SUPPLEMENTAL FIGURE LEGENDS

Figure S1, related to Figure 2. Rsr and PNPase interact via the Y RNA.

(A) ^{32}P-labeled *D. radiodurans* Y RNA was mixed with no protein (lanes 1 and 5), Rsr purified from either baculovirus-infected insect cells (lane 2) or *E. coli* (lane 6), PNPase (lanes 3 and 7) or both Rsr and PNPase (lanes 4 and 8) and fractionated in nondenaturing gels.

(B) To confirm the identities of the RNPs, bands labeled **a**, **b**, **c**, **d**, and **e** were excised and subjected to SDS-PAGE and Western blotting to detect Rsr and PNPase. As standards, PNPase (lane 1) and Rsr (lane 2) were run in parallel in a separate gel and visualized by Western blotting. The two gels were joined at the lines.

(C) Domain structure of PNPase.

(D) Molecular surface representations of *S. antibioticus* PNPase (PDB 1E3P). Portions of the KH and S1 domains visualized in this structure are colored dark gray and black, respectively.

(E) Because PNPase mutants lacking the KH or S1 domains do not bind the Y RNA (Figure 2E), we confirmed that the mutants formed trimers using nondenaturing gels. Proteins were visualized by staining with Coomassie blue. Lane 1, molecular size standards.

(F) Full-length and mutant PNPases were examined for trimer formation using negative stain electron microscopy. From left to right, raw micrographs of wild-type PNPase and mutants lacking the KH or S1 domains. Scale bar, 50 nm.

Figure S2, related to Figure 3. Single particle EM of the Rsr/Y RNA/PNPase RNP.

(A) Representative raw electron micrograph tilt-pairs of negatively stained Rsr/Y RNA/PNPase complexes. Individual corresponding particles are marked in yellow circles. Scale bar, 100 nm.

(B) Random conical tilt (RCT) class volume showing the *ab initio* structure of the RNP. Scale

bar, 5 nm.

(C) Initial model generated by projection matching refinement of the reference-free class averages of the RNP to the RCT volume. This initial model was used for refinement of the raw particle images of the RNP. Scale bar, 5 nm.

(D) Reference-free class averages of the RNP in complex with a monoclonal antibody directed towards the N-terminal His_6-tag of Rsr. The three major components of the assembly are labeled for clarity in the first class average. Scale bar, 10 nm.

(E) Final EM map of the RNP segmented based on the antibody-labeling results in (E). The two major components of the assembly are colored as in Figure 3. PNPase, blue; Ro, purple. Scale bar, 5 nm.

(F) Reprojections of the Rsr/Y RNA/PNPase three-dimensional reconstruction (even columns) and corresponding reference-free class averages (odd columns), as matched by cross-correlation. Scale bar, 10 nm.

(G) Euler angle distribution map for the Rsr/Y RNA/PNPase RNP reconstruction. The size of each spot is proportional to the number of particles that belong to that specific view.

(H) Fourier shell correlation (FSC) curve for the reconstruction showing the resolution of the map to be ~25 Å using the 0.5 criterion.

Figure S3, related to Figure 4. Rsr and Y RNA, as part of a Rsr/Y RNA/PNPase RNP, enhance degradation of a structured RNA but do not influence poly(A) tail addition.

(A) Sequences and proposed secondary structures of the RNA substrates used in Figure 4. The misfolded *X. laevis* pre-5S rRNA contains the minimal six nucleotide 3’ extension necessary for Ro binding as well as several mutations (boxed) that cause it to misfold into a structure

recognized by Ro (Shi et al., 1996; Fuchs et al., 2006). Note that the weak 5 bp stem formed by pairing the 5' and 3' ends is not a barrier to PNPase degradation (Figure 4B).

(B) The activity of the RNP complex on the 5'-labeled SL7-N_{35} substrate (lanes 3-7) was compared with that of PNPase (lanes 8-12) and PNPase when Rsr (lanes 13-17) or mutant Rsr-H189S was added together with equimolar amounts of Y RNA (lanes 18-22). Under these conditions, which omit the 30^{o}C incubations used for EMSAs (Figure 2), formation of the RNP complex is very inefficient. Also shown are the effects of adding equimolar amounts of Rsr and Rsr-H189S to PNPase in the absence of Y RNA (lanes 23-27 and 28-32, respectively). Aliquots were removed at intervals and fractionated in 8% polyacrylamide, 8.3 M urea gels. To generate size markers, SL7-N_{35} was subjected to alkaline hydrolysis (lane 1) and T1 RNase digestion (lane 2). The complex stalls ~1 nt closer to the stemloop of the SL7-N_{35} RNA (arrow). Addition of equimolar amounts of bovine serum albumin or *Sulfolobus solfataricus* ribosomal protein L7 also did not enhance PNPase degradation (data not shown).

(C) The RNAI substrate used previously to assay *E. coli* and *Streptomyces coelicolor* PNPases (Sohlberg et al., 2003).

(D) The activity of the Rsr/Y RNA/PNPase RNP and PNPase in polyadenylation was compared on RNAI (left) and RNAI containing a 30 nt poly(A) tail (right panel). RNAs were fractionated in 8% polyacrylamide, 8.3 M urea gels. Although neither enzyme was effective at polyadenylating the RNAI substrate, PNPase and the complex exhibited similar activity on the $(A)_{30}$-containing RNA. Similar effects were seen when RNAI contained a poly(U) tail.

(E) Results from five independent polyadenylation experiments using RNAI-A_{30} were graphed. Data are represented as mean values ± SEM.

(F) To detect A-rich tails, RNA from wild-type *D. radiodurans* (lane 1) and strains lacking Rsr

(lanes 2, 4, 6, 8), the Y RNA (lanes 3, 4, 7, 8) or PNPase (lanes 5-8) was labeled at the 3' end with [^{32}P]pCp, digested with RNase A (which cuts after C and U) and RNase T1 (which cuts after G), and fractionated in a 12% polyacrylamide, 8.3 M urea gel. Because *D. radiodurans*, like most bacteria, lacks a dedicated poly(A) polymerase (Martin and Keller, 2004), the long poly(A) tails detected in *E. coli* are replaced by short oligo(A) tails. Sequencing of similar tails from *S. coelicolor* revealed that they were heteropolymeric and included oligo(A) stretches (Bralley et al., 2006). Arrows, A-rich tails that require PNPase for formation.

Figure S4, related to Figure 5. Activity of PNPase and the Rsr/Y RNA/PNPase RNP on RNA duplexes.

(A) To examine the requirement for a single-stranded 3' end, substrates containing 17 bp duplexes and 3' poly(U) extensions of varying length (ds17-U_N) were tested. The overhang-containing strand was labeled at the 5' end.

(B) The activity of PNPase and the RNP was compared on substrates containing 10, 20 and 30 nt overhangs. Both PNPase and the RNP stall ~9-11 nt 3' to the duplex.

(C) Shorter duplexes were generated by annealing 9-17 nt top strands to the 30 nt overhang-containing strand.

(D) and (E) The activity of PNPase and the complex was assayed on substrates containing (D) 15 bp and (E) 13 bp duplexes. Aliquots were removed at intervals and fractionated in 15% polyacrylamide, 8.3 M urea gels. Both PNPase and the RNP degrade the 13 bp duplex, but not the 15 bp duplex, as evidenced by the appearance of limit oligonucleotides.

(F) To confirm that duplexes formed, their migration in nondenaturing gels was compared to that of the ss17-U_{30} bottom strand.

Figure S5, related to Figure 6. The presence of *S.* Typhimurium HA-Rsr and YrlA in anti-FLAG eluates is dependent on PNPase.

In parallel with the *FLAG$_3$-pnp HA-rsr* lysates shown in Figure 6F, *HA-rsr* lysates were subjected to affinity purification using anti-FLAG, followed by sedimentation of the eluates in glycerol gradients. HA-Rsr and PNPase were detected by immunoblotting with anti-HA and anti-PNPase antibodies, respectively. YrlA was detected by Northern blotting. Samples were analyzed in multiple gels that were joined at the lines. Positions of ovalbumin (44 kD), bovine γ-globulin (158 kD) and thyroglobulin (670 kD) run in parallel gradients are shown.

EXTENDED EXPERIMENTAL PROCEDURES

Generation of *D. radiodurans* Strains

To generate the strain carrying *pnp-FLAG$_3$* and *proteinA-TEV-rsr* (strain PTR17), we first generated a *D. radiodurans* strain carrying *pnp-FLAG$_3$* (strain B11). To this end, approximately 1 kb of the C-terminal coding sequence of PNPase was amplified from genomic DNA using 5'-CCCTACGTGATTCGCGTGGTGG-3' and 5'-TAATCACCGTCATGGTCTTGTAGTCGTCCTCGCGTCTCGGGAAGAC-3'. The 3XFLAG epitope was amplified from pcDNA3FLAG (a gift of Dr. Soyeong Sim, Yale University) using 5'-GACTACAAAGACCATGACGGTGATTA-3' and 5'-CATGGCCCTCAGGCCCTCGCTCAATCGTCATCCTTGTAATCGATATCAT-3' and fused to the PNPase C-terminus using PCR. The p*kat-hyg* cassette was amplified from pTNK104 (Tanaka et al., 2004) with 5′-GCGAGGGCCTGAGGGCCAT-3′ and 5′-TCAGGCGCCGGGGGCGGTGTC-3′ as described (Chen et al., 2007) and fused to ~1 kb of

PNPase 3' flanking sequence that had been amplified with 5'-GACACCGCCCCCGGCGCCTGAGCGAAGCAGGAGATTTATCTTTAG-3' and 5'-GGCAGGCCCAACTGACCCTCC. After joining the two fragments using PCR, cloning into pCR-Blunt resulted in plasmid BFH2. After linearization and transformation, integration at the *pnp* locus was confirmed by PCR. To generate strain PTR17 carrying *pnp-FLAG*$_3$ and *proteinA-TEV-rsr* (strain PTR17), the Protein A domain and TEV site were amplified with 5'-CCAAAACCATGGTGGACAACAAATTCAAC-3' and 5'-ATGGCACGGAGCAAGTTCTTTTCCATCGAACCCTGAAAATACAAATTCTCGGC-3' from pPFDR12 (Chen et al., 2007). Rsr was also amplified from pPFDR12 with 5'-ATGAAGAACTTGCTCCGTGCCATCAACC-3' and 5'-CCCAAAGGATCCTCAAACCTCGCCCCGCGCAAAAGC-3'. After overlapping PCR, the DNA containing Protein A-TEV-Rsr was cleaved with NcoI/Bam HI and used to replace the Protein A-TEV-Flag-Rsr allele in the *cat*-containing pPFDR12 (Chen et al., 2007) to generate plasmid pPTR11. Transformation of strain B11, followed by chloramphenicol selection (Chen et al., 2000), resulted in strain PTR17 expressing Protein A-TEV-Rsr and PNPase-FLAG$_3$.

Purification of *D. radiodurans* Rsr, PNPase and Y RNA for Electrophoretic Mobility Shift Assays (EMSAs)

For baculovirus expression, Rsr was amplified from *D. radiodurans* DNA and inserted into the BamHI/HindIII sites in pFastbac1 (Invitrogen). Recombinant baculovirus was obtained and Rsr purified as described for *X. laevis* Ro (Stein et al., 2005). For *E. coli* expression, plasmid pET28b-Rsr expressing His-tagged Rsr (Ramesh et al., 2007) was provided by Drs. Arati Ramesh and James Sacchettini (Texas A&M University, College Station, TX). His-tagged Rsr

was purified under native conditions using Ni-NTA beads (Qiagen), eluted with 250 mM imidazole and dialyzed into enterokinase buffer (50 mM Tris-HCl pH8.0, 1 mM $CaCl_2$, 0.1 mM Tween-20). After removal of the His-tag with EKMax Enterokinase (Invitrogen), the protein was loaded onto a Superdex 200 gel filtration column (GE Healthcare) in 25 mM Tris-HCl pH 7.5, 100 mM NaCl, 5% glycerol, 0.1 mM EDTA and 1 mM DTT. Peak fractions were pooled and concentrated using Amicon Ultra-15 centrifugal filter devices (Millipore).

Because the *E. coli* and baculovirus-expressed Rsr behaved identically in EMSAs (Figure S1), the Rsr-H189S mutation was generated using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) from the pET28b-Rsr template, expressed in *E. coli*, purified and subjected to cleavage of the His-tag as above. Thus in Figure 2A, the binding of *E. coli*-made Rsr and Rsr-H189S to the Y RNA was compared, while the experiments in Figures 2C, 2D and 2E all used baculovirus-expressed Rsr.

The expression and purification of His-tagged *D. radiodurans* PNPase was as described (Chen et al., 2007) using Ni-NTA agarose beads under native conditions. After cleaving the tag with enterokinase (Invitrogen) and gel filtration on Superdex 200 (GE Healthcare), PNPase fractions were stored at -80°C. Mutants ΔKH (deletion of amino acids 589-653) and ΔS1 (deletion of amino acids 656-723) were generated using PCR. Trimer formation was assayed by fractionation in 5-20% nondenaturing gels (Jarrige et al., 2002) and by EM (see below).

To generate wild-type and mutant Y RNAs, PCR was used to place these RNAs behind the T7 promoter. Transcripts were generated using T7 RNA polymerase according to the manufacturer's instructions (Promega).

Co-expression of *D. radiodurans* Rsr, PNPase and Y RNA in *E. coli*

Rsr was amplified from *D. radiodurans* genomic DNA using 5'-AATTGGATCCATGAAGAACTTGCTCCGTGCCATCAAC-3' and 5'-AACCCCAAGCTTTCAAACCTCGCCCCGCGCAAAAG-3', digested with BamHI and HindIII and inserted into pRSFDuet-1 (Novagen) to generate pRSFDuet-HisRsr. Strep-tagged PNPase was amplified from genomic DNA using oligonucleotides 5'-TACATATGTGGAGCCACCCGCAGTTCGAAAAACCGCAGCTTAAAGGCCGG-3' and 5'-GGTTCGAACCGGTACCTCAGTCCTCGCGTCTCGGGAAG-3', digested with NdeI and KpnI and inserted into pRSFDuet-HisRsr, generating pRSFDuet-HisRsr-StrepPNPase. The *D. radiodurans* Y RNA was amplified from genomic DNA with 5'-GCTCTAGAGCTCTGGGCCGAAGTGGTC-3' and 5'-GAATTCCAAGACCCGTTTAGAGGCCCCAAGGGGTTATGCTAGAGTGCTCTGGACAA GGGT-3', digested with XbaI and EcoRI and inserted into pETDuet-1, generating pETDuet-DrYRNA. In this construct, the 5' end of the Y RNA contained 35 extra nt, which include lac operator sequences to repress T7 RNA polymerase transcription, while the 3' end of the Y RNA was fused to a T7 terminator. Next, a hammerhead ribozyme (Link et al., 2007) was added between the Y RNA and the T7 terminator to generate a 3' end similar to the *D. radiodurans* RNA. To this end, 5'-GCACTGTCCTGGATTCCACGGTACATCCAGCTGATGAG-3' and 5'-ACACTGTTTCGTCCTATTTGGGACTCATCAGCTGGATGTAC-3', which contain hammerhead sequences, were annealed and then amplified with 5'-ACCCTTGTCCAGAGCACTGTCCTGGATTCCAC-3' and 5'- CCCAAGGGGTTATGC TAGACACTGTTTCGTCCTATTTG-3' to generate a DNA fragment containing the last 18 nt of the Y RNA, followed by hammerhead sequences and the T7 terminator. This DNA was annealed to the plasmid pETDuetDrYRNA, amplified, digested with Dpn I to degrade the parent plasmid

and transformed into DH5α to generate plasmid pETDuet-ymrz. In this construct, *E. coli* expression generates a Y RNA containing 35 extra nt at the 5' end and three extra nt at the 3' end. As a subsequent construct in which the upstream sequences were removed did not yield stable Y RNA accumulation when co-expressed with His-Rsr, pETDuet-yrmz was used for co-expression. To co-express His-Rsr, Strep-PNPase and the Y RNA, plasmids RSFDuet-HisRsr-StrepPNPase and pETDuet-ymrz were transformed into *E.coli* BL21(gold) to generate strain RRP3-4. To express PNPase alone, Strep-PNPase was amplified as described above, digested with NdeI and KpnI and inserted into pRSFDuet-1 (Novagen), and the resulting plasmid (pRSFDuet-StrepPNPase) transformed into *E.coli* BL21 (gold).

Negative Stain EM Image Processing, Three-dimensional Reconstruction and Analysis

For two-dimensional analysis, EMAN (Ludtke et al., 1999) was used to perform semi-automatic particle picking and to box particles from the raw micrographs into boxes of 160 x 160 square pixels. The particles were normalized and high- and low-pass filtered prior to processing. ~2,200, ~4,000 and ~1,000 raw particles of PNPase, Rsr/Y RNA/PNPase and the RNP-antibody complex were subjected to reference-free alignment and classification using multivariate statistical analysis and multi-reference alignment in IMAGIC (van Heel et al., 1996) to a total of 50, 90 and 100 classes, respectively.

For the three-dimensional reconstruction of Rsr/Y RNA/PNPase RNP, we generated an initial starting model by the random conical tilt method (RCT) (Radermacher et al., 1987) with the RCT module (Voss et al., 2010) in the Appion image-processing pipeline (Lander et al., 2009). Briefly, particles were selected and correlated between tilt-pairs semi-automatically using TiltPicker (Voss et al., 2009), binned by a factor of 2, high- and low- pass filtered and extracted

from raw micrographs. The untilted particle images were subjected to 15 iterations of maximum-likelihood two-dimensional classification and averaging using the ml2d command (Scheres et al., 2005) in Xmipp (Scheres et al., 2008) and RCT volumes were calculated for each class average using back-projection in SPIDER (Frank et al., 1996) based on the angles and shifts in the alignment of untilted particles and the angle between tilt-pairs of micrographs. The majority of the resulting 3D densities showed a smaller globular density connected to a larger globular density. One RCT class volume in particular showed striking features and appeared to be suffering less from artifacts associated with the missing cone than other volumes (Figure S2B).

This RCT model was low-pass filtered and subjected to iterative projection matching refinement using the reference-free class averages obtained from the untilted particle images in EMAN (Ludtke et al., 1999). This reconstruction (Figure S2C) served as the initial model for three-dimensional reconstruction using iterative projection matching refinement with libraries from the EMAN2 and SPARX software packages (Hohn et al., 2007; Tang et al., 2007) as described (Wiedenheft et al., 2011; Lander et al., 2012). Reprojections of the final three-dimensional reconstruction showed excellent agreement with the reference-free class averages (Figure S2F). The final reconstruction showed structural features to 25 Å resolution based on the 0.5 Fourier shell correlation criterion and displayed a large distribution of Euler angles, despite some preferential orientations of the particles on the carbon film (Figure S2G and S2H). In an alternative approach, we used a low-pass filtered model of the trimeric PNPase crystal structure as an initial model for the above-mentioned projection matching refinement. This led to an EM density very similar to the final model presented (Figure 3), except more globular due to initial model bias.

The final reconstruction was segmented (Figure S2E) manually using Chimera (Pettersen

et al., 2004) based on inspection of the antibody-labeling results. The atomic coordinates of *Xenopus laevis* Ro (PDB 1YVP and 2I91) and *S. antibioticus* PNPase [PDB 1E3P; (Symmons et al., 2000)] arranged into a trimer using COOT (Emsley et al., 2010) were docked into the map manually using information from the biochemical characterization, map segmentation and by searching for the optimal cross-correlation coefficient between the low-pass filtered crystal structure (at 25 Å resolution) and the EM map using Chimera (Pettersen et al., 2004).

Preparation of Substrates for Exonuclease and Polyadenylation Assays

To generate the SL7-N_{35} substrate, the pSP64-SL11R (a gift of Dr. E. Wurtmann, Yale University), which contains a similar RNA with an 11 GC base-pair stem under control of a T7 promoter, was subjected to site-directed mutagenesis to generate the 7 GC basepair stem. After linearization with HindIII, transcription with T7 polymerase results in an RNA with the sequence 5'-GGGAAUUCGAGCUCGGUACCCGGGCCUUUGCCCGGGGGAUGGACUAGAGUCGACCUGCAGGCAUGCAAGCU-3'. In this RNA, which resembles the SL7R substrate used to assay *E. coli* PNPase (Spickler and Mackie, 2000), the underlined sequences basepair to form the 7 GC stem. The plasmid allowing transcription of the misfolded pre-5S rRNA was described (Fuchs et al., 2006). To prepare the 16S rRNA 3' end substrate, the T7 promoter-containing oligonucleotide 5'-GGCGGCGAATTCTAATACGACTCACTATAGGAAAAAAAATAAAAAATAAATCCGCCCGTCACACCATGGGAG-3' and 5'-GGCGGCGGATCCTTTAAAGGAGGTGATCCAACCGC-3' were used to amplify *D. radiodurans* genomic DNA. After cleavage with EcoRI and BamHI, the DNA was cloned into

the EcoRI/BamHI sites of pSP64. After cleavage with DraI, transcription with T7 polymerase results in a 160 nt RNA containing 20 nt of 5' unstructured sequence (GGAAAAAAAAUAAAAAAUAA) followed by the last 140 nt of *D. radiodurans* 16S rRNA. The SL7-N35, pre-5S and 16S rRNA substrates were transcribed with T7 RNA polymerase, 5' labeled with [γ-^{32}P]ATP and purified by denaturing gel electrophoresis. Purified RNAs were resuspended in 20 mM Tris-HCl pH 7.5, 50 mM KCl, 1 mM $MgCl_2$, incubated at 95ºC for 3 min, frozen on dry ice and thawed on ice.

Duplex substrates were modeled on those used previously (Cheng and Deutscher, 2005). Because *X. laevis* Ro has a slight preference for RNAs with poly(U) tails (Fuchs et al., 2006), our initial substrates contained these tails. Oligonucleotides (Dharmacon) were purified from 15% polyacrylamide, 8.3 M urea gels, labeled with [γ-^{32}P]ATP and re-purified from gels. Duplexes were formed by mixing labeled (overhang-containing strand) oligonucleotides with their unlabeled complementary (top-strand) oligonucleotides in a 1:1.3 molar ratio in 20 mM Tris-HCl pH 7.5, 50 mM KCl, 1 mM $MgCl_2$. After incubating at 95ºC for 3 min, RNAs were allowed to cool to room temperature.

A DNA fragment allowing transcription of RNAI was made using 5'-GCGGCTAATACGACTCACTATA GGGACAGTATTTGGTATCTGCGCTC-3' and 5'-AACAAAAAAACCACCGCTACCAGCGGTG-3' to amplify sequences from the plasmid pRS314 (Sikorski and Hieter, 1989). Because this RNA was a poor substrate for PNPase, we generated a second fragment allowing transcription of a form of this RNA containing 30 additional adenines [RNAI-$(A)_{30}$], using 5' TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTAACAAAAAAACCACCGCTACCAGCG-3' as the reverse primer.

Degradation Assays

To examine the requirement for Y RNA-mediated tethering (Figure S3), 174 fmol of refolded Y RNA was mixed with 174 fmol Rsr in 20 mM Tris-HCl, 50 mM NaCl, 10% glycerol, 2% DTT and incubated at 4^{o}C for 10 min to form an Rsr/Y RNA complex. Next, 174 fmol PNPase was added to either this mixture or to the buffer alone, and incubated an additional 10 min at 4^{o}C in a final volume of 21.7 µl. Under these conditions, which use shorter incubation times and lower temperatures than those used for EMSAs in order to maintain the enzymatic activity of PNPase, formation of the Rsr/Y RNA/PNPase complex is even less efficient than that shown in Figure 2A. To assay degradation, 20 fmol of either the PNPase or the Rsr, Y RNA and PNPase mixture were incubated with 94 fmol SL7-N_{35} RNA in 20 mM Tris-HCl, 50 mM KCl, 1 mM MgCl2, 2 mM DTT and 10 mM phosphate.

Polyadenylation Assays

Polyadenylation assays (Sohlberg et al., 2003) contained 140 fmol 5'-labeled RNA and 70 fmol PNPase or Rsr /Y RNA/PNPase RNP in 50 mM Tris-HCl pH 8.0, 5 mM $MgCl_2$, 50 mM NaCl, 1 mM DTT, and 0.5 mM ADP at 30ºC. At intervals, aliquots were mixed with equal volumes of 90% formamide, 1 mM EDTA, 0.25% SDS, 0.03% bromophenol blue, 0.03% xylene cyanol and fractionated in 8% polyacrylamide/8.3 M urea gels. The fraction of polyadenylated substrate was determined with a PhosphorImager (Molecular Dynamics). To detect oligo(A) tails *in vivo*, total RNA was extracted from *D. radiodurans* and labeled with [^{32}P]pCp and T4 RNA ligase as described (Chen et al., 2000). 5 µg of labeled RNA was digested with 6.25 µg of RNase A and 100 units of RNase T_1 in 20 mM Tris-HCl (pH 8.0), 500 mM NaCl, 10 mM EDTA for 3 h at

37ºC. Afterwards, the RNA was extracted with phenol:chloroform:isoamyl alcohol (25:24:1), precipitated with ethanol and fractionated in 12% polyacrylamide/8.3 M urea gels.

S. Typhimurium Strains and Plasmids

All *Salmonella* strains used in this study are derivatives of the wild-type *Salmonella enterica* serovar Typhimurium strain SL1344 (Hoiseth and Stocker, 1981). Genetic manipulations were made using standard recombinant DNA techniques and allelic exchange methods (Kaniga et al., 1994) featuring the conjugative transfer of R6K-based suicide vectors using the donor strain *E. coli* β-2163 Δ*nic*35 (Demarre et al., 2005). To generate a strain carrying chromosomally integrated *FLAG$_3$-pnp*, ~800 bp of *pnp*$^+$ upstream sequence was amplified from genomic DNA with 5'-AAAATGGCAACCCGGTTCG-3' and 5'-TCATGGTCTTTGTAGTCCATAATAATATCCTTTCCAATTTTTGACG-3'. This fragment was fused to 3 copies of the FLAG epitope amplified from plasmid pcDNA3Flag (a gift of S. Sim, Yale University) using 5'-ATGGACTACAAAGACCATGAC-3'and 5'-ATCGTCATCCTTGTAATCGATATC-3'. The resulting fragment was joined to 800 bp of PNPase coding sequence using 5'-TCGATTACAAGGATGACGATTTGCTTAATCCGATCGTTCG-3' and 5'-TAGCGCTCTTGTTTATCGG-3'. The resulting DNA fragment was cloned into the SmaI site of pSB890 (Palmer et al., 1998) to generate pSB890-PNPase6. To generate the *HA-rsr* allele, 800 bp of *rsr*$^+$ upstream sequence was amplified from genomic DNA using 5'-GCGACGCGCTCAATTTGC-3' and 5'-AGCGTAATCTGGAACATCGTATGGGTACATTTGTTTTTCCCATTTCCGTTATTC-3', and fused to 800 bp of Rsr coding sequence using 5'-

CGATGTTCCAGATTACGCTATGGCTAATCCACTTTTGTTC-3' and 5'-GCGTTGCATTCTCAAACAC-3'. The amplified DNA was cloned into the SmaI site of pSB890 to generate pSB890-HA-Rsr19. The plasmids were introduced individually into *E. coli* β-2163 Δ*nic*35 which was used as a donor for conjugation with the parent strain *Salmonella* SB300.

To overexpress N-terminally truncated RtcR, the oligonucleotides 5'-GGCGGCCCATGGCGCTCAACTTCCTGAAGTCCGGCATTGC-3' and 5'-GGCGGCTCTAGATTAATTCTGTAAAACGTCCCACGTCAG-3' were used to amplify DNA encoding amino acids 179-527 of RtcR from *S.* Typhimurium genomic DNA. Digestion with NcoI and XbaI and cloning into the same sites of pBAD24 resulted in plasmid pRtcRΔN.

Characterization of Rsr-containing RNPs in *S.* Typhimurium

For immunoprecipitations, overnight cultures of strains carrying either the empty vector pBAD24 or pRtcRΔN were diluted 1:200 into 400 ml of fresh LB containing 75 µg/ml ampicillin and 0-0.2% arabinose and grown for 3 hours at 37^{o}C with shaking to OD_{600}=0.5. Cells were pelleted by centrifugation, and resuspended in 10 ml of buffer D [20 mM Tris-HCl pH 7.5, 100 mM NaCl, 5% glycerol, 1 mM $MgCl_2$, 2 mM β-mercaptoethanol, 0.5 mM PMSF, 1x protease inhibitor cocktail (Roche)] supplemented with 1.25 mM vanadyl ribonuclase complex. After lysing by passing through a French press, cell debris was removed by centrifuging at 13,000 rpm in a Beckmann TA-14-50 rotor for 15 min, followed by 18,000 rpm in a Sorvall SS34 rotor for 20 min. To immunoprecipitate Rsr, supernatants were mixed with rabbit antibodies against amino acids 1-24 of *S.* Typhimurium Rsr (raised by Yenzym Antibodies, LLC, South San Francisco, CA) that was previously conjugated to protein A Sepharose (GE Healthcare). After

incubating for 1 hour at 4^{o}C, beads were washed with Buffer D, and RNAs extracted as described (Wolin and Steitz, 1984). To prepare cDNAs, RNA bands were excised from denaturing gels, ligated to the RNA linkers L5 (5'-OH AGGGAGGACGAUGCGG 3'-OH) and L3 (5'-P GTGTCAGTCACTTCCAGCGG 3'-puromycin), reverse transcribed with DNA oligonucleotide P3 (5'-CCGCTGGAAGTGACTGACAC-3'), and subjected to PCR with P3 and P5 (5'-AGGGAGGACGATGCGG-3') (Ule et al., 2005). Secondary structures (Figure 6D) were derived using MFOLD (Zuker, 2003).

To isolate PNPase-containing complexes, clarified lysates were mixed with anti-FLAG M2 agarose (Sigma) for 1 h at 4ºC, washed with buffer D, and $FLAG_3$-PNPase complexes eluted with 1 mg/ml 3xFLAG peptide in buffer D. The eluate was layered on a 10-40% glycerol gradient in 20 mM Tris-HCl pH 7.5, 50 mM NaCl, 1 mM $MgCl_2$, 2 mM β-mercaptoethanol and 1 mM Pefabloc (Sigma), and sedimented at 36,000 rpm in a Beckman SW41 rotor for 20 h at 4ºC. Molecular weight standards (Bio-Rad) were run in parallel gradients. Northern blots to detect YrlA were probed with a 1:1 mixture of 5'-ACAAATGTAGATCCACAGGCATTCGCCAA-3' and 5'-GTGGCGACAAGTTTATGTGAATACACCGCTCTACC-3'. To detect YrlB, blots were probed with a 1:1 mixture of 5'-GATACGCATCGGGAAAGATGTAA-3' and 5'-TGCCTTCAGCGTTGCGCGAC-3'. The 5S rRNA was detected with 5'-CACTTCTGAGTTCGGCATGGGG-3'. For Western blotting, HA-Rsr was detected with Anti-HA-Peroxidase (Roche), while PNPase was detected with an antibody against *E. coli* PNPase (a gift of A. Carpousis, Université Paul Sabatier, Toulouse, France).

SUPPLEMENTAL REFERENCES

Bralley, P., Gust, B., Chang, S., Chater, K.F., and Jones, G.H. (2006). RNA 3'-tail synthesis in *Streptomyces*: *in vitro* and *in vivo* activities of RNase PH, the *SC03896* gene product and polynucleotide phosphorylase. Microbiology *152*, 627-636.

Demarre, G., Guerout, A.M., Matsumoto-Mashimo, C., Rowe-Magnus, D.A., Marliere, P., and Mazel, D. (2005). A new family of mobilizable suicide plasmids based on broad host range R388 plasmid (IncW) and RP4 plasmid (IncPalpha) conjugative machineries and their cognate Escherichia coli host strains. Research in microbiology *156*, 245-255.

Emsley, P., Lohkamp, B., Scott, W.G., and Cowtan, K. (2010). Features and development of Coot. Acta Crystallogr D Biol Crystallogr *66*, 486-501.

Frank, J., Radermacher, M., Penczek, P., Zhu, J., Li, Y., Ladjadj, M., and Leith, A. (1996). SPIDER and WEB: processing and visualization of images in 3D electron microscopy and related fields. J Struct Biol *116*, 190-199.

Hohn, M., Tang, G., Goodyear, G., Baldwin, P.R., Huang, Z., Penczek, P.A., Yang, C., Glaeser, R.M., Adams, P.D., and Ludtke, S.J. (2007). SPARX, a new environment for Cryo-EM image processing. J Struct Biol *157*, 47-55.

Hoiseth, S.K., and Stocker, B.A. (1981). Aromatic-dependent Salmonella typhimurium are non-virulent and effective as live vaccines. Nature *291*, 238-239.

Jarrige, A., Brechemier-Baey, D., Mathy, N., Duche, O., and Portier, C. (2002). Mutational analysis of polynucleotide phosphorylase from *Escherichia coli*. J Mol Biol *321*, 397-409.

Kaniga, K., Bossio, J.C., and Galan, J.E. (1994). The Salmonella typhimurium invasion genes invF and invG encode homologues of the AraC and PulD family of proteins. Mol Microbiol *13*, 555-568.

Lander, G.C., Estrin, E., Matyskiela, M.E., Bashore, C., Nogales, E., and Martin, A. (2012). Complete subunit architecture of the proteasome regulatory particle. Nature *482*, 186-191.

Lander, G.C., Stagg, S.M., Voss, N.R., Cheng, A., Fellmann, D., Pulokas, J., Yoshioka, C., Irving, C., Mulder, A., Lau, P.W., *et al.* (2009). Appion: an integrated, database-driven pipeline to facilitate EM image processing. J Struct Biol *166*, 95-102.

Link, K.H., Guo, L., Ames, T.D., Yen, L., Mulligan, R.C., and Breaker, R.R. (2007). Engineering high-speed allosteric hammerhead ribozymes. Biol Chem *388*, 779-786.

Ludtke, S.J., Baldwin, P.R., and Chiu, W. (1999). EMAN: semiautomated software for high-resolution single-particle reconstructions. J Struct Biol *128*, 82-97.

Martin, G., and Keller, W. (2004). Sequence motifs that distinguish ATP(CTP):tRNA nucleotidyl transferases from eubacterial poly(A) polymerases. RNA *10*, 899-906.

Palmer, L.E., Hobbie, S., Galan, J.E., and Bliska, J.B. (1998). YopJ of Yersinia pseudotuberculosis is required for the inhibition of macrophage TNF-alpha production and downregulation of the MAP kinases p38 and JNK. Mol Microbiol *27*, 953-965.

Pettersen, E.F., Goddard, T.D., Huang, C.C., Couch, G.S., Greenblatt, D.M., Meng, E.C., and Ferrin, T.E. (2004). UCSF Chimera--a visualization system for exploratory research and analysis. J Comput Chem *25*, 1605-1612.

Radermacher, M., Wagenknecht, T., Verschoor, A., and Frank, J. (1987). Three-dimensional reconstruction from a single-exposure, random conical tilt series applied to the 50S ribosomal subunit of Escherichia coli. Journal of microscopy *146*, 113-136.

Scheres, S.H., Nunez-Ramirez, R., Sorzano, C.O., Carazo, J.M., and Marabini, R. (2008). Image processing for electron microscopy single-particle analysis using XMIPP. Nature protocols *3*, 977-990.

Scheres, S.H., Valle, M., Nunez, R., Sorzano, C.O., Marabini, R., Herman, G.T., and Carazo, J.M. (2005). Maximum-likelihood multi-reference refinement for electron microscopy images. J Mol Biol *348*, 139-149.

Sikorski, R.S., and Hieter, P. (1989). A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccaromyces cerevisiae*. Genetics *122*, 19-27.

Tanaka, M., Earl, A.M., Howell, H.A., Park, M.J., Eisen, J.A., Peterson, S.N., and Battista, J.R. (2004). Analysis of *Deinococcus radiodurans'* transcriptional response to ionizing radiation and desiccation reveals novel proteins that contribute to extreme radioresistance. Genetics *168*, 21-33.

Tang, G., Peng, L., Baldwin, P.R., Mann, D.S., Jiang, W., Rees, I., and Ludtke, S.J. (2007). EMAN2: an extensible image processing suite for electron microscopy. J Struct Biol *157*, 38-46.

Ule, J., Jensen, K., Mele, A., and Darnell, R.B. (2005). CLIP: a method for identifying protein-RNA interaction sites in living cells. Methods *37*, 376-386.

van Heel, M., Harauz, G., Orlova, E.V., Schmidt, R., and Schatz, M. (1996). A new generation of the IMAGIC image processing system. J Struct Biol *116*, 17-24.

Voss, N.R., Lyumkis, D., Cheng, A., Lau, P.W., Mulder, A., Lander, G.C., Brignole, E.J., Fellmann, D., Irving, C., Jacovetty, E.L., *et al.* (2010). A toolbox for ab initio 3-D reconstructions in single-particle electron microscopy. J Struct Biol *169*, 389-398.

Voss, N.R., Yoshioka, C.K., Radermacher, M., Potter, C.S., and Carragher, B. (2009). DoG Picker and TiltPicker: software tools to facilitate particle selection in single particle electron microscopy. J Struct Biol *166*, 205-213.

Wiedenheft, B., Lander, G.C., Zhou, K., Jore, M.M., Brouns, S.J., van der Oost, J., Doudna, J.A., and Nogales, E. (2011). Structures of the RNA-guided surveillance complex from a bacterial immune system. Nature *477*, 486-489.

Wolin, S.L., and Steitz, J.A. (1984). The Ro small cytoplasmic ribonucleoproteins: Identification of the antigenic protein and its binding site on the Ro RNAs. Proc Natl Acad Sci USA *81*, 1996-2000.

Zuker, M. (2003). Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Res *31*, 3406-3415.