Supplemental Material

RtcB is the RNA ligase component of an *Escherichia coli* RNA repair operon

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Recombinant RtcB. The *rtcB* ORF was amplified by PCR from *E. coli* genomic DNA with primers designed to introduce a BamHI site at the ATG start codon and an XhoI site immediately downstream of the stop codon. The PCR product was digested with BamHI and XhoI and then inserted between the BamHI and XhoI sites of pET28b-His_{10}Smt3. The plasmid insert was sequenced completely to exclude the acquisition of unwanted changes during amplification and cloning. The pET28b-His_{10}Smt3-RtcB plasmid was transformed into *E. coli* BL21-CodonPlus(DE3). A 1-liter culture derived from a single transformant was grown at 37°C in LB medium containing 50 μg/ml kanamycin until the A_{600} reached 0.6 to 0.8. The culture was chilled on ice for 30 min and then adjusted to 0.1 mM isopropyl β-D-thiogalactoside. Incubation was continued at 17°C for 16 h with constant shaking. Cells were harvested by centrifugation and stored at −80°C. All subsequent procedures were performed at 4°C. The cell pellet was suspended in 50 ml buffer A (50 mM Tris-HCl, pH 7.4, 250 mM NaCl, 10% sucrose) and lysozyme was added to 0.2 mg/ml. After mixing for 30 min, the cell suspension was adjusted to 0.1% Triton X-100 and incubated for 15 min. The lysate was sonicated to reduce viscosity and insoluble material was removed by centrifugation at 20,000 g for 30 min. The soluble lysate was mixed for 1 h with 12 ml of a 50% slurry of Ni-NTA agarose (QIAGEN) that had been equilibrated in buffer A. The resin was recovered by centrifugation and resuspended in 25 ml buffer B (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10% glycerol) containing 25 mM imidazole. The cycle of centrifugation and resuspension of the resin in buffer B was repeated three times, after which the washed agarose beads were poured into a column. The column was washed with 25 ml of 2 M KCl in 50 mM Tris-HCl (pH 7.4) and then eluted stepwise with 7-ml aliquots of 100, 200, 300, 400 and 500 mM imidazole in buffer B. The elution profile was monitored by SDS-PAGE. The 300 mM imidazole eluate containing His_{10}Smt3-RtcB was treated with the Smt3-specifc protease Ulp1 (at a His_{10}Smt3-RtcB:Ulp1 ratio of 500:1) during overnight dialysis against buffer B. The dialysate was mixed with 3 ml of 50% slurry of Ni-NTA agarose that had been equilibrated in buffer B and the mixture was poured into a column. The column was washed with buffer B and bound material was eluted with 500 mM imidazole. The tag-free RtcB protein was recovered in the flow-through fractions, whereas the cleaved His_{10}Smt3 was bound to the resin and recovered in the imidazole eluate. The tag-free RtcB preparation was concentrated by centrifugal ultrafiltration (Amicon Ultra-4, 10 kDa cut-off; Millipore) to a

concentration of 16 mg/ml (in 500 µl) then gel-filtered through a 120 ml 16/60 HiLoad Superdex 200 pg column (GE Healthcare) equilibrated with buffer C (10 mM Tris-HCl, pH 8.0, 350 mM NaCl, 1 mM DTT) at a flow rate of 1.0 ml/min, while collecting 2-ml fractions. The peak RtcB-containing fractions (Fig. S1) were pooled and concentrated by centrifugal ultrafiltration. Protein concentration was determined by using the Biorad dye reagent with bovine serum albumin as the standard. The yield of RtcB was 5 mg from a 1-liter bacterial culture.

Other recombinant proteins. *K. lactis* gamma toxin was produced in *E. coli* and purified as described (*1*). *Arabidopsis thaliana* tRNA ligase (AtRNL) was produced in *E. coli* and purified as described (*2*).

RNA repair substrates. Synthetic RNA oligonucleotides R39 and R30 containing the anticodon step-loop of yeast $tRNA^{Glu(UUC)}$ (purchased from Dharmacon) are shown below with the anticodon trinucleotide underlined and the site of incision by *K. lactis* γ-toxin flanking the wobble uridine indicated by "p".

R39: 5'-UGGCUCCGAUAUCACGCUUpUCACCGUGGUAUCGGAGCGC

R30: 5'-UGGCUCCGAUAUCACGCUUpUCACCGUGGUA

R39 and R30 were 5' ^{32}P-labeled by reaction with T4 Pnkp and [γ^{32}P]ATP and then purified by preparative PAGE. The labeled tRNA-like stem-loops were cleaved by reaction with purified recombinant *K. lactis* γ-toxin (*1*). Cleavage reaction mixtures (40 µl) containing 20 mM Tris-HCl (pH 7.5), 2 M trimethylamine oxide, 20 µM 5' ^{32}P-labeled R39 or R30, and 20 µM γ-toxin were incubated at 4˚C for 2 h. The products of R39 cleavage – a 5' ^{32}P-labeled 19-mer (R19) and an unlabeled 20-mer (R20) – were co-purified free of residual R39 by preparative PAGE. The 5' ^{32}P-labeled 19-mer (R19) produced by incision of R30 was purified free of the unlabeled 11-mer product and residual R30 by preparative PAGE. A synthetic R20 oligonucleotide (5'-UCACCGUGGUAUCGGAGCGC; from Dharmacon) was employed to reconstituted the tRNA-like stem loop by mixture with the 5' ^{32}P-labeled R19 single strand. Prior to use in ligation assays, the cold R20 oligonucleotide was subjected to preparative PAGE in order to eliminate the short oligonucleotide contaminants.

1. Keppetipola, N., Jain, R., Meineke, B., Diver, M., and Shuman, S. (2009) *RNA* **15**, 1036-1044.
2. Nandakumar, J., Schwer, B., Schaffrath, R., and Shuman, S. (2008) *Mol. Cell* **31**, 278-286.

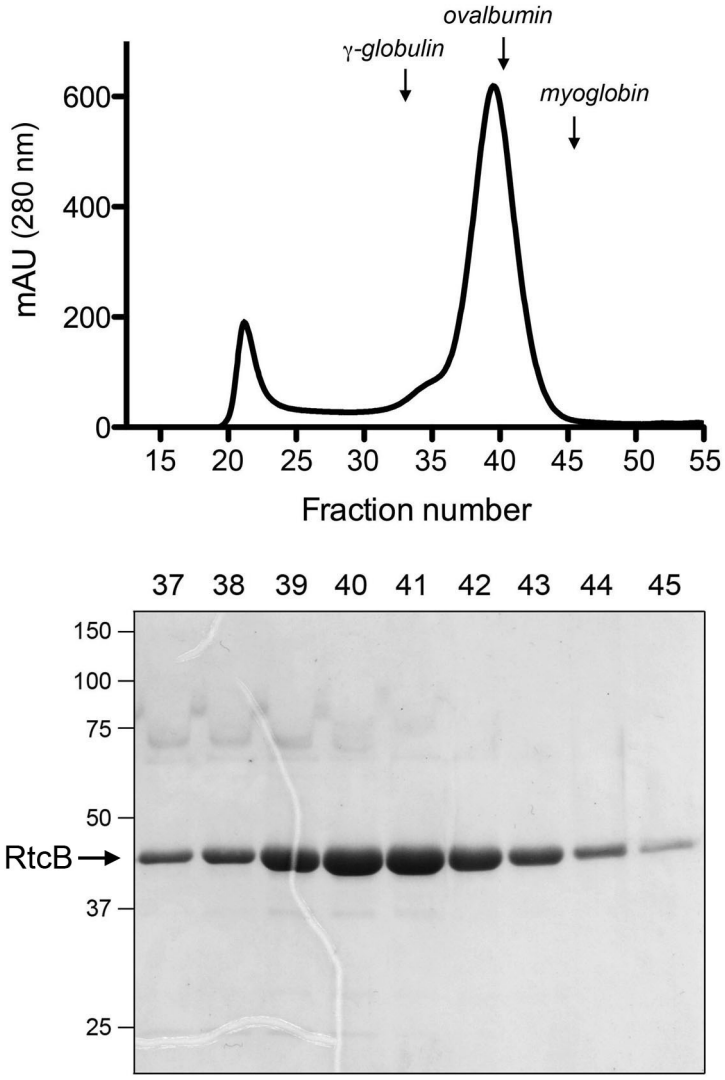

Figure S1. **Gel filtration of purified RtcB**. RtcB was analyzed by Superdex 200 gel filtration as described under Methods. (*Top panel*) The protein elution profile was monitored continuously by UV absorbance. A_{280} is plotted as a function of fraction number. The column was calibrated by tracking the elution profiles of marker proteins of known native size. The peak positions of the markers aldolase (158 kDa), ovalbumin (43 kDa), and myoglobin (17 kDa) are indicated by arrows. (*Bottom panel*) Aliquots (5 µl) of the indicated column fractions were analyzed by SDS-PAGE. The Coomassie blue-stained gel is shown. The positions and sizes (kDa) of marker proteins are indicated on the left. The elution profile of the RtcB protein tracked with the major A_{280} peak.

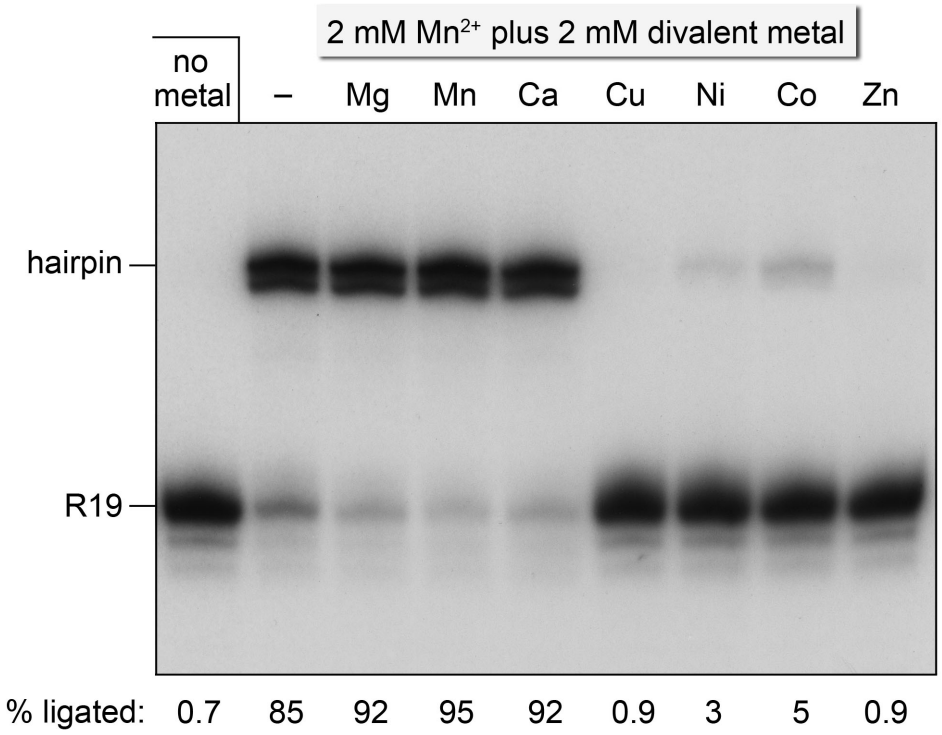

Figure S2. **Insights to divalent cation specificity from a metal mixing experiment**. Reaction mixtures (10 µl) containing 50 mM Tris-HCl (pH 7.4), 0.1 µM 5' ^{32}P-labeled R19 strand, 1 µM cold R20 strand, 100 µM GTP, 1 µM RtcB, 2 mM $MnCl_2$, and 2 mM additional divalent cation as specified (either 2 mM $MgCl_2$, $MnCl_2$, $CaCl_2$, $CuCl_2$, $NiCl_2$, $CoCl_2$, or $ZnCl_2$) were incubated at 37˚C for 30 min. Manganese was omitted from an RtcB-containing control reaction (no metal). The products were resolved by denaturing PAGE and visualized by autoradiography. The extents of ligation were quantified by scanning the gel with a phosphorimager and are specified below the lanes.

Figure S3. ***E. coli* RtcB and its *Pyrococcus* homolog**. The amino acid sequences of *E. coli* (Eco) RtcB and *Pyrococcus horikoshii* (Pho) RtcB (from the crystal structure in pdb ID IUC2) are aligned. The 187 positions of side chain identity/similarity are indicated by ●. Gaps in the alignment are indicated by –. The conserved histidines and cysteine that are clustered together in the crystal structure and may comprise a metal-binding site are highlighted in yellow.