucleotide and a divalent cation cofactor (28–31).

The PrrC NTPase domain contains a consensus Walker A-box motif (AxxGxGKT[47]) found in many nucleotide-dependent phosphotransferases. The A-box is situated between the first β-strand and the first α-helix of the NTPase module and forms a classical P-loop structure in which the main-chain amide nitrogens and the signature lysine side chain (Lys46 in PrrC) coordinate the NTP phosphate oxygens (Supplementary Figure S1). The signature threonine/serine side chain vicinal to the lysine (Thr47 in PrrC) coordinates the divalent cation cofactor that bridges the β and γ phosphates (Supplementary Figure S1). Lys46 and Thr47 were both essential for PrrC toxicity, according to the alanine scan. Lys46 was strictly essential, insofar as neither arginine nor glutamine was active in its stead. Thr47 was also strictly essential; neither serine not valine could sustain *Eco*PrrC toxicity in yeast, implying that, in addition to the imputed coordination of magnesium by Thr-Oγ, the Thr47-Cγ makes an important contact as well. [In the case of human CFTR ABC protein (pdb 2PZE; 31), the equivalent A-box threonine makes a close van der Waals contact to the metal-binding aspartate of the Walker B-box.] The *Eco*PrrC Arg48 side chain flanking the A-box was also strictly essential for toxicity in yeast; neither lysine nor glutamine was functional. It is possible that Arg48 engages in bidentate hydrogen bonding or ionic interactions that lysine does not sustain. Arg48 is conserved in *Nme*PrrC and *Smu*PrrC, but is replaced by glutamine in *Xca*PrrC (Figure 2A).

The ABC proteins are homodimers, arranged head-to-tail, with two composite NTPase active sites formed by motifs derived from the *cis* protomer (which provides the A-box and B-box) and the *trans* protomer, which interacts with the P-loop of the *cis* protomer and also directly coordinates the NTP γ phosphate (Supplementary Figure S1). The *Eco*PrrC peptide segment [215]DDPVSSLDDNH[225], which embraces eight essential side chains, is composed of two distinct ABC motifs that form the active site: a Walker B-box (YVFIDD[216]) derived from the *cis* protomer and a 'D loop' motif (SSLD[222] in *Eco*PrrC) derived from the
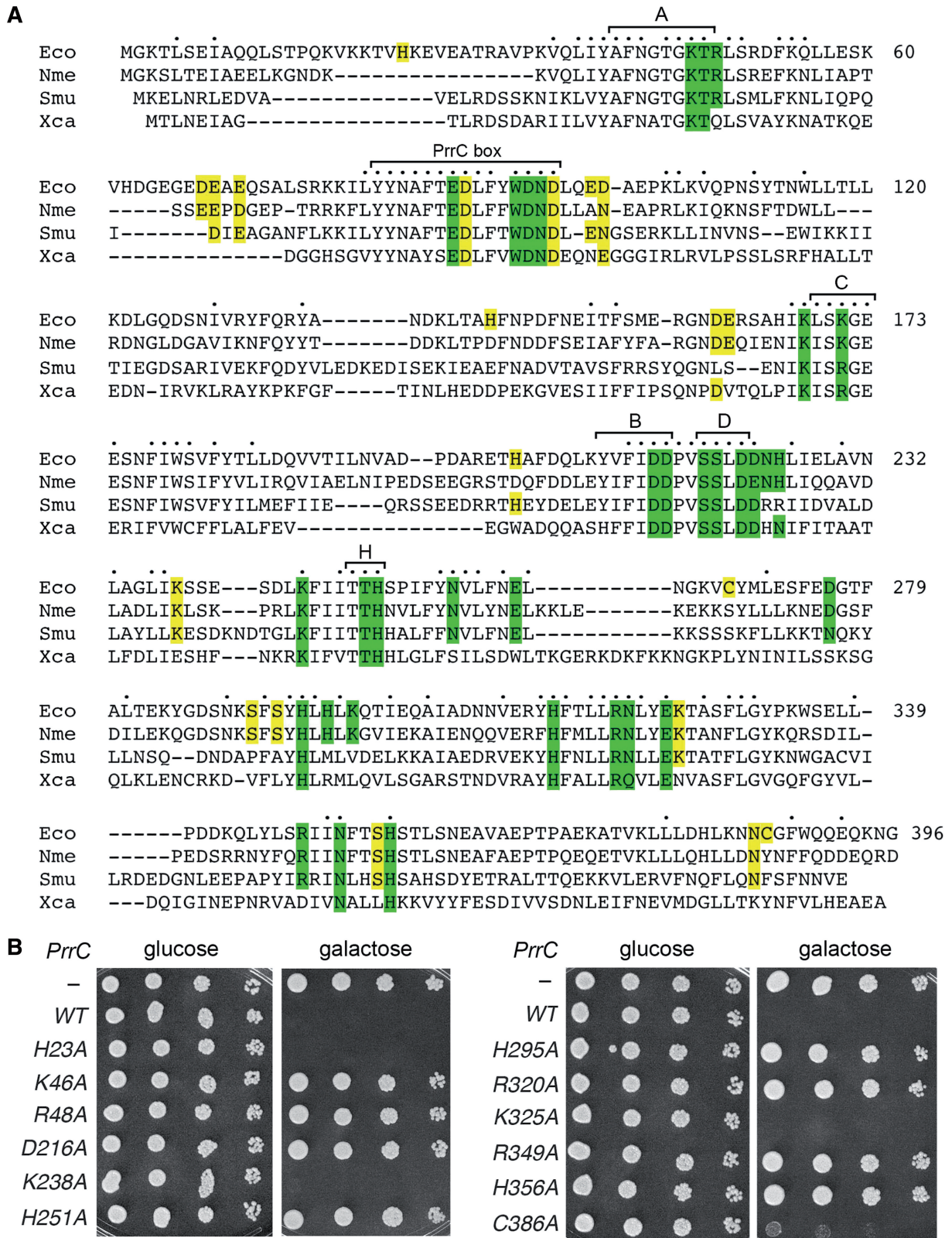
Figure 2. Homology-guided alanine-scanning mutagenesis of *Eco*PrrC. (**A**) The amino acid sequence of *Eco*PrrC is aligned to sequences of the *Nme*PrrC, *Smu*PrrC, and *Xca*PrrC proteins. Positions of side-chain identify/similarity in all four proteins are indicated by a filled black circle above the alignment. The conserved peptide motifs of the N-terminal NTPase domain are demarcated by brackets. The 54 amino acids targeted in the alanine scan are highlighted. Residues defined as essential for yeast toxicity by the alanine scan are shaded green; nonessential residues are shaded yellow. (**B**) Exemplary toxicity tests for wild-type *Eco*PrrC and EcoPrrC-Ala mutants are shown, in which serial 5-fold dilutions of yeast cells bearing the indicated *CEN GAL1-prrC* plasmid were spotted on –Leu agar plates containing 2% glucose or galactose as specified.

Table 1. Mutational effects on PrrC ribotoxin activity in yeast

PrrC allele	Toxicity	PrrC allele	Toxicity	PrrC allele	Toxicity	PrrC allele	Toxicity
WT	++	E162A	++	K238A	++	H315A	–
H23A	++	K168A	–	K245A	–	H315N	–
K46A	–	K168R	–	K245R	++	H315Q	–
K46R	–	K168Q	–	K245Q	–	R320A	–
K46Q	–	K171A	–	T250A	–	R320K	–
T47A	–	K171R	++	T250S	–	R320Q	–
T47S	–	K171Q	–	H251A	–	N321A	–
T47V	–	H204A	++	H251N	–	N321Q	–
R48A	–	D215A	–	H251Q	–	N321D	–
R48K	–	D215E	–	N257A	–	E324A	–
R48Q	–	D215N	–	N257Q	++	E324D	–
D68A	++	D216A	–	N257D	–	E324Q	–
E69A	++	D216E	–	E262A	–	K325A	++
E71A	++	D216N	–	E262Q	–	R349A	–
E88A	–	S219A	–	E262D	++	R349K	–
E88D	–	S219T	++	C268A	++	R349Q	–
E88Q	++	S219C	++	D276A	–	N352A	–
D89A	++	S220A	–	D276N	–	N352D	–
W93A	–	S220T	–	D276E	–	N352Q	++
W93Y	–	S220C	–	S291A	++	S355A	++
W93H	–	D222A	–	S293A	++	H356A	–
D94A	–	D222E	–	H295A	–	H356N	–
D94E	–	D222N	–	H295N	–	H356Q	–
D94N	–	D223A	–	H295Q	–	N385A	++
N95A	–	D223E	++	H297A	–	C386A	++
N95Q	–	D223N	–	H297N	–		
N95D	–	N224A	–	H297Q	–		
D96A	++	N224Q	++	K299A	–		
E99A	++	N224D	–	K299R	–		
D100A	++	H225A	–	K299Q	–		
H144A	++	H225N	++				
D161A	++	H225Q	–				

trans protomer (Supplementary Figure S1). The B-box provides two key carboxylates to the phosphohydrolase active site. The proximal aspartate is a component of the metal coordination complex. The vicinal Asp/Glu coordinates the water nucleophile. The corresponding Asp215 and Asp216 residues in *Eco*PrrC were both strictly essential; neither could be functionally substituted with asparagine or glutamate, signifying that a carboxylate is essential at both positions and that the putative PrrC active site cannot accommodate the longer main-chain to the carboxylate linker of Glu versus Asp.

In the ABC family, the eponymous aspartate side chain of the D loop caps an α-helix and makes a cross-protomer hydrogen bond with a phosphate-binding main chain amide of the A-box (Supplementary Figure S1). We find that the D loop Asp222 residue of *Eco*PrrC was essential and irreplaceable by Asn or Glu (Table 1). The two serines preceding the D loop aspartate were both essential for PrrC toxicity, but they displayed different structure-activity relations. Toxicity was restored when Ser219 was replaced by threonine or cysteine, signifying that hydrogen bonding of the Oγ atom is the functionally relevant property at this position and the extra methyl group of threonine is benign. The equivalent D loop serine in ABC proteins Sav1866 and HylB donates a hydrogen bond to the main-chain carbonyl of the essential B-box acidic residue located three residues upstream (corresponding to *Eco*PrrC Asp216) (30,31) (Supplementary

Figure S1). We surmise that PrrC Ser219 plays a structural role in stabilizing the conformation of the loop that contains the contiguous B and D motifs. At Ser220 of *Eco*PrrC, the S220T and S220C mutants were inactive *in vivo* (Table 1), which suggests a tight steric constraint on the Ser220 side chain that does not tolerate the extra bulk of the threonine-Cγ or even the larger atomic radius of the cysteine-Sγ versus serine-Oγ. The essential Asp223, Asn224 and His225 residues flanking the *Eco*PrrC D loop are not generally conserved among ABC proteins. We conclude that the carboxylate functional group is the pertinent property at position 223, because the D223E mutant was toxic in yeast while D223N was nontoxic (Table 1). Asp223 is conserved as Asp/Glu in the *Smu*, *Nme* and *Xca* PrrCs (Figure 2A). At Asn224, the amide group was critical for function, i.e. glutamine restored toxicity while aspartate did not. At His225, asparagine supported PrrC activity though glutamine did not, which suggests that hydrogen bonding by His225-Nδ is the relevant property of this residue.

The KFIITTH[251] motif of *Eco*PrrC that spans three essential side chains is the counterpart of the conserved 'H loop' motif of ABC-type NTPases (also called the 'switch' motif). The H loop connects a β-strand to an α-helix. The signature histidine donates a hydrogen bond to an NTP γ phosphate oxygen (28) that would stabilize the phosphohydrolase transition state. The H loop His251 of *Eco*PrrC is strictly essential for its activity *in vivo*,

insofar as the H251N and H251Q mutants were nontoxic (Table 1). The vicinal threonine-Oγ (corresponding to Thr250 in PrrC) makes a hydrogen bond to the main-chain amide of the H loop residue on the carboxyl side of the histidine (28) and thereby stabilizes the loop conformation. Loss of toxicity of the T250S mutant indicated that threonine is strictly essential in this position. The upstream Lys245 is essential for *Eco*PrrC and conserved in other PrrC homologs (albeit not in other ABC proteins). Positive charge appeared to suffice at this position, because K245R was active in yeast whereas K245Q was not (Table 1). Two important residues downstream of the H loop in *Eco*PrrC, Asn257 and Glu262, are conserved in *Nme*PrrC and *Smu*PrrC, but not in *Xca*PrrC or ABC proteins generally. The amide group of Asn257 is the key property, because mutant N257Q was toxic in yeast, whereas N257D was inactive (Table 1). At Glu262, the carboxylate was critical, i.e. E262D was toxic while E262Q was not. The C loop motif of ABC proteins (also called the ABC-signature motif) is conserved in *Eco*PrrC as LSKGE[173] (Figure 2A). A C loop derived from the *trans* protomer packs closely against the nucleoside and γ phosphate of the NTP substrate. The C loop and the A box P-loop of the *cis* protomer together form an oxyanion hole for the NTP γ phosphate (Supplementary Figure S1). Here we found that Lys171 within the C loop and Lys168 immediately preceding the loop were both essential for *Eco*PrrC toxicity. These two lysine residues are conserved among the PrrC homologs (Figure 2A), but they displayed different structure–activity relations. Whereas Lys168 was strictly essential (i.e. arginine and glutamine rendered PrrC nontoxic), Lys171 could be replaced functionally by arginine, but not glutamine, signifying that positive charge sufficed for activity at this position. The ABC protein Sav1866 has a lysine at the position corresponding to Lys168 in PrrC; the Sav1866 structure shows that the lysine packs over the adenine base of the bound NTP, with which it makes multiple van der Waals contacts (31; pdb 2ONJ).

Finally, we identified four essential residues (Glu88, Trp93, Asp94 and Asn95) within the *Eco*PrrC segment [82]YYNAFYEDLFYWDND[96] of the NTPase domain that has been dubbed the 'PrrC box' by the Kaufmann laboratory (19) in light of its strong conservation among bacterial PrrC homologs (Figure 2A). There is little primary structure similarity between the PrrC box and the corresponding segments of ABC proteins, though it is possible that the PrrC box is a divergent analog of the ABC Q-loop motif. The Q loop is a mobile hinge that is sensitive to the presence of NTP and metal ligands. The eponymous glutamine side chain of the Q loop (e.g. Gln90 in the MJ0796 protein) makes direct contacts with metal and the nucleophilic water in the phosphohydrolase active site (28). Replacing PrrC box residue Glu88 with glutamine supported toxicity in yeast, whereas aspartate did not. This result signifies that hydrogen bonding, not negative charge, is the key property of this residue and that the distance from the main-chain to the terminal functional group of Glu/Gln is critical, accounting for why retraction of this distance in aspartate leads to loss of PrrC activity.

Trp93 appeared to be strictly essential, in that function was not revived by installation of alternative aromatic (tyrosine) or planar hydrogen bonding (histidine) residues (Table 1). The flanking Asp94 and Asn95 residues were also strictly essential, i.e. the respective conservative mutants D94E, D94N, N95Q and N95D were nontoxic (Table 1).

Structure–activity relationships at essential residues in the nuclease domain

The C-terminal nuclease domains of PrrC and RloC proteins have no discernible primary structure similarity to any other tRNA ribotoxins, or to any known ribonucleases, phosphotransferases or tRNA-binding proteins. The Kaufmann laboratory has proposed two functional components of the *Eco*PrrC nuclease domain: (i) a triad comprising Arg320, Glu324 and His356 that they implicate in chemical catalysis of transesterification at the wobble nucleotide to generate 2′,3′-cyclic phosphate and 5′-OH product strands (19); and (ii) a putative lysine anticodon recognizing peptide (LARP) motif, [284]KYGDSNKSFSY[294] (33). The Arg–Glu–His triad is conserved among PrrC and RloC homologs, consistent with a catalytic function. In contrast, the LARP motif, mutations of which affect the tRNA substrate preference of *Eco*PrrC (16,17), is found only in a subset of PrrC proteins and is absent from RloC (20,33). Thus it is conceivable that LARP is a *bona fide* determinant of the target specificity of a subset of PrrC proteins that contains this motif. However, LARP is unlikely be the decisive factor with respect to yeast toxicity of bacterial PrrCs, insofar as the *Eco*PrrC LARP is well conserved (10/11 identical residues) in *Nme*PrrC, which is not toxic in yeast, yet LARP is not conserved (3/11 identical residues) in *Smu*PrrC, which is toxic in yeast (Figure 2A). Two alanine mutations in the *Eco*PrrC LARP motif tested presently (at Ser291 and Ser293, which are conserved in *Nme*PrrC) had no effect on cytotoxicity in yeast.

In mutagenizing the nuclease domain, we adopted an agnostic view and mainly targeted residues we deemed most likely to be involved in catalysis of phosphoryl transfer (histidine, lysine, arginine, glutamate) or RNA binding (lysine, arginine), based on general principles and the specific mechanisms of other well-studied ribonucleases that generate 2′,3′-cyclic phosphodiesters: e.g. RNase A, RNase T1, colicin E5 and tRNA splicing endonuclease (34–37). Our alanine scan identified 11 essential amino acids in the nuclease domain, at which we assessed structure-activity relationships with 22 conservative mutations. The results are summarized in Table 1 and discussed below in light of the catalytic mechanisms and structures of analogous ribonucleases that leave 2′,3′-cyclic phosphate and 5′-OH ends.

The 'classical' mechanism of RNA cleavage by transesterification exemplified in RNase A relies on two histidine side chains that serve, respectively, as: (i) a general base catalyst that abstracts a proton from the attacking ribose 2′-OH, and (ii) a general acid catalyst that donates a proton to the ribose 5′-OH leaving group (34). We replaced His295, His297, His315 and His356 in the

*Eco*PrrC nuclease domain with alanine, glutamine and asparagine and found that each histidine was strictly essential for toxicity (Table 1). Three of them—His295, His315 and His356—are conserved among PrrC homologs, and are therefore plausible candidates for a catalytic role, possibly as acid–base catalysts. Two of these three histidines (His295 and His356) are also conserved in RloC (20). In contrast, His297, though retained as histidine in nontoxic *Nme*PrrC, is replaced by methionine in the toxic *Smu*PrrC protein (Figure 2A), a scenario that makes it unlikely that His297 acts as a general acid–base catalyst.

Structural and functional studies of RNase A highlight a single essential lysine that interacts with the scissile phosphodiester and stabilizes the pentacoordinate transition state (34). None of the nine lysines in the nuclease domain of *Eco*PrrC is conserved in all three of the other PrrC homologs aligned in Figure 2A. Of the two lysines that we chose for the alanine scan, Lys325 was unessential and Lys299 was essential. PrrC was nontoxic when Lys299 was replaced conservatively by arginine or glutamine (Table 1). Yet, because this lysine is replaced by a valine and leucine in *Smu*PrrC and *Xca*PrrC, we think it unlikely that Lys299 plays a direct catalytic role in *Eco*PrrC.

Arginines classically play a role in ground-state binding and transition-state stabilization during phosphoryl transfer reactions by making bidentate contacts to the phosphate oxygens. Several of the transesterifying ribonucleases with known structures assimilate a catalytic arginine in their active sites. For example, the colicin E5 active site includes an arginine that coordinates both nonbridging oxygens of the scissile phosphodiester (37). The active site of RNase T1 also has an arginine that contacts the scissile phosphodiester (38). The active site of barnase includes two arginines that contact the scissile phosphodiester (39). Here we identified two arginines in the nuclease domain (Arg320 and Arg349) as strictly essential for *Eco*PrrC toxicity, i.e. Ala, Lys and Gln mutants thereof were inactive (Table 1). Both of these arginines are conserved in the toxic *Smu*PrrC homolog (Figure 2A) and in RloC (18) and are therefore plausible candidates for a catalytic role.

Glu324 was strictly essential for *Eco*PrrC toxicity (Table 1). This position is conserved as glutamate in the *Nme*, *Smu* and *Xca* homologs (Figure 2A) and also in RloC. Glutamate acts as a general base catalyst of RNA transesterification by RNase T1 and barnase (35,39,40).

We identified two essential asparagines in the *Eco*PrrC nuclease domain. Asn321 was strictly essential (Ala, Gln and Asp mutants were inactive; Table 1). This residue is conserved as Asn or Gln among PrrC homologs (Figure 2A). At Asn352, the Ala and Asp changes eliminated toxicity, but the conservative N352Q mutant retained toxicity. This result attests to the importance of the amide functional group at position 352 and tolerance by *Eco*PrrC of the longer main-chain to amide distance in Gln versus Asn. Asn352 is conserved as Asn in PrrC homologs (Figure 2A) and in RloC (18).

In sum, our mutational study of the nuclease domain verifies the importance of several residues studied by Kaufmann and colleagues (19), while identifying new candidate constituents of the active site and establishing structure–activity relationships for each of 11 essential residues. A definitive interpretation of the mutational data awaits an atomic structure of the nuclease domain.

*Eco*PrrC is toxic in the absence of a modified tRNA mcm⁵U wobble base

The target specificity of many tRNA anticodon nucleases is achieved via recognition of modified nucleobases in the anticodon loop, especially the wobble base. For example, colicin E5 cleaves bacterial tRNA^{Tyr}, tRNA^{His}, tRNA^{Asn} and tRNA^{Asp} that contain the wobble base queosine (37). *K. lactis* γ-toxin specifically cleaves yeast tRNA$^{Glu(UUC)}$ containing the modified wobble base mcm⁵s²U (5-methoxycarbonylmethyl-2-thiouridine; Figure 3C) (3,4). *Pichia acaciae* toxin (PaT) exerts its toxicity by incising a different yeast tRNA containing the mcm⁵s²U wobble base: tRNA$^{Gln(UUG)}$ (5).

*Eco*PrrC incises bacterial tRNA$^{Lys(UUU)}$ at a single phosphodiester 5′ of the modified wobble base mnm⁵s²U (5-methylaminomethyl-2-thiouridine) (16) (Figure 3C). The mnm⁵U wobble modification does not exist in eukaryal tRNAs, which instead have mcm⁵s²U in their tRNA$^{Lys(UUU)}$ (and also in tRNA^{Glu} and tRNA^{Gln}). *Kluyveromyces lactis* γ-toxin requires the mcm⁵s²U modification in its tRNA^{Glu} target, such that yeast *tot3Δ(elp3Δ)* and *trm9Δ* mutants, which either fail to modify the C5 atom or fail to add the terminal methyl group (Figure 3C), are resistant to γ-toxin's effects (41). Consequently, galactose-induced intracellular expression of γ-toxin, which prevents growth of wild-type *S. cerevisiae*, had no effect on the growth of *tot3Δ* and *trm9Δ* cells (Figure 3A). In contrast, we found that *Eco*PrrC was toxic to *tot3Δ* and *trm9Δ* cells (Figure 3A). Thus, we infer that: (i) the target specificity of PrrC in yeast differs from that of γ-toxin; and (ii) if PrrC exerts its toxicity in yeast by cleaving tRNA^{Lys}, then it does so without strict need for the mcm⁵s²U wobble modification.

Is yeast tRNA^{Lys} a target of *Eco*PrrC?

If an intracellular ribotoxin exerts its effect by breaking a specific cellular RNA target, then one might expect to reverse the toxicity by overexpressing the RNA target. This is demonstrated nicely for *K. lactis* γ-toxin, whereby overexpression of its specific target tRNA$^{Glu(UUC)}$ protects yeast from toxin-induced growth arrest (25) (Figure 3B). Here we screened various yeast tRNAs on multicopy 2 μ plasmids for their ability to protect yeast from the toxicity of *Eco*PrrC. Increased gene dosage of tRNA$^{Glu(UUC)}$ afforded no protection from *Eco*PrrC, but neither did overexpression of the presumptive target tRNA$^{Lys(UUU)}$, the isoacceptor tRNA$^{Lys(CUU)}$ or a combination of both tRNA$^{Lys(UUU)}$ and tRNA$^{Lys(CUU)}$ (Figure 3B). High-copy plasmids expressing other tRNAs with wobble uridines (tRNA^{Gln}, tRNA^{Arg}, tRNA^{Leu} or tRNA^{Gly}) or tRNA$^{Tyr(GUA)}$ were also ineffective (data not shown). Several possibilities come to mind to explain the negative results of the tRNA rescue experiment: (i) tRNA^{Lys} is not a PrrC target in yeast; (ii) tRNA^{Lys} is a PrrC target, but so are
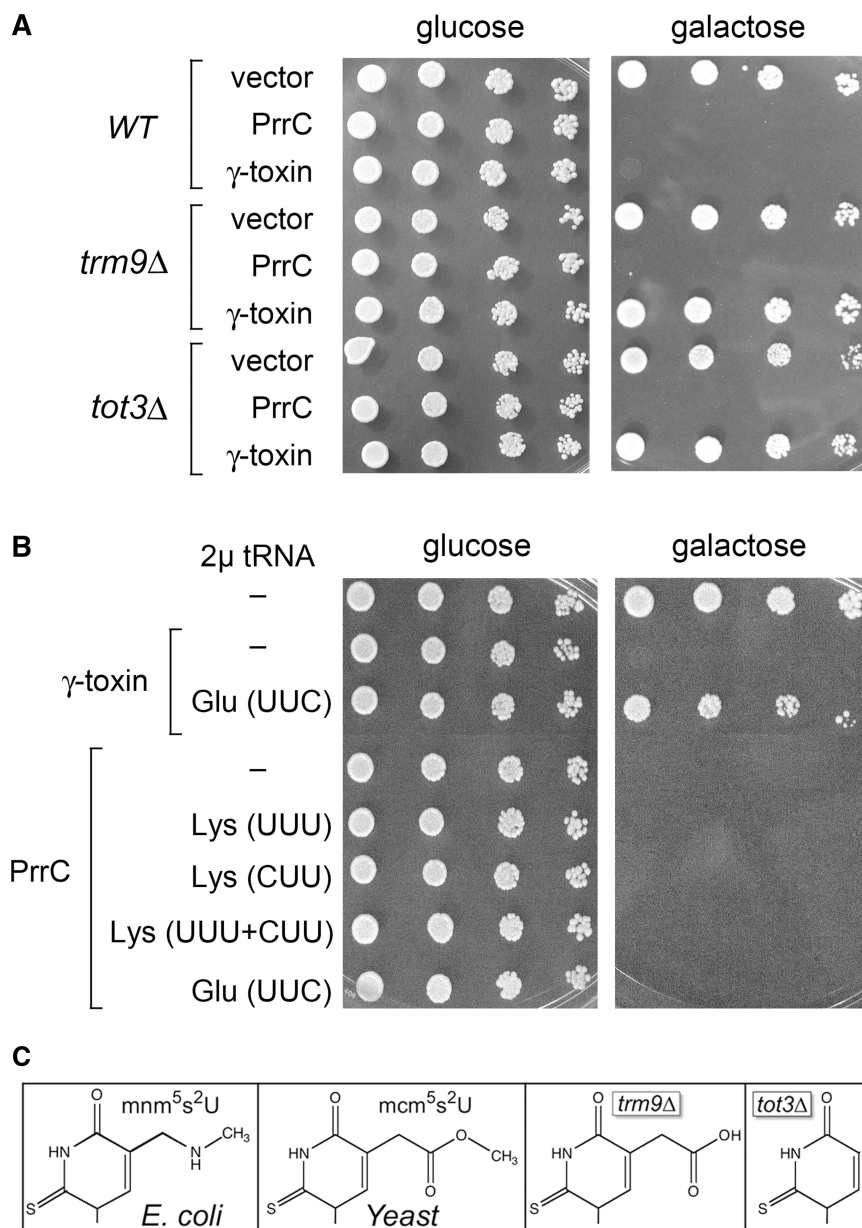
Figure 3. *Eco*PrrC is toxic in the absence of a modified tRNA mcm^{5}U wobble base. (**A**) Serial 5-fold dilutions of wild-type (WT), *trm9*Δ, and *tot3*Δ yeast cells bearing a *CEN* plasmid encoding galactose-regulated *Eco*PrrC or *K. lactis* γ-toxin were spotted on –Leu agar plates containing 2% glucose or galactose as specified. (**B**) Serial dilutions of wild-type yeast cells bearing a *CEN* plasmid encoding galactose-regulated *Eco*PrrC or *K. lactis* γ-toxin plus a 2 μ plasmid carrying the indicated yeast tRNA genes were spotted on –Leu–Ura agar plates containing glucose or galactose. (**C**) Structures of the wobble uridine modifications found in tRNA$^{Lys(UUU)}$ of *E. coli* (mnm^{5}s^{2}U), wild-type yeast (mcm^{5}s^{2}U) and yeast mutants *trm9*Δ (cm^{5}s^{2}U; 5-carboxymethyl-2-thiouridine) and *tot3*Δ (s^{2}U; 2-thiouridine).

other essential yeast RNAs or (iii) tRNA^{Lys} is the principal PrrC target, but the level of anticodon nuclease activity in the PrrC-producing yeast cells is sufficient to cleave the target tRNA pool even when the tRNA gene is present in high copy.

To interrogate the third option, we surveyed several of our collection of 'active' PrrC mutants for rescue of toxicity by increased tRNA^{Lys} gene dosage. We focused especially on two possibly hypomorphic mutants, C386A and S219T, that were clearly toxic, but reliably yielded faint spots when cells bearing the *CEN prrC-C386A* and *prrC-S219T* plasmids were plated on galactose agar at low

dilution. We found that overexpression of tRNA$^{Lys(UUU)}$ partially protected yeast cells from the toxic effect of both of these PrrC mutants, whereas overexpression of tRNA$^{Lys(CUU)}$ or tRNA$^{Glu(UUC)}$ did not (Figure 4A). This instructive result indicated that tRNA$^{Lys(UUU)}$ is a physiologic target of PrrC toxicity in yeast, as it is in *E. coli*.

The inferred hypomorphic quality of *Eco*PrrC S219T and C386A was reinforced by the results of experiment shown in Figure 4B, wherein we analyzed their effect on growth of *trm9*Δ cells. Whereas wild-type *Eco*PrrC was profoundly toxic in *trm9*Δ, neither of the mutants
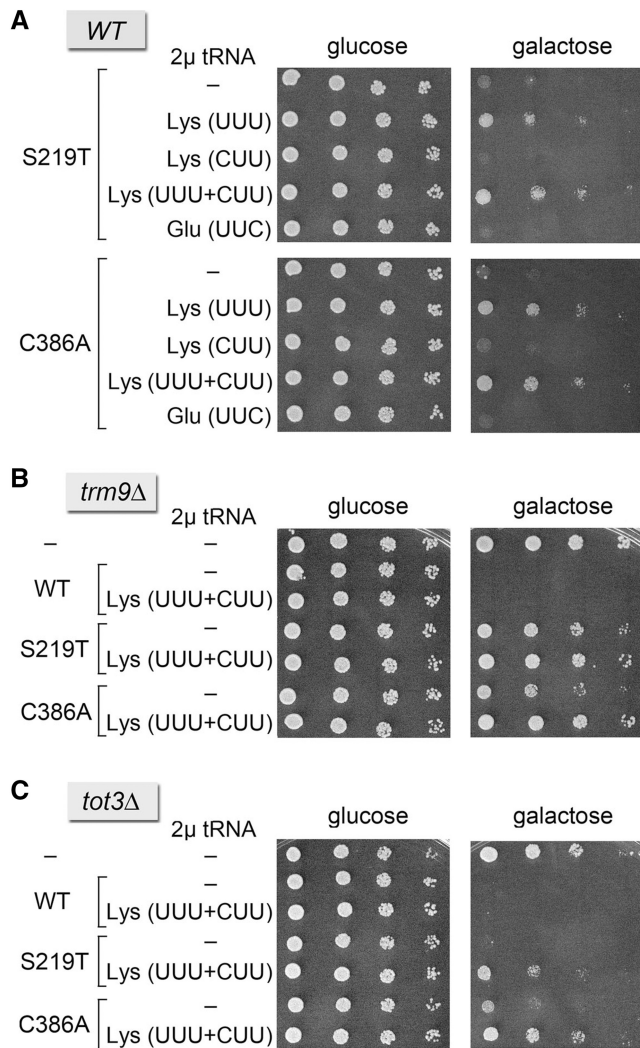
Figure 4. Rescue of *Eco*PrrC S217 and C386A toxicity by 2 μ tRNA^{Lys(UUU)}. (**A**) Serial dilutions of wild-type yeast cells bearing a *CEN* plasmid encoding galactose-regulated *Eco*PrrC mutants S217A or C386A plus a 2 μ plasmid carrying the indicated yeast tRNA genes (or an empty 2 μ vector, denoted by a dash) were spotted on –Leu–Ura agar plates containing glucose or galactose. (**B**) Serial dilutions of yeast *trm9*Δ cells bearing a *CEN* plasmid encoding galactose-regulated wild-type *Eco*PrrC or mutants S217A or C386A plus a 2 μ plasmid carrying the indicated yeast tRNA genes (or an empty 2 μ vector, denoted by a dash) were spotted on agar plates containing glucose or galactose. (**C**) Serial dilutions of yeast *tot3*Δ cells bearing a *CEN* plasmid encoding galactose-regulated wild-type *Eco*PrrC or mutants S217A or C386A plus a 2 μ plasmid carrying the indicated yeast tRNA genes (or an empty 2 μ vector, denoted by a dash) were spotted on agar plates containing glucose or galactose.

prevented growth of the *trm9*Δ cells on galactose. Rather, they had a modest effect on growth rate, as gauged by colony size compared to the vector control (Figure 4B). Moreover, while the toxicity of wild-type *Eco*PrrC in *trm9*Δ cells was unabated by increased dosage of tRNA^{Lys}, the same maneuver sufficed to reverse the slow-growth phenotypes of the S219T and C386A mutants (Figure 4B). In contrast, the yeast *tot3*Δ mutation *per se* afforded no protection against the toxic effect of the S219T mutant, and only minimally protection

against C386A (evident at low dilution), notwithstanding that partial protection of *tot3*Δ cells against both mutants was still conferred by tRNA^{Lys} overexpression (Figure 4C).

We surmise from these experiments that: (i) *Eco*PrrC can target a wobble uridine with either bacterial mnm⁵ or eukaryal mcm⁵ modifications *in vivo*; and (ii) *Eco*PrrC can also target a wobble uridine with no modifications at the C5 atom (*tot3*Δ). However, an incompletely modified eukaryal wobble uridine lacking the terminal methyl group (*trm9*Δ; Figure 3C) is relatively resistant *in vivo* to *Eco*PrrC S219T and C386A. Our findings resonate with *in vitro* studies from the Kaufmann laboratory (16,17) that showed a hierarchy of wobble base-modification effects on the cleavage of anticodon stem-loop structures by extracts of *E. coli* expressing a different hypomorphic *Eco*PrrC mutant (D222E). The order of wobble U preferences was: mnm^{5}s^{2}U > s^{2}U > mcm^{5}s^{2}U.

Dominant negative effects of PrrC mutants

PrrC and RloC are the only known ribotoxins with an ABC-like NTPase domain. Whereas the present mutational scan of the *Eco*PrrC NTPase fortified the conclusion that NTP binding and/or hydrolysis are essential for PrrC toxicity, the mechanism by which the PrrC NTPase activates the PrrC nuclease is unknown. It has been suggested that one role of NTPase domain could be the regulation the nuclease-masking interaction PrrC with the EcoPrrI DNA restriction enzyme (18). This would not be a factor in yeast toxicity studied presently. Structural studies of other ABC domains highlight the general theme that formation or stability of the head-to-tail ABC dimer is influenced by NTP occupancy of the phosphohydrolase active site. If it is true for PrrC that NTP binding acts as an allosteric switch, then the key issue is whether the NTP switch activates the nuclease directly (by inducing an active state of the inherently latent nuclease domain) or indirectly (by relieving the constitutively repressive effects of the NTPase domain on an inherently competent nuclease domain).

One prediction of a purely anti-repression model is that removal of the NTPase domain might lead to a constitutively active nuclease. However, this was not the case in yeast, insofar as we found that induced expression of the isolated C-terminal nuclease domain of *Eco*PrrC had no effect on cell growth, even when the nuclease domain was expressed from a multicopy 2 μ plasmid (data not shown). In addition, we found that induced coexpression of the *Eco*PrrC NTPase and nuclease domains in *trans* (from separate genes on *CEN* or 2 μ plasmids) also had no effect on yeast growth (data not shown). We surmised from these results that the NTPase and nuclease domains must be linked in *cis* for *Eco*PrrC to exert its toxicity in yeast.

A plausible direct activation model invokes NTP-triggered dimerization of the NTPase domain to yield a PrrC quaternary structure in which the previously latent nuclease module is now functional for tRNA target recognition and scission. One prediction of an obligatory oligomerization model is that overexpression of inactive

mutants of PrrC might dampen the activity of wild-type PrrC, by forcing the assembly of defective hetero-oligomers via mass action. We evaluated this scenario by installing several of our nontoxic *prrC*-Ala alleles on 2μ plasmids under the control of a *GAL1* promoter. After verifying that these mutants were nontoxic when the 2μ plasmid-bearing cells were plated on galactose-containing agar (Figure 5A; PrrC–), we tested them for a dominant-negative effect on the galactose-induced growth arrest caused by wild-type *Eco*PrrC on a *CEN* plasmid (Figure 5A; PrrC+). Spotting tests revealed distinct levels of effects exerted by overexpression of the mutants in *trans*. For example, mutants K46A, T250A and H251A in the NTPase domain partially suppressed the toxicity of wild-type *Eco*PrrC and allowed the cells to form colonies on galactose agar, albeit smaller colonies than the 'PrrC–' controls (Figure 5A). In contrast, mutants H315A, R320A and E324A in the nuclease domain had essentially no impact on the toxicity of wild-type *Eco*PrrC (Figure 5A).

To quantify the dominant negative effect, we grew liquid cultures of yeast strains bearing a *CEN* wild-type *Eco*PrrC plasmid (or empty *CEN* control) plus 2μ plasmids expressing 15 different nontoxic PrrC-Ala mutants (or an empty 2μ vector control). Equal aliquots of cells from the cultures were then transferred to liquid medium containing galactose to induce PrrC expression. Measurement of A_{600} after overnight growth in galactose $\pm$ wild-type *Eco*PrrC revealed that the PrrC–culture bearing the empty 2μ vector had grown to saturation ($A_{600} > 4.0$), while the PrrC+ culture was arrested completely (A_{600} 0.26; compare black and white bars in Figure 5B, 'vector'). Six of the PrrC mutants (H295A, H315A, R320A, E324A, R349A and H356A) had little or no impact on the toxicity of wild-type PrrC, i.e. cells expressing these mutant alone grew to saturation in

galactose ($A_{600} > 4.0$), whereas cells coexpressing wild-type PrrC grew to A_{600} of 0.3–0.6 (Figure 5). In contrast, the toxicity of wild-type PrrC was clearly ameliorated in cells overexpressing mutants K46A, T47A, R48A, K168A, D215A, D216A, S220A, T250A and H251A, such that the cultures grew to A_{600} values of 1–2 (Figure 5B). It was most striking that all of the dominant negative mutations were in the putative active-site motifs of the NTPase domain, whereas the mutations that did not have a negative effect in *trans* were all in located in the nuclease domain.

These results are consistent with a model for the physical organization of the active *Eco*PrrC toxin, in which: (i) antiparallel head-to-tail dimerization of the NTPase domain is required for PrrC toxicity in yeast; and (ii) toxicity requires two fully functional NTP-binding phosphohydrolase active sites (Figure 6A). Consequently, the dominant negative effect of the NTPase mutants results from the formation of mixed dimers in which one of the active sites is defective (Figure 6B). In contrast, we infer that just one active nuclease module suffices for PrrC toxicity in yeast, provided that proper dimerization has occurred, thereby accounting for why overexpression of PrrC with a nuclease-inactivating mutation (but an intact NTPase domain) does not diminish the toxicity of wild-type *Eco*PrrC (Figure 6C). (A caveat to this interpretation of the absence of dominant negative effects of the nuclease mutants is that we have not directly gauged the steady-state levels of the mutant PrrC proteins expressed in yeast from the 2μ plasmids. Thus we cannot exclude a scenario in which all of the mutations surveyed in the nuclease domain destabilize PrrC in yeast while none of the mutations in the NTPase domain have this effect.) The model in Figure 6 focuses on dimerization as a key quaternary-structure trigger, but is equally adaptable to alternative
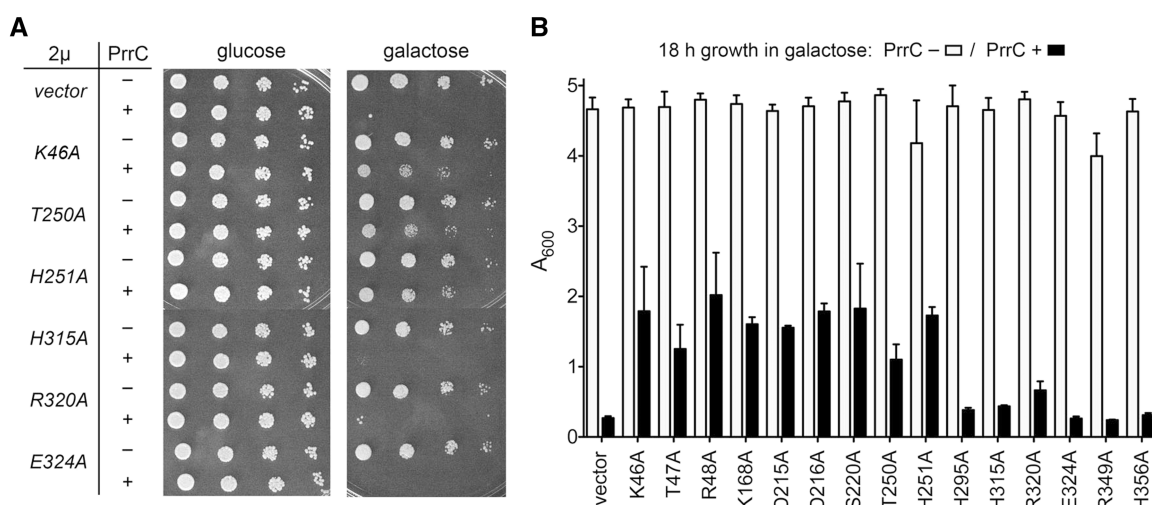

Figure 5. Dominant negative effects of PrrC-Ala mutants. (**A**) Serial dilutions of yeast cells bearing a *CEN* plasmid encoding wild-type *Eco*PrrC (PrrC+) or the empty *CEN* vector (PrrC–) plus a 2μ plasmid encoding the indicated PrrC-Ala mutants (or the empty 2μ vector) were spotted on –Leu–His agar plates containing glucose or galactose. (**B**) Yeast cells bearing a *CEN* plasmid encoding wild-type *Eco*PrrC (PrrC+) or the empty *CEN* vector (PrrC–) plus a 2μ plasmid encoding the indicated PrrC-Ala mutants (or the empty 2μ vector) were inoculated into SD–Leu–His medium containing 2% galactose to attain an A_{600} of 0.1. The cultures were incubated at 30°C for 18 h, at which time A_{600} was measured. Each datum is the average of three independent galactose-induction experiments $\pm$ SEM.
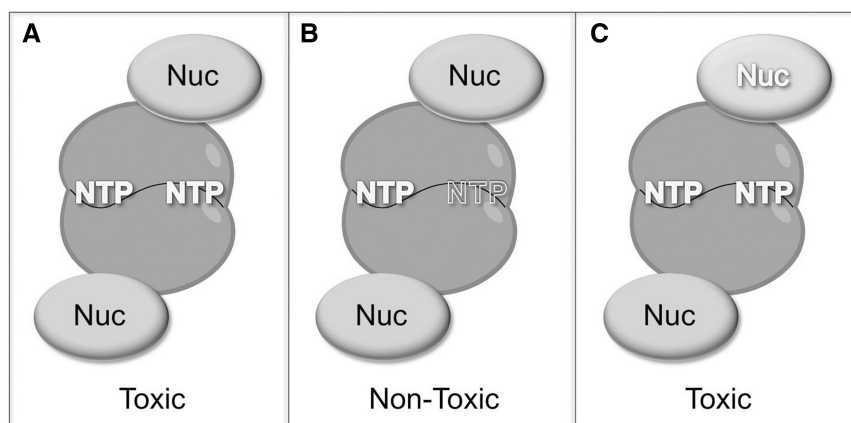
Figure 6. Model for oligomeric domain organization in *Eco*PrrC in light of mutational data and dominant negative effects of NTPase mutations. (**A**) Toxicity of wild-type *Eco*PrrC is contingent on head-to-tail dimerization of the ABC-like NTPase domains and the consequent formation of two composite NTP phosphohydrolase active sites ('NTP' in white bold type), which in turn activates the C-terminal nuclease domains ('Nuc' in black). (**B**) A mixed PrrC dimer of one wild-type protomer and one NTPase-defective protomer ('NTP' in gray) is nontoxic, because only one of the two composite NTPase sites is intact. (**C**) A mixed dimer of one wild-type protomer and one nuclease-defective protomer ('Nuc' in black) is toxic, because it is able to execute the proposed dual NTP-dependent activation of the one intact nuclease module.

oligomerization models, variously invoking hexameric or tetrameric states of *Eco*PrrC (18,19,33), wherein all NTPase sites, but not necessarily all nuclease sites, must be intact to exert toxicity.

CONCLUSIONS

Programmed RNA damage is a common feature of cellular responses to virus infection, whether it be tRNA restriction by PrrC in *E. coli* (14) or innate immune signaling via RNase L-damaged RNAs in mammalian cells (42). There is also a growing consensus that programmed RNA damage—especially tRNA damage—is a common feature of eukaryal cellular stress responses, wherein the broken tRNAs molecules *per se* can have a signaling role at levels of tRNA damage that do not significantly deplete the pool of the tRNA target (43–51). This suggests a possible therapeutic niche for enzymatic and chemical ribotoxins, predicated either on: (i) depleting an essential RNA or (ii) eliciting an 'RNA damage response' (e.g. reduced and altered protein synthesis, altered gene expression, cell-cycle arrest, apoptosis, etc.) analogous to DNA damage responses. As an example of the former mode, Kaufmann *et al.* have suggested PrrC as a means of interdicting HIV infection via depletion of the pool of human tRNA^{Lys} that serves as primer for HIV reverse transcriptase (17,52).

Cytotoxic ribonucleases from bacteria and eukarya exemplify a new modality of cancer therapy (53). Onconase, an RNase A-like ribonuclease elaborated by frogs, has been studied extensively as an anticancer agent (54). Onconase cytotoxicity is facilitated by its ready uptake by mammalian cells, its resistance to the cellular RNase inhibitor protein and its induction of damage to tRNAs in human tumor cells (55). Bacterial tRNA ribotoxins have much higher selectivity for specific tRNA target sites than does Onconase. The present demonstration that PrrC is

fungicidal in yeast, together with recent reports that colicins D and E5 are also growth suppressive in yeast (22,23), suggest practical applications for bacterial tRNA anticodon nucleases in eukarya. Because fungi appear not to have an endogenous RNA repair system capable of rectifying the tRNA anticodon breaks (56), they would be vulnerable to ribotoxins as antifungals, provided one could devise a way to modify the toxin to promote its cellular uptake. Cancer cells might also be sensitized to tRNA ribotoxins, alone or in combination with other chemotherapeutics.

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