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Rho-dependent transcriptional switches regulate the bacterial response to cold shock

Mildred Delaleau¹, Nara Figueroa-Bossi², Thuy Duong Do^{1,3}, Patricia Kerboriou², Eric Eveno¹,
Lionello Bossi², and Marc Boudvillain^{1,3,4}

¹Centre de Biophysique Moléculaire, CNRS UPR4301, affiliated with Université d'Orléans, rue Charles Sadron, 45071 Orléans cedex 2, France.

²Université Paris-Saclay, CEA, CNRS, Institut de Biologie Intégrative de la Cellule (I2BC), 91190 Gif-sur-Yvette, France.

³ED 549, Sciences Biologiques & Chimie du Vivant, Université d'Orléans, France.

⁴Corresponding author (marc.boudvillain@cnrs.fr)

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Running title: Cold-shock Rho-dependent riboswitches

30 **ABSTRACT**

31 **Rho-dependent transcription termination plays a pivotal role in the regulation of bacterial**
32 **gene expression. Binding of the Rho helicase to the nascent transcript triggers termination**
33 **in response to cellular signals that directly or indirectly modulate mRNA structure and the**
34 **accessibility of Rho utilization (*Rut*) sites. Despite the impact of temperature on RNA**
35 **structure, Rho-dependent termination has never been associated to the bacterial response**
36 **to temperature shifts. Here, we show that Rho is in fact a central player in the cold shock**
37 **response (CSR), challenging the conventional view that CSR is primarily a posttranscriptional**
38 **program. We identify *Rut* sites in the 5' untranslated regions of key CSR genes/operons**
39 **(*cspA*, *cspB*, *cspG*, *nsrR-rnr-yjfHI*) that trigger premature termination by Rho at 37°C but not**
40 **at 15°C, *in vitro* and *in vivo*. High concentration of the RNA chaperone CspA or base-pair**
41 **mutations in the *cspA* mRNA leader can shift the balance between termination and**
42 **antitermination, revealing how Rho can be controlled through RNA restructuring to activate**
43 **CSR genes during the cold shock, and then to silence these genes during cold acclimation.**
44 **Overall, the work presented here sets a new paradigm for how RNA thermosensors can**
45 **modulate gene expression.**

46 **INTRODUCTION**

47 The cold shock response (CSR) is a complex and highly orchestrated set of molecular events
48 that enables bacteria to cope with sudden temperature downshifts and to maintain cellular
49 homeostasis under low temperature conditions. In *Escherichia coli* and various other
50 mesophilic bacteria, an abrupt decrease in temperature causes the arrest of cell growth and
51 a drastic reduction of mRNA translation due, among others, to increased structuring of the
52 mRNAs. The CSR proteins escape this general inhibition and are involved in a variety of cold
53 acclimation processes such as the remodeling of cell membrane composition or the facilitation
54 of nucleic acid transactions thermodynamically constrained by the lower temperature. Some
55 CSR proteins are upregulated transiently during cold acclimation while others are produced at
56 high levels for as long as cold persists. Cold acclimation can take several hours during which
57 bulk protein synthesis gradually increases until cells are sufficiently cold-adapted to resume
58 growth, albeit at a slower rate (reviewed in ref. ¹⁻³).

59 In *E. coli*, two CSR proteins appear to play a central role in cold acclimation by
60 dynamically regulating gene expression at the posttranscriptional level. The RNA chaperone
61 CspA helps unfold mRNA structures that are formed at low temperatures and impede
62 translation while the [3'→5'] exoribonuclease RNase R, which is not inhibited by RNA
63 secondary structures, stimulates the degradation and turnover of mRNAs.⁴ The expression of
64 RNase R (encoded by *rnr*) is induced 8-15 fold upon cold shock, an increase attributed to cold-
65 induced folding and increased mRNA stability against degradation.^{5,6} The expression of CspA
66 is also stimulated following a cold shock (by a factor that varies with growth stage), in part
67 through greater protection of the *cspA* mRNA against ribonucleases^{7,8}, but also largely through
68 the regulation of *cspA* translation.^{4,9,10} Indeed, contrary to most RNAs in the cell, the *cspA*
69 mRNA folds into a structure favoring efficient translation at low temperatures (e.g. 15°C)
70 whereas, at 37°C, the mRNA adopts a conformation inhibiting translation.^{4,9} Notably, binding
71 of CspA to the “15°C-conformation” can trigger an mRNA rearrangement less favorable to
72 translation⁴ thereby providing a negative feedback control on CspA expression during the
73 CSR.¹¹ Conversely, Binding of CspA to the “37°C conformation” stimulates *cspA* translation.¹²
74 This may contribute to the basal expression and abundance of CspA during early exponential
75 growth at 37°C.^{13,14} Sequence elements critical for the posttranscriptional regulation of *cspA*

76 have been identified in its 5' untranslated region (5'UTR) and in the initial section of its coding
77 sequence (CDS).^{4,9,10,12,15,16}

78 There are nine Csp (cold shock protein) homologues in *E. coli* but only expression of
79 CspA, CspB, CspG, and CspI is induced by cold shock (**Supplementary Figure 1**).¹⁻³ Although
80 some of these proteins share regulatory features and have partially redundant functions^{4,17-}
81 ¹⁹, CspA stands out as the primary contributor to the CSR due to its remarkable abundance.
82 Upon cold shock, CspA production can indeed represent close to 15% of total protein
83 synthesis.^{11,20} Thus, despite a modest affinity for RNA (K_d in μM range)^{12,21}, the high
84 concentration of CspA in cold-shocked cells ($\sim 100 \mu\text{M}$)²² and its fairly relaxed sequence
85 specificity² enables its widespread binding to and unfolding of cellular RNA. This process
86 largely contributes to the reduction of global mRNA structure and to the recovery of bulk
87 translation during the cold acclimation phase.⁴

88 While the regulatory interplay between mRNA structure, translation, and decay during
89 cold acclimation is relatively well established¹⁻³, the contribution of transcription, in particular
90 its elongation phase, remains uncertain despite early work suggesting its potential
91 significance. In one instance, incremental deletions of the long 5'UTR of *cspA* were shown to
92 strongly stimulate the production of the mRNA independently of cold shock.²³ Since the
93 deletions did not change the rate of decay of the *cspA* mRNA, the authors postulated that the
94 5'UTR might be "involved in negative regulation of the *cspA* gene expression at the level of
95 transcription". Transcription initiation is not the regulated step⁷ but CspA binding to the 5'-
96 leader of its own mRNA stimulates transcriptional pausing in the 5'UTR, which could play a
97 role.²⁴ However, CspA inhibits other pause signals or intrinsic transcription terminators, most
98 likely by preventing the formation of the corresponding RNA hairpin structures²⁵, and is thus
99 usually regarded as a positive rather than negative transcription regulator.¹⁻³

100 A source of negative transcriptional regulation in bacteria is Rho-dependent
101 termination (**Figure 1**), which silences hundreds of genomic loci in *E. coli*²⁶⁻²⁸ and has been
102 implicated in several conditional attenuation mechanisms (reviewed in ref. ²⁹⁻³¹). However, to
103 our knowledge, the involvement of Rho-dependent termination in the CSR has not been
104 investigated. This is somewhat surprising given that cold shock is expected to produce
105 widespread decoupling of translation from transcription^{32,33} and that Rho-dependent
106 termination is the main mechanism of surveillance of transcription-translation coupling

107 **(Figure 1**, bottom; reviewed in ref. ^{34,35}). In addition, increased mRNA structuring at low
108 temperature may hide Rho uttilization (*Rut*) sites and inhibit termination by preventing Rho
109 access to the transcript (**Figure 1**, top right) in a manner reminiscent of the riboswitch-like
110 mechanisms used by some Rho-dependent transcriptional attenuators.^{36,37}

111 Here, we present comprehensive evidence for the involvement of Rho in the cold shock
112 response. We identify *Rut* sites in the 5'UTRs of genes or operons involved in the CSR (*cspA*,
113 *cspB*, and *cspG* genes and *nsrR-rnr-yjfHI* operon) and show that transcription of these
114 genes/operons is prematurely terminated by Rho at 37°C but not at 15°C, *in vitro* and *in vivo*.
115 We also demonstrate that CspA can restore Rho-dependent termination at 15°C, implying that
116 dual-input, antagonistic control (T° vs Csp concentration) of transcriptional attenuation
117 contributes to the dynamic regulation of cold shock genes during the acclimation phase.
118 Altogether, our results unveil the existence of an upper layer of regulation in the bacterial
119 response to cold stress and a novel mechanism through which RNA thermosensors modulate
120 gene expression.

121

122 **RESULTS AND DISCUSSION**

123 ***Identification of Rut sites in the 5'UTRs of cold shock genes***

124 In a previous study, we mapped putative *Rut* sites along the genome of *E. coli* using a new
125 global screening approach that we named "Helicase-SELEX".³⁸ Close examination of the
126 Helicase-SELEX data revealed that a number of putative *Rut* sites localized within the 5'UTRs
127 of genes involved in the CSR, namely *cspA*, *cspB*, *cspG*, *lpxP* (encoding a palmitoleoyl-
128 transferase involved in cold adaptation of the membrane), and *nsrR* (at the start of the *nsrR*-
129 *rnr-yjfHI* operon) as well as in the initial section of the *rnr* CDS (**Figure 2A**). Interestingly, these
130 putative *Rut* sites matched some of the so-called bicyclomycin-sensitive regions previously
131 detected in genome-wide analyses of the transcriptional response to Rho inhibitor
132 bicyclomycin (see supporting data in ref. ^{26,27} and **Figure 2A**). These regions were not discussed
133 specifically in these previous studies and have remained overlooked until now.

134 To substantiate the presence of Rho-dependent terminators in these regions of the *E.*
135 *coli* genome, we first calculated the percentages of C and G residues along the coding DNA
136 strand, in search of large C>G biases (aka C>G bubbles) characteristic of Rho-dependent

137 terminators.^{39,40} In each case, a C>G bubble overlapping with the putative *Rut* site uncovered
138 by Helicase-SELEX was indeed found (**Figure 2B**). Next, we performed *in vitro* transcription
139 experiments with DNA templates encoding the upstream sections of the relevant transcription
140 units (5'UTR and first ~200-400 nucleotides [nt] of the CDS) under control of the strong T7A1
141 promoter.⁴⁰ We also used a DNA template encoding the Rho-dependent λ TR1 terminator as
142 positive control.

143 Rho-dependent termination signals were detected in all instances when the
144 transcription reactions were carried out at 37°C in presence of Rho (**Figure 2**, 'Term'). Addition
145 of transcription factor NusG stimulated the production of Rho-dependent truncated
146 transcripts, including at sites closer to the promoter, as observed under similar conditions for
147 other Rho-dependent terminators (e.g. in ref. ^{37,41-43}). Importantly, the termination regions
148 detected *in vitro* (**Figure 3A-E**) are located immediately downstream from the C>G bubbles
149 and putative *Rut* sites (**Figure 2B**), consistent with Rho-dependent termination initiating in the
150 5'-leader regions of the cold shock genes. A notable exception is *lpxP*, which has a shorter
151 5'UTR (67 base pairs [bp]) than the other cold shock genes (146-161 bp) and for which the
152 termination region starts ~50 bp downstream from the start codon (**Figure 3E**).

153 A strong *in vitro* Rho-dependent termination signal was also detected in the CDS of the
154 *rnr* gene, ~100 bp downstream from the short (38 bp) intergenic *nsrR-rnr* region
155 (**Supplementary Figure 2A**). This intragenic signal complements the upstream termination
156 signal located in the 5'UTR of the *nsrR-rnr-yjfHI* operon (**Figure 3D**), in good agreement with
157 observations from genome-wide studies (**Figure 2**). Altogether, these data support the
158 presence of Rho-dependent termination signals in the 5'UTRs of *cspA*, *cspB*, *cspG*, and *nsrR*
159 and in the upstream parts of the *lpxP* and *rnr* CDSs.

160

161 ***Temperature governs Rho-dependent transcriptional attenuation of cold shock genes***

162 Results were radically different for some of the DNA templates when the transcription
163 reactions were carried out at 15°C instead of 37°C. Most notably, the formation of Rho-
164 dependent truncated transcripts was strongly inhibited with templates *cspA*, *cspB*, and *cspG*
165 (**Figure 3A-C**, right gels). With template *nsrR*, termination within the 5'UTR was also strongly
166 inhibited at 15°C while a new site of strong Rho-dependent termination became apparent

167 farther downstream in the *nsrR* CDS (**Figure 3D**, star). By comparison, the Rho-dependent
168 termination profiles obtained with templates *lpxP* (**Figure 3E**), λ T1 (**Figure 3F**), and *rnr*
169 (**Supplementary Figure 2A**) were much less affected by the decrease in reaction temperature.
170 Taken together, these data strongly suggest that the 5'-leaders of the *cspA*, *cspB*, *cspG*, and
171 *nsrR* genes adopt structures at 15°C that prevent Rho-dependent termination, most likely
172 because they hide the *Rut* sites from Rho (**Figure 1**, top right). By contrast, RNA folding at 15°C
173 does not effectively shield the *lpxP*, *rnr*, and λ T1 terminators nor the downstream *nsrR*
174 terminator (*) from Rho action. Thus, temperature may regulate the expression of *cspA*, *cspB*,
175 *cspG*, and *nsrR-rnr-yjfHI* by a conditional Rho-dependent attenuation mechanism. This
176 transcriptional regulation, working alongside the established posttranscriptional
177 mechanisms⁴⁻¹⁰, is expected to ensure the tightest form of control in a manner akin to the
178 multilayered *modus operandi* of some riboswitches.³¹ Finally, the experiment in Figure 3
179 shows that CspA, at the sub-saturating concentration of 0.5 μ M ($_{low}[CspA]$), has no effect on
180 Rho-dependent transcription termination with the tested DNA templates at both 15°C and
181 37°C (see more about this point below).

182 To determine if the above findings hold true *in vivo*, we first compared the amounts of
183 *cspA* transcripts formed in WT (*rho*⁺) and *rho*^{R66S} strains of *E. coli* MG1655. The *rho*^{R66S} mutant
184 is viable but defective in the termination of many transcripts^{44,45}, presumably because the
185 R66S mutation weakens Rho binding to *Rut* sites.⁴⁶ Total RNA was extracted from log phase
186 cultures of the *rho*⁺ and *rho*^{R66S} strains after they were either cold-shocked at 15°C or kept at
187 37°C for 30 min. To detect the full-length *cspA* mRNA (*cspA*-FL, hereafter) and potentially
188 shorter species, we performed northern blots with a ³²P-labeled oligonucleotide probe
189 hybridizing to the +3 to +48 region of the *cspA* 5'leader (+1 being the transcription start site).

190 Formation of *cspA*-FL was readily detected in the cold-shocked cells, with no significant
191 difference in *cspA*-FL abundance between the *rho*⁺ and *rho*^{R66S} strains (**Figure 4A**; $p = 0.41$,
192 unpaired, two-tailed Student's t-test). Results were dramatically different for cells cultured at
193 37°C throughout. In this case, *cspA*-FL was weakly expressed in the *rho*^{R66S} strain and almost
194 undetectable in the *rho*⁺ strain (**Figure 4A**). Overall, *cspA*-FL was around two orders of
195 magnitude more abundant in the *rho*^{R66S} mutant than in the *rho*⁺ strain at 37°C but still two
196 orders of magnitude less abundant than in the cold-shocked cells ($p < 0.05$; unpaired, two-
197 tailed Student's t-tests). Moreover, the proportions of truncated *cspA* transcripts were

198 comparatively much lower in the cold-shocked cells (**Figure 4A**, graphs). These dramatic
199 effects cannot be explained by concomitant changes in the cellular levels of Rho or RNA
200 polymerase, which do not vary much upon cold shock (**Supplementary Figure 1**).⁴ Rather, they
201 are consistent with the *in vitro* data from above and support the scenario in which Rho-
202 dependent termination downregulates *cspA* in exponentially growing cells at 37°C whereas
203 cold shock triggers anti-termination and thus stimulates *cspA* expression at the co-
204 transcriptional level.

205 Similar Northern Blot experiments were performed with oligonucleotide probes
206 hybridizing to the 5'UTRs of *cspB* and *cspG*. In the case of *cspB*, signals were weaker than for
207 *cspA* but the trend was similar, i.e. full-length *cspB* mRNA was barely detected at 37°C, and
208 only in the *rho*^{R66S} mutant, but was present in considerably higher amounts in both *rho*⁺ and
209 *rho*^{R66S} strains after cold-shock (**Supplementary Figure 3A**). Moreover, the signal for the full-
210 length *cspB* mRNA in the *rho*^{R66S} mutant was more easily detected when cells were cultured
211 at 30°C instead of 37°C, which is consistent with the lower temperature of induction of *cspB*
212 as compared to *cspA*.⁴⁷ Similarly, we detected a weak signal for the full-length *cspG* mRNA at
213 30°C, but not at 37°C, in the *rho*^{R66S} mutant only, whereas we observed a strong induction of
214 *cspG* after cold-shock in both *rho*⁺ and *rho*^{R66S} strains (**Supplementary Figure 3B**). Note that in
215 contrast to *cspA* and *cspB*, the optimal temperature of induction for *cspG* has not been
216 determined.

217 To probe the polycistronic *nsrR-rnr-yjfHI* transcript products, we used denaturing 1.5%
218 agarose (instead of 8% polyacrylamide) electrophoresis and two distinct oligonucleotide
219 probes for Northern blotting: probe A hybridizes to the 5'UTR of *nsrR* while probe B hybridizes
220 to the intergenic region between the *nsrR* and *rnr* genes. With both probes, we detected two
221 diffuse bands (**Figure 4B**) reflecting the complex biogenesis and posttranscriptional processing
222 of the *nsrR-rnr-yjfHI* transcripts.^{5,6} The intensities of the two bands were higher after cold-
223 shock (**Figure 4B**), confirming the stabilization of the transcripts under this condition.^{5,6} The
224 intensities of the bands were also higher in the *rho*^{R66S} mutant than in the *rho*⁺ strain,
225 supporting Rho involvement in the regulation of *nsrR-rnr-yjfHI* expression. The effect of the
226 *rho*^{R66S} mutation was particularly marked for the longest transcript species (slowest migrating
227 band) and was still noticeable for this species at 15°C. These observations support a scenario
228 where Rho-dependent polarity along the *nsrR-rnr-yjfHI* transcription unit is mediated by

229 several Rho-dependent signals such as those detected *in vitro* in the 5'UTR and in the CDS of
230 *nsrR* and that operate at high and low temperatures, respectively (**Figure 3D**). Of note,
231 translation of the *nsrR* gene is very inefficient⁴⁸ and may thus not adequately mask the
232 intragenic termination signal (**Figure 3D**, star) *in vivo*. By contrast, the downstream *rnr* gene is
233 efficiently translated, in particular at low temperature⁴⁹, which should mask the Rho-
234 dependent signal in the *rnr* CDS (**Supplementary Figure 2A**).

235 To confirm our *in vivo* observations, we performed quantitative RT-PCR experiments
236 with pairs of primers hybridizing in the coding regions of *cspA*, *cspB*, *cspG*, *nsrR*, *rnr*, and *lpxP*.
237 As a control, we first performed RT-PCR quantification of the *rho* gene, which is regulated by
238 Rho-dependent attenuation⁵⁰ The RT-qPCR signal for *rho* was indeed higher in the *rho*^{R66S}
239 mutant than in the *rho*⁺ strain and this difference was highly significant at both 37°C and 15°C
240 (**Figure 4C**), suggesting that the Rho-dependent attenuator controlling *rho* expression is not
241 much affected by cold shock. For each strain, the differences in *rho* signals between 37°C and
242 15°C were modest (less than 5-fold), confirming that *rho* mRNA levels do not vary much upon
243 cold shock.⁴ By contrast, we observed dramatic increases of the RT-qPCR signals for *cspA*, *cspB*,
244 and *cspG* upon cold shock and large differences between the signals for the *rho*⁺ and *rho*^{R66S}
245 strains at 37°C (15-40 fold), which however were cancelled out at 15°C (**Figure 4D**). These
246 results are in good agreement with the Northern blot experiments (**Figure 4A** and
247 **Supplementary Figure 3**) and further support that Rho-dependent attenuators sensitive to
248 cold regulate *cspA*, *cspB*, and *cspG*.

249 The increases of RT-qPCR signals upon cold shock were smaller for *nsrR* and *rnr*, as
250 were the differences between *rho*⁺ and *rho*^{R66S} signals at 37°C (**Figure 4D**). These differences
251 were further reduced but not fully abrogated at 15°C ($p < 10^{-2}$, unpaired, two-tailed Student's
252 t-tests), which is also consistent with Northern blot observations (**Figure 4B**). Thus, Rho-
253 dependent attenuation appears to be less important for the temperature-dependent
254 regulation of the *nsrR* and *rnr* genes than for *cspA*, *cspB*, and *cspG*. Alternatively, a
255 temperature lower than 15°C may be required to fully switch off Rho-dependent attenuation
256 of the *nsrR-rnr-yjfHI* operon. Interestingly, RT-qPCR probing of *lpxP* suggests that this gene is
257 also regulated by Rho in a temperature-dependent manner (**Figure 4D**) even though *in vitro*
258 Rho-dependent termination in *lpxP* is not strongly affected by temperature (**Figure 3E**). *In vivo*,
259 however, the intragenic *lpxP* terminator (**Figures 2B** and **3E**) should be regulated by cold-shock

260 stimulated *lpxP* translation.⁴ Increased translation may also better protect the *lpxP* mRNA
261 from degradation by ribonucleases, thereby further contributing to the higher level of the
262 mRNA at 15°C versus 37°C (**Figure 4D**).

263 Taken together, our *in vitro* and *in vivo* data reveal a new facet of the intricate
264 regulation of cold-shock genes based on the modulation of Rho-dependent attenuation. Two
265 alternative mechanisms appear to be involved. On one hand, an increase in the efficiency of
266 translation upon cold shock contributes to shield intragenic *Rut* sites from Rho action (e.g. as
267 for *lpxP* and, possibly, *rnr*; **Figure 1**, top left). On the other hand, an increase in the secondary
268 structure of the mRNA at the lower temperature prevents Rho access to a *Rut* site located in
269 the 5'-leader portion of the mRNA (e.g. as for *cspA*, *cspB*, and *cspG*; **Figure 1**, top right).

270

271 ***Mutations that stabilize the cold-shock conformation of the cspA mRNA leader inhibit Rho-*** 272 ***dependent attenuation***

273 Paralogs corresponding to six of the nine Csp proteins found in *E. coli* are also present in
274 *Salmonella enterica*. Notably, *Salmonella* CspA shares 100% identity with its *E. coli*
275 counterpart, and the two genes exhibit a 91% identity in their 5'UTRs, which are 164 and 160
276 nt long, respectively (**Supplementary Figure 4A**). Drawing from the analogy with *E. coli*, where
277 the structure of the 5'UTR of *cspA* was determined by DMS mapping *in vivo*⁴, the *Salmonella*
278 *cspA* sequence is predicted to adopt two alternative conformations: a more 'open'
279 conformation at 37°C and a folded one containing a long hairpin-like structure at lower
280 temperature (**Supplementary Figure 4B**). Conceivably, the latter structure might sequester
281 residues required for Rho binding and thus promote readthrough transcription following the
282 cold shock.

283 To evaluate the role of Rho in the cold-shock regulation of *cspA* in *Salmonella*, we
284 engineered a strain carrying a second copy of the 5'UTR of *cspA* fused to the coding sequence
285 of the *lacZ* gene (in a *lacY*⁺ background) at an ectopic location under the control of the TetR-
286 regulated P^{tet} promoter. An isogenic derivative of this strain carrying *rho* allele Y80C, which
287 causes a termination defect at *Rut* sites^{43,51}, was subsequently constructed through phage P22
288 transduction. Measurement of β -galactosidase activity 30 minutes (**Figure 5A**) and 180
289 minutes (**Figure 5B**) after the addition of the P^{tet} inducer, anhydrotetracycline (AHTc), revealed

290 a significant increase in *lacZ* expression caused by the Rho mutation at 37°C, but not at 15°C
291 (**Figures 5A** and **5B**). This is consistent with the *cspA* leader sequence containing a Rho-
292 dependent terminator active only at 37°C. Notably, the mutant-to-wild-type expression ratio
293 at 37°C exhibited a gradual rise during the 30 to 180-minute interval (from approximately 4-
294 fold to 30-fold), suggesting that termination efficiency is enhanced when the bacteria enter
295 stationary phase. Enhanced termination can explain the early finding that *cspA* mRNA levels
296 undergo an abrupt decline in *E. coli* cells transitioning from mid-to-late exponential growth,
297 as reported in early studies.^{13,14}

298 The P^{tet} promoter fusion utilized in these experiments contains only one of the two
299 operator sequences normally found at the 5' end of the *tetA* gene (see Materials and Methods
300 for details).⁵² Consequently, TetR-mediated repression exhibits some degree of "leakiness",
301 resulting in low-level transcription in the absence of the inducer. However, due to termination
302 in the *cspA* leader region, expression of the *cspA-lacZ* fusion at 37°C is insufficient to support
303 bacterial growth on a medium containing lactose as the sole carbon source. As a result, the
304 strain is Lac⁻. The introduction of the *rho*^{Y80C} mutation confers a Lac⁺ phenotype, most likely by
305 allowing transcription to read through the *cspA* leader region (**Figure 5C**). This observation
306 suggested that the lactose-based medium could be used to positively select mutants evading
307 Rho-dependent termination in the *rho*⁺ background. Colonies growing on minimal lactose
308 plates (see example in **Figure 5C**, upper left spot) were isolated and characterized. This led to
309 the identification of four mutants carrying single base-pair substitutions in the 5'UTR of *cspA*:
310 C41A, A55G, C73U, and C76U. Remarkably, all four changes allow additional base pairing in
311 the hairpin structure predicted to form at low temperature, thereby stabilizing the structure
312 (**Figure 5D** and **Supplementary Figure 4B**).

313 Measurements of *lacZ* expression in strains carrying each of the four *cspA* alleles alone
314 or in combination with *rho*^{Y80C} confirmed the *cspA* changes to be completely epistatic to the
315 Rho mutation, indicating that they act in the same pathway as Rho (**Figures 5E** and **5F**). This
316 supports the conclusion that the changes in the *cspA* leader RNA prevent Rho from
317 terminating transcription at 37°C because they enable the hairpin structure to form at this
318 higher temperature.

319 We selected two of the mutations, C41A and A55G, for further characterization *in vitro*.
320 We prepared ³²P-labeled *Salmonella cspA* leader transcripts carrying either mutation, no

321 mutation (WT), or the G54U substitution expected to destabilize rather than stabilize the
322 hairpin structure (**Supplementary Figure 4B**). We probed the transcript conformations by
323 native polyacrylamide gel electrophoresis (PAGE). The C41A and A55G transcripts migrated
324 faster in the PAGE gel than the WT transcript (**Supplementary Figure 5A**), indicating more
325 compact conformations consistent with the stabilization of the hairpin structure by the
326 mutations. By contrast, the G54U mutation markedly reduced the electrophoretic mobility of
327 the transcript, consistent with a more open conformation enacted by hairpin destabilization.
328 Furthermore, we observed different *in vitro* transcription termination profiles with DNA
329 templates encoding the WT *Salmonella cpsA* or mutant sequences. Notably, in defined
330 conditions, the WT and G54U sequences could sustain premature Rho-dependent termination
331 in the 5'UTR whereas the C41A and A55G mutant sequences could not (**Supplementary Figure**
332 **5B**), thereby linking termination efficiency to the stability of the leader hairpin structure. Thus,
333 both our *in vitro* and *in vivo* observations support the conclusion that the temperature-
334 dependent folding pathway of the *cspA* mRNA leader not only governs *cspA* translation^{4,9} but
335 also finely controls Rho-dependent attenuation of *cspA* expression.

336

337 ***CspA stimulates Rho-dependent termination at csp terminators***

338 Since CspA contributes to regulate cold shock genes (including *cspA* itself) during cold
339 acclimation, we wondered if this could proceed, at least in part, through the modulation of
340 Rho-dependent attenuation. To test this possibility, we performed *in vitro* transcription
341 experiments in the presence of CspA. As noted above, we first tested a concentration (0.5 μ M)
342 just below the dissociation constants measured for CspA with model oligonucleotides at 20°C
343 (1-10 μ M range).^{12,21} At this sub-saturating concentration, CspA had no effect on Rho-
344 dependent transcription termination with the tested DNA templates at both 15°C and 37°C
345 (**Figure 3**). Results were different when we tested CspA at its concentration in cold-shocked
346 cells (~100 μ M).²² At this high (saturating) concentration, CspA was able to both stimulate
347 Rho-dependent termination and activate upstream termination sites (**Figure 6A, B** and
348 **Supplementary Figure 2B-D**).

349 The upstream shift of the termination window was particularly marked with the *cspA*
350 template for which Rho-dependent termination prevailed in the 5'UTR or farther in the CDS
351 in presence or absence of CspA, respectively (**Figure 6A**). Although these features are

352 reminiscent of the action of NusG, in particular at suboptimal Rho-dependent terminators^{37,41-}
353 ⁴³, CspA and NusG appear to have additive rather than mutually exclusive effects (**Figure 6A,**
354 **B** and **Supplementary Figure 2B-D**). More importantly, CspA was able to activate Rho-
355 dependent termination at 15°C with the *csp* templates and at the upstream *nsrR* terminator,
356 this activation being particularly efficient when NusG is also present (**Figure 6A, B** and
357 **Supplementary Figure 2B, D**). CspA had a more modest effect at the *rnr*, *lpxP*, and control
358 λtR1 terminators and did not shift or enlarge the termination window in these cases (**Figure**
359 **6C** and **Supplementary Figure 2A, C**). Thus, CspA (and possibly other Csp) can stimulate Rho-
360 dependent termination *in vitro*, presumably by disrupting RNA structures that block Rho
361 access to *Rut* sites (**Figure 1**). This effect seems particularly relevant for cold shock genes as it
362 could contribute to the dynamic regulation of their expression during cold acclimation.

363 To test this hypothesis in bacterial cells, we performed Northern blots and RT-qPCR
364 analyses of the *cspA* mRNA levels in the *rho*⁺ and *rho*^{R66S} strains at different times during the
365 cold acclimation phase. Both analyses confirmed that Rho-dependent attenuation of *cspA* is
366 switched off shortly after cold shock, resulting in similarly high mRNA levels in both *rho*⁺ and
367 *rho*^{R66S} strains (**Figure 6D**, compare 0 h and 0.5 h time points). After 1 h at 15°C, however, *cspA*
368 mRNA became less abundant in the *rho*⁺ strain than in the *rho*^{R66S} mutant and this difference
369 remained significant after 2 h of incubation at 15°C despite the decrease in *cspA* mRNA levels
370 in both strains (**Figure 6D**). After 5 h of incubation at 15°C, *cspA* mRNA levels were even lower
371 and the difference between *rho*⁺ and *rho*^{R66S} strains was no longer statistically significant.
372 These data are consistent with Rho-dependent attenuation of *cspA* being reactivated during
373 the cold acclimation phase, a process that is correlated to the sharp increase of the
374 concentrations of Csp proteins during this phase^{3,4,22} and that is congruent with our *in vitro*
375 observations (**Figure 6A**). Thus, the sequential but antagonistic effects of cold temperature
376 and Csp action on the Rho-dependent attenuator of *cspA* trigger a burst of *cspA* expression
377 upon cold shock (~70-fold burst for *rho*⁺ cells; **Figure 6D**).⁴⁷ By contrast, *cspA* expression
378 decreases if the exponentially growing cells remain at 37°C (**Supplementary Figure 6**)^{13,14}, an
379 effect that we ascribe to increased termination by Rho (see discussion of **Figure 5A, B** above),
380 further accentuating the difference between the low and high temperature situations
381 (compare WT levels in **Figures 5A** and **5B** or in **Figures 4A, D** and **6D**).

382

383 CONCLUSIONS

384 Growing evidence marks Rho-dependent termination of transcription as a pivotal mechanism
385 in the bacterial response to environmental changes and stresses. Relevant examples involve
386 Rho in the response to genotoxic and oxidative stresses^{53,54}, exposure to toxic substances such
387 as copper⁵⁵ or antibiotics⁵⁶, nutriment fluctuations^{37,43} or variations in the levels of key
388 metabolites^{36,45,57-61}, and even in the synthesis of the general stress sigma factor RpoS.⁶² Rho
389 involvement often hinges on a Rho-dependent terminator whose efficiency is tuned by a
390 specific cytoplasmic signal affecting the structure or translatability of the mRNA (**Figure 1**).
391 Notably, Rho-dependent regulatory mechanisms have been linked to the dynamic remodeling
392 of mRNA leader regions upon binding of small molecule ligands^{36,63}, regulatory small RNAs^{43,62},
393 and proteins.³⁷ Here, we provide evidence that a physical signal – temperature – can also
394 remodel Rho access to mRNAs and regulate transcription termination.

395 Our data unveil the existence of thermosensing Rho-dependent terminators in the
396 5'UTRs of transcripts encoding key CSR proteins, namely CspA, CspB, CspG, and Rnase R. The
397 transcription terminators are functional at 37°C and contribute to repress the synthesis of the
398 CSR proteins alongside the known posttranscriptional mechanisms (**Figure 7**, left). Upon a
399 temperature downshift, extensive restructuring of the 5'-leader of the mRNA, which is well
400 documented for *cspA* and *cspB*^{4,9}, hinders Rho action and allows transcription to proceed into
401 the structural genes (**Figure 7**, right). Once the Csp proteins have reached a sufficiently high
402 concentration, binding of these proteins to the mRNA brings the 5'-leader back to a silencing-
403 prone conformation. This negative feedback mechanism down-regulates *csp* expression at
404 both transcriptional and posttranscriptional levels during the acclimation phase (**Figure 7**).

405 Previous work supports the implication of transcriptional regulation in the CSR. Early
406 studies suggested that *cspA* transcription is regulated by sequences present in the leader
407 portion of the gene and by the interaction of the 5'-leader RNA with Csp proteins^{17,18,23,24,47},
408 although the exact mechanism remained unidentified. More recently, genome-wide studies
409 unveiled Rho-dependent signals in the 5'UTRs and CDSs of genes/operons involved in the CSR,
410 including *CspA* (**Figure 2A**).^{26,27,38} Besides the representative cases studied here, genome-wide
411 studies suggest that several other CSR genes could be regulated in a Rho-dependent manner
412 (**Table S4**). This regulation may be based on RNA thermosensors located in 5'-leaders, as it is
413 the case for *cspA/B/G* genes (**Figure 7**) or on intragenic *Rut* sites whose accessibility is

414 modulated by the efficiency of translation, as for *lpxP* (see above). The presence of *Rut* peaks
415 upstream from the promoters of some CSR genes (**Table S4**) suggests an additional, yet
416 untested scenario whereby regulation would stem from read-through of terminators that
417 normally insulate upstream transcription units (i.e. thermosensing terminators located in
418 mRNA 3'UTRs).

419 The regulation of *rpoS* by the cold-induced sRNA DsrA⁶⁴ provides another compelling
420 example of a Rho-dependent switch operating during cold acclimation. Indeed, DsrA binding
421 to the 5'-leader of *rpoS* enhances the translatability of the mRNA at low temperature⁶⁵ but
422 also inhibits Rho-dependent attenuation of *rpoS*.⁶² The latter is likely to contribute to the
423 increased expression of *rpoS* at low temperatures.⁶⁶ Conversely, one might envision that
424 activation of Rho-dependent termination participates in the repression of non-CSR proteins
425 upon cold shock. Translational repression associated to the increased structuring of bulk
426 mRNA⁴ is thought to trigger widespread decoupling of translation from transcription.^{32,33}
427 Whether this favors Rho-dependent termination (contributing to gene silencing) or is
428 compensated by increased structural shielding of intragenic *Rut* sites (**Figure 1**) awaits
429 experimental testing. Regardless, the work described here defines Rho-dependent
430 transcription termination as an important component of the bacterial response to cold stress
431 and sets a new paradigm for how RNA remodeling by antagonistic factors, here temperature
432 and Csp chaperones, can modulate transcription termination.

433

434 **MATERIALS AND METHODS**

435 **Materials**

436 Unless specified otherwise, chemicals and enzymes were from Merck and New England
437 Biolabs, respectively. Radionucleotides were obtained from PerkinElmer and synthetic
438 oligonucleotides (**Table S1**) from Eurogentec or Merck.

439 DNA templates for *in vitro* transcription were prepared by standard PCR amplification of
440 genomic DNA⁶⁷ with the forward (fwd) and reverse (rev) primer pairs listed in **Table S1**. In a
441 second round of PCR, the forward primer was replaced by primer T7A1_univ or pT7-univ_A to
442 introduce the full sequence of the T7 ϕ 6.5 (for T7 transcription) or T7A1 promoter (for
443 transcription with *E. coli* RNAP), respectively. Sequences of the DNA templates are provided
444 in **Table S3**.

445 Plasmid pET28b-CspA was obtained by subcloning the *E. coli cspA* CDS between the NcoI and
446 XhoI sites of plasmid pET28b(+) (Novagen). The CspA protein was overexpressed in BL21(DE3)
447 cells harboring the pET28b-CspA plasmid and purified as described previously.⁶⁸ The Rho and
448 NusG proteins were prepared as described previously.⁶⁷ Rho concentrations are expressed in
449 hexamers throughout.

450

451 **Bacterial strains and growth conditions**

452 Bacterial strains are listed in **Table S2**. *E. coli* MG1655 *rho*⁺ and *rho*^{R66S} strains were kindly
453 provided by Pr. Joseph T. Wade (New York state University at Albany). *Salmonella* strains are
454 described in the next sections. For cold shock experiments, bacteria were cultured in LB broth
455 at 37°C until absorbance at 600 nm (*A*₆₀₀) reached 0.5. Culture aliquots (50 mL) were then
456 withdrawn and either kept at 37°C or transferred to a 15°C water bath. Cultures were further
457 incubated at 37°C or 15°C for the indicated times.

458

459 **Construction of an ectopic *cspA-lacZY* fusion in *Salmonella***

460 *Salmonella* strain MA14009 harbors a tripartite *tetR*-P^{tet}-*ccdB-cat* cassette⁶⁹ inserted in the
461 region upstream of the *chiPQ* promoter in the background of *chiP-lacZY* translational fusion.⁴³
462 This strain was made recombineering-proficient by introduction of lambda *Red* plasmid
463 pKD46.⁷⁰ The resulting strain was transformed with a DNA fragment spanning the *cspA* leader
464 region amplified from wild-type *Salmonella* with primers AN13 and AN16 (**Table S1**). These
465 primers carry 5' extensions designed to recombine within the P^{tet} promoter region on one end
466 (AN13), and with the sequence immediately downstream from the initiation codon of *lacZ* on
467 the opposite end (AN16), so as to replace the entire segment between P^{tet} and the beginning
468 of *lacZ* open reading frame, with the *cspA* sequence. Recombinants were obtained selecting
469 for AHTc-resistance as described.⁶⁹ One of these recombinants, strain MA14627, which thus
470 carries the 5'UTR of *cspA* directly fused to the *lacZY* operon under the control of the *tetR*-P^{tet}
471 module was used for the experiments described here.

472

473 **Isolation of *Salmonella cspA* leader mutants**

474 Two cultures of strain MA14627 inoculated from separate single colonies were grown
475 overnight at 37°C. 0.1 ml aliquots were spread on the surface of two minimal (NCE) lactose
476 plates⁴³, and incubated at 37°C. Lac⁺ colonies typically developed during a 36-to-48-hour

477 incubation. A number of colonies were picked, purified by streaking, and subjected to colony
478 PCR (primers ppZ18 – AB24). The amplified fragments were subjected to Sanger sequencing.
479 Out of 14 clones analyzed, 7 carried changes in the *cspA* leader sequence: the four point-
480 mutants characterized in this study, C41A, A55G, C73U (three occurrences), C76U and one
481 carrying the deletion of the first 80 bp of the 5'UTR. The latter was not analyzed any further.

482

483 **RNA extraction and Northern blot analyses**

484 Total RNA was extracted from *E. coli* cell cultures by the acid hot phenol procedure as
485 described previously⁷¹, with minor modifications. Briefly, bacterial cultures were mixed with
486 one tenth of volume of ice cold Ethanol:Phenol [95:5] solution to quench RNA degradation.
487 Bacteria were pelleted, suspended in RNA extraction buffer (20 mM sodium acetate pH 5.2,
488 2% SDS, and 0.3 M sucrose), and boiled for 2 min. The mixtures were mixed with one volume
489 of acidic phenol, left at room temperature for 30 sec, and incubated on ice for 2 min. Mixtures
490 were then centrifuged at 12,000 g for 5 min. The aqueous phases were collected and extracted
491 thrice with a solution of Phenol:Chloroform:Isoamyl Alcohol [25:24:1] and twice with
492 chloroform. RNA preparations were purified on RNA Clean & Concentrator-25 columns
493 following manufacturer (Zymo Research) instructions and stored at -20°C in ME buffer (10 mM
494 MOPS, PH 6.5, 1 mM EDTA) before use. For Northern blotting, total RNA was fractionated on
495 a denaturing 8 % polyacrylamide/urea gel or a 1.5 % agarose MOPS/formaldehyde gel and
496 electro-transferred to a Hybond-N⁺ membrane (Cytiva). Blots were hybridized to ³²P-end
497 labeled DNA oligonucleotide probes complementary to the mRNA region of interest and to 5S
498 ribosomal RNA (**Table S1**). Hybridization signals were detected by phosphorimaging with a
499 Typhoon FLA-9500 instrument equipped with ImageQuant TL v8.1 software (Cytiva). Signals
500 for the 5S rRNA were used for normalization of the mRNA signals.³⁷

501

502 **Reverse transcription-quantitative PCR (RT-qPCR) analyses**

503 Residual DNA was removed from RNA samples by treatment with Turbo DNase I (Thermo
504 Fisher Scientific), following manufacturer's instructions. Then, 0.5 µg of total RNA was used
505 for cDNA synthesis with 2.5 µM random hexamer primer, 0.5 mM of each dNTP, and 200 U of
506 Maxima H-minus reverse transcriptase (Thermo Fisher Scientific) upon incubation for 5 min at
507 25°C and 1 h at 50°C. For quantitative PCR, each cDNA was mixed with the Power SYBR® Green
508 PCR Master Mix (Applied Biosystems™) and forward and reverse primers (0.2 µM each; **Table**

509 **S1**). Mixtures were placed in a LightCycler®480 qPCR instrument (Roche) and amplified with
510 the following conditions: initial denaturation at 95°C for 10 min followed by 40 cycles of 15 s
511 of denaturation at 95°C, and 1 min of hybridization/elongation at 60°C. Sample melting curves
512 were used to monitor the specificity of cDNA amplification at each cycle. The threshold cycle
513 'Ct' was calculated with the instrument software (LightCycler®480 v1.5.0) using the second
514 derivative maximum method. Data analysis was performed by the relative standard curve
515 method, as recommended in Applied Biosystems User Bulletin #2. Sample signals were then
516 normalized using *rrsB* (which encodes 16S rRNA).⁶¹ Statistical analyses were performed with
517 Kaleidagraph 4.1 (Synergy software) using the unequal variance option of the unpaired two-
518 tailed Student's t-test.

519

520 **Measurement of β -galactosidase activity**

521 β -galactosidase activity was assayed in toluene-permeabilized *Salmonella* cells as described
522 by Miller⁷² and is expressed in Miller units. All measurements were performed three times,
523 each time on duplicate cultures, as specified in the legend to Figure 5. Statistical significance
524 was determined as specified in the legend to Figure 5 using GraphPad Prism 9 software.

525

526 **Transcription termination experiments**

527 Standard transcription termination experiments were performed as described⁴⁰, with minor
528 modifications. Briefly, DNA template (0.1 pmol), *E. coli* RNAP (0.3 pmol), Rho (1.4 pmol), NusG
529 (0 or 2.8 pmol), Superase-In (0.5U/ μ L), and CspA (0, 10, or 2000 pmol) were mixed in 18 μ L of
530 transcription buffer (20 mM HEPES, pH 7.5, 150 mM potassium acetate, 10 mM NaCl, 100 nM
531 CaCl₂, 5 mM MgCl₂, 1.5 mM DTT) and incubated for 10 min at 37°C. Then, 2 μ L of initiation mix
532 (2 mM ATP, GTP, and CTP, 0.2 mM UTP, 2.5 μ Ci/ μ L of ³²P- α UTP, and 250 μ g/mL rifampicin in
533 transcription buffer) were added to reaction mixtures before incubation for 20 min at 37°C.
534 Transcription reactions were stopped with 4 μ L of EDTA (0.5 M), 6 μ L of tRNA (0.25mg/mL),
535 and 80 μ L of sodium acetate (0.42 M) before extraction with a phenol:chloroform:isoamyl
536 alcohol [25:24:1] mixture and precipitation at -20°C with 330 μ L of ethanol. Reaction pellets
537 were dissolved in denaturing loading buffer (95% formamide, 5 mM EDTA), and analyzed by
538 denaturing 8% PAGE and Typhoon FLA-9500 phosphorimaging.

539

540 **Electrophoretic mobility shift assay (EMSA)**

541 DNA templates encoding the wild-type mRNA leader of *Salmonella cspA* (nt 1-164) and single-
542 point mutants C41A, A55G, and G54U were transcribed with T7 RNA polymerase as described
543 previously⁷³, in the presence of ³²P- α UTP. The ³²P-labeled transcripts were purified by
544 denaturing 8% PAGE and stored at -20°C in ME buffer. Trace amounts (~400 β -ray counts per
545 minute) of each transcript were dissolved in EMSA buffer (10 mM MOPS, pH 6.5, 1 mM EDTA,
546 and either 50 mM KCl or 150 mM potassium acetate) and incubated for 3 min at 95°C and
547 then for 15 min at 37°C. Samples were mixed with Ficoll-400 (8% v/v final) and analyzed by
548 native 6% PAGE (4 h migration at 20°C and 10 V/cm) and Typhoon FLA-9500 phosphorimaging.

549

550 **Bioinformatics analyses**

551 Percentages of C and G residues along the *E. coli* MG1655 genome were computed with
552 EMBOSS freak 5.0.0 (step: 1; averaging window: 78) and visualized with Kaleidagraph 4.1.
553 Log₁₀ fold enrichment (FE) profiles were determined from Helicase-SELEX data³⁸ deposited in
554 the European Nucleotide Archive under accession #PRJEB50170. Curated reads were mapped
555 on the MG1655 genome (NC_000913.3) with Bowtie2 (v2.4.2, default options). Coverage and
556 RPM normalization were performed with Bedtools v2.29.2. The Log₁₀FE profiles were obtained
557 with MACS2 bdgcmp v2.1.1 using the deposited R10+ (+NusG) or R10- (-NusG) sample as input
558 and R0 sample as control. Peak calling was performed with MACS2 bdgpeakcall v2.1.1 (cutoff:
559 0; Min peak length: 30; Max gap: 30). Log₁₀FE profiles were visualized with the JBrowse
560 genome browser (jbrowse.org).⁷⁴

561

562 **AUTHOR CONTRIBUTIONS**

563 MD performed Northern blot, RT-qPCR, and *in vitro* transcription experiments; NF-B
564 constructed the *Salmonella* strains used in this study; TDD prepared DNA templates and
565 performed Northern blot and EMSA experiments; PK and LB performed β -galactosidase
566 assays; EE and MB performed computational mining of published Helicase-SELEX and genomic
567 data; LB isolated *Salmonella cspA* leader mutants; MB prepared proteins and wrote the paper
568 with help from MD, NF-B, and LB; MD, NF-B, LB, and MB conceived the study. All authors
569 participated in data analysis.

570

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575

576 **COMPETING INTERESTS**

577 The authors declare no competing interests

578 **FIGURE LEGENDS**

579 **Figure 1:** Rho-dependent termination. Rho binds to free *Rut* sites within nascent transcripts
580 and then uses its ATP-dependent motor activity to catch up with RNA polymerase (RNAP)
581 along the RNA chain and to trigger the dissociation of the transcription elongation complex
582 (bottom).⁷⁵ Rho action is prevented by the presence of translating ribosomes or by secondary
583 structures in the nascent transcript that block *Rut* sites (top). In an alternative termination
584 pathway, Rho binds RNAP early on during transcription and detects accessible *Rut* sites in the
585 nascent transcript from this RNAP-bound position⁷⁶, which is not represented here for
586 simplicity.

587

588 **Figure 2:** Putative *Rut* sites in the 5'-UTRs of *E. coli* genes involved in the CSR. **(A)** Fold
589 enrichment (FE; log₁₀ scale) profiles for Helicase-SELEX experiments performed with Rho in
590 presence or absence of NusG were determined from published data.³⁸ Cyan bars highlight the
591 positions of the enrichment peaks (median log₁₀FE ≥ 0.3) suggestive of *Rut* sites in the 5'UTRs.
592 Regions where transcription is upregulated *in vivo* in presence of the Rho inhibitor
593 Bicyclomycin, as determined by ChIP-chip (orange arrows)²⁷ or microarrays and RNAseq
594 (purple bars)²⁶ are also shown. Note that different scales along the genome coordinates (x-
595 axis) are used for the various panels. **(B)** C>G bubbles (black arrows) overlap with the *Rut* peaks
596 detected by Helicase-SELEX (cyan lines) and are present upstream from the *in vitro*
597 transcription termination regions (brown lines), a feature characteristic of Rho-dependent
598 terminators. Percentages of C and G residues were calculated using a sliding window of 78 nt.
599 Termination boundaries were estimated with the molecular size calibration/calculation
600 function of ImageQuant software using all *in vitro* transcription gel lanes.

601

602 **Figure 3:** Cold shock genes contain Rho-dependent terminators in their 5'UTRs.
603 Representative gel images show the *in vitro* transcription profiles obtained with DNA
604 templates encoding the upstream sections of the *E. coli cspA*, *cspB*, *cspG*, *lpxP* and *nsrR*
605 transcription units **(A-E)** or the λtR1 terminator **(F)**. Transcription reactions were performed at
606 15 or 37°C with or without Rho (20 nM), NusG (70 nM), or CspA (0.5 μM), as indicated. Bands
607 corresponding to runoff (RO) and termination (Term) transcripts are indicated. Since
608 transcripts are labeled by incorporation of α³²P-UTP during transcription, band intensities

609 reflect both the amounts and sizes of the transcripts. A downstream *nsrR* termination site (*)
610 is detected at 15°C (panel D).

611

612 **Figure 4:** Transcription of cold shock genes is regulated by Rho *in vivo*. *E. coli* log phase cultures
613 ($A_{600} = 0.5$) were incubated for 30 min at 15°C or 37°C before RNA extraction. **(A, B)** Northern
614 blot analysis of the expression of *cspA* and *nsrR* in WT (*rho*⁺) and *rho*^{R66S} (R66S) strains.
615 Phosphorimager *cspA* counts were normalized to 5S RNA and the resulting values were
616 divided by that obtained for WT at 37°C. The [*cspA*] values are (mean ± SD) values from three
617 independent biological replicates. Pairwise comparisons of the normalized WT and R66S
618 signals along gel lanes are provided on the right of the *cspA* membrane images (FL: full-length
619 transcripts). Polycistronic *nsrR-rnr-yjfHI* transcripts are subjected to complex
620 posttranscriptional processing^{5,6} and were not quantified. **(C, D)** RT-qPCR confirms Rho-
621 dependent regulation of cold shock genes. For each gene, signals are normalized to the
622 condition *rho*⁺, 37°C. Four biological replicates were analyzed by RT-qPCR, each time in
623 triplicate (open circles represent the triplicate means). Statistical significance was determined
624 by unpaired two-tailed Student's t-tests (ns, $p > 0.05$; *, $p < 0.05$; **, $p < 10^{-2}$; ***, $p < 10^{-3}$;
625 ****, $p < 10^{-4}$).

626

627 **Figure 5.** Mutations in the *cspA* leader RNA prevent Rho-dependent transcription termination
628 in *Salmonella*. **(A, B)** Impact of Rho mutation Y80C on *cspA-lacZY* expression. Isogenic strains
629 carrying the *cspA-lacZY* fusion under control of the P^{tet} promoter and either *rho*⁺ or *rho*^{Y80C}
630 were cultured in LB at 37°C until reaching mid-exponential phase ($DO_{600} \approx 0.4 - 0.5$). At this
631 point, expression of the *cspA-lacZY* fusion was induced by adding the P^{tet} inducer AHTc. The
632 cultures were then divided, with one half transferred to 15°C and the other maintained at
633 37°C, and both were further incubated for **(A)** 30 and **(B)** 180 min. Cells were then processed
634 for the determination of β-galactosidase activity. **(C)** Lac phenotype of strains carrying the P^{tet}-
635 *cspA-lacZY* fusion in combination with either *rho*⁺ (strain MA14635) or *rho*^{Y80C} (strain
636 MA14636). Bacteria were cultured to stationary phase and 10 μl were spotted on minimal-
637 lactose plates ±AHTc (37°C). The strain carrying *rho*^{Y80C} is Lac⁺ under non-inducing conditions
638 (-AHTc) whereas the *rho*⁺ strain is Lac⁻. The yellow arrow highlights a Lac⁺ colony forming of
639 the lawn of *rho*⁺ cells. **(D)** Position of mutations suppressing the inhibitory effect of Rho on

640 *cspA-lacZ* expression at 37°C. **(E, F)** Effect of the mutations in **D** on the expression of the *cspA-*
641 *lacZ* fusion in the presence or absence of Rho Y80C at 37°C **(E)** and at 15°C **(F)**. Cells were
642 cultured and treated as described in **(A, B)** with a time of incubation of 30 min after AHTc
643 induction. Throughout this experiment, β -galactosidase activity was assayed with the method
644 of Miller⁷² on three independent replicates of the bacterial cultures, with each assay carried
645 out in duplicate. Statistical significance was determined by one-way ANOVA with Šidák's
646 multiple comparisons test (ns, $p > 0.05$; ***, $p \leq 10^{-3}$; ****, $p \leq 10^{-4}$).

647

648 **Figure 6:** A high concentration of CspA stimulates Rho-dependent termination at *E. coli csp*
649 terminators. Transcription experiments were performed with the *cspA* **(A)**, *cspB* **(B)** and
650 control $\lambda tR1$ **(C)** DNA templates as described in the legend to Figure 3, except that a 200-fold
651 higher concentration of CspA was used (100 vs 0.5 μ M). **(D)** Northern blot and RT-qPCR
652 analyses of Rho-dependent attenuation of *cspA* as a function of the duration of the incubation
653 at 15°C following cold shock. Four biological replicates were analyzed by RT-qPCR, each time
654 in triplicate (open circles represent the triplicate means). Statistical significance was
655 determined by unpaired two-tailed Student's t-tests (ns, $p > 0.05$; *, $p < 0.05$; **, $p < 10^{-2}$; ***,
656 $p < 10^{-3}$).

657

658 **Figure 7:** Model for regulation of *csp* genes. **(Left)** Rho-dependent attenuation of transcription
659 in the 5'UTR inhibits *csp* expression at 37°C. Poor translatability of the transcripts that escape
660 termination further contributes to *csp* down-regulation directly^{4,9,10} and, possibly indirectly,
661 by facilitating intragenic transcription termination or RNA degradation or both.⁷ **(Right)**
662 Restructuring of the 5'-leader upon the cold shock hides the *Rut* site and prevents premature
663 transcription termination, at the same time enhancing the accessibility of the ribosome
664 binding site. Protection of the CDS by translating ribosomes may contribute to stabilize the
665 mRNA. During cold acclimation, the concentration of Csp proteins increases until it becomes
666 sufficient to promote Csp binding to the mRNA. Csp binding drives the mRNA into a
667 conformation that resembles the "37°C-conformation"⁴ and thereby restores attenuation.

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Gene expression

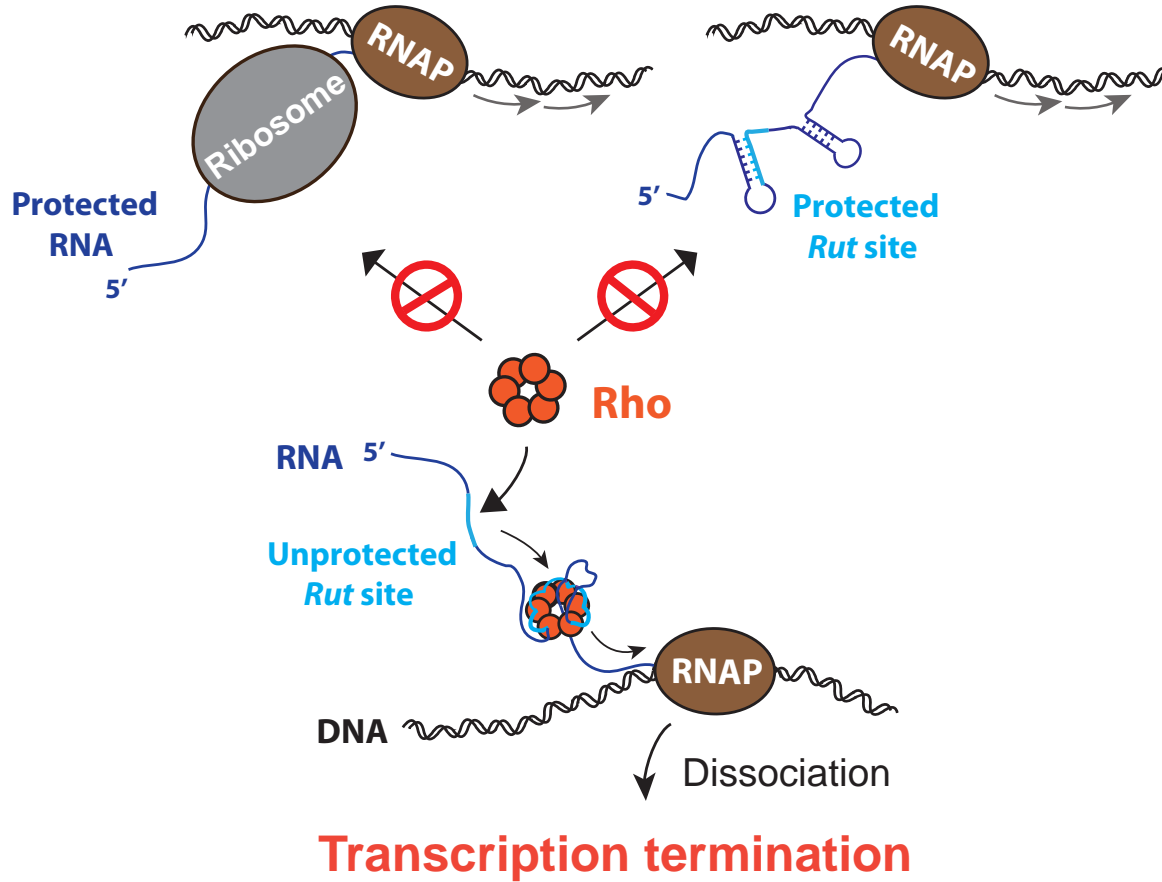


Figure 1: Rho-dependent termination. Rho binds to free *Rut* sites within nascent transcripts and then uses its ATP-dependent motor activity to catch up with RNA polymerase (RNAP) along the RNA chain and to trigger the dissociation of the transcription elongation complex (bottom) (Molodtsov et al., 2023). Rho action is prevented by the presence of translating ribosomes or by secondary structures in the nascent transcript that block *Rut* sites (top). In an alternative termination pathway, Rho binds RNAP early on during transcription and detects accessible *Rut* sites in the nascent transcript from this RNAP-bound position (Hao et al., 2021), which is not represented here for simplicity.

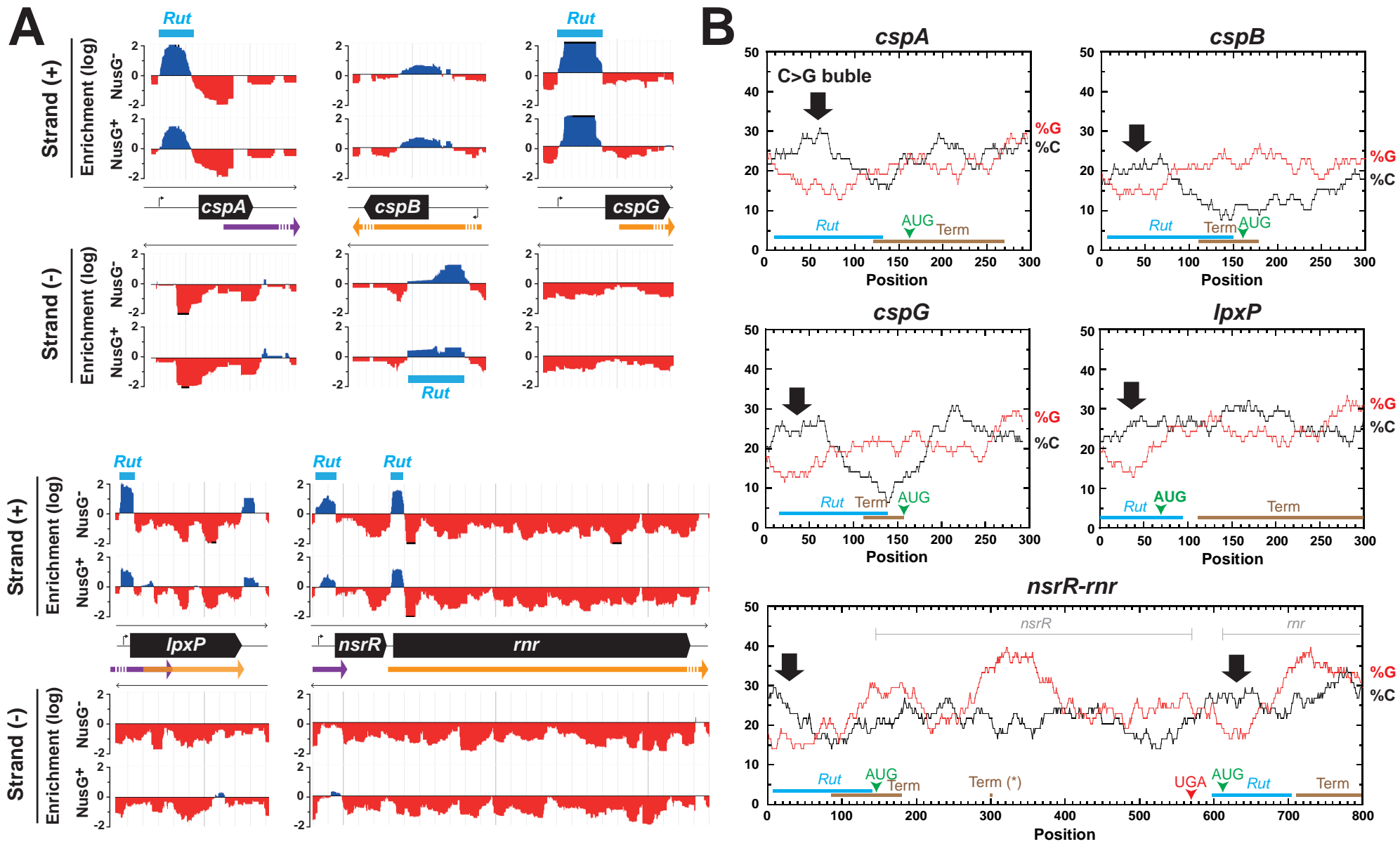


Figure 2: Putative *Rut* sites in the 5'-UTRs of *E. coli* genes involved in the CSR. **(A)** Fold enrichment (FE; \log_{10} scale) profiles for Helicase-SELEX experiments performed with Rho in presence or absence of NusG were determined from published data (Delaleau et al., 2022). Cyan bars highlight the positions of the enrichment peaks (median \log_{10} FE ≥ 0.3) suggestive of *Rut* sites in the 5'UTRs. Regions where transcription is upregulated *in vivo* in presence of the Rho inhibitor Bicyclomycin, as determined by ChIP-chip (orange arrows; (Peters et al., 2009)) or microarrays and RNAseq (purple bars; (Peters et al., 2012)) are also shown. Note that different scales along the genome coordinates (x axis) are used for the various panels. **(B)** C>G bubbles (black arrows) overlap with the *Rut* peaks detected by Helicase-SELEX (cyan lines) and are present upstream from the *in vitro* transcription termination regions (brown lines), a feature characteristic of Rho-dependent terminators. Percentages of C and G residues were calculated using a sliding window of 78 nucleotides. Termination boundaries were estimated with the molecular size calibration/calculation function of ImageQuant software using *in vitro* transcription gel lanes.

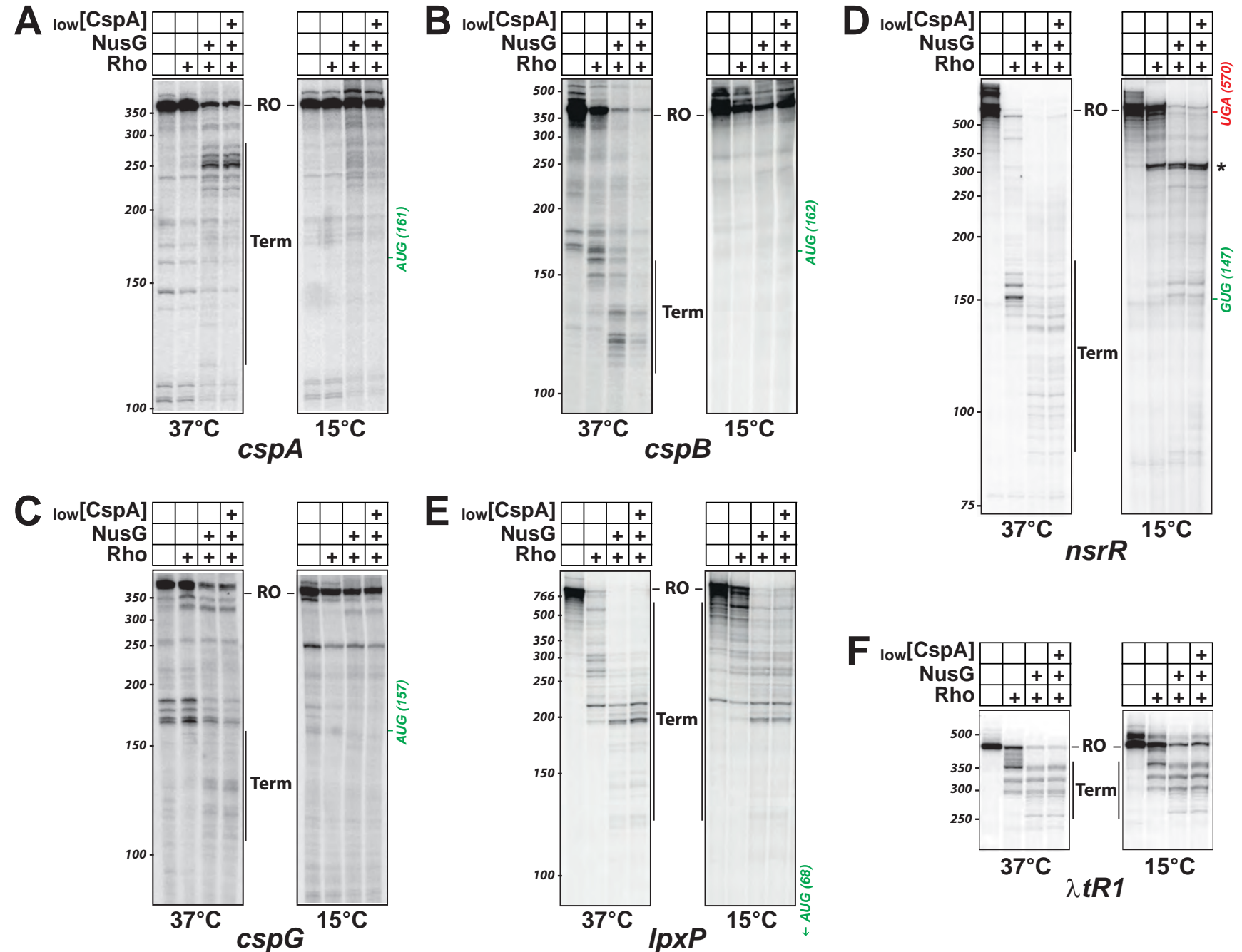


Figure 3: Cold shock genes contain Rho-dependent terminators in their 5'UTRs. Representative gel images show the *in vitro* transcription profiles obtained with DNA templates encoding the upstream sections of the *E. coli* *cspA*, *cspB*, *cspG*, *lpxP* and *nsrR* transcription units (**A-E**) or the $\lambda tR1$ terminator (**F**). Transcription reactions were performed at 15 or 37°C with or without Rho (20 nM), NusG (70 nM), or CspA (0.5 μ M), as indicated. Bands corresponding to runoff (RO) and termination (Term) transcripts are indicated. Since transcripts are labeled by incorporation of $\alpha^{32}P$ -UTP during transcription, band intensities reflect both the amounts and sizes of the transcripts. A downstream *nsrR* termination site (*) is detected at 15°C (panel D).

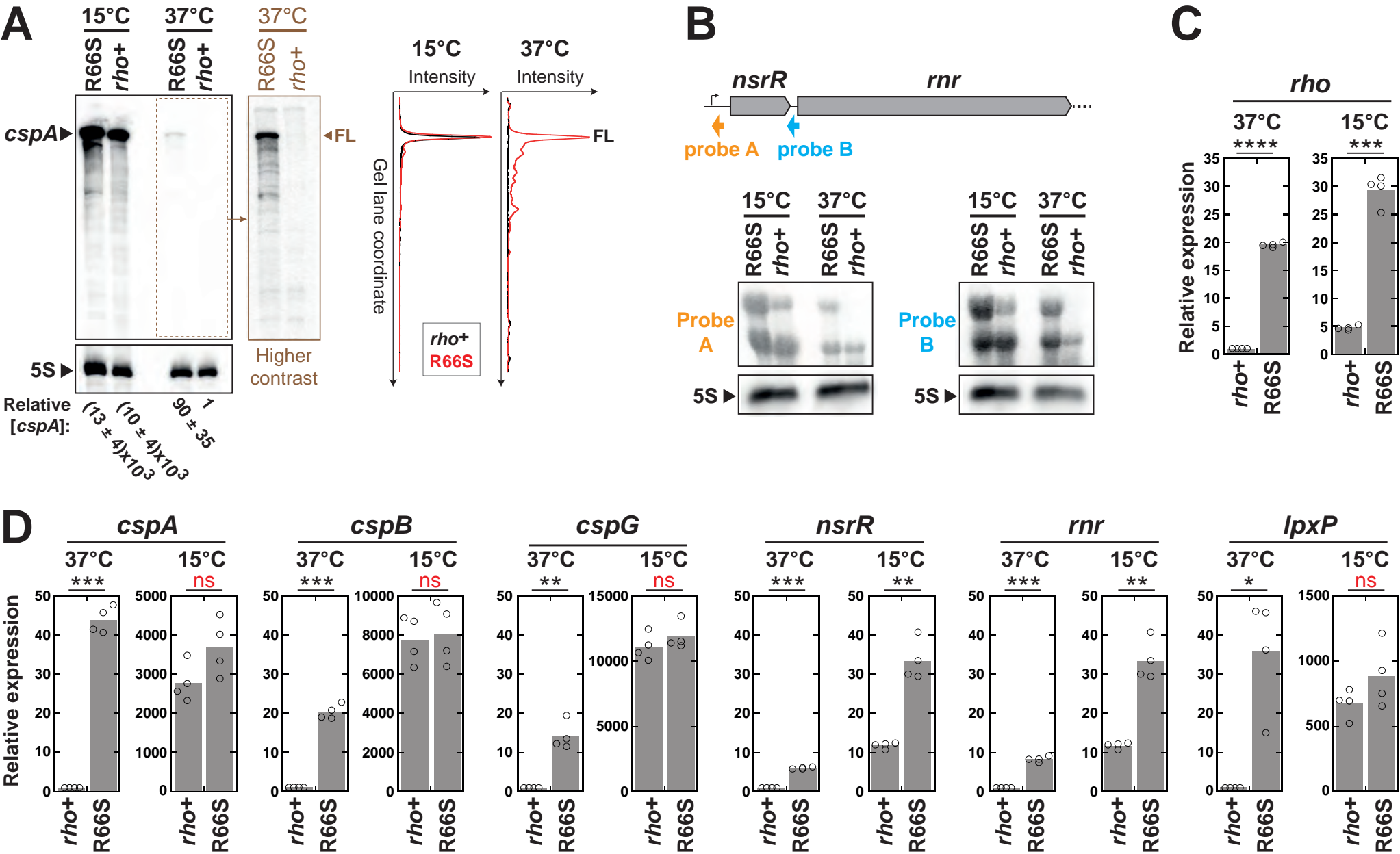


Figure 4: Transcription of cold shock genes is regulated by Rho *in vivo*. *E. coli* log phase cultures ($A_{600} \sim 0.5$) were incubated for 30 min at 15°C or 37°C before RNA extraction. **(A, B)** Northern blot analysis of the expression of *cspA* and *nsrR* in WT (ρ^{ho+}) and ρ^{R66S} (R66S) strains. Phosphorimager *cspA* counts were normalized to 5S RNA and the resulting values were divided by that obtained for WT at 37°C. The [cspA] values are (mean \pm SD) values from three independent biological replicates. Pairwise comparisons of the normalized WT and R66S signals along gel lanes are provided on the right of the *cspA* membrane images (FL: full-length transcripts). Polycistronic *nsrR-rnr-yjfhI* transcripts are subjected to complex posttranscriptional processing (Cairrao and Arraiano, 2006; Cairrao et al., 2003) and were not quantified. **(C, D)** RT-qPCR confirms Rho-dependent regulation of cold shock genes. For each gene, signals are normalized to the condition ρ^{ho+} , 37°C. Four biological replicates were analyzed by RT-qPCR, each time in triplicate (open circles represent the triplicate means). Statistical significance was determined by unpaired two-tailed Student's t-tests (ns, $p > 0.05$; *, $p < 0.05$; **, $p < 10^{-2}$; ***, $p < 10^{-3}$; ****, $p < 10^{-4}$).

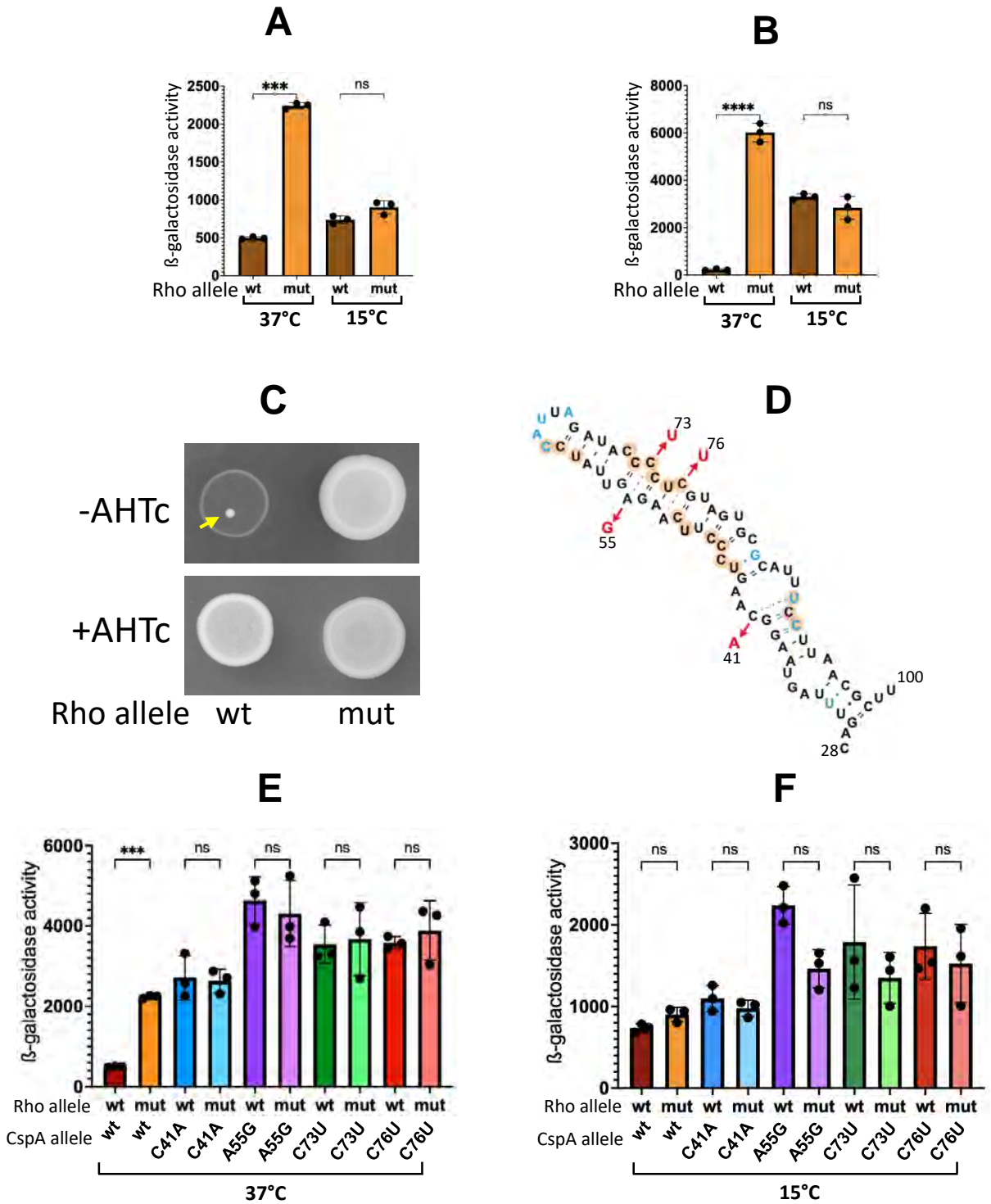


Figure 5. Mutations in the *cspA* leader RNA prevent Rho-dependent transcription termination in *Salmonella*. (A, B) Impact of Rho mutation Y80C on *cspA-lacZY* expression. Isogenic strains carrying the *cspA-lacZY* fusion under control of the P^{tet} promoter and either rho^+ or rho^{Y80C} were cultured in LB at 37°C until reaching mid-exponential phase ($DO_{600} \approx 0.4 - 0.5$). At this point, expression of the *cspA-lacZY* fusion was induced by adding the P^{tet} inducer AHTc. The cultures were then divided, with one half transferred to 15°C and the other maintained at 37°C, and both were further incubated for (A) 30 and (B) 180 min. Cells were then processed for the determination of β -galactosidase activity. (C) Lac phenotype of strains carrying the P^{tet} -*cspA-lacZY* fusion in combination with either rho^+ (strain MA14635) or rho^{Y80C} (strain MA14636). Bacteria were cultured to stationary phase and 10 μ l were spotted on minimal-lactose plates \pm AHTc (37°C). The strain carrying rho^{Y80C} is Lac⁺ under non-inducing conditions (-AHTc) whereas the rho^+ strain is Lac⁻. The yellow arrow highlights a Lac⁺ colony forming of the lawn of rho^+ cells. (D) Position of mutations suppressing the inhibitory effect of Rho on *cspA-lacZ* expression in the presence or absence of Rho Y80C at 37°C (E) and at 15°C (F). Cells were cultured and treated as described in (A, B) with a time of incubation of 30 min after AHTc induction. Throughout this experiment, β -galactosidase activity was assayed with the method of Miller (Miller, 1992) on three independent replicates of the bacterial cultures, with each assay carried out in duplicate. Statistical significance was determined by one-way ANOVA with Šidák's multiple comparisons test (ns, $p > 0.05$; ***, $p \leq 10^{-3}$; ****, $p \leq 10^{-4}$).

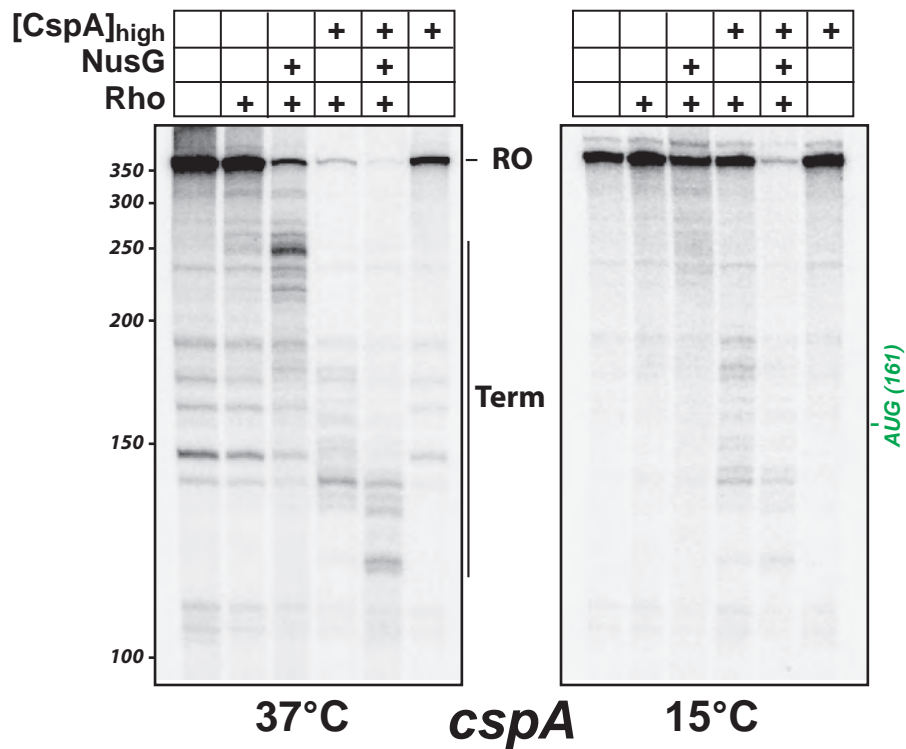
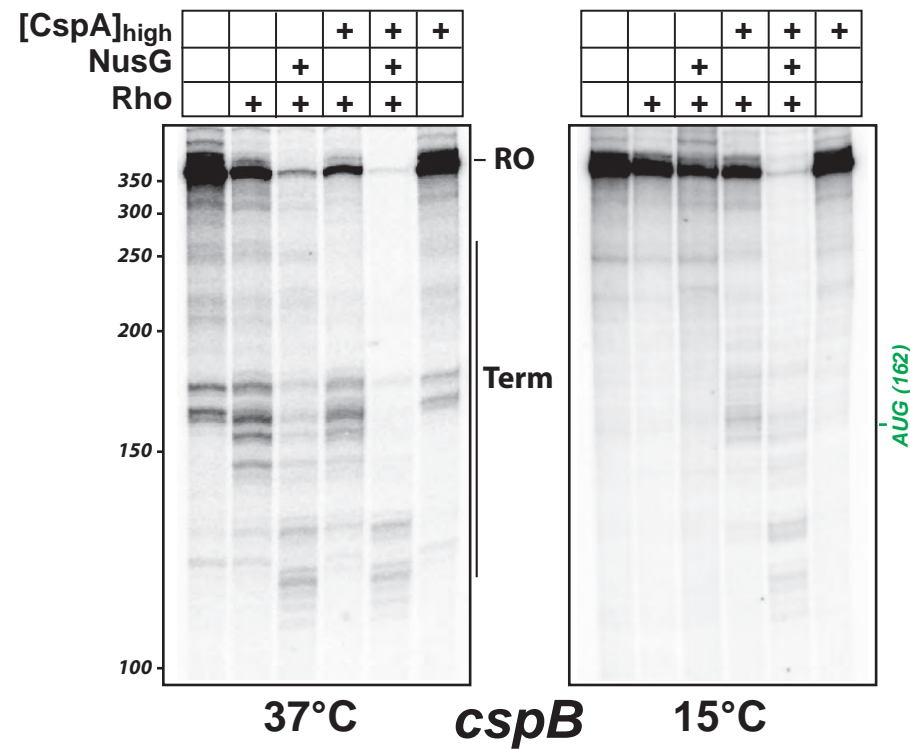
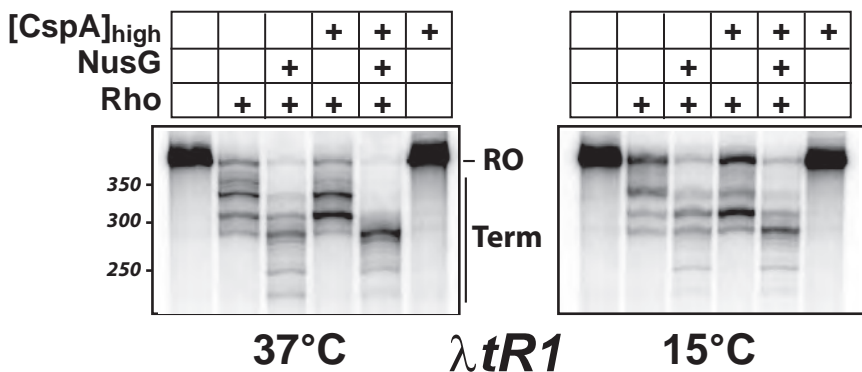
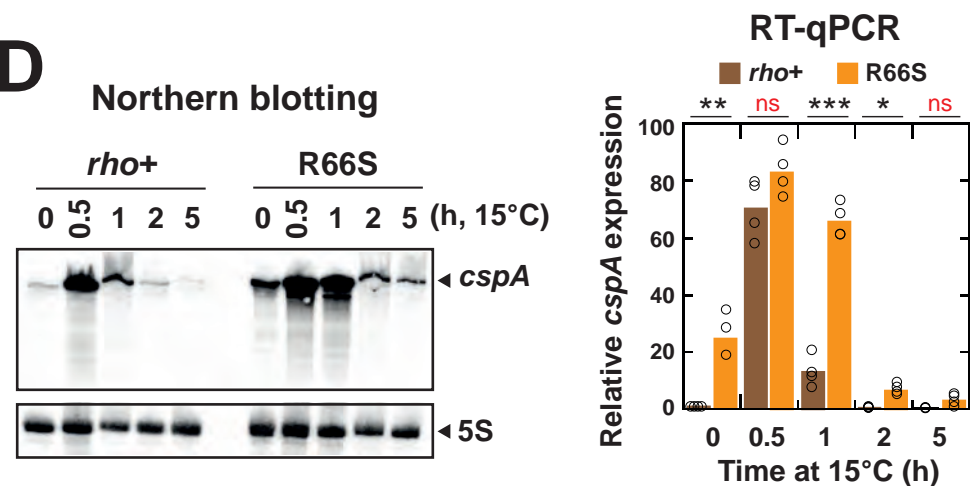
A**B****C****D**

Figure 6: A high concentration of CspA stimulates Rho-dependent termination at *E. coli* *csp* terminators. Transcription experiments were performed with the *cspA* (A), *cspB* (B) and control $\lambda tR1$ (C) DNA templates as described in the legend to Figure 3, except that a 200-fold higher concentration of CspA was used (100 vs 0.5 μ M). (D) Northern blot and RT-qPCR analyses of Rho-dependent attenuation of *cspA* as a function of the duration of the incubation at 15°C following cold shock. Four biological replicates were analyzed by RT-qPCR, each time in triplicate (open circles represent the triplicate means). Statistical significance was determined by unpaired two-tailed Student's t-tests (ns, $p > 0.05$; *, $p < 0.05$; **, $p < 10^{-2}$; ***, $p < 10^{-3}$).

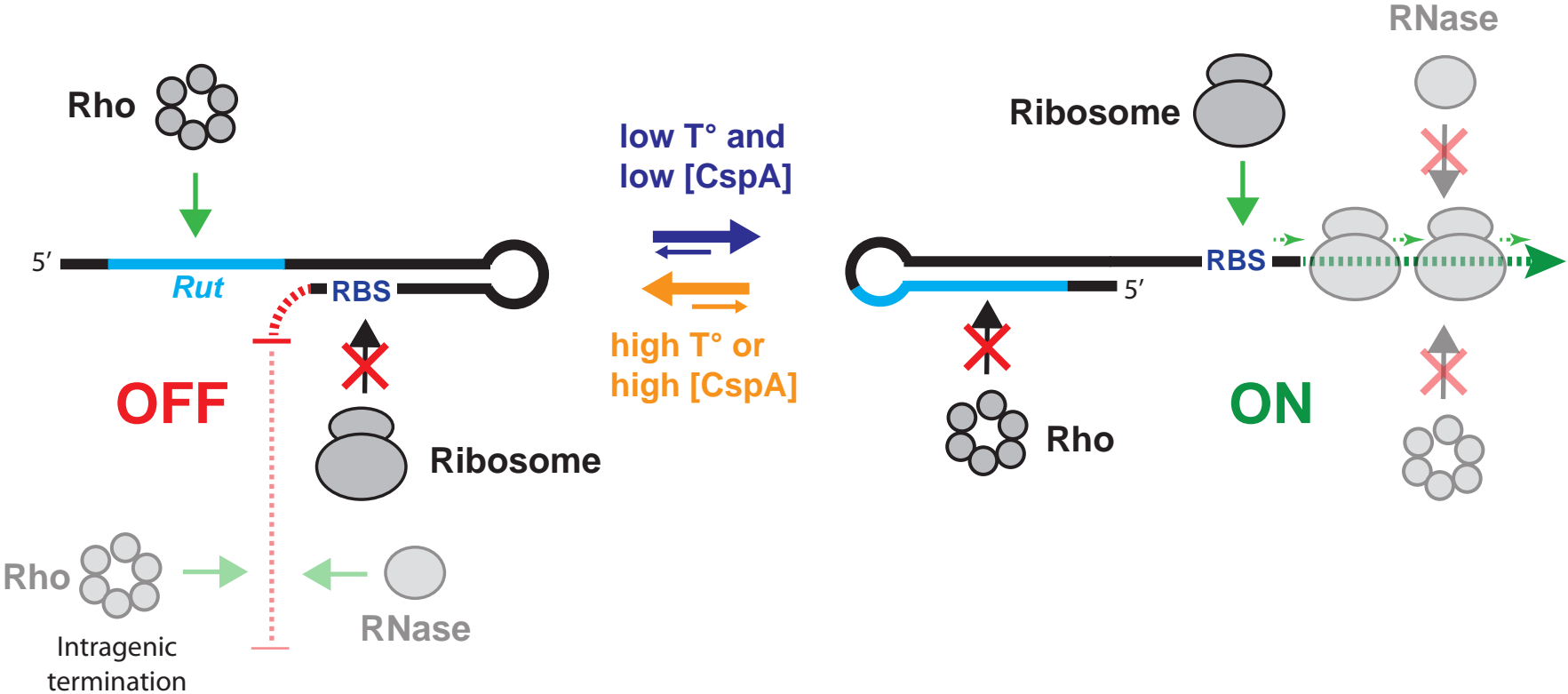


Figure 7: Model for regulation of *csp* genes. **(Left)** Rho-dependent attenuation of transcription in the 5'UTR inhibits *csp* expression at 37°C. Poor translatability of the transcripts that escape termination further contributes to *csp* down-regulation directly (Giuliodori et al., 2010; Yamanaka et al., 1999; Zhang et al., 2018) and, possibly indirectly, by facilitating intragenic transcription termination or RNA degradation or both (Fang et al., 1997). **(Right)** Restructuring of the 5'-leader upon the cold shock hides the *Rut* site and prevents premature transcription termination, at the same time enhancing the accessibility of the ribosome binding site. Protection of the CDS by translating ribosomes may contribute to stabilize the mRNA. During cold acclimation, the concentration of Csp proteins increases until it becomes sufficient to promote Csp binding to the mRNA. Csp binding drives the mRNA into a conformation that resembles the “37°C-conformation” (Zhang et al., 2018) and thereby restores attenuation.