Dinucleoside polyphosphates act as 5'-RNA caps in *Escherichia coli*

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Dinucleoside polyphosphates (Np_nNs), discovered more than 50 years ago,[1] are pleiotropic molecules present in almost all types of cells.[2] It has been shown that their intracellular concentration can under stress conditions increase from the µM to mM range [2,3]. However, the cellular roles and mechanisms of action of Np_nNs are still speculative[4,5]. They have never been considered as part of the RNA, even though they have similar chemical structures as already known RNA caps, such as the nicotinamide adenine dinucleotide (NAD)[6-8] and 7-methylguanylate cap[9]. Here, we show that both methylated and non-methylated Np_nNs serve as RNA caps in *Escherichia coli* (*E. coli*). Np_nNs are excellent substrates for T7 and *E. coli* RNA polymerases (RNAP) and efficiently initiate transcription. Further, we demonstrate that the *E. coli* decapping enzyme RNA 5' pyrophosphohydrolase (RppH) is able to remove the Np_nNs-cap from the RNA. RppH was, however, not able to cleave the methylated forms of the Np_nN-caps, suggesting that the methylation adds an additional layer to the RNA stability regulation. Our work introduces an original perspective on the chemical structure of RNA in prokaryotes and the function of RNA caps. This is the first evidence that small molecules like Np_nNs can act in cells via their incorporation into RNA and influence the cellular metabolism.

The role and chemical structure of the 5'-end of prokaryotic RNA is still unclear. The discovery of NAD[6,10] and Coenzyme A (CoA)[11] as 5' RNA caps changed the perception of the RNA structure. 5'-caps are usually cleaved by NudiX enzymes (NudC[6,12,13], RppH[14,15]), which can, besides their decapping role (eukaryotic Nudt16 and Dcp2[16,17]), cleave nucleoside diphosphate linked to another moiety (*e.g.* Np_nNs[2,18]). To investigate whether Np_nNs (Figure 1a) can serve as non-canonical initiating nucleotides (NCINs) similarly to NAD and CoA[19], we performed *in vitro* transcription in the presence of different Np_nNs ($Ap_{3-6}A$, $Ap_{4-5}G$, Gp_4G, Figure 1b) with T7 RNAP. The resulting RNA was a mixture of capped and uncapped RNA (step 1 in Figure 1b, Extended data Fig. 1a). The presence of the cap was confirmed by electrophoretic analysis after subsequent treatment with 5'-polyphosphatase and Terminator™ 5´-phosphate-dependent exonuclease (terminator, step 2 and 3 in Figure 1b). The former dephosphorylated the 5'-triphosphate RNA (5'-ppp RNA) but not any capped RNA. The terminator then digested all RNA with 5'-monophosphate termini (5'-p RNA) and left the capped RNA intact. We observed that all tested Np_nNs were excellent substrates for T7 RNAP and served as NCINs for *in vitro* transcription (Figure 1c).

Subsequently, we tested *E. coli* RNAP that is known to accept NAD as NCIN *in vitro* and *in vivo*[19-21] (Extended data Fig. 1b). To confirm the existence of 5'-capped RNA products, we also treated them with 5'-polyphosphatase and the terminator. We found that Np_nNs are superior initiating substrates compared to NAD. The absence of NAD-capping (Figure 1d) was likely due to the different promoter sequence than previously published[19].

To identify the best substrate for RNAP, we varied the concentrations of the Np_nNs in the presence of a constant (1 mM) ATP and GTP concentration (Figure 1e, Extended data Fig. 1c,d). The amount of capped RNA increased linearly with the concentration of Np_nN. When the ratio of ATP (GTP) to Np_nN was 1, we observed between 27% (for Ap_6A) and 46% (for Ap_4G) capped products. The NAD-capped RNA was produced in lower amounts compared to the majority of Np_nNs.

Next, we wanted to determine whether Np_nNs exist as 5'-RNA caps *in vivo* in *E. coli*. We established an LC-MS method for their detection in RNA. Because the intracellular concentration of Np_nNs is known to grow under stress conditions[2,3,22], we collected cells in exponential (EXP, OD=0.3) and late stationary phase of growth (STA). We focused on short RNA (sRNA) where NAD-cap[10] and CoA-cap[11] have also been detected. The RNA was washed to remove all non-covalently interacting molecules and digested by Nuclease P1 into the form of nucleotides (Figure 2a). The negative control samples, where the addition of Nuclease P1 was omitted, did not show any signals of nucleotides or Np_nNs, which excluded the possibility of non-covalently bound contamination.

In all the digested sRNA, we observed signals of Ap_3A ($[M-H]^-$ at *m/z* 755.077), Ap_3G ($[M-H]^-$ at *m/z* 771.065) and Ap_5A ($[M-2H]^{2-}$ at *m/z* 457.008) (Extended data Fig. 2a,b,c). We also observed significant signals of mono- and dimethylated forms of Np_nNs, specifically methyl-Ap_3A ($[M-H]^-$ at *m/z* 769.080), dimethyl-Gp_4G ($[M-2H]^{2-}$ at *m/z* 447.018) and methyl-Ap_5G ($[M-2H]^{2-}$ at *m/z* 472.018) (Extended data Fig. 3a,b,c). Besides the previously mentioned caps, in the STA, we detected signals of methyl-Ap_4G ($[M-2H]^{2-}$ at *m/z* 432.016), methyl-Ap_5A ($[M-2H]^{2-}$ at *m/z* 463.992) and dimethyl-Ap_5G ($[M-2H]^{2-}$ at *m/z* 479.003) (Figure 2b, Extended data Fig. 4a,b,c). We compared the amounts of dimethyl-Gp_4G-RNA at various growth stages. The level of this cap was more than two-fold higher in the STA compared to EXP (Figure 2c). In general, a higher number of Np_nNs were detected in this phase. This may indicate that the cells in the STA lack the nutrients and methylate the Np_nN-caps to preserve RNA.

To confirm the structure of the detected Np_nN-caps, we grew the *E. coli* in minimal media with the sole source of nitrogen from either $^{14}NH_4Cl$ or $^{15}NH_4Cl$. We detected only three caps: methyl-Ap_3A, Ap_3G, and dimethyl-Gp_4G (Figure 2b,d,e,f Extended data Fig. 5a,b,c), because this type of growth represents another type of stress. This experiment confirmed the presence of ten nitrogen atoms in every detected molecule. To further verify the chemical structure of Np_nNs, we compared the LC-MS properties of standard Gp_4G with the isomeric p_3GpG. While half of the p_3GpG was fragmented in the ionization source to p_2GpG, the Gp_4G stayed intact (Extended data Fig. 6a,b). The same behavior was observed for the dimethyl-Gp_4G in the *E. coli* RNA sample, proving the internal polyphosphate chain. By linear ion trap LC-MS, we detected an intact triphosphate chain of Ap_3A confirming its structure (Extended data Fig. 7).

Since Np_nN-capped RNAs are produced in *E. coli*, degradation mechanisms of the capped RNA in *E. coli* must exist. $Ap_{4-6}A$ have been reported to be *in vitro* substrates for the *E. coli* NudiX enzyme NudH (RppH[18,23]). RppH is an *E. coli* decapping enzyme of 5'-triphosphate[4,14,24] and 5'-diphosphate RNA[15]. To assess whether $Ap_{4-6}A$-capped RNA can be an RppH substrate *in vivo*, we prepared Np_nN- and NAD-capped RNAs by *in vitro* transcription and tested the products as substrates for RppH. First, we added RppH to transform 5'-cappped RNA into 5'-p RNA, which then served as substrate for the terminator (Figure 3a). Electrophoretic analysis showed that Np_nN-capped RNAs are cleaved into 5'-p RNA and subsequently degraded (Figure 3b,c), suggesting that $Ap_{4-6}A$ and $Ap_{4-5}G$-capped RNAs are excellent substrates for RppH *in vitro*. However, Ap_3A and NAD[6] were cleaved less efficiently. Surprisingly, the Np_nN-capped RNAs were

always superior substrates for RppH compared to 5'-ppp RNAs. To understand the substrate specificity of RppH, we performed kinetic study of Np_nN-capped RNAs. The decapping reaction of $Ap_{4,5}N$ RNA was almost quantitative within 5 min while the corresponding 5'-ppp RNA was decapped only by 50% within 40 min (Figure 3d,e, Extended data Fig 8).

As Np_nN RNAs are excellent substrates for RppH *in vitro*, we constructed a mutant *E. coli* strain KS47 (Δ*rpph)* with knock-down RppH to enhance the *in vivo* concentration of Np_nN RNAs. The RNA isolated from the mutant strain did not show any significant difference in Np_nN-capping compared to wild type, possibly due to the redundancy of NudiX enzymes.[25]

To reveal the effect of Np_nN-caps methylation on the RNA, we performed molecular dynamics (MD) simulations of the interaction between RppH and Np_nNs-capped RNA (Gp_4G-G and $2mGp_4G$-G). The LC-MS analysis confirmed the presence of one methyl group per guanosine moiety in $2mGp_4G$ (Extended data Fig. 9). We speculated that these methylations could be in the positions N^7 (m^7G) and O2' (Gm) similarly to eukaryotic RNA caps. We observed in MD simulations that the interactions with the arginines R28 and R86 were lost when the methylations were present (Figure 4a). These arginines are responsible for purines binding via cation-π stacking. This finding demonstrates that methylation of Np_nNs-caps in RNA can hamper decapping by RppH.

To experimentally prove the MD findings, we added RppH into the mixture of isolated sRNA with a spiked model Gp_4G-RNA or Gp_5A-RNA to compare the activity of RppH on methylated and non-methylated substrates. We found that majority of the model capped RNAs were cleaved within 1 h while the amount of naturally present methyl-Ap_5G-RNA slightly decreased and dimethyl-Gp_4G/Ap_5G-RNA remained unchanged (Figure 4b,c) This confirms that the methylation stabilizes the Np_nN capped RNAs against cleavage by RppH.

In summary, we identified Np_nNs as novel 5'-RNA caps, which are incorporated into RNA by RNAPs. We found that Np_nN RNAs were cleaved by the *E. coli* RppH decapping enzyme. Caps with long polyphosphate chains were cleaved most efficiently and are better substrates than 5'-ppp RNAs. This suggests that they may be the main cellular targets of RppH. In the cell, we detected the presence of Np_nNs in sRNA, including their methylated variants. MD simulations and experiments revealed that the methylation protects the caps from the RppH cleavage. The amounts of caps and their methylations increased in the STA. Hence, we propose that bacteria use methylated caps to stabilize some RNAs under stress (Figure 4d). In the exponential phase, the metabolism is efficient and the turnover of the macromolecules is fast. Therefore, the methylated caps are not necessary and were detected in low amount. In the stationary phase, cells lack nutrients so they need to find a strategy to save macromolecules. The methylation of the Np_nN-caps can be the way to preserve important RNA molecules. It is intriguing to conceive that many functions of Np_nNs can be explained via their RNA capping potential. Moreover, we show that RNA possesses at its 5'-termini previously unknown structures that may interact with a wide range of cellular partners and influence *e.g.* cellular reaction to starvation. Besides searching for methyltransferases responsible for the methylation of the Np_nN-caps, the biggest challenges lie in the development of specific techniques for identification of Np_nN-capped RNAs.

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Supplementary Information is available in the online version of the paper

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Author Contributions O.H., R.B., M.H. and H.C. conceived the study, designed the experiments. O.H. and R.B. performed the experiments. J.C. L.R. and H.C. supervised the work. M.C. performed molecular dynamics study. O.H., R.B., M.C. and H.C. wrote the paper.

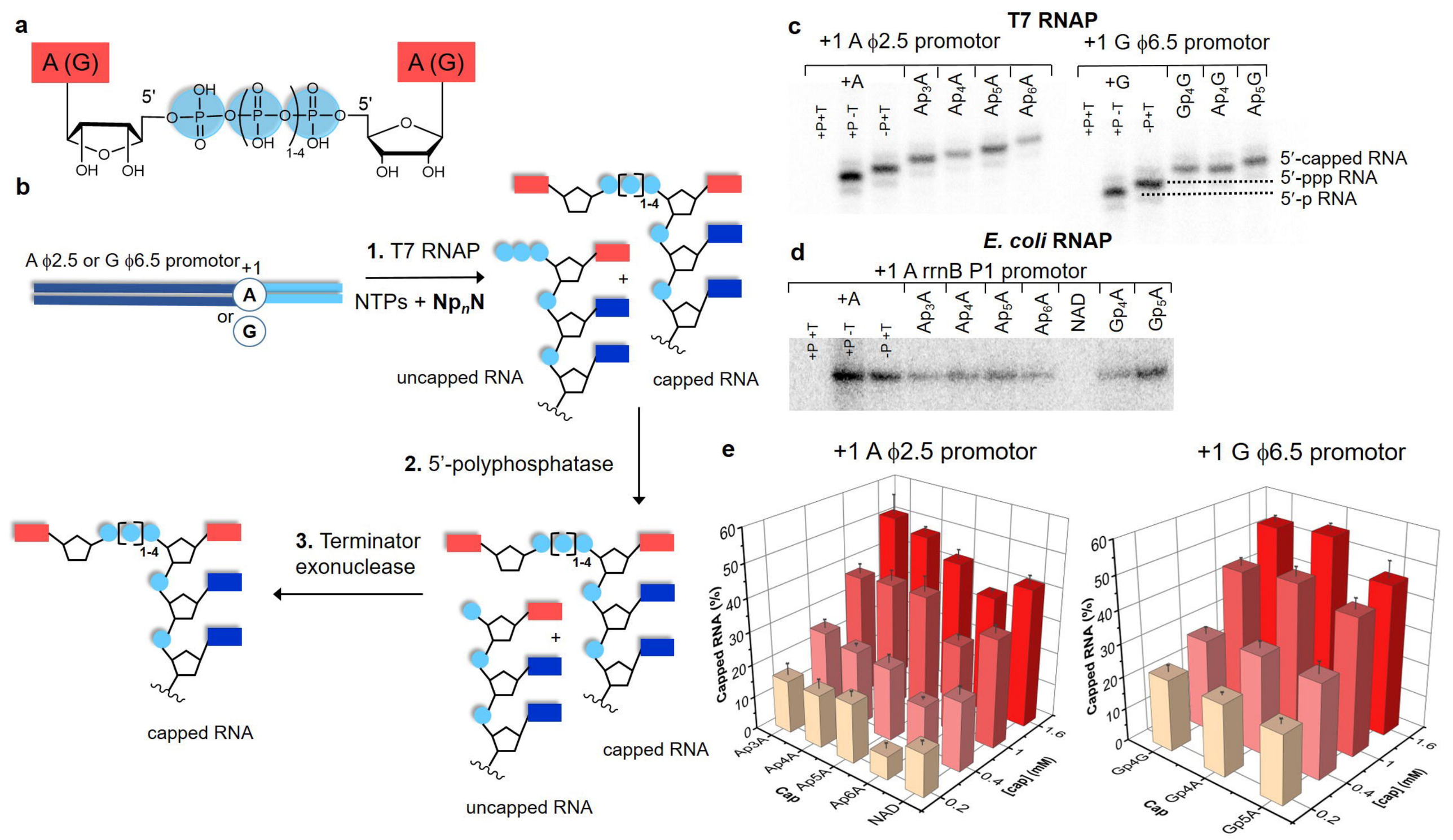
Author Information The authors declare no competing financial interests. Correspondence and requests for materials should be addresses to H.C. (cahova@uochb.cas.cz)

Figure 1: Np_nNs are excellent substrates for RNAP. **a,** The chemical structure of Np_nNs. **b,** Scheme showing the *in vitro* transcription using T7 RNAP in the presence of Np_nNs (1.6 mM) and template DNA with A ϕ 2.5 and G ϕ 6.5 promotors yielding 35mer RNA with A or G at the 5'-end. The first step resulted in a mixture of capped and uncapped RNAs, which is then treated by 5'-polyphosphatase (P) cleaving a diphosphate group from the 5'-ppp RNA leaving intact the capped RNA. In the third step the 5'-p RNA is degraded by a terminator exonuclease (T). Red (dark blue) rectangles correspond to purine base, and light blue spots depict phosphates **c,** Polyacrylamide gel electrophoretic (PAGE) analysis of the products of the *in vitro* transcription with T7 RNAP followed by P and/or T treatment. If not specified, samples were treated with both P and T enzymes. **d,** PAGE analysis of the products of *in vitro* transcription with *E. coli* RNAP and template plasmid 458 with promotor rrnB P1, which lead to the production of 144 nt long RNA having A as the first nucleotide, followed by P and/or T treatment. **e,** Percentage of different types of capped RNAs produced by *in vitro* transcription with T7 RNAP calculated from PAGE analysis. The depth axis represents various concentrations of Np_nNs (0.2, 0.4, 1 and 1.6 mM, different shades) at a constant concentration of ATP (1 mM) and GTP (1 mM). The left panel shows the percentage of $Ap_{3-6}A$ and NAD capped RNA, the right panel shows the percentage of $Ap_{4-5}G$ and Gp_4G.

Figure 2: LC-MS detection of naturally occurring Np_nN-RNA in *E. coli.* **a,** Scheme showing the RNA preparation for comparative LC-MS measurements. RNA was isolated from *E. coli* in various stages of growth. Short RNA was separated from long RNA by the RNAzol protocol and analyzed by Tape station. sRNA was separated from non-covalently bound small molecules by size exclusion chromatography and divided into two parts. One part was treated by Nuclease P1, the other was treated under identical conditions without addition of Nuclease P1 as negative control. Both samples were subjected to size exclusion chromatography again and the fraction of small molecules was analyzed by LC-MS. **b,** Table of detected *m/z* values in LC-MS analysis of digested sRNA from *E. coli* harvested in the exponential phase (EXP), the late stationary phase (STA) and after growth in minimal media with the sole source of nitrogen from $^{14}NH_4Cl$ (^{14}N) or $^{15}NH_4Cl$ (^{15}N). **c,** Relative quantification of dimethylated-Gp_4G in RNA from EXP and STA growth of *E. coli.* **d-f,** Structures of different RNA caps and MS spectra of the detected *m/z* in RNA from *E. coli* growth in minimal media containing ^{14}N or ^{15}N of methyl-Ap_3A (**d,** in ^{14}N *m/z* 769.085 was shifted to *m/z* 779.056 in ^{15}N), Ap_3G (**e,** in ^{14}N *m/z* 771.069 was shifted to *m/z* 781.035 in ^{15}N), dimethyl-Gp_4G (**f,** in ^{14}N *m/z* 447.021 was shifted to *m/z* 452.006 in ^{15}N).

Figure 3: RppH cleavage of Np_nN-capped RNA. **a,** Scheme showing the degradation of capped and uncapped RNA from *in vitro* transcription using RppH and terminator exonuclease. **b,** PAGE analysis of *in vitro* transcribed RNA treated with RppH (+R) or without RppH (-R). **c,** PAGE analysis of RNA from **b** after the reaction with the terminator (T). **d,e,** Kinetic studies of RppH cleavage of Np_nN-capped and uncapped RNA stopped after 30 s, 1, 2, 5, 10, 20 and 40 min and analysed by PAGE. **d,** Ap_nA-RNAs in comparison with pppA-RNA. **e,** Gp_nA-RNAs in comparison with pppA-RNA.

Figure 4: Role of RppH in the cleavage of Np_nN-capped RNA in *E. coli*. **a,** Snapshots from molecular dynamics simulation of the interaction of RppH with Gp_4G-G and m^7Gp_4Gm-G after 200 ns. **b,c** Relative abundance of non-methylated Np_nNs (left) and methylated Np_nNs (right) as derived from EIC in the sRNA fraction spiked with Gp_4G-RNA before (blue) and after 1 h RppH treatment (red) **b** and spiked with Gp_5A-RNA before (blue) and after 1 h RppH treatment (red) **c. d,** Hypothetic cellular processing of RNA in *E. coli* at different stages of growth.


a
A (G)
A (G)
5'
5'
1-4
b
A ϕ2.5 or G ϕ6.5 promotor
+1
A
or
G
1. T7 RNAP
NTPs + Np$_n$N
uncapped RNA
capped RNA
2. 5'-polyphosphatase
3. Terminator exonuclease
capped RNA
uncapped RNA
capped RNA
c
T7 RNAP
+1 A ϕ2.5 promotor
+1 G ϕ6.5 promotor
+A
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+P+T
+P -T
-P+T
Ap$_3$A
Ap$_4$A
Ap$_5$A
Ap$_6$A
Gp$_4$G
Gp$_4$A
Gp$_5$G
5'-capped RNA
5'-ppp RNA
5'-p RNA
d
E. coli RNAP
+1 A rrnB P1 promotor
+A
+P +T
+P -T
-P +T
Ap$_3$A
Ap$_4$A
Ap$_5$A
Ap$_6$A
NAD
Gp$_4$A
Gp$_5$A
e
+1 A ϕ2.5 promotor
+1 G ϕ6.5 promotor
Capped RNA (%)
0
10
20
30
40
50
60
Cap
Ap3A
Ap4A
Ap5A
Ap6A
NAD
Gp4G
Gp4A
Gp5A
[cap] (mM)
0.2
0.4
1
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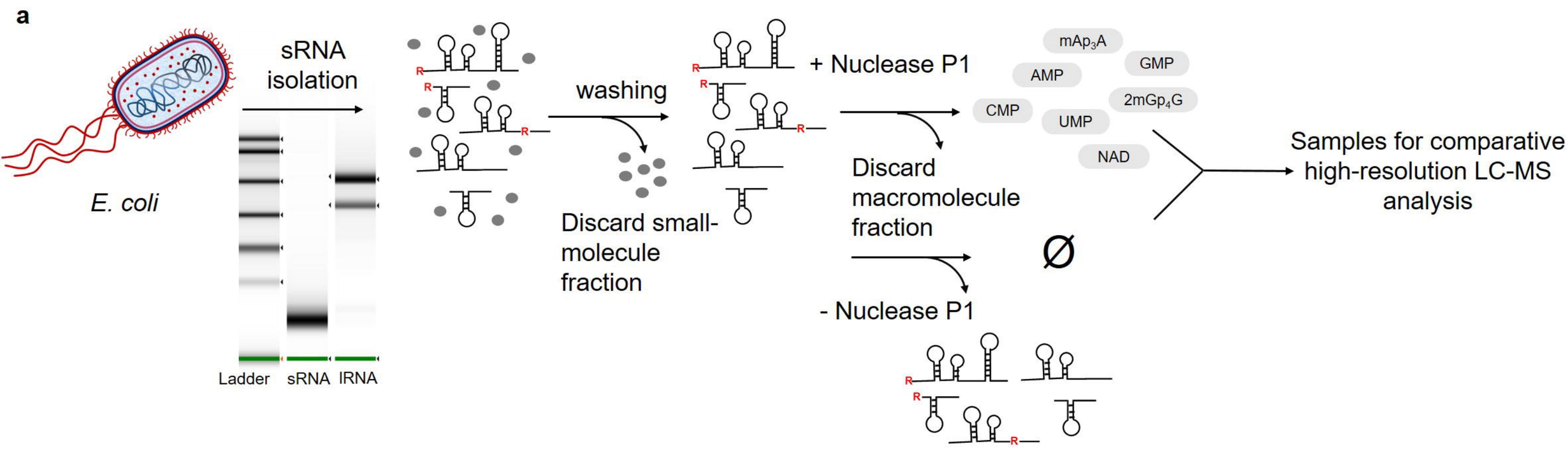
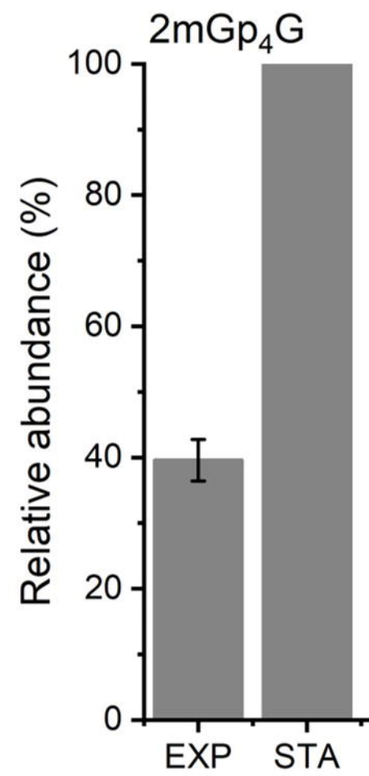


b

RNA cap	Calculated mass	Detected mass		^{15}N Calculated mass	^{14}N Detected mass	^{15}N Detected mass
		EXP	STA		Minimal medium	
Ap_3A	755.074	755.077 ± 0.001	755.078 ± 0.007			
m Ap_3A	769.090	769.080 ± 0.006	769.078 ± 0.010	779.06	769.085 ± 0.011	779.056 ± 0.01
Ap_3G	771.069	771.065 ± 0.001	771.060 ± 0.010	781.039	771.069 ± 0.006	781.035 ± 0.013
m Ap_4G	432.022[a]		432.016 ± 0.004[a]			
2m Gp_4G	447.027[a]	447.018 ± 0.001[a]	447.021 ± 0.005[a]	452.012	447.020 ± 0.001	452.005 ± 0.001
Ap_5A	457.000[a]	457.008 ± 0.003	457.000 ± 0.013[a]			
m Ap_5A	464.007[a]		463.992 ± 0.004[a]			
m Ap_5G	472.005[a]	472.018 ± 0.001[a]	472.020 ± 0.002[a]			
2m Ap_5G	479.013[a]		479.003 ± 0.010[a]			

c



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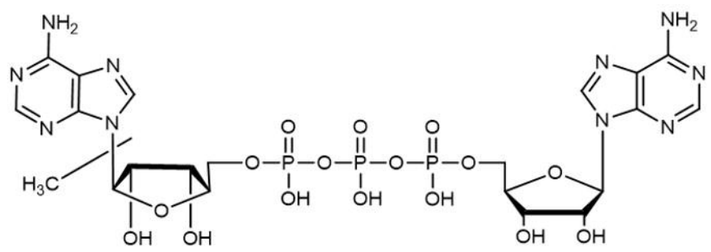
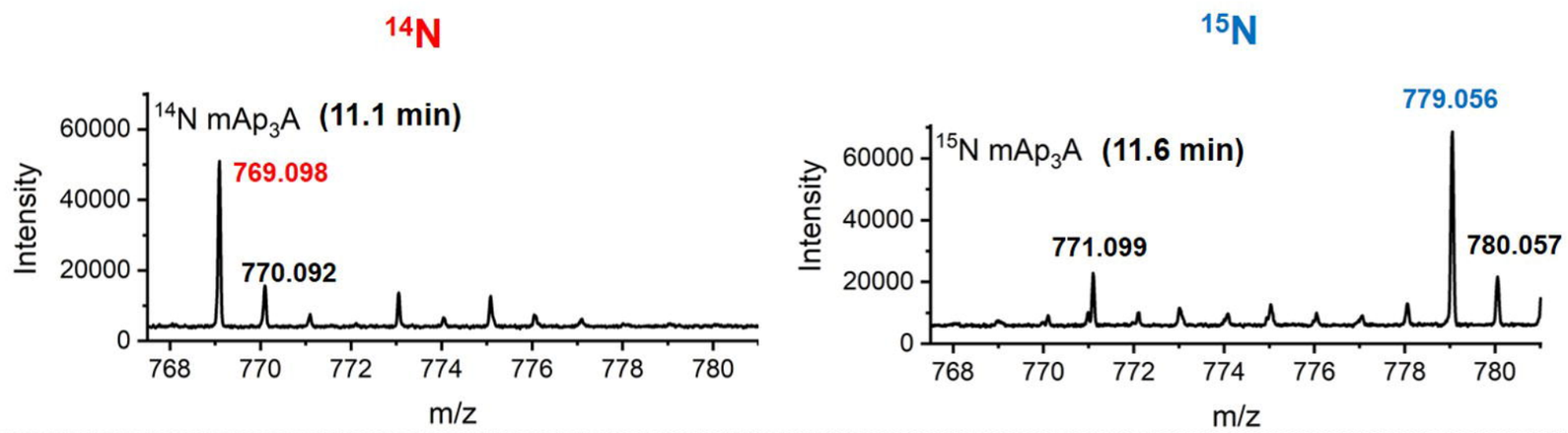
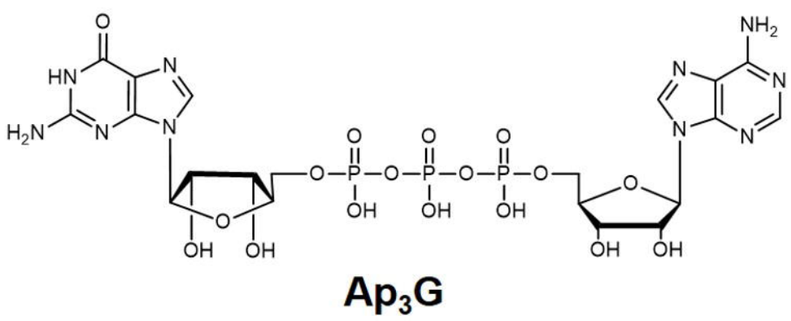
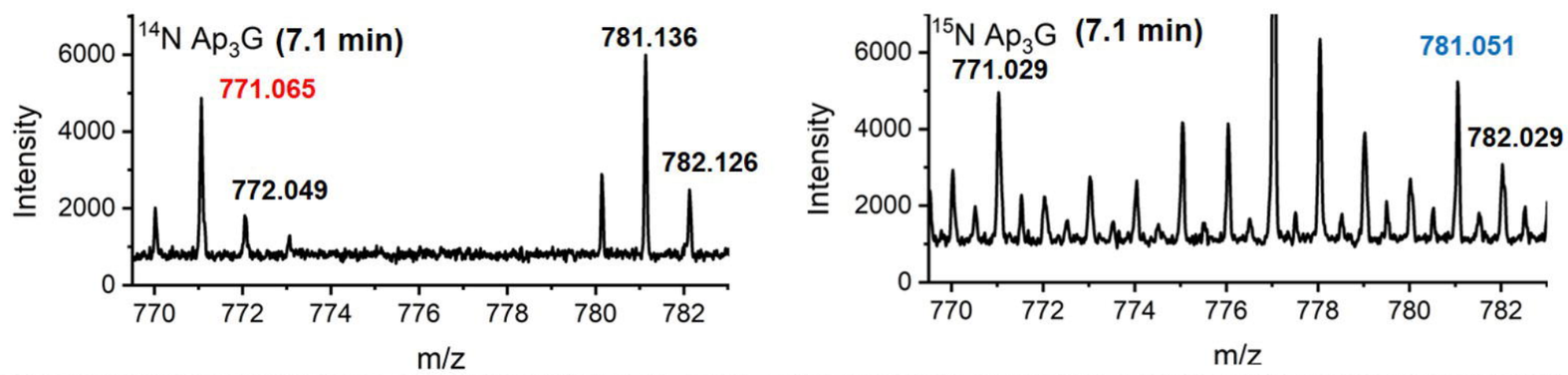




e





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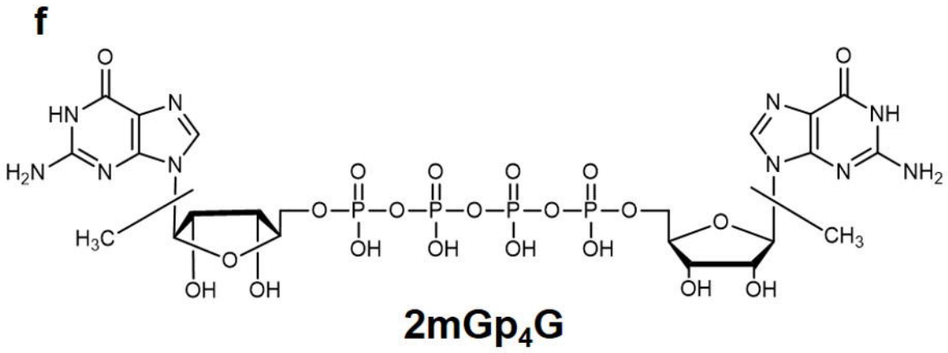
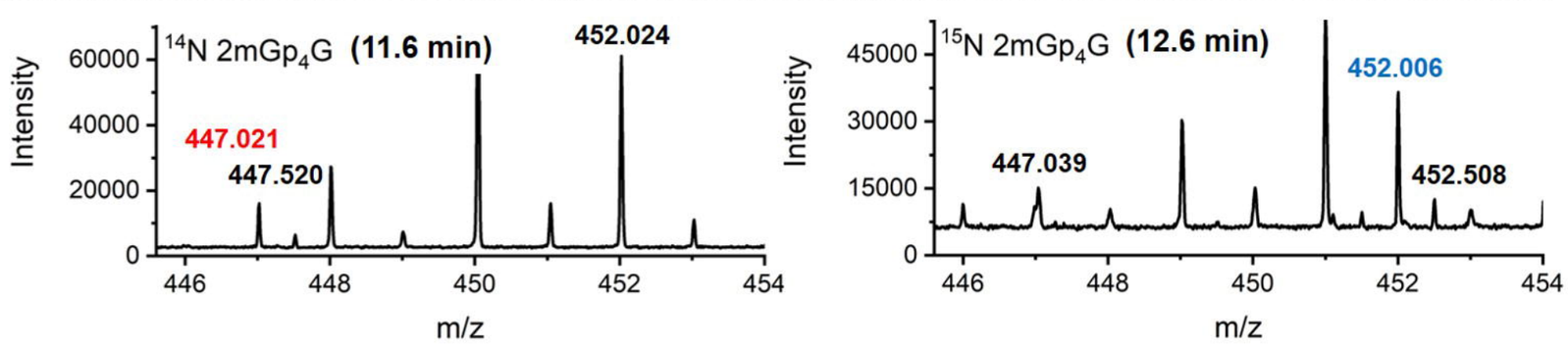
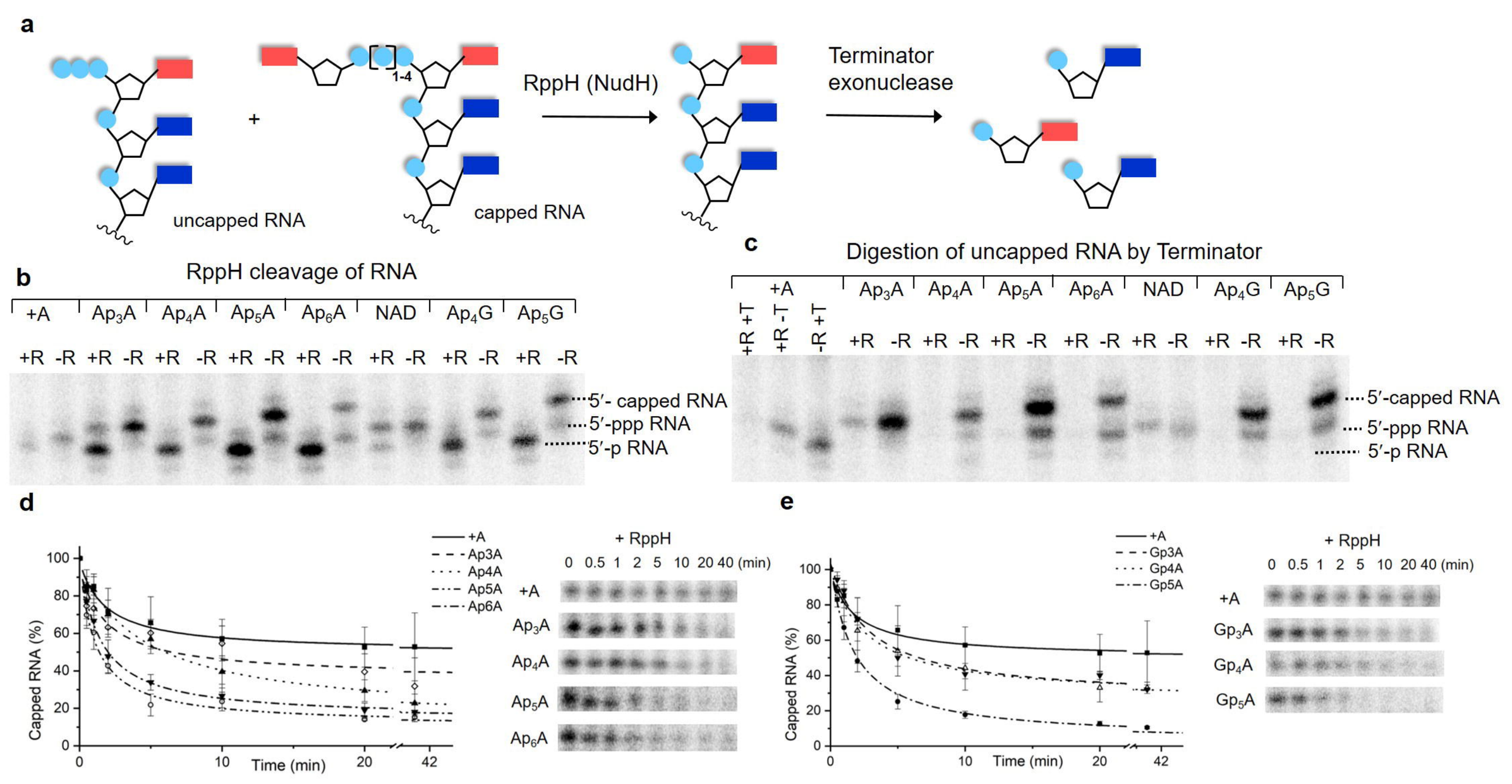
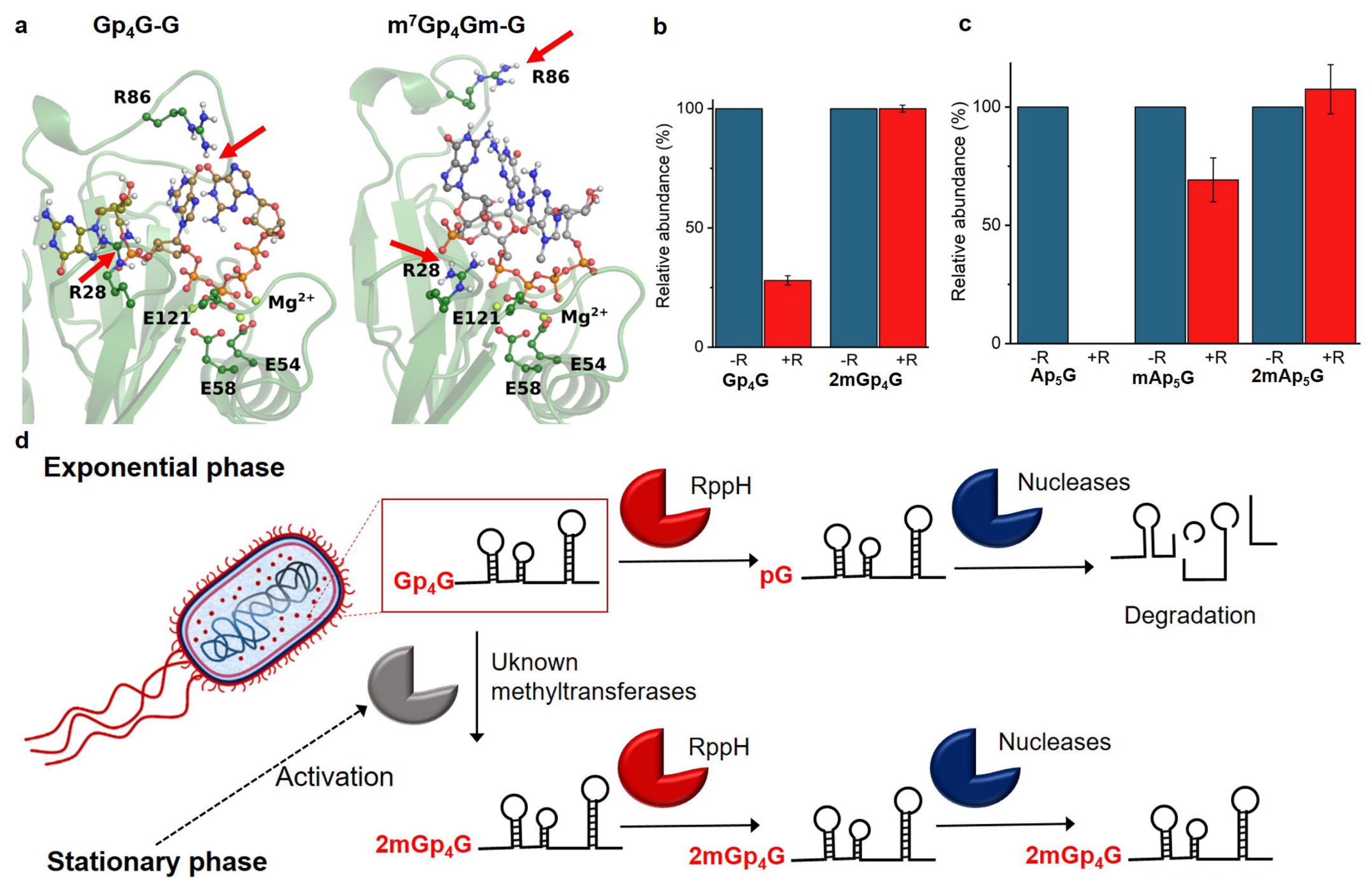





a
uncapped RNA
+
1-4
capped RNA
RppH (NudH)
Terminator exonuclease
b
RppH cleavage of RNA
+A
Ap3A
Ap4A
Ap5A
Ap6A
NAD
Ap4G
Ap5G
+R -R +R -R +R -R +R -R +R -R +R -R +R -R +R -R
5′- capped RNA
5′-ppp RNA
5′-p RNA
c
Digestion of uncapped RNA by Terminator
+A
+R +T
+R -T
-R +T
Ap3A
Ap4A
Ap5A
Ap6A
NAD
Ap4G
Ap5G
+R -R +R -R +R -R +R -R +R -R +R -R +R -R
5′-capped RNA
5′-ppp RNA
5′-p RNA
d
Capped RNA (%)
Time (min)
+A
Ap3A
Ap4A
Ap5A
Ap6A
+ RppH
0 0.5 1 2 5 10 20 40 (min)
+A
Ap3A
Ap4A
Ap5A
Ap6A
e
Capped RNA (%)
Time (min)
+A
Gp3A
Gp4A
Gp5A
+ RppH
0 0.5 1 2 5 10 20 40 (min)
+A
Gp3A
Gp4A
Gp5A


a
Gp4G-G
m7Gp4Gm-G
R86
R28
E121
Mg2+
E54
E58
b
Relative abundance (%)
100
50
0
-R
+R
Gp4G
2mGp4G
c
Relative abundance (%)
Ap5G
mAp5G
2mAp5G
d
Exponential phase
Gp4G
RppH
pG
Nucleases
Degradation
Uknown
methyltransferases
Activation
Stationary phase
2mGp4G
Stable RNA