Figure S1

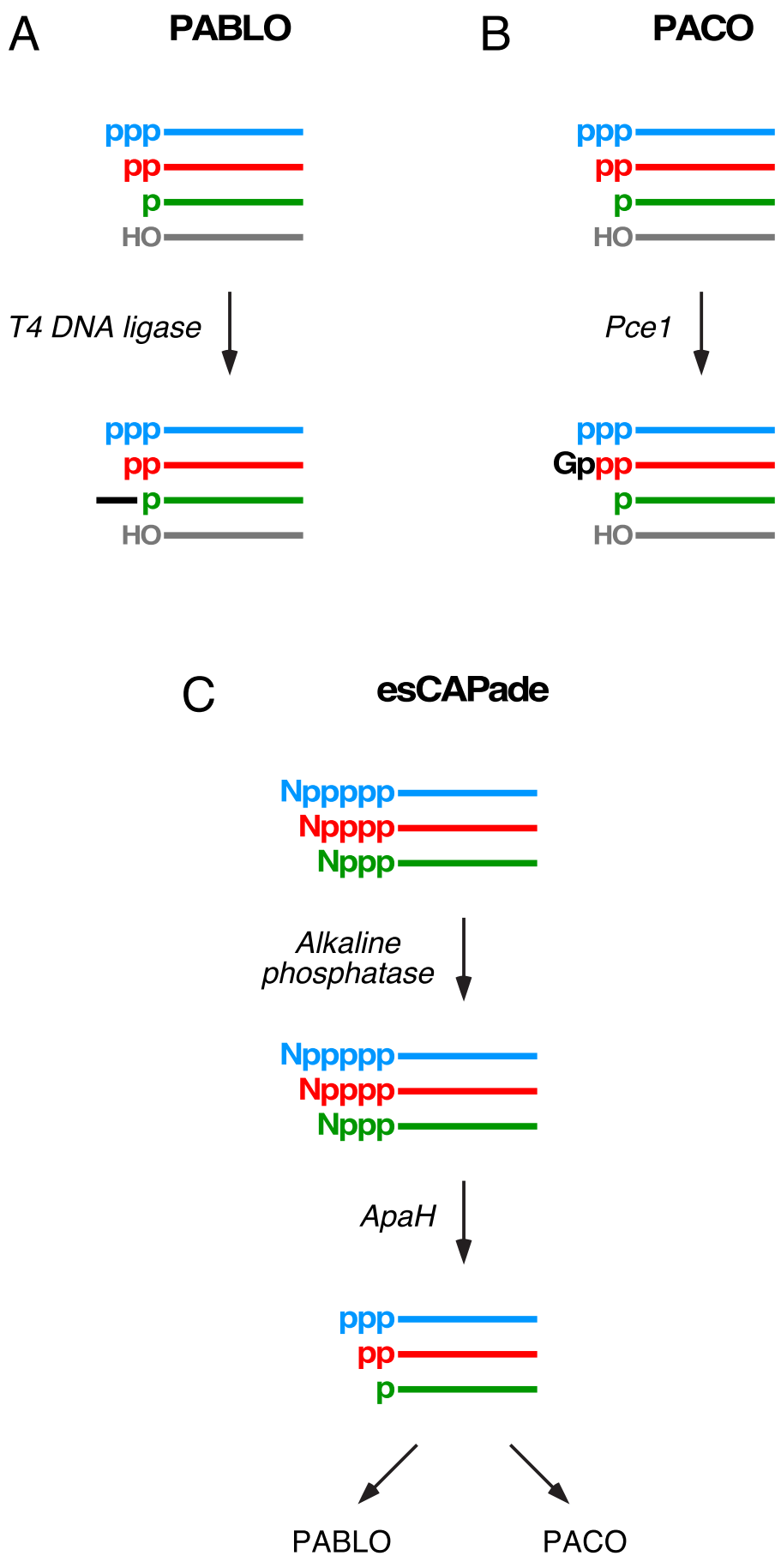
A
PABLO
ppp
pp
p
HO
T4 DNA ligase
ppp
pp
p
HO
B
PACO
ppp
pp
p
HO
Pce1
ppp
Gppp
p
HO
C
esCAPade
Npppppp
Nppppp
Nppp
Alkaline phosphatase
Npppppp
Nppppp
Nppp
ApaH
ppp
pp
p
PABLO
PACO

**Figure S1. Examination of RNA 5′ termini by PABLO, PACO, and esCAPade** (related to Figs. 1, 3, and 5).

(A) Detection of monophosphorylated 5′ ends by PABLO. PABLO (Phosphorylation Assay By Ligation of Oligonucleotides) is a splinted ligation assay in which monophosphorylated RNA 5′ ends are selectively ligated to the 3′ end of a DNA oligonucleotide when the two are precisely juxtaposed by simultaneous base pairing to a bridging DNA oligonucleotide and joined by T4 DNA ligase. The ligation product and its unligated counterpart are then separated by electrophoresis on a denaturing gel and detected by Northern blotting. The differential mobility of these two bands can be enhanced by site-specific RNA cleavage with a deoxyribozyme (not shown). ppp, 5′ triphosphate; pp, 5′ diphosphate; p, 5′ monophosphate; HO, 5′ hydroxyl.

(B) Detection of diphosphorylated 5′ ends by PACO. PACO (Phosphorylation Assay by Capping Outcome) detects diphosphorylated RNA 5′ ends on the basis of their reactivity with *Schizosaccharomyces pombe* Pce1, a guanylyltransferase that preferentially caps RNA 5′ ends bearing two phosphates. The cap added by Pce1 is detected by its ability to retard the electrophoretic migration of RNA on a boronate gel after site-specific cleavage with a deoxyribozyme to generate a 5′-terminal fragment bearing a 2′,3′ cyclic phosphate at its 3′ end (not shown).

(C) Determining the number of cap-associated phosphates by esCAPade. esCAPade (Enumeration of Shielded Cap-Associated Phosphates by Analysis of Decapped Ends) is a method for ascertaining how many phosphates are associated with the cap of a specific transcript. The capped RNA is first treated with alkaline phosphatase to convert any uncapped 5′ termini to unreactive hydroxyls and then with *E. coli* ApaH to release the cap as a nucleoside diphosphate. As a result, caps containing five, four, or three phosphates (Npppp, Nppp, or Nppp) are converted to triphosphorylated, diphosphorylated, or monophosphorylated 5′ ends, respectively. The phosphorylation state of the decapped products is then examined by PABLO and PACO to determine the percentage bearing a 5′ monophosphate or diphosphate (the remainder are assumed to be triphosphorylated). In this manner, the number phosphates associated with the cap of a cellular RNA can be determined by comparison to a set of capped standards synthesized by *in vitro* transcription.

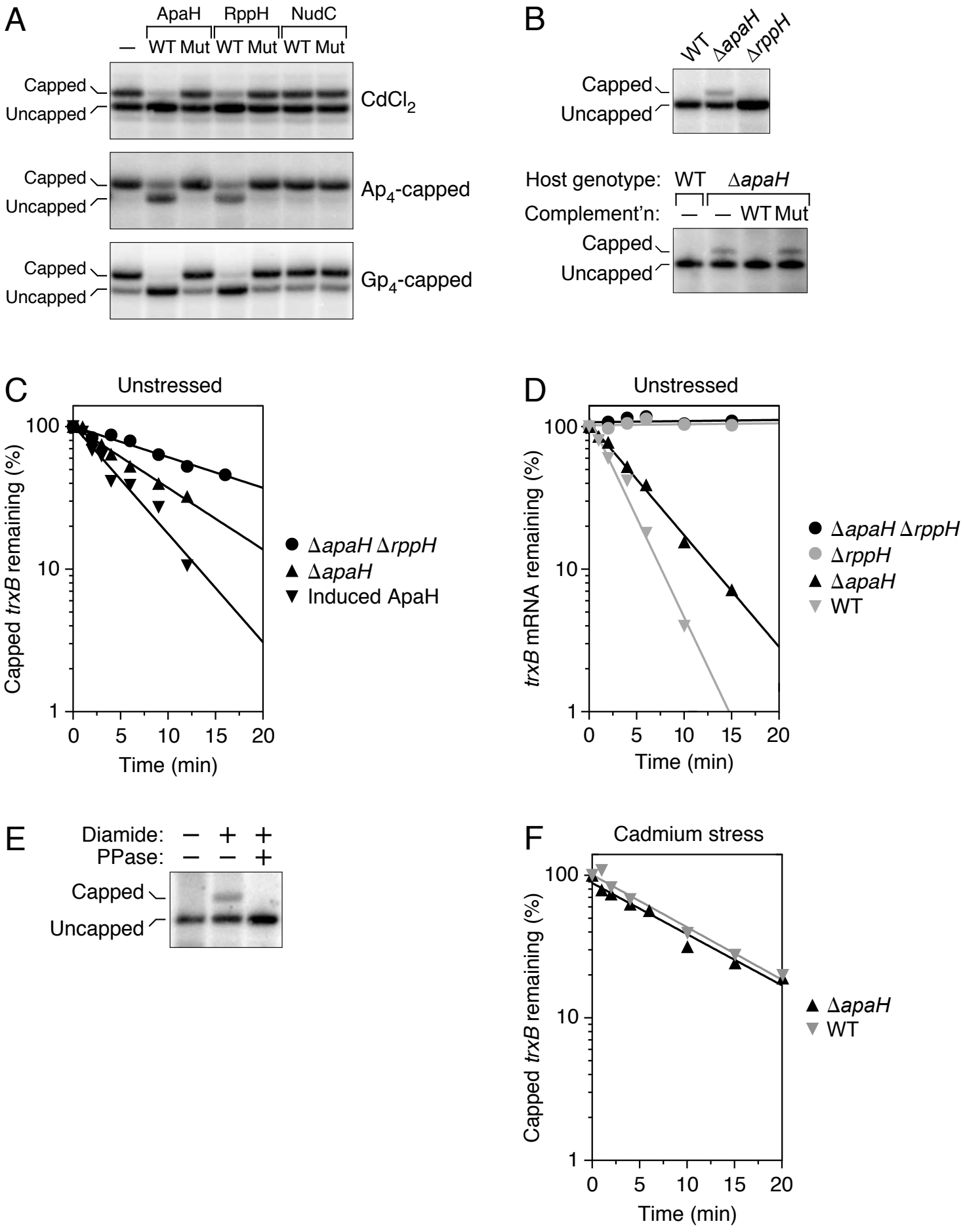
A
ApaH
RppH
NudC
—
WT Mut WT Mut WT Mut
Capped
Uncapped
CdCl2
Ap4-capped
Gp4-capped
B
WT
ΔapaH
ΔrppH
Host genotype: WT ΔapaH
Complement'n: — — WT Mut
C
Unstressed
Capped trxB remaining (%)
Time (min)
ΔapaH ΔrppH
ΔapaH
Induced ApaH
D
Unstressed
trxB mRNA remaining (%)
Time (min)
ΔapaH ΔrppH
ΔrppH
ΔapaH
WT
E
Diamide: — + +
PPase: — — +
Capped
Uncapped
F
Cadmium stress
Capped trxB remaining (%)
Time (min)
ΔapaH
WT

**Figure S2. Parallel analysis of *trxB* mRNA** (related to Figs. 2, 5, and 6).

(A) Decapping *in vitro*. Purified ApaH, RppH, and NudC and catalytically inactive mutants thereof were each tested for their ability to decap *trxB* mRNA that had been extracted from cadmium-treated cells ($CdCl_2$) or had been synthesized with a 5′-terminal $Ap_4$ or $Gp_4$ cap by *in vitro* transcription. Decapping was detected by boronate gel electrophoresis and blotting. WT, wild-type enzyme; Mut, mutant enzyme lacking catalytic activity; –, no enzyme.

(B) Presence of capped *trxB* mRNA in cells lacking ApaH but not in cells lacking RppH. Total RNA was extracted from cultures of unstressed wild-type, Δ*apaH*, or Δ*rppH* cells (top) or from Δ*apaH* cells that contained a plasmid encoding wild-type or catalytically inactive ApaH (bottom), and the presence of capped *trxB* mRNA was examined by boronate gel electrophoresis and Northern blotting. WT, wild-type *apaH* allele; Mut, mutant *apaH* allele encoding a catalytically inactive enzyme; –, no plasmid.

(C) Rate of loss of capped *trxB* mRNA after arresting transcription in *E. coli apaH* and *rppH* mutants. Cultures of the indicated strains growing without stress in LB were treated with rifampicin, and equal amounts of total RNA extracted at time intervals thereafter were analyzed by boronate gel electrophoresis and blotting. Data from representative experiments are shown.

(D) Effect of an *apaH* deletion on the aggregate decay rate of *trxB* mRNA in the presence or absence of RppH. Cultures of the indicated strains growing without stress in MOPS-glucose were treated with rifampicin, and equal amounts of total RNA extracted at time intervals thereafter were analyzed by electrophoresis on ordinary polyacrylamide gels. Data from representative experiments are shown.

(E) Presence of capped *trxB* mRNA in diamide-treated cells. Total RNA from *E. coli* cells that had or had not been treated with diamide was analyzed by boronate gel electrophoresis and Northern blotting, with or without prior decapping by RppH (PPase).

(F) Slow decapping of *trxB* mRNA in cells experiencing disulfide stress. The indicated strains of *E. coli* growing in MOPS-glucose were treated first with cadmium chloride and then with rifampicin, and equal amounts of total RNA extracted at time intervals thereafter were analyzed by boronate gel electrophoresis and blotting. Data from representative experiments are shown.

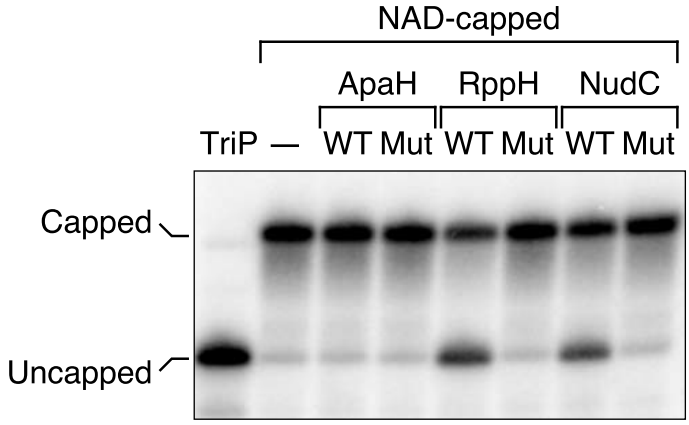


**Figure S3. Deprotection of NAD-capped RNA** (related to Fig. 2).

Purified ApaH, RppH, and NudC and catalytically inactive mutants thereof were each tested for their ability to decap *yeiP* RNA that had been synthesized with a 5'-terminal NAD cap by *in vitro* transcription. Decapping was detected by boronate gel electrophoresis and blotting. WT, wild-type enzyme; Mut, mutant enzyme lacking catalytic activity; –, no enzyme; TriP, uncapped *yeiP* marker.

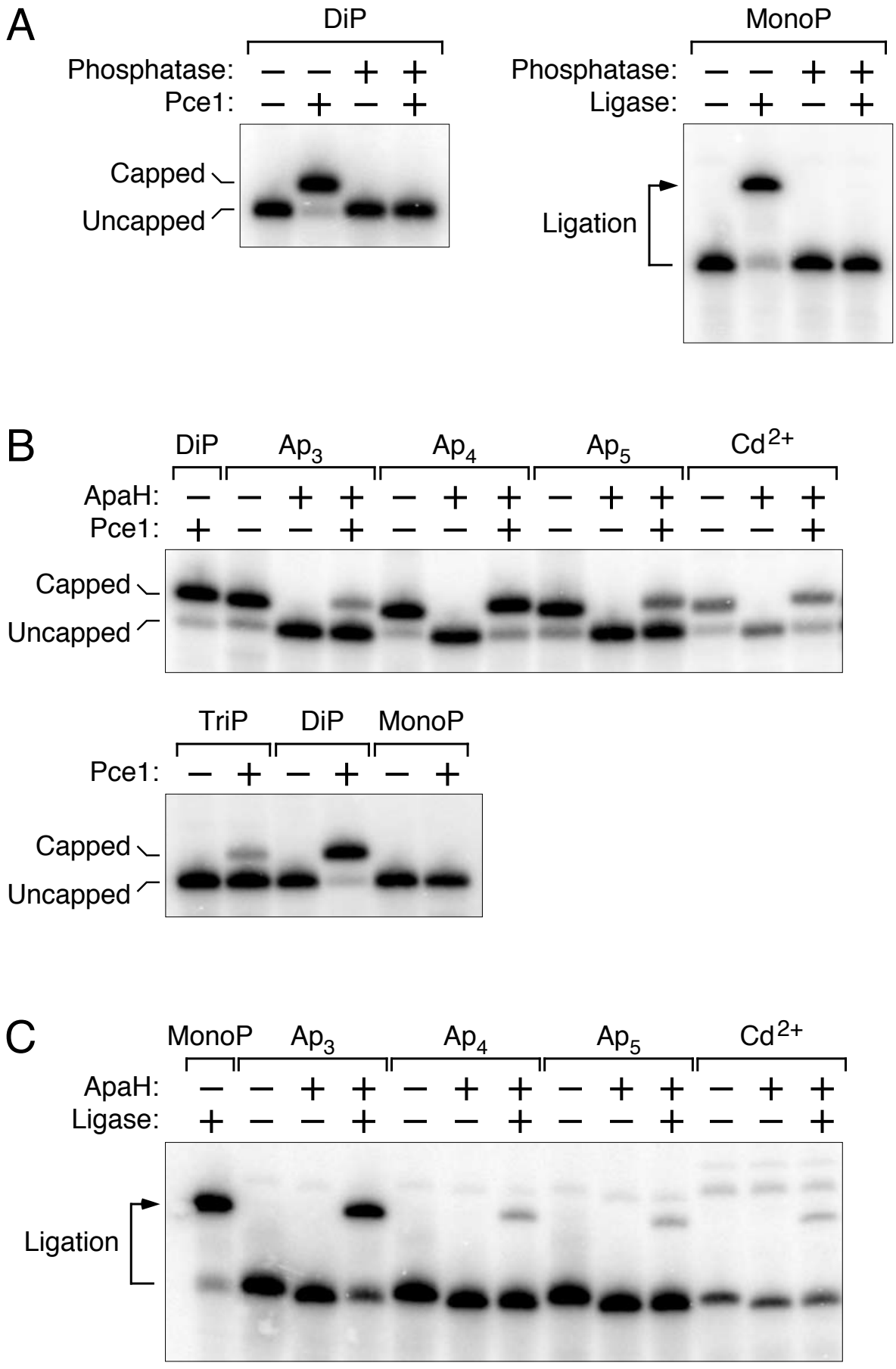
A
DiP
Phosphatase: – – + +
Pce1: – + – +
Capped
Uncapped
MonoP
Phosphatase: – – + +
Ligase: – + – +
Ligation
B
DiP
Ap3
Ap4
Ap5
Cd2+
ApaH: – – + + – + + – + + – + +
Pce1: + – – + – – + – – + – – +
Capped
Uncapped
TriP
DiP
MonoP
Pce1: – + – + – +
Capped
Uncapped
C
MonoP
Ap3
Ap4
Ap5
Cd2+
ApaH: – – + + – + + – + + – + +
Ligase: + – – + – – + – – + – – +
Ligation

**Figure S4. Enumeration of cap-associated phosphates by esCAPade** (related to Fig. 3).

(A) Efficacy of exposed phosphate removal by alkaline phosphatase. *In vitro* transcribed *yeiP* RNA bearing a 5′-terminal diphosphate (DiP, left) or monophosphate (MonoP, right) was mixed with total *E. coli* RNA from Δ*yeiP* cells, and the ability of alkaline phosphatase treatment to prevent subsequent capping by Pce1 (Luciano et al., 2017) or splinted ligation (Celesnik et al., 2007), respectively, was examined.

(B) PACO analysis of the products of *yeiP* decapping by ApaH. (Top) *In vitro* transcribed *yeiP* RNA bearing an $Ap_3$, $Ap_4$, or $Ap_5$ cap or *yeiP* RNA extracted from cadmium-treated cells ($Cd^{2+}$) was treated with alkaline phosphatase and then with ApaH, and the ability of the decapped products to undergo capping by purified Pce1 was analyzed by boronate gel electrophoresis and blotting (Luciano et al., 2017; Luciano and Belasco, 2019). The *in vitro* transcripts were each analyzed in the presence of total *E. coli* RNA from Δ*yeiP* cells to simulate the decapping and recapping of a cellular transcript. (Top left and Bottom) Pce1-mediated capping of *in vitro* transcribed *yeiP* RNA bearing a 5′-terminal triphosphate (TriP), diphosphate (DiP), or monophosphate (MonoP) was examined in parallel.

(C) PABLO analysis of the products of *yeiP* decapping by ApaH. The ability of the decapped products analyzed in panel (B) to undergo splinted ligation (Celesnik et al., 2007) was examined. Splinted ligation of *in vitro* transcribed *yeiP* RNA bearing a 5′-terminal monophosphate (MonoP) was examined in parallel.

Figure S5

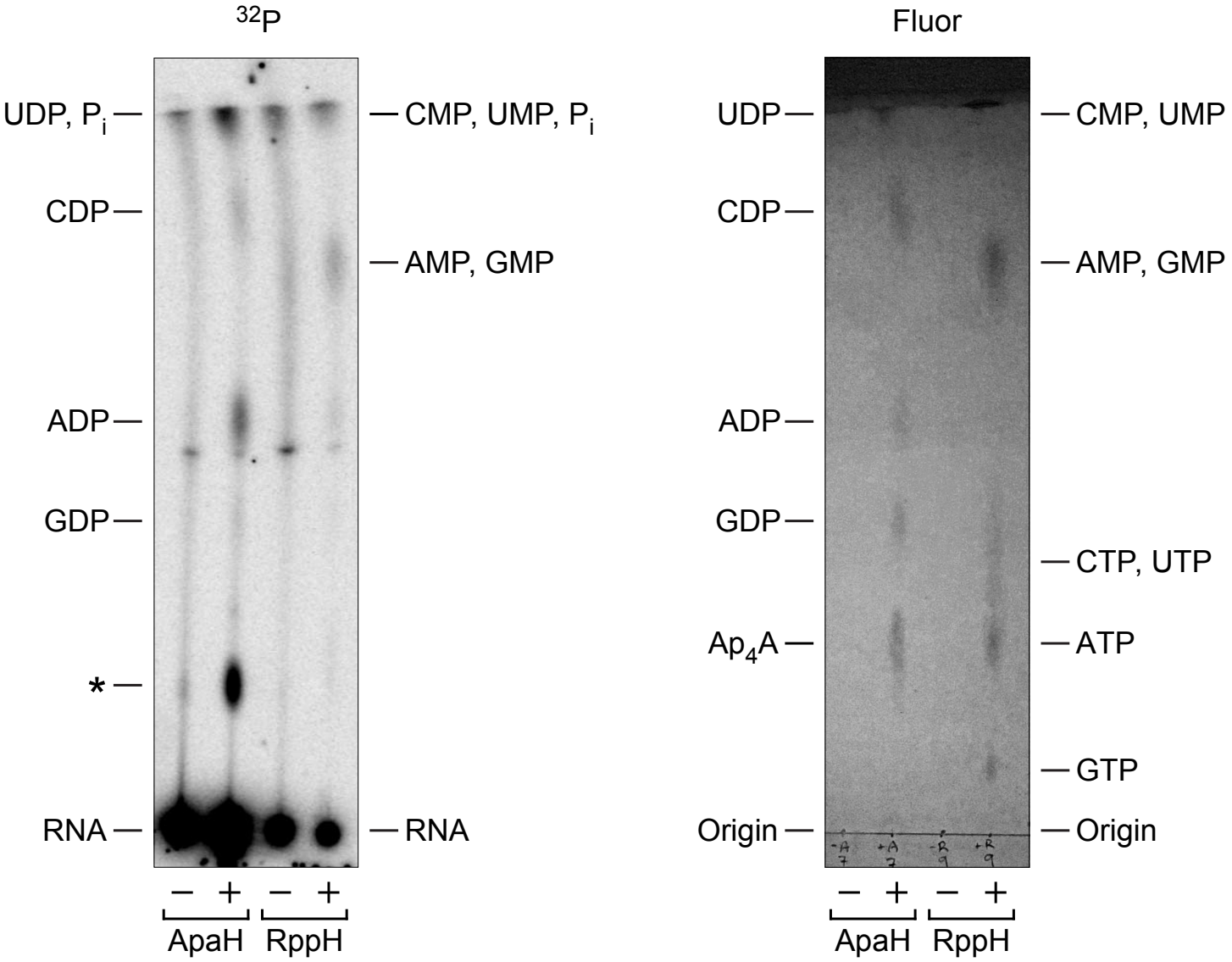
32P
UDP, Pi
CDP
ADP
GDP
*
RNA
CMP, UMP, Pi
AMP, GMP
RNA
− + − +
ApaH RppH
Fluor
UDP
CDP
ADP
GDP
Ap4A
Origin
CMP, UMP
AMP, GMP
CTP, UTP
ATP
GTP
Origin
− + − +
ApaH RppH

**Figure S5. Characterization of the cap nucleobase** (related to Fig. 3).

Total RNA extracted from *E. coli* cells that had been radiolabeled with [$^{32}$P] phosphate and exposed to cadmium chloride was treated with alkaline phosphatase to convert uncapped 5′ ends to hydroxyls and hydrolyze any contaminating mononucleotides. It was then fractionated by sucrose-gradient centrifugation, and RNAs 100-1,400 nt long were further separated from nucleotide contaminants by size-exclusion chromatography on Sephadex G-25. The RNA was subsequently treated with either ApaH or RppH or mock treated, and the radiolabeled nucleotides thereby released were purified by another round of size-exclusion chromatography, mixed with sets of unlabeled nucleotide standards, and analyzed by thin-layer chromatography on fluorescent polyethylenimine-cellulose plates. $^{32}$P, radioactivity; Fluor, fluorescence quenched by nucleotide standards; ✻, unidentified nucleotide.

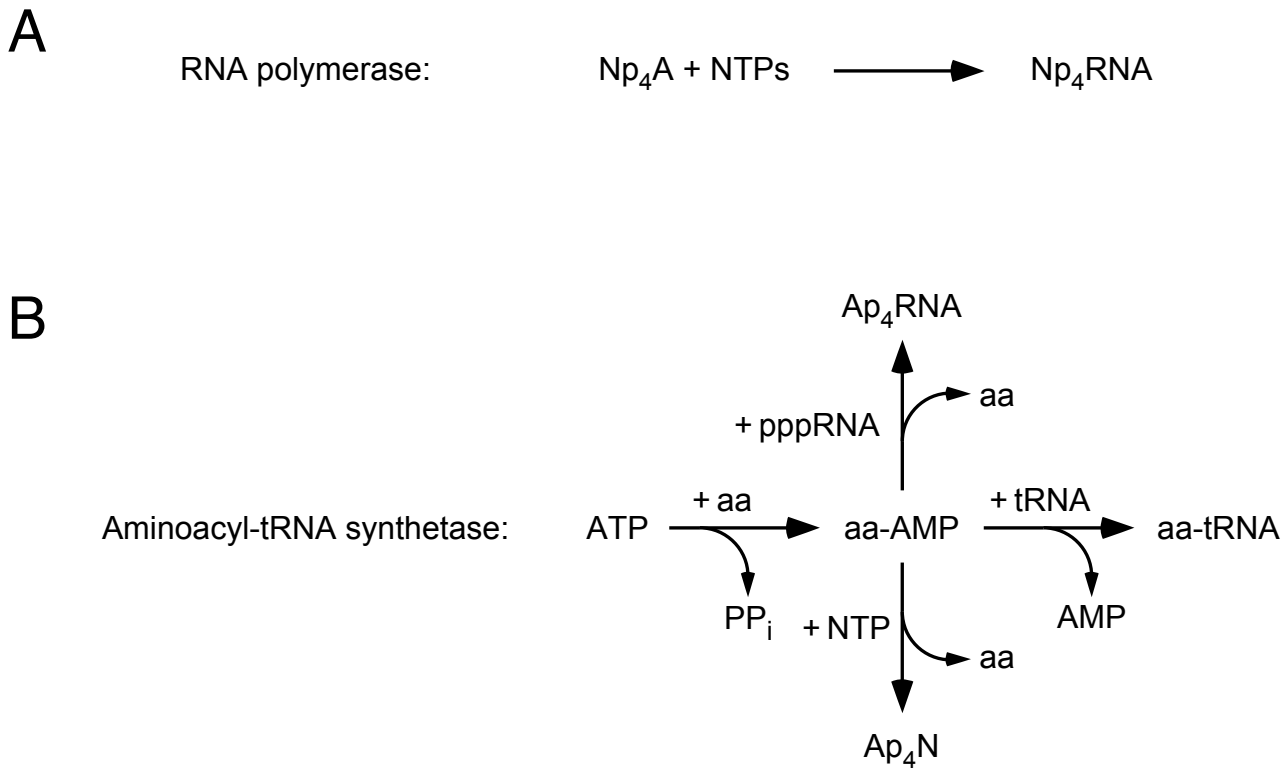


**Figure S6. Reaction pathways for $Np_4$ cap addition *in vitro*** (related to Fig. 4).

(A) Incorporation of $Np_4A$ during transcription initiation by *E. coli* RNA polymerase. $Np_4RNA$, $Np_4$-capped transcript.

(B) Post-transcriptional $Np_4$ capping by an aminoacyl-tRNA synthetase. $PP_i$, pyrophosphate; aa, amino acid; aa-AMP, aminoacyl-adenylate; aa-tRNA, aminoacyl-tRNA. pppRNA, triphosphorylated transcript; $Ap_4RNA$, $Ap_4$-capped transcript. Note that $Ap_4N$ and $Np_4A$ are synonymous due to molecular symmetry.

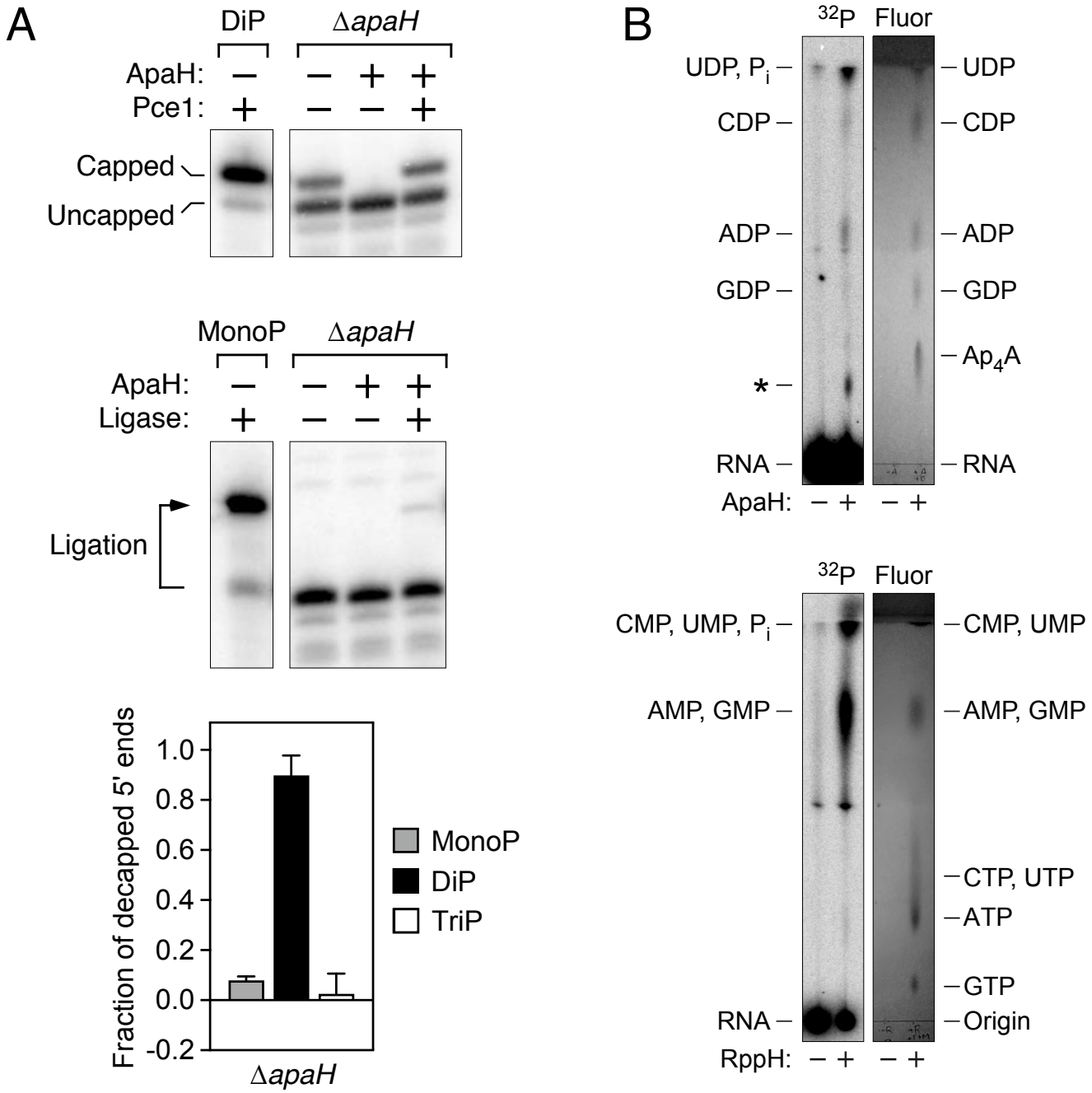
A
DiP
ΔapaH
ApaH:
Pce1:
Capped
Uncapped
MonoP
ΔapaH
ApaH:
Ligase:
Ligation
Fraction of decapped 5' ends
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
MonoP
DiP
TriP
ΔapaH
B
32P
Fluor
UDP, Pi
CDP
ADP
GDP
*
RNA
UDP
CDP
ADP
GDP
Ap4A
RNA
ApaH:
32P
Fluor
CMP, UMP, Pi
AMP, GMP
CTP, UTP
ATP
GTP
Origin
RNA
RppH:
C
ApaH
RppH
NudC
WT Mut WT Mut WT Mut
yeiP
Capped
Uncapped
trxB
Capped
Uncapped

**Figure S7. Analysis of capped mRNA in cells lacking ApaH** (related to Figs. 2, 3, and 5).

(A) Enumeration of cap-associated phosphates in unstressed Δ*apaH* cells by esCAPade. (Top) PACO analysis of the products of *yeiP* decapping by ApaH. Total RNA extracted from unstressed Δ*apaH* cells was treated with alkaline phosphatase and then with ApaH, and the ability of the decapped *yeiP* products to undergo capping by purified Pce1 was examined by boronate gel electrophoresis and blotting beside diphosphorylated *yeiP* RNA synthesized by *in vitro* transcription (DiP). (Middle) PABLO analysis of the products of *yeiP* decapping by ApaH. The ability of the same decapped *yeiP* products to undergo splinted ligation was examined beside monophosphorylated *yeiP* RNA synthesized by *in vitro* transcription (MonoP). (Bottom) Calculated fraction of capped *yeiP* 5′ ends from unstressed Δ*apaH* cells that bore a monophosphate (MonoP), diphosphate (DiP), or triphosphate (TriP) after decapping by purified ApaH. Data from three biological replicates were used to calculate each value (Tables S1-S6). Error bars correspond to a confidence level of 68.3%.

(B) Characterization of the cap nucleobase. RNA from unstressed *E. coli* Δ*apaH* cells that had been radiolabeled with [$^{32}$P] phosphate was purified as in Fig. 3D and then decapped with either ApaH (top) or RppH (bottom) as in Fig. S5. The radiolabeled nucleotides thereby released were mixed with a set of unlabeled nucleotide standards and analyzed by thin-layer chromatography on fluorescent polyethylenimine-cellulose plates. $^{32}$P, radioactivity; Fluor, fluorescence quenched by nucleotide standards; ✻, unidentified nucleotide.

(C) Decapping *in vitro*. Purified ApaH, RppH, and NudC and catalytically inactive mutants thereof were each tested for their ability to decap *yeiP* and *trxB* mRNA that had been extracted from unstressed Δ*apaH* cells. Decapping was detected by boronate gel electrophoresis and blotting. WT, wild-type enzyme; Mut, mutant enzyme lacking catalytic activity; –, no enzyme.

**Table S1. Measurements used to calculate the PABLO ligation efficiencies and PACO capping efficiencies of *yeiP* standards** (related to Figs. 3 and 5).

| Measured values | | | | | 5′ homogeneity | |
|---|---|---|---|---|---|---|
| PABLO | | PACO | | | | |
| $E_{LM}$ | $E_{LD}$ | $Y_{CM}$ | $Y_{CD}$ | $Y_{CT}$ | $M_S$ | $D_S$ |
| | | | | | | |
| 0.910 ± 0.005 | 0.011 ± 0.004 | 0.000 ± 0.000 | 0.918 ± 0.005 | 0.109 ± 0.015 | 0.978 | 0.930 |

$E_{LM}$ and $E_{LD}$ are the fractional PABLO ligation yields obtained for the monophosphorylated and diphosphorylated standards, respectively. $Y_{CM}$, $Y_{CD}$, and $Y_{CT}$ are the fractional PACO capping yields obtained for the monophosphorylated, diphosphorylated, and triphosphorylated standards, respectively. The E and Y values correspond to the mean and standard deviation of three independent measurements. $M_S$ and $D_S$ are the calculated fractions of those standards that are diphosphorylated and monophosphorylated, respectively.

**Table S2. Calculated PACO capping efficiencies of the *yeiP* standards** (related to Figs. 3 and 5).

| Capping efficiencies | | |
|---|---|---|
| $E_{CM}$ | $E_{CD}$ | $E_{CT}$ |
| | | |
| $^{+\ 0.000}_{-\ 0.002}$ $^{}_{-\ 0.000}$ | $^{+\ 0.005}_{0.979}$ $^{}_{-\ 0.006}$ | $^{+\ 0.017}_{0.107}$ $^{}_{-\ 0.013}$ |
| | | |

$E_{CM}$, $E_{CD}$, and $E_{CT}$ are the fractional capping efficiencies calculated for monophosphorylated, diphosphorylated, and triphosphorylated RNA, respectively, with 0 being no capping and 1 being complete capping. Each efficiency determined by Monte Carlo simulation corresponds to the peak of an asymmetric distribution, and the error range (±) corresponds to a confidence level of 68.3%, equivalent to one standard deviation. A minor discrepancy versus the theoretical minimum (0.000) resulted from the small mathematical correction factors that were used.

**Table S3. Measurements used to calculate the phosphorylation state of the capped *yeiP* standards after phosphatase treatment and decapping by ApaH** (related to Figs. 3 and 5).

| $Ap_3$-capped | | | $Ap_4$-capped | | | $Ap_5$-capped | | |
|---|---|---|---|---|---|---|---|---|
| $P_3$ | $Y_{L3}$ | $Y_{C3}$ | $P_4$ | $Y_{L4}$ | $Y_{C4}$ | $P_5$ | $Y_{L5}$ | $Y_{C5}$ |
| | | | | | | | | |
| 0.867 ± 0.005 | 0.624 ± 0.054 | 0.161 ± 0.007 | 0.890 ± 0.014 | 0.030 ± 0.017 | 0.842 ± 0.005 | 0.835 ± 0.004 | 0.024 ± 0.005 | 0.272 ± 0.007 |

$P_3$, $P_4$, and $P_5$ are the fraction of the $Ap_3$-, $Ap_4$-, and $Ap_5$-capped standards, respectively, that actually bore a cap, as determined by boronate gel electrophoresis. $Y_{L3}$, $Y_{L4}$, and $Y_{L5}$ are the PABLO ligation yields of the $Ap_3$-, $Ap_4$-, and $Ap_5$-capped standards after decapping by ApaH. $Y_{C3}$, $Y_{C4}$, and $Y_{C5}$ are the PACO capping yields of the $Ap_3$-, $Ap_4$-, and $Ap_5$-capped standards after decapping by ApaH. Each value corresponds to the mean and standard deviation of three independent measurements.

**Table S4. Normalized phosphorylation state of the capped *yeiP* standards after phosphatase treatment and decapping by ApaH** (related to Figs. 3 and 5).

| $Ap_3$-capped | | | $Ap_4$-capped | | | $Ap_5$-capped | | |
|---|---|---|---|---|---|---|---|---|
| $M_3/P_3$ | $D_3/P_3$ | $T_3/P_3$ | $M_4/P_4$ | $D_4/P_4$ | $T_4/P_4$ | $M_5/P_5$ | $D_5/P_5$ | $T_5/P_5$ |
| | | | | | | | | |
| + 0.072<br>0.787<br>– 0.069 | + 0.013<br>0.171<br>– 0.014 | + 0.070<br>0.042<br>– 0.073 | + 0.021<br>0.026<br>– 0.022 | + 0.017<br>0.951<br>– 0.018 | + 0.028<br>0.022<br>– 0.027 | + 0.006<br>0.029<br>– 0.008 | + 0.018<br>0.229<br>– 0.020 | + 0.022<br>0.742<br>– 0.019 |
| | | | | | | | | |

$M_n/P_n$, $D_n/P_n$, and $T_n/P_n$ (where n = 3, 4, or 5) are the fractional yields of monophosphorylated, diphosphorylated, and triphosphorylated products, respectively, obtained by decapping the $Ap_3$-, $Ap_4$-, and $Ap_5$-capped standards, normalized to the fraction of each standard that actually bore a cap. The fractional yields of monophosphorylated and diphosphorylated products determined by Monte Carlo simulation correspond to the peak of an asymmetric distribution, and the error range (±) corresponds to a confidence level of 68.3%, equivalent to one standard deviation. The fractional yield of triphosphorylated products was calculated by substraction ($T_n/P_n = 1 – D_n/P_n – M_n/P_n$).

**Table S5. Measurements used to calculate the phosphorylation state of capped *E. coli yeiP* mRNA after phosphatase treatment and decapping by purified ApaH** (related to Figs. 3 and 5).

| Wild-type + $CdCl_2$ | | | Δ*apaH* | | |
|---|---|---|---|---|---|
| C | $Y_L$ | $Y_C$ | C | $Y_L$ | $Y_C$ |
| | | | | | |
| 0.756 ± 0.008 | 0.098 ± 0.005 | 0.709 ± 0.030 | 0.359 ± 0.006 | 0.029 ± 0.005 | 0.381 ± 0.017 |

C is the fraction of *yeiP* mRNA that was capped either in wild-type *E. coli* cells experiencing cadmium stress or in unstressed *E. coli* Δ*apaH* cells. $Y_L$ is the fractional PABLO ligation yield after decapping by ApaH. $Y_C$ is the fractional PACO capping yield after decapping by ApaH. The values listed correspond to the mean and standard deviation of three biological replicates.

**Table S6. Normalized phosphorylation state of decapped *E. coli yeiP* mRNA and normalized fraction of *E. coli yeiP* caps that contain three, four, or five bridging phosphates** (related to Figs. 3 and 5).

| Wild-type + $CdCl_2$ | | | Δ*apaH* | | |
|---|---|---|---|---|---|
| $M_C/C$ | $D_C/C$ | $T_C/C$ | $M_C/C$ | $D_C/C$ | $T_C/C$ |
| | | | | | |
| $0.131^{+0.009}_{-0.007}$ | $0.931^{+0.048}_{-0.050}$ | $-0.062^{+0.050}_{-0.049}$ | $0.078^{+0.016}_{-0.016}$ | $0.897^{+0.051}_{-0.080}$ | $0.024^{+0.082}_{-0.053}$ |
| | | | | | |
| $P_{3C}/C$ | $P_{4C}/C$ | $P_{5C}/C$ | $P_{3C}/C$ | $P_{4C}/C$ | $P_{5C}/C$ |
| | | | | | |
| $0.136^{+0.034}_{-0.031}$ | $0.980^{+0.072}_{-0.069}$ | $-0.109^{+0.069}_{-0.091}$ | $0.070^{+0.032}_{-0.036}$ | $0.919^{+0.084}_{-0.101}$ | $0.033^{+0.089}_{-0.112}$ |

$M_C/C$, $D_C/C$, and $T_C/C$ are the fractional yields of monophosphorylated, diphosphorylated, and triphosphorylated products, respectively, obtained by decapping *yeiP* mRNA from *E. coli*, normalized to the fraction of *yeiP* mRNA that was capped. The fractional yield of triphosphorylated products was calculated by substraction ($T_C/C = 1 - D_C/C - M_C/C$). $P_{3C}/C$, $P_{4C}/C$, and $P_{5C}/C$ are the calculated fraction of *yeiP* caps in *E. coli* that contain three, four, or five phosphates, normalized to the total fraction of *yeiP* mRNA that was capped. Each fractional value determined by Monte Carlo simulation corresponds to the peak of an asymmetric distribution, and the error range (±) corresponds to a confidence level of 68.3%, equivalent to one standard deviation. Minor discrepancies versus the theoretical minimum (0.000) resulted from the small mathematical correction factors that were used and are nearly within the margin of error.