LETTERS

The bacterial enzyme RppH triggers messenger RNA degradation by 5′ pyrophosphate removal

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The long-standing assumption that messenger RNA (mRNA) degradation in *Escherichia coli* begins with endonucleolytic cleavage has been challenged by the recent discovery that RNA decay can be triggered by a prior non-nucleolytic event that marks transcripts for rapid turnover: the rate-determining conversion of the 5′ terminus from a triphosphate to a monophosphate[1]. This modification creates better substrates for the endonuclease RNase E, whose cleavage activity at internal sites is greatly enhanced when the RNA 5′ end is monophosphorylated[2,3]. Moreover, it suggests an explanation for the influence of 5′ termini on the endonucleolytic cleavage of primary transcripts, which are triphosphorylated[4–8]. However, no enzyme capable of removing pyrophosphate from RNA 5′ ends has been identified in any bacterial species. Here we show that the *E. coli* protein RppH (formerly NudH/YgdP) is the RNA pyrophosphohydrolase that initiates mRNA decay by this 5′-end-dependent pathway. *In vitro*, RppH efficiently removes pyrophosphate from the 5′ end of triphosphorylated RNA, irrespective of the identity of the 5′-terminal nucleotide. *In vivo*, it accelerates the degradation of hundreds of *E. coli* transcripts by converting their triphosphorylated 5′ ends to a more labile monophosphorylated state that can stimulate subsequent ribonuclease cleavage. That the action of the pyrophosphohydrolase is impeded when the 5′ end is structurally sequestered by a stem-loop helps to explain the stabilizing influence of 5′-terminal base pairing on mRNA lifetimes. Together, these findings suggest a possible basis for the effect of RppH and its orthologues on the invasiveness of bacterial pathogens. Interestingly, this master regulator of 5′-end-dependent mRNA degradation in *E. coli* not only catalyses a process functionally reminiscent of eukaryotic mRNA decapping but also bears an evolutionary relationship to the eukaryotic decapping enzyme Dcp2.

We reasoned that a protein with RNA pyrophosphohydrolase activity might previously have been identified as an enzyme able to remove pyrophosphate from mononucleotides. Because several members of the Nudix protein family have been shown to possess mononucleotide pyrophosphohydrolase activity *in vitro*[9], we purified 12 Nudix proteins from *E. coli* and tested them individually for their ability to remove pyrophosphate from the 5′ end of triphosphorylated RNA. This screening revealed that RppH functions *in vitro* as an efficient RNA pyrophosphohydrolase. When added to RNA bearing a 5′-terminal γ-^{32}P label and an internal fluorescein label, this enzyme removed the radiolabelled γ-phosphate from the 5′ end without degrading the transcript (Fig. 1a). No such activity was observed for an RppH mutant with a substitution at an essential active-site residue (E53A)[10]. To demonstrate that RppH removes both the γ- and β-phosphates, we prepared a triphosphorylated RNA substrate (GA(CU)$_{13}$) bearing a single radiolabelled phosphate at either the 5′-terminal α position or between the first and second nucleotides. Alkaline hydrolysis of either of these substrates produced pppGp as the major radiolabelled product, as determined by thin-layer chromatography (TLC) (Fig. 2, and Supplementary Fig. S1). After treatment with wild-type RppH, the principal radiolabelled product of alkaline hydrolysis was pGp, as expected for an enzyme that is able to convert triphosphorylated RNA 5′ ends to monophosphorylated 5′ ends (Fig. 2). Little, if any, ppGp was produced. Treatment with inactive RppH-E53A had no effect. Additional experiments demonstrated that RppH is also active on triphosphorylated RNAs that begin with A, C or U (Supplementary Fig. S2). To ascertain whether this enzyme removes the γ- and β-phosphates in a single step or sequentially, the radiolabelled products generated by

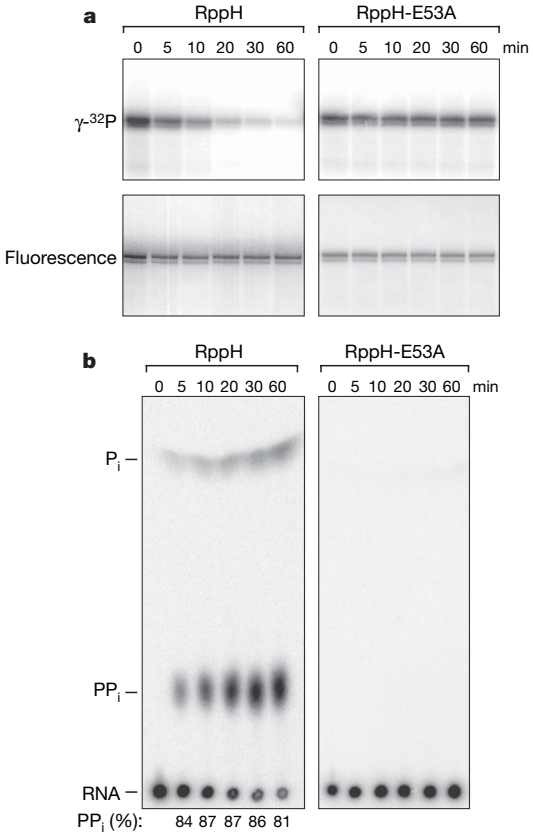

Figure 1 | RNA pyrophosphohydrolase activity of purified RppH. **a**, Electrophoretic assay demonstrating γ-phosphate removal. Triphosphorylated *rpsT* P1 RNA bearing a γ-^{32}P label and an internal fluorescein label was treated with purified RppH or RppH-E53A. **b**, TLC assay demonstrating pyrophosphate production. The products of the reaction shown in **a** were analysed by TLC and autoradiography. PP_i, pyrophosphate; P_i, orthophosphate. PP_i (%) = 100 × PP_i/(PP_i + P_i).

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treating the γ-^{32}P end-labelled transcript with RppH were monitored by TLC as a function of time. Almost all of the radiolabel was released as pyrophosphate, although a small but invariant fraction (16 ± 3%) was released as orthophosphate (Fig. 1b).

To examine the biological significance of the RNA pyrophosphohydrolase activity of RppH, we tested the effect of a chromosomal *rppH* deletion (*ΔrppH*) on the 5′ phosphorylation state of a transcript of the *E. coli rpsT* gene, which encodes ribosomal protein S20. This gene is transcribed from two promoters to generate a pair of transcripts (P1 and P2) that are degraded by an RNase E-dependent mechanism[11–14]. Previous studies have shown that the *rpsT* P1 transcript can be stabilized by replacing its 5′-terminal triphosphate with a hydroxyl, a finding indicative of a 5′-end-dependent decay mechanism[1]. Consistent with the view that this decay mechanism involves pyrophosphate removal as the initial step, a substantial portion of *rpsT* P1 mRNA in *E. coli* is monophosphorylated at steady-state. This was judged from an assay (PABLO analysis[1]) in which the 5′ phosphorylation state was determined from the ability of monophosphorylated (but not triphosphorylated) 5′ ends to undergo splinted ligation with P1-specific DNA oligonucleotides (Fig. 3a). In contrast,

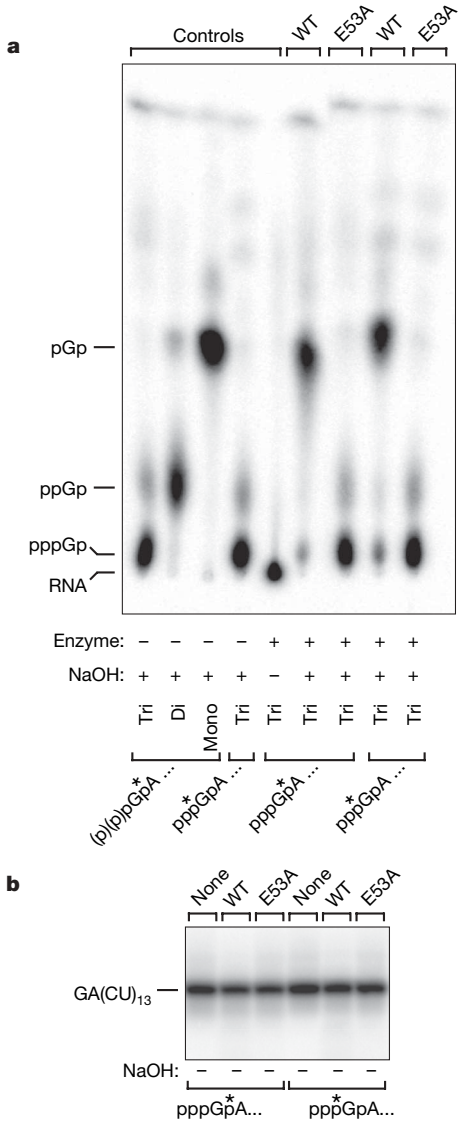

Figure 2 | Triphosphate-to-monophosphate conversion by purified RppH. A triphosphorylated transcript ($GA(CU)_{13}$) bearing a single ^{32}P label at the 5′-terminal α position (ppp*GpA …) or between the first and second nucleotides (pppGp*A …) was treated with purified RppH (WT) or RppH-E53A (E53A). The radiolabelled products were either subjected to alkaline hydrolysis and then analysed by TLC (**a**) or examined by gel electrophoresis without hydrolysis to confirm RNA integrity (**b**). Markers were generated by hydrolysing triphosphorylated (Tri), diphosphorylated (Di) or monophosphorylated (Mono) $GA(CU)_{13}$ without prior RppH treatment. (See Supplementary Fig. S1 for a representation of this assay.)

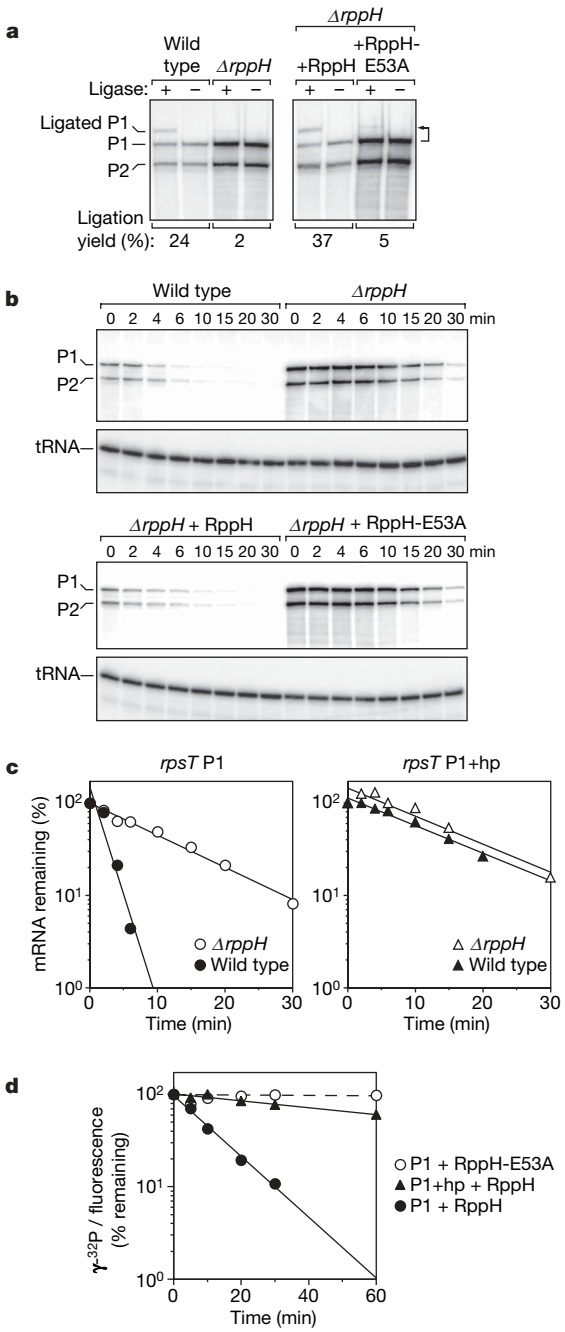

Figure 3 | RNA pyrophosphohydrolase activity of RppH in *E. coli*. **a**, Effect of RppH on the 5′ phosphorylation state of the *rpsT* P1 transcript, as determined by PABLO analysis[1] of cellular RNA with P1-specific oligonucleotides. **b**, Effect of RppH on the decay rate of *rpsT* mRNA, as determined by northern blot analysis of cellular RNA extracted at time intervals after inhibiting transcription. **c**, RppH-independent decay of *rpsT* mRNA bearing a 5′-terminal stem-loop. The three plasmid-encoded *rpsT* transcripts (P1, P1+hp and P2) were detected by probing for a sequence tag inserted into the 3′ untranslated region. Data from representative experiments are shown. **d**, Inhibition of purified RppH by a 5′-terminal stem-loop. Triphosphorylated *rpsT* P1 and P1+hp RNAs bearing a γ-^{32}P label and an internal fluorescein label were treated *in vitro* with RppH or RppH-E53A, and representative rates of pyrophosphate removal were plotted. The first three nucleotides of each transcript (AGC) were identical.

Table 1 | Influence of RppH on selected transcripts in *E. coli*

Transcript	Fold increase in mRNA concentration			mRNA half-life (min)	
	Microarray (E53A/wild type)	Northern blot (E53A/wild type)	Northern blot (*ΔrppH*/*rppH*$^+$)	*rppH*$^+$	*ΔrppH*
efp	2.9 ± 0.1	11.3 ± 1.5	9.8 ± 0.6	1.6 ± 0.2	5.3 ± 0.3
ppa	1.9 ± 0.1	4.8 ± 0.1	6.6 ± 1.4	1.5 ± 0.2	8.0 ± 0.3
rpsT P1	2.4 ± 0.2	7.8 ± 1.0	5.4 ± 0.2	1.3 ± 0.3	6.9 ± 0.6
rpsT P2	2.4 ± 0.2	5.3 ± 0.6	4.3 ± 0.4	2.2 ± 0.4	6.9 ± 1.4
slyB	2.8 ± 0.2	14.9 ± 1.0	5.9 ± 1.2	1.9 ± 0.1	9.8 ± 0.4
trxB	3.1 ± 0.2	4.9 ± 0.4	7.1 ± 1.0	2.6 ± 0.8	28.5 ± 2.1
yeiP	5.9 ± 0.5	8.9 ± 0.7	17.3 ± 0.4	1.6 ± 0.1	10.8 ± 1.0

Transcript concentrations and half-lives were compared in isogenic wild-type (*rppH*$^+$) and *ΔrppH* strains and in a *ΔrppH* strain complemented with plasmid-encoded wild-type or inactive (E53A) RppH, either by northern blotting or by microarray analysis. The two *rpsT* transcripts were indistinguishable in microarrays. Errors indicate s.d.

few of the P1 transcripts are monophosphorylated in *E. coli* cells lacking RppH. This defect in the *ΔrppH* strain can be fully complemented in trans by a plasmid-borne copy of the wild-type *rppH* gene but not by a mutant allele (*rppH-E53A*). We conclude that RppH is the enzyme principally responsible for pyrophosphate removal from *rpsT* P1 transcripts in *E. coli*.

To investigate the significance of RppH-catalysed pyrophosphate removal for the decay of the *rpsT* P1 transcript, we compared its degradation rate in cells containing or lacking the *rppH* gene. Both the P1 and P2 transcripts were stabilized 3- to 5-fold in the absence of RppH (Fig. 3b), demonstrating the importance of that enzyme for their decay. Rapid turnover was restored in the *ΔrppH* strain by complementation with wild-type RppH but not RppH-E53A. Together, these findings indicate that pyrophosphate removal by RppH triggers rapid degradation of *rpsT* mRNA in *E. coli*.

Previous evidence that *E. coli* transcripts are often stabilized by a 5′-terminal stem-loop[1,4–6,8] suggests that such a structure may exert its influence, at least in part, by hindering pyrophosphate removal by RppH. Consistent with this hypothesis, deletion of the *rppH* gene did not further stabilize an *rpsT* P1 mRNA variant whose lifetime in wild-type cells had been prolonged by adding a 5′-terminal hairpin (P1+hp in Fig. 3c, and Supplementary Fig. S3). To determine whether the RNA pyrophosphohydrolase activity of RppH requires an unpaired 5′ end, we compared the ability of purified RppH to remove pyrophosphate from triphosphorylated *rpsT* P1 and P1+hp transcripts. The release of pyrophosphate from the transcript with an unpaired 5′ end was nine times faster *in vitro* (Fig. 3d, and Supplementary Fig. S4). Because previous data have shown that RNase E activation by a 5′ monophosphate also requires a single-stranded 5′ terminus[2], we conclude that the ability of a 5′ stem-loop to stabilize mRNA in *E. coli* is a consequence of both impaired pyrophosphate removal and slow RNase E cleavage caused by sequestration of the 5′ end. Whether pyrophosphate removal is also influenced by translating ribosomes remains an open question.

The increased concentration of the *rpsT* transcripts in *E. coli* cells lacking RppH suggested that other targets of this enzyme could be identified by microarray analysis. Triplicate samples of total cellular RNA were isolated from isogenic *ΔrppH E. coli* strains complemented by plasmids encoding either wild-type RppH or inactive RppH-E53A and used to probe microarrays representing all the known protein-coding genes of *E. coli* K-12. The abundance of 382 gene transcripts was found to increase significantly (FDR < 0.05) in cells containing RppH-E53A versus wild-type RppH (Supplementary Table S1). As expected, these included *rpsT* mRNA.

To validate that the observed concentration increases were due to impaired mRNA degradation in the absence of active RppH, the longevity and concentration of several of these transcripts were compared in isogenic wild-type (*rppH*$^+$) and *ΔrppH E. coli* strains by northern blotting. In every case, the half-life of the message increased 3- to 11-fold in *ΔrppH* cells, and its steady-state concentration increased 4- to 17-fold (Table 1). That the enhanced longevity of these transcripts in the absence of RppH resulted from impaired pyrophosphate removal was verified for *yeiP* mRNA by showing that its sevenfold greater stability in *ΔrppH* cells was accompanied by a marked reduction in the percentage of that message that was monophosphorylated (Supplementary Fig. S5). The substantially greater effect of RppH that was measured by blotting versus microarrays suggests that the 382 transcripts shown by gene array analysis to be degraded by an RppH-dependent mechanism may be an underestimate of the actual total.

The half-lives of the transcripts in Table 1 also increased upon RNase E inactivation (1.4- to 3.4-fold; Supplementary Table S2), indicating a role for that endonuclease in degrading the monophosphorylated intermediates produced by RppH. That the absence of RppH caused greater stabilization suggests that those intermediates may each decay by multiple pathways, including some that are independent of RNase E. For example, certain mRNAs sensitive to RppH (such as *yeiP*) are also known targets of RNase G, a minor 5′-monophosphate-dependent RNase E paralogue[15–17], whereas others might undergo 3′ exoribonuclease attack facilitated by 3′ oligoadenylate tails added by poly(A) polymerase, another 5′-monophosphate-dependent enzyme[18]. That RppH is not essential for *E. coli* cell growth despite functioning as the master regulator of 5′-end-dependent mRNA decay attests to the availability of alternative, 5′-end-independent degradation pathways.

The biological function of RppH has not previously been defined, even though homologous proteins are widespread among prokaryotic organisms. Genetic experiments with pathogenic bacteria have indicated important roles for this enzyme and its orthologues in invasiveness and virulence[19–22], which we now suspect may be manifestations of the influence of these proteins on patterns of gene expression. Although studies of purified RppH have shown that it can convert diadenosine oligophosphates into mononucleotides (for example, A[5′]pppp[5′]A → ATP + AMP)[23], the biological importance of that *in vitro* activity has not been established. No other catalytic activity of RppH has been reported until now. We therefore propose that this protein (formerly designated NudH/YgdP) and its gene be named RppH to reflect its biological function as an RNA pyrophosphohydrolase.

The ability of RppH to trigger bacterial RNA decay by removing a protective structure at the 5′ terminus bears a striking resemblance to the removal of cap structures (m^7Gppp) from the 5′ ends of eukaryotic mRNAs. In each case, a 5′-terminal or 5′-proximal triphosphate is cleaved to produce a monophosphorylated intermediate vulnerable to attack by a 5′-monophosphate-dependent ribonuclease (for example, the endonuclease RNase E in *E. coli* or the 5′ exonuclease Xrn1 in eukaryotes)[1,24]. Interestingly, the protein responsible for cap removal in eukaryotic cells (Dcp2) is itself a member of the Nudix family[25,26]. Thus, despite significant structural differences between *E. coli* and human mRNAs, the enzymes that de-protect their 5′ termini appear to have evolved from a common ancestor.

METHODS SUMMARY

Pyrophosphate release from γ-^{32}P-labelled RNA and α-^{32}P-labelled RNA. Synthetic RNAs were prepared by *in vitro* transcription from a class III ϕ2.5 T7 promoter[27], gel-purified and incubated with affinity-purified RppH or RppH-E53A. The products of reactions that contained *rpsT* P1 or P1+hp RNA bearing a 5′-terminal γ-^{32}P label and an internal fluorescein label were analysed by electrophoresis on a polyacrylamide–urea gel or by TLC on

PEI-cellulose. The products of reactions that contained triphosphorylated $GA(CU)_{13}$, $AG(CU)_{13}$, $CG(A)_{26}$ or $UG(A)_{26}$ bearing a single ^{32}P label at either the 5′-terminal α position or between the first and second nucleotides were examined both by gel electrophoresis to confirm the integrity of the RNA and by alkaline hydrolysis and TLC to test for pyrophosphate removal. Hydrolysed monophosphorylated and diphosphorylated forms of the same α-labelled RNAs served as TLC standards.

Analysis of RNA extracted from *E. coli*. RNA lifetimes and phosphorylation states were analysed at 37 °C in *E. coli* K-12 strain BW25113 and its isogenic derivative JW2798*Δkan*, which bears an in-frame deletion of the *rppH* coding region[28], or at 44 °C in strain TA1025 (*rne*$^{+}$) and its isogenic derivative TA1026[29], which has a temperature-sensitive RNase E allele (*rne-1*). Total cellular RNA was harvested, as previously described[30], from cells growing exponentially in MOPS medium containing glucose, uracil and thiamine. In some experiments, isopropyl-β-D-thiogalactoside (IPTG) (10 μM) was included to induce synthesis of plasmid-encoded RppH. The 5′ phosphorylation state of specific transcripts was determined by PABLO analysis, as described[1]. mRNA decay rates were measured after inhibiting transcription with rifampicin. Microarray analysis using *E. coli* Genome 2.0 arrays (Affymetrix) was performed with total cellular RNA extracted from triplicate cultures of JW2798*Δkan* containing either pPlacRppH or pPlacRppH-E53A and growing exponentially in the presence of IPTG.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions A.D., H.C. and J.G.B. planned the studies, interpreted the data and wrote the manuscript. A.D. and H.C. performed the experiments.

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METHODS

Plasmids. Plasmid pET-RPST1, when linearized with *Not*I, allows *in vitro* synthesis of an RNA identical to the *rpsT* P1 transcript but for a U → G substitution at the second nucleotide[1]. This substitution facilitates transcription by T7 RNA polymerase from a class III φ2.5 T7 promoter that efficiently produces A-initiated transcripts[27]. Plasmid pET-RPST1 + hp was constructed from pET-RPST1 by inserting the sequence AGCGCCGCTCGAGCGGCGCT at the beginning of the transcribed region. Plasmid pRPST1 contains a complete copy of the *E. coli rpsT* gene along with a unique sequence tag inserted into the 3′ untranslated region (UTR)[1]. Plasmid pRPST1 + hp is a derivative of pRPST1 in which the *rpsT* P1 promoter has been replaced by a *bla* promoter and an inverted repeat (ATCGCCGCTCGAGCGGCGAT) has been added to the 5′ end of the P1 transcriptional unit. Plasmid pPlacRppH6 is a pRNGL3[17] derivative that encodes amino-terminally hexahistidine-tagged RppH under the control of an IPTG-inducible *lacUV5* promoter. pPlacRppH6-E53A was constructed from pPlacRppH6 by a codon substitution (GAA → GCA) at position 53. Removal of the six histidine codons generated plasmids pPlacRppH and pPlacRppH-E53A, which were used to test complementation of the *ΔrppH* phenotype of JW2798*Δkan*.

Affinity purification of RppH. A 1–2 litre culture of *E. coli* JW2798*Δkan* containing pPlacRppH6 or pPlacRppH6-E53A was induced with IPTG (1 mM) for 4 h and harvested. The bacterial pellets were resuspended in 10 ml of buffer E (10 mM HEPES pH 7.6, 300 mM NaCl, 0.25% Genapol, 0.1 mM PMSF) containing protease inhibitors (Complete, EDTA-free; Roche) and lysed by passage through a French press. The lysate was treated for 1 h with DNase I (0.1 mg; Roche) in the presence of $MgSO_4$ (20 mM) and cleared by centrifugation at 14,500*g* for 30 min. The hexahistidine-tagged RppH protein was then attached to TALON beads (1–2 ml; BD Biosciences) by incubation for 1 h at 4 °C, washed with buffer E supplemented with protease inhibitors and containing 20 mM imidazole, and eluted with buffer E supplemented with protease inhibitors and containing 250 mM imidazole (pH 7.6). After overnight dialysis against buffer E, the protein was purified a second time with TALON beads, concentrated by centrifugal ultrafiltration, and stored at −20 °C in buffer E containing 25% (by volume) glycerol.

Detection of pyrophosphate release from γ-^{32}P-labelled RNA. Doubly labelled *rpsT* P1 or P1+hp RNA bearing a 5′-terminal γ-^{32}P label and an internal fluorescein label was synthesized by *in vitro* transcription in a mixture (40 μl) containing Tris-Cl (40 mM, pH 7.9), $MgCl_2$ (6 mM), NaCl (10 mM), dithiothreitol (10 mM), spermidine (2 mM), GTP (1 mM), CTP (1 mM), UTP (1 mM), ATP (0.5 mM), fluorescein-UTP (0.25 mM; Roche), [γ-^{32}P]ATP (50 μCi, 0.28 μM), RNasin (20 units; Promega), plasmid pET-RPST1 or pET-RPST1+hp linearized by *Not*I cleavage (0.13 pmol), and T7 RNA polymerase (40 units; New England Biolabs). (These DNA templates each contained a class III φ2.5 T7 promoter for efficient production of A-initiated transcripts[27].) After incubation for 4 h at 37 °C, the RNA was gel-purified.

The doubly labelled RNA (16,000 c.p.m., 18.2 nM) was incubated with purified RppH (75 nM) in a solution (160 μl) containing HEPES (20 mM, pH 7.6), $MgCl_2$ (5 mM), dithiothreitol (1 mM) and glycerol (1%) for 0–60 min at 37 °C. Reaction samples (20 μl) were quenched at time intervals with 5 μl of EDTA (100 mM, pH 8.0) and analysed by electrophoresis on a 6% polyacrylamide–8 M urea gel or by TLC on PEI-cellulose (J. T. Baker) developed with potassium phosphate buffer (0.3 M, pH 7.5). Band or spot intensities were quantified and compared by using a Molecular Dynamics Storm 820 PhosphorImager or a Molecular Dynamics FluorImager 575 and ImageQuant software.

Detection of pyrophosphate release from α-^{32}P-labelled RNA. Triphosphorylated $GA(CU)_{13}$, $AG(CU)_{13}$, $CG(A)_{26}$ and $UG(A)_{26}$ oligoribonucleotides bearing a single ^{32}P label at either the 5′-terminal α position or between the first and second nucleotides were synthesized for 6–8 h at 37 °C in a mixture (40 μl) containing Tris-Cl (40 mM, pH 7.9), $MgCl_2$ (6 mM), spermidine (2 mM), dithiothreitol (20 mM), rRNasin (40 units, Promega), T7 RNA polymerase (100 units; New England Biolabs), Ampliscribe T7-Flash enzyme solution (2 μl; Epicentre), the appropriate DNA template bearing a class III φ2.5 T7 promoter[27] (30 pmol), GTP (1 mM unlabelled or 50 μCi = 0.42 μM α-^{32}P-labelled), ATP (1 mM unlabelled or 50 μCi = 0.42 μM α-^{32}P-labelled), CTP (1 mM unlabelled or 50 μCi = 0.42 μM α-^{32}P-labelled) and UTP (1 mM unlabelled). Monophosphorylated and diphosphorylated forms of the same RNAs were synthesized by replacing the 5′-terminal NTP in the transcription reaction with either NMP or NDP (10 mM). Each RNA was gel-purified.

The α-labelled RNA (40,000 c.p.m., 0.3 nM) was incubated with purified RppH (75 nM) in a solution (20 μl) containing HEPES (20 mM, pH 7.6), $MgCl_2$ (5 mM), dithiothreitol (1 mM) and glycerol (1%) for 2 h at 37 °C. The reaction was stopped by adding 5 μl of EDTA (100 mM, pH 8.0). To confirm the integrity of the RNA, 15 μl of the reaction product was examined by electrophoresis on a 16% polyacrylamide–8 M urea gel. The remaining 10 μl was subjected to alkaline hydrolysis by incubation with 5 μl of NaOH (0.2 M) at 95 °C for 15 min. After neutralization with 5 μl of formic acid (3 M), the reaction products were analysed by TLC on a PEI-cellulose plate (J. T. Baker) developed with potassium phosphate buffer (0.3 M, pH 3.3), and radiolabelled products were detected with a Molecular Dynamics Storm 820 PhosphorImager.

RNA extraction from *E. coli*. Measurements of RNA lifetime and phosphorylation state were performed in *E. coli* K-12 strain BW25113[31] and its isogenic derivative JW2798*Δkan*, which bears an in-frame deletion of all but the first codon and last six codons of the *rppH* coding region, or in strain TA1025 (rne^+) and its isogenic derivative TA1026[29], which has a temperature-sensitive RNase E allele (*rne-1*). JW2798*Δkan* was constructed by excising the *kan* cassette that had replaced the *rppH* gene of JW2798[28], a Keio strain provided by the National Institute of Genetics (Japan).

Total cellular RNA was harvested, as previously described[30], from *E. coli* growing exponentially at 37 °C in MOPS medium containing glucose (0.2%), uracil (20 μg ml^{-1}) and thiamine (1 μg ml^{-1}), or 10 min after a temperature increase from 30 to 44 °C in the case of TA1025 and TA1026. In some experiments, the culture medium also contained IPTG (10 μM) to induce synthesis of plasmid-encoded RppH.

PABLO analysis. The 5′ phosphorylation state of RNA in *E. coli* was determined by PABLO analysis, as described[1]. The oligonucleotides used in these experiments were $Y_{rpsT\text{-}P1}$ (5′-ACTCGTTACGTAGTGATCAAGTTATCATTCATATTGTTC-3′), Y_{yeiP} (5′-AGTCGAAAATGTCAAAAATATCAAGTTATCATTCATATTGTTC-3′) and X_{90} (5′-CCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCGAACAATATGAATGATAACTTG-3′). The 5′ end of *yeiP* mRNA (5′-AUAUUUUUG …) maps to a site 35 base pairs (bp) upstream of the initiation codon and 7 bp downstream of the *yeiP* promoter (TTGCCC–17 bp–TACTTT), whose identity was confirmed by mutation. The radioactive probes used to detect *rpsT* and *yeiP* mRNA were a 5′-end-labelled oligonucleotide complementary to a 5′ UTR segment shared by the *rpsT* P1 and P2 transcripts (5′-GTCCAACTCCCAAATGTGTTC-3′) and an internally labelled probe generated by random priming (High Prime, Roche) on *yeiP* DNA.

Measurements of RNA half-life. Total cellular RNA was extracted from *E. coli* at time intervals after inhibiting transcription with rifampicin (0.2 mg ml^{-1}), and equal amounts (10 μg) were subjected to gel electrophoresis on polyacrylamide (6% or 4.5%) containing 8 M urea. The RNA was transferred to a Hybond-XL membrane (Amersham) by electroblotting and ultraviolet crosslinking, and probed with an internally radiolabelled probe generated by random priming (High Prime, Roche) on the coding region of *yeiP*, *ppa*, *efp*, *slyB* or *trxB*, or with a 5′-radiolabelled oligonucleotide probe complementary to a 5′ UTR segment shared by the *rpsT* P1 and P2 transcripts (5′-GTCCAACTCCCAAATGTGTTC-3′), a sequence tag inserted into the 3′ UTR of *rpsT* (5′-CAAAGATCGGGGTGGGGGTCTAAG-3′), an internal region of *yeiP* (5′-GCCGTTGTAATTCAGTACCA-3′), an internal region of *ppa* (5′-TCTGCGTTAGCCGGGATCTC-3′), 5S rRNA (5′-ACTACCATCGGCGCTACGGC-3′) or *cysT* tRNA (5′-GGAGTCGAACCGGACTAGACGG-3′). Radioactive bands were visualized with a Molecular Dynamics Storm 820 PhosphorImager, and band intensities were quantified by using ImageQuant software. RNA half-lives were calculated by linear regression analysis of data, which were obtained from at least two or three independent experiments.

Microarray analysis. Total cellular RNA was extracted from triplicate cultures of JW2798*Δkan* containing either pPlacRppH or pPlacRppH-E53A and growing exponentially in the presence of IPTG (10 μM) to induce RppH synthesis. Complementary DNA was prepared by random-primed reverse transcription with SuperScript II (Invitrogen), fragmented with DNase I (GE Biosciences), biotin-labelled with the GeneChip DNA labelling reagent (Affymetrix) and terminal deoxynucleotidyl transferase (Promega), and used to probe *E. coli* Genome 2.0 arrays (Affymetrix). The microarrays were scanned with an Affymetrix GeneChip Scanner 3000, and the raw data were scaled and quantified with Affymetrix GCOS software. Calculations of relative mRNA concentrations, including normalization, were performed with the MAS 5 algorithm.

31. Datsenko, K. A. & Wanner, B. L. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl Acad. Sci. USA* **97**, 6640–6645 (2000).