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Intrinsic and Rho-dependent termination cooperate for efficient transcription termination at 3' untranslated regions

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ABSTRACT

The intrinsic, and the Rho-dependent mechanisms of transcription termination are conserved in bacteria. Generally, the two mechanisms have been illustrated as two independent pathways occurring in the 3′ ends of different genes with contrasting requirements to halt RNA synthesis. However, a majority of intrinsic terminators terminate transcription inefficiently leading to transcriptional read-through. The unwanted transcription in the downstream region beyond the terminator would have undesired consequences. To prevent such transcriptional read-through, bacteria must have evolved ways to terminate transcription more efficiently at or near the termination sites. We describe the participation of both the mechanisms, where intrinsic terminator and Rho factor contribute to prevent transcriptional read-through. Contribution from both the termination processes is demonstrated at the downstream regions of the genes both *in vitro* and *in vivo* in mycobacteria. Distinct patterns of cooperation between the two modes of termination were observed at the 3' untranslated regions of the genes to ensure efficient termination. We demonstrate similar mode of operation between the two termination processes in *Escherichia coli* suggesting a likely prevalence of this cooperation across bacteria. The reporter system developed to assess the Rho – intrinsic termination collaboration *in vivo* for mycobacteria and *E. coli* can readily be applied to other bacteria.

1. Introduction

Accurate and efficient transcription termination is an important step to generate functional transcripts. The two distinct transcription termination mechanisms in bacteria are intrinsic and Rho-dependent termination (RDT). Intrinsic termination is guided by the sequences that form a G/C-rich hairpin stem followed by a U-tract, which stimulates transcription elongation complex to pause and dissociate [1]. RDT involves the well-conserved termination factor Rho that halts RNA synthesis downstream of the coding sequences to prevent unnecessary transcription read-through [2]. While intrinsic termination is conserved in all bacterial species, RDT is found in a majority of the bacteria. However, Rho seems to be essential for survival only in some bacterial species indicating its importance in these species [3,4]. In organisms where termination has been studied, there seems to be a preference either for intrinsic termination or RDT as a predominant mode of transcription termination [5,6]. In *E. coli,* intrinsic termination appears to be the major mode; and about 20–30% of its genes are terminated by Rho [7,8]. Nevertheless, Rho is found to be an essential gene in *E. coli* [9]. In contrast, Rho is not essential in *Bacillus subtilis* and the organism seems to be overly reliant on intrinsic termination [10,11]. In mycobacteria, intrinsic terminators are underrepresented and Rho is essential [4,12], suggesting their dependence on RDT.

Efficient transcription termination is important for the regulation of gene expression. Barring intrinsic terminators of rRNA and a few others, a majority of the genes terminate transcription inefficiently [13–15]. This inefficiency appears to arise from the weaker terminator structure. A canonical intrinsic terminator consists of a strong G/C-rich stem followed by 7–9 U's whereas a non-canonical intrinsic terminator is comprised of fewer number of U residues and secondary structures with higher ΔG [16,17]. The inefficiency of intrinsic terminators is exacerbated in mycobacteria. As such,

Abbreviations: RDT- Rho-dependent termination, RNAP- RNA Polymerase; CKD, Conditional knockdown; KO, Knockout; aTc, anhydrotetracycline; BCM, Bicyclomycin.

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mycobacteria suffer from the paucity of intrinsic terminators which is further compounded by the lack of canonical terminators [5,12]. Inefficient transcription termination and read-through can have deleterious consequences to cells due to unwanted transcription of downstream genes [4,15,18]. Hence organisms have evolved mechanisms to bring about more efficient termination when intrinsic terminators are by themselves inefficient. Our previous study suggested suppression of transcription read-through *in vivo* despite the presence of weak intrinsic terminators in mycobacteria, hinting at the role of additional players in termination [13].

In this study, we have explored the possibility of the two different modes of termination coming together for better control of transcription termination. We demonstrate that both intrinsic and RDT processes function together to ensure more efficient transcription termination in mycobacteria and *E. coli*.

2. Materials and methods

2.1. Bacterial strains, culture conditions, plasmids, chemicals and enzyme

Mycobacterium tuberculosis (Mtb) H37Rv, *Mycobacterium smegmatis* (Msm) mc^{2}155, *E. coli* AMO14 [19] and RS1309 (Δrho Δrac) [20] strains were used for transcription termination studies. *E. coli* DH10B and BL21 λDE3 were used for cloning and protein overexpression, respectively (Table S1). Msm and Mtb were cultured in Middlebrook 7H9 broth supplemented with 0.5% albumin, 0.2% dextrose, 0.085% NaCl, 0.2% glycerol and 0.05% Tween 80, and with antibiotics when appropriate (50 μg/ml hygromycin and 25 μg/ml kanamycin). Mtb and Msm Rho-conditional knockdown (CKD) strains were grown at 37 °C and 30 °C respectively with shaking at 120 rpm. For induction of small guide RNA (sgRNA) and *dcas9* expression from pRH2521 and pRH2502 plasmids respectively, Msm and Mtb Rho-CKD cultures were supplemented with anhydrotetracycline (aTc) to a final concentration of 200 ng/ml as described [21]. When grown on Middlebrook 7H11 agar supplemented with 0.5% glycerol and 10% oleic acid-albumin-dextrose-catalase, aTc (100 ng) was added on a paper disc when indicated. *E. coli* AMO14 and RS1309 strains were grown in LB broth having appropriate antibiotics [19,20]. Kanamycin (50 μg/ml) and chloramphenicol (30 μg/ml) were used for AMO14. Tetracycline (30 μg/ml), ampicillin (100 μg/ml), and IPTG (1 mM) were added for *E. coli* Rho (EcRho) expression in RS1309.

DNA fragments carrying intrinsic terminators were cloned in pUC18 plasmid downstream of the T7A1 promoter (Table S2) [13]. Oligonucleotides were synthesized by Sigma (Table S3). α^{32}P UTP was purchased from the Board of Radiation and Isotope Technology, Mumbai. DNA modifying enzymes were purchased either from NEB or Roche. Sigma A (SigA) enriched His-tagged RNA polymerase (RNAP) was purified from Msm SM07 strain as described before [22] and was used for *in vitro* transcription assays. MtbRho and its mutant that lacks the Walker B motif, Q-loop, and R-loop (ΔWQRRho) of the ATPase domain were purified by polyethyleneimine and ammonium sulphate precipitation, elution from Heparin and SP-sepharose chromatography columns as described previously [23]. EcRho with a C-terminal His-tag was expressed in *E. coli* BL21 λDE3 cells. The lysate was passed through the Ni-NTA column, eluted with 500 mM imidazole, and further purified using the Hitrap-heparin column [23].

2.2. Construction of Rho-CKD strains

To generate Rho-CKD in Msm and Mtb, Rho-specific sgRNAs were designed and cloned in pRH2521 vector. Protospacer adjacent motif (PAM) sequence was identified in the proximal coding region for both the strains of mycobacteria. These vectors were transformed into Msm/Mtb cells containing pRH2502 (a vector expressing an inactive version of *dcas9*) [21].

2.3. mRuby fluorescence assay to estimate transcription termination in vivo

DNA fragment containing the *mRuby* gene [24] was cloned in the pMV261 vector downstream hsp60 promoter. Intrinsic terminators from *tuf, cyp144* and *bfrB* genes of Mtb were cloned upstream of *mRuby* (Table S1 and S2). The DNA fragment containing hsp60 promoter-intrinsic terminator-*mRuby* was excised from pMV261 and cloned in pRH2521 vector. This pRH2521 vector contains MsmRho specific sgRNA. Msm cells carrying pRH2502 vector expressing dCas9 protein were electroporated with pRH2521-*mRuby*/intrinsic terminator-*mRuby* and colonies from each of these strains were grown till O.D 0.5 in Middlebrook-7H9 media at 30 °C. Cultures were washed and resuspended in 1X phosphate-buffered saline. The fluorescence readouts were normalized against the O.D. 300 μl of 0.5 O.D culture from these strains were used to measure mRuby fluorescence. The fluorescence was estimated using excitation and emission wavelength of 558 nM and 605 nM, respectively in the infiniteM200PRO-TECAN.

DNA fragments containing intrinsic terminators *aroG, ycbL* and *rrnBT1* from *E. coli* K-12 MG1655 were cloned into pRH2521 vector upstream of *mRuby* gene and transformed into *E. coli* AMO14 and RS1309 strains. mRuby fluorescence assays were carried out by growing respective test and control strains in LB media until 0.5 O.D. at 37 °C. For EcRho-knockout (KO) strain RS1309, mRuby fluorescence was measured at 0.5 O.D. For the temperature-sensitive *E. coli* AMO14 strain, where the genomic *Rho* gene is inactivated and a functional copy of *E. coli Rho* is supplied on a temperature-sensitive plasmid [19], mRuby fluorescence assay was performed after shifting and incubating the cultures to non-permissive temperature (42 °C) as described [19].

2.4. Western blot analysis

The concentration of proteins from the lysates Mtb and Msm were estimated by the Bradford method for both the wild type and Rho-CKD strains, in the presence and absence of aTc. Proteins were separated on 8% SDS-PAGE. Rho was detected by immunoblotting with 1/20000 dilutions of anti-MtbRho antisera and visualized by chemiluminescence. Blots were also stained with Ponceau S to depict the protein in each lane.

2.5. RNA isolation and real-time-quantitative PCR (RT-qPCR)

RNA was isolated using RNAZol-RT reagent (Sigma) from exponentially growing Mtb H37Rv/Msm mc^{2}155 and its Rho CKD strain. cDNA was synthesized by the Applied Bioscience cDNA synthesis kit following the manufacturer's instructions using the gene-specific reverse primer for *bfrB* and *mkl* and random primers for MtbRho, MsmRho, mRuby, and hygromycin resistant gene (Hyg^R). RT-qPCR was carried out in a BioRad CFX96 Touch Real-Time PCR Machine. To quantify the terminated and read-through transcripts, two primer pairs were designed targeting upstream and downstream region of the *bfrB* and *mkl* intrinsic terminators [13,25]. A standard curve was generated using the known concentration of *in vitro* transcribed *bfrB* and *mkl* RNA (Figure S1 and Table S2). Terminated and read-through transcripts of *bfrB* and *mkl* control and MtbRho-CKD strains were interpolated from the standard curve. RT-qPCR to compare the RNA levels of MtbRho, MsmRho, mRuby, and Hyg^R were carried out for control and Rho-CKD strains. Quantitation of Hyg^R in various conditions served as an indicator of

plasmid copy number [26]. 16SrRNA was used as an internal control.

2.6. ATPase assays

ATPase assays were carried with polycytidylic acid (poly C - 40 ng) or *in vitro* transcribed 3' untranslated region (UTR) of *mutT1, Rv3183, mkl, bfrB, metE, cyp144,* and *tuf* (300 μM) by T7 RNAP in the presence of 2 mM rNTP (Table S2). RNA substrates were incubated in T-Buffer (Tris HCl pH8 50 mM, MgOAc 3 mM, potassium glutamate 100 mM, DTT 0.1 mM, EDTA 0.1 mM, BSA 0.1 mg/ml, Glycerol 5%) in the presence of 100 nM MtbRho with or without 600 μM bicyclomycin (BCM) or ΔWQRRho. The reactions were initiated by the addition of 1 mM of ATP with 100 nCi of γ^{32}P-ATP followed by incubation at 37 °C for 30 min. The reactions were stopped by adding chloroform. Following centrifugation, the aqueous phase was used to estimate the release of inorganic phosphate (Pi) by resolving on polyethyleneimine thin-layer chromatography sheets using LiCl (1.2 M) and EDTA (0.1 mM) as the mobile phase and visualized with the Typhoon 9500(GE) Phosphorimager.

2.7. In vitro transcription

Intrinsic terminators of *mutT1, Rv3183, mkl, bfrB, metE, cyp144,* and *tuf* genes of Mtb, and from *E. coli,* intrinsic terminators of *aroG* and *ycbL* genes were selected for *in vitro* transcription assays (Table S2). DNA templates for transcription reactions were prepared as described [13]. 10 nM DNA template and 200 nM RNAP from Msm or *E. coli* were incubated in T-Buffer, first on ice followed by 10 min at 37 °C. MtbRho (100 nM) or MtbRho pre-incubated BCM (600 μM) or EcRho (100 nM) were added to the reaction mixture and incubated at 37 °C for 5 min. Transcription was initiated with the addition of 200 μM rNTPs (200 μM mix of rATP, rCTP, rGTP, and 20 μM rUTP) and 10 μCi α-^{32}P-UTP, incubated at 37 °C for 30 min and terminated by the addition of phenol-chloroform. Following centrifugation, an equal volume of gel-loading dye (95% deionized Formamide, 0.05% Bromophenol Blue, and 0.05% Xylene cyanol) was added to the aqueous phase, heated at 90 °C for 2 min, and resolved on 8% 8 M urea-PAGE. The gels were scanned and quantified using Typhoon 9500(GE) Phosphorimager and Multi Gauge V2.3 software respectively.

2.8. Termination assays using immobilized DNA templates

Assays were carried out using immobilized DNA templates as described [13]. Reactions were set up in duplicates. 10 nM biotinylated templates were incubated with streptavidin-coated agarose beads preequilibrated with T-buffer as described at 37 °C for 15 min. *In vitro* transcriptions were carried out in the presence and absence of 100 nM MtbRho at 37 °C for 30 min. One set was separated into supernatant and beads before processing and the other set was directly processed as described [13].

3. Results

3.1. Rho contributes to transcription termination at intrinsic terminators

The participation of RDT at an intrinsic terminator or preceding it to prevent transcriptional read-through at the 3′ UTR of genes has not yet been addressed. We surmised that a combined action of the two processes at a site is of importance especially when intrinsic terminators are inefficient by themselves to cause termination. First, we estimated the contribution of intrinsic terminator and Rho in termination by using a mRuby reporter fused downstream of an intrinsic terminator in a vector transformed in a Rho-CKD strain as described in Materials and Methods (Fig. 1A). For the analysis we employed non-canonical intrinsic terminators from Mtb (Table S2) [13]. To investigate Rho's contribution to termination with these terminators, the Msm Rho-CKD strain was constructed (Figure S2). In this *dcas9* based system, depleting the levels of Rho by increasing aTc concentration caused growth retardation and arrest (Figure S2B), confirming the essentiality of the gene in mycobacteria [4]. In the Rho depleted strain high levels of mRuby expression is observed in absence of intrinsic terminator. However, in presence of intrinsic terminator upstream of the reporter gene, the expression was significantly reduced indicating that intrinsic termination is decreasing mRuby expression (Fig. 1B, C, D, and Figure S3A) reducing the transcription read-through. The possibility that plasmid copy number is altered in Rho depleted cells contributing to the alteration in mRuby levels was examined by estimating Hyg^R transcripts in control and Rho depleted strain. Hyg^R transcripts remained unaltered in these experimental conditions indicating that the decrease in mRuby expression is due to the introduction of the terminator (Figure S3B). Employing this reporter system, we measured the termination efficiency contributed by both intrinsic terminator and Rho at the 3′ UTR of *tuf, cyp144,* and *bfrB* genes of Mtb. The difference in the percentage of mRuby expression in the presence and absence of intrinsic terminator in the normal and reduced level of Rho indicate the individual contribution of both the modes of termination **(**Figure S3C**).** For *tuf* intrinsic terminator, the termination efficiency was estimated to be 78 ± 2.7%. However, at depleted Rho levels, the efficiency reduced to 52.4 ± 3.9% (Fig. 1E), indicating the contribution of Rho in the termination of *tuf* gene. Rho-contributed increase in total transcription termination was also observed for *cyp144* and *bfrB* genes. In contrast, *rrnBT1* which harbours a strong intrinsic terminator [14] did not show any change in termination efficiency in the Rho-CKD background indicating that Rho has rather an insignificant role in transcription termination at strong terminators (Fig. 2 and Figure S4).

3.2. Rho prevents transcriptional read-through in genes with weak intrinsic terminators

To verify the above results, transcription read-through was assessed by RT-qPCR in MtbH37Rv. The experimental strategy is depicted in Fig. 3A and details are described in Materials and Methods. We estimated the terminated and read-through transcripts of *bfrB* and *mkl* genes which have intrinsic terminators that lack U-tract [13]. The levels of their transcripts were compared between wild type and Rho-CKD strain. By depleting Rho (Fig. 3B and C), the quantity of transcripts increased at the 3′ UTR of *bfrB* and *mkl* genes (Fig. 3D), pointing towards the role of Rho in suppressing transcriptional read-through at their 3' ends.

3.3. Rho activity at the 3' UTR of mutT1, Rv3183, mkl, bfrB, metE, cyp144, and tuf

Rho initiates its function by binding to *rut* (rho utilization) site. EcRho *rut* site essentially comprises C-rich, unstructured RNA [2]. Mycobacteria as such have G/C-rich genome and the nature of its *rut* site is not known. Our efforts to identify *rut* site have not provided any unambiguous sequence preference. Nevertheless, like *E. coli*, MtbRho shows ATPase activity with poly C substrates (Figure S5). To examine MtbRho activity with RNA substrates carrying intrinsic terminator, ATPase assays were carried out as described in Materials and Methods. The results presented in Fig. 4 show that MtbRho can hydrolyse ATP in the presence of these RNA substrates. The Rho specific ATPase activity was inhibited by BCM and ΔWQRRho showed no ATP hydrolysis (Fig. 4). These results

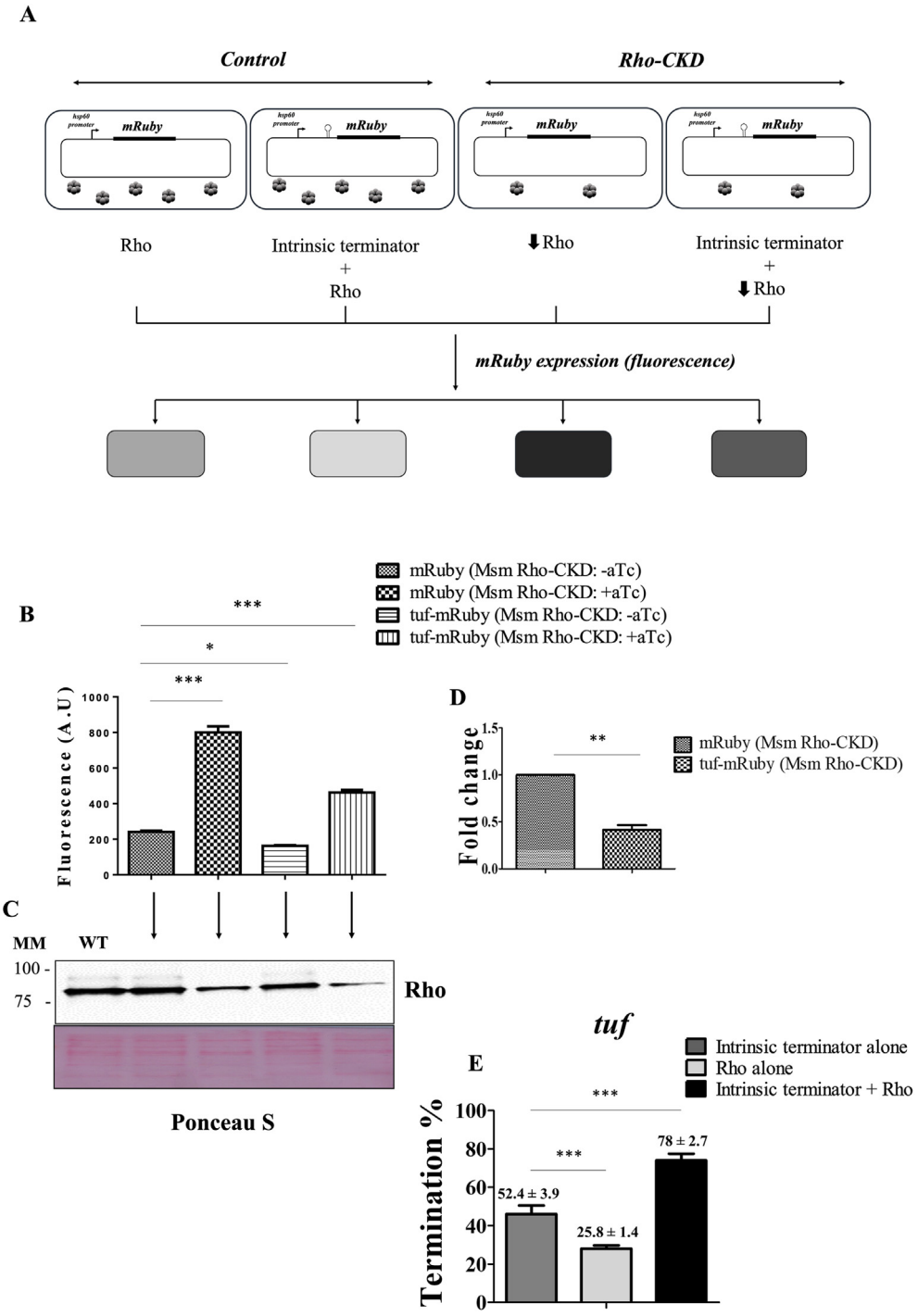
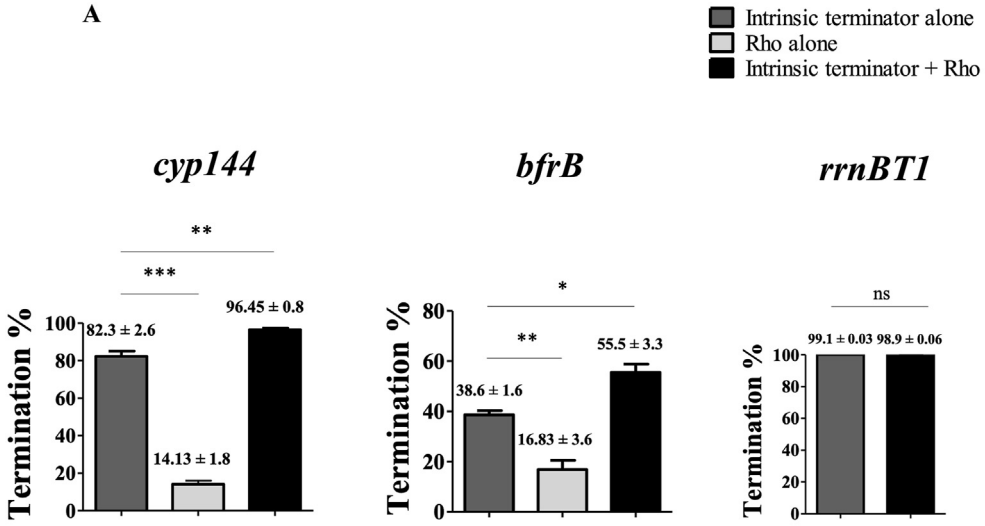


Fig. 1. Reporter assay to determine the contribution of intrinsic and RDT (A) Schematic representation of experimental strategy to estimate intrinsic and Rho-dependent termination (RDT) using mRuby fluorescence as a reporter. Dark to light shade in the schematic represents the intensity of mRuby expression in bacteria in the given conditions. represents Rho depletion. (B) Comparison of mRuby expression level in the presence and absence of *tuf* intrinsic terminator in Msm Rho-CKD strain at normal and depleted levels of Rho. (C and D) Immunoblotting and qRT-PCR representing Rho protein and mRuby RNA levels respectively. MM represents molecular marker (kDa) and Ponceau S staining is shown as a loading control. (E) Contribution of intrinsic terminator and Rho in transcription termination of *tuf* was estimated as a percentage of mRuby fluorescence from transcriptional read-through in Rho-CKD background, in the presence and absence of intrinsic terminator (mRuby Msm Rho-CKD + aTc) − (*tuf*-mRuby Msm Rho-CKD + aTc or − aTc)/(mRuby Msm Rho-CKD + aTc). The data represented is mean ± standard deviation (SD) from the three independent experiments. The statistics were calculated using one-way ANOVA followed by Tukey multiple-comparisons test, *p < 0.05, **p < 0.001,***p < 0.0001, 'ns' stands for not significant. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

A



B

Gene	% Termination efficiency	
	Intrinsic terminator	Intrinsic terminator + Rho
tuf	52.4 ± 3.9	78 ± 2.7
cyp144	82.3 ± 2.6	96 ± 0.8
bfrB	38.6 ± 1.6	55.5 ± 3.3
rrnBT1	99.1 ± 0.1	98.9 ± 0.1

Fig. 2. Transcription termination by intrinsic terminator and Rho at 3′UTR (A) Assessment of transcription termination efficiency for *cyp144, bfrB,* and *rrnBT1* genes using the mRuby fluorescence. The data represents the contribution of intrinsic terminator and Rho individually and together in transcription termination. The mean ± SD calculation and statistical analysis is performed same as described in Fig. 1E (B) The table depicts the impact of intrinsic terminator alone and with Rho in transcription termination for above genes.

suggest that MtbRho can use RNA substrates harboring an intrinsic terminator.

3.4. Rho-dependent transcription termination augments intrinsic termination

From the above results, it is apparent that Rho imparts its effect downstream of the 3' end of genes with non-canonical intrinsic terminator and transcription termination *in vivo* could be a culmination of contribution by Rho. To investigate the participation of Rho in transcription termination in templates containing intrinsic terminators, *in vitro* transcriptions were carried out with Msm RNAP and templates having T7A1 promoter (Fig. 5A). *In vitro*, termination efficiency solely depends on the strength of an intrinsic terminator in absence of Rho. When Rho is present, the additional contribution by the factor can be assessed.

In these assays, MtbRho substantially increased the transcription termination for all the tested templates (Fig. 5B). In templates containing *cyp144* intrinsic terminator, RDT was observed at the site of intrinsic termination. With *mutT1, and mkl* templates, RDT was observed downstream of the stop codon at different locations in addition to the intrinsic termination site. With *mkl*, RDT signals were found between the stop codon and the intrinsic terminators. However, with *mutT1*, RDT signals were also seen on either side of the intrinsic terminator, indicating wide landscape in the 3' UTR for Rho action. In the case of *Rv3183, bfrB, metE,* and *tuf* templates, intrinsic termination and RDT signals appeared at independent locations, with RDT preceding the structure. Nevertheless, their additive impact resulted in increased transcription termination (Fig. 5B). Rho inhibitor BCM reduced the RDT signals without affecting the intrinsic termination in these reactions. In assays with Rho mutant ΔWQRRho, no change in termination efficiency was observed (Figure S6A). To verify whether these RDT events result in the dissociation of transcripts, transcript release experiments were carried as described [23,24]. In assays with streptavidin bead-bound DNA templates, transcript release was observed in the supernatant fraction. The fraction of terminated transcripts increased in the presence of MtbRho (Figure S6B).

3.5. Intrinsic terminator and Rho collaborate for efficient termination in E. coli

Generally, the two modes of transcription termination are treated as stand-alone processes in *E. coli*. To assess their participation together, the above-described assays were performed in *E. coli*. mRuby fluorescence and *in vitro* transcription assays were

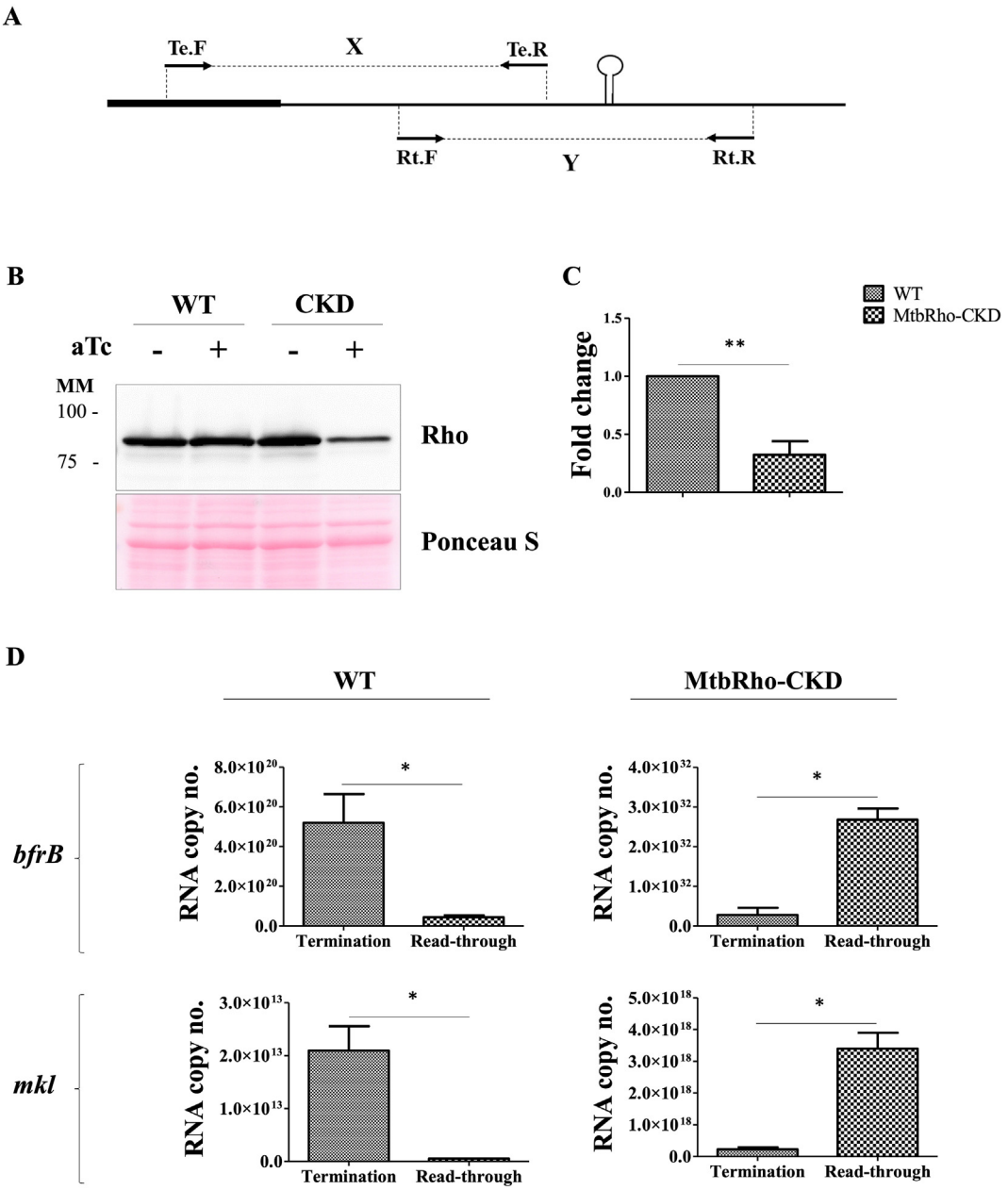


Fig. 3. Rho suppresses transcriptional read-through at intrinsic terminators. (A) Schematic depiction of the RT-qPCR experiment to quantify the terminated (X) and read-through (Y) transcripts in genes carrying intrinsic terminator. Te.F–Te.R and Rt.F-Rt.R represent the primer sets to measure terminated and read-through transcripts respectively. (B and C) Comparison of protein and RNA level of Rho between wild type (WT) and Rho-CKD strain of MtbH37Rv by western blotting and RT-qPCR. (D) Differential levels of the *bfrB* and *mkl* terminated and read-through transcripts between WT and Rho-CKD strains. The data represented is mean ± SD from the three independent experiments. The P-values were calculated by using unpaired, two-tailed *t*-test, *p < 0.05, **p < 0.005.

carried out with *E. coli* intrinsic terminators in the presence and absence of Rho. In addition to *rrnBT1*, previously identified *E. coli* intrinsic terminators *aroG* and *ycbL* [8] were chosen for these assays. The strength of these intrinsic terminators was assessed *in vivo* as the percentage of mRuby fluorescence in EcRho temperature-sensitive strain AMO14 [19] and KO strain RS1309 [20]. In these strains, mRuby expression was higher in the absence of Rho and/or when intrinsic terminator was not introduced (Fig. 6A and Figure S7A). However, when intrinsic terminators were introduced, mRuby expression was markedly reduced. Further reduction in the fluorescence signal in the presence of Rho suggest that transcription at the 3' end of *aroG* and *ycbL* genes is decreased by the additive effect of their intrinsic terminator and Rho (Fig. 6B and Figure S7B). In contrast, *rrnBT1* terminator show no dependence on Rho for termination. The difference in the mRuby fluorescence level in these two *E. coli* strains before the introduction of terminators appears to be due to the strain difference. While mRuby expression is driven by hsp60 promoter in both the strains, Rho induction in RS1309 is by IPTG, whereas in AMO14 it is thermo inducible. As a result high mRuby expression is seen in AMO14.

In vitro transcription assays with *E. coli* RNAP and DNA templates carrying *aroG* and *ycbL* intrinsic terminators showed a substantial increase in termination at the site of intrinsic termination when EcRho was added (Fig. 6C). The presence of BCM reduced the RDT signals. Together, the results from the *in vivo* fluorescence assays and *in vitro* transcription assays indicate that intrinsic terminator

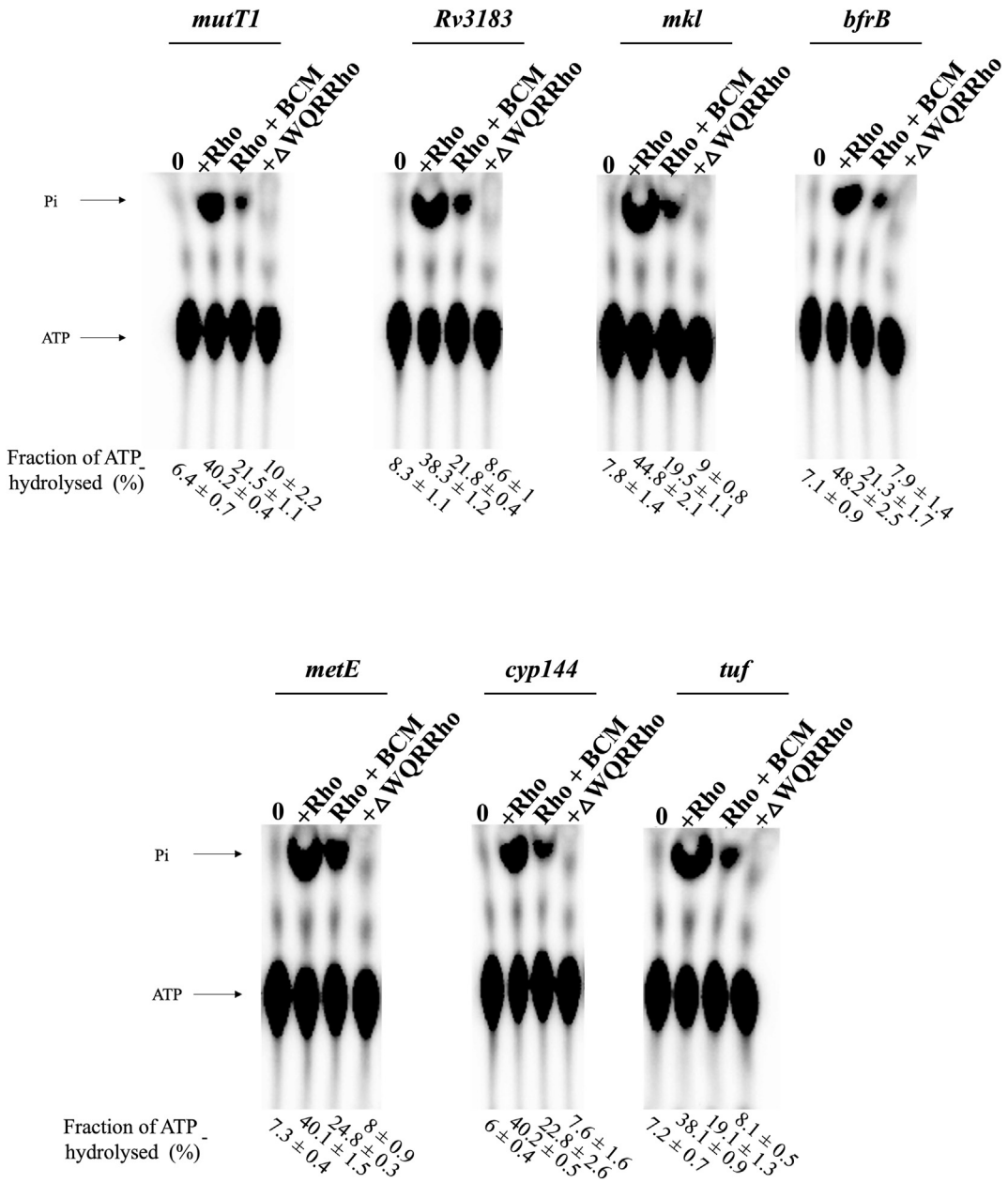


Fig. 4. ATP hydrolysis by MtbRho with RNA carrying intrinsic terminator. ATPase assay with MtbRho (100 nM) in the presence of *in vitro* transcribed RNA (300 μM) containing intrinsic terminators as described in Materials and Methods. Percentage fraction of inorganic phosphate (Pi) release was calculated and SDs were determined from three independent experiments.

and Rho function together to prevent unwanted transcriptional read-through in *E. coli* as well.

4. Discussion

We describe participation of both the modes of transcription termination wherein intrinsic terminators and Rho function together for more effective stop of RNA synthesis in a given 3′ UTR. The need for such collaboration is apparent as a majority of intrinsic terminators are inefficient in terminating transcription [13–15]. The interplay of the two processes seems necessary to enhance the overall efficiency of termination at the 3' end of genes to prevent transcriptional read-through. The *in vitro* transcription and *in vivo* reporter assays described here show that Rho participates in transcription termination at the intrinsic terminators or in the neighbouring regions.

Now it is apparent that suboptimal and weak terminators are found in *Bacillus* [15,27], *E. coli* [28] mycobacteria [13,14] and other species (Table S4) [6,29]. Variation in the length of stem, its strength (ΔG), and the number of U residues of any intrinsic terminator can influence its termination efficiency [30,31]. Hence, in addition to mycobacteria we also extended our studies to *E. coli* to see whether intrinsic terminator and Rho functional interaction is a more widespread phenomenon.

With their distinct mode of termination the two conserved mechanisms have been generally regarded as stand-alone mechanisms [1,2,32,33]. However, terminator inefficiency and the consequent read-through pointed at the contribution of additional

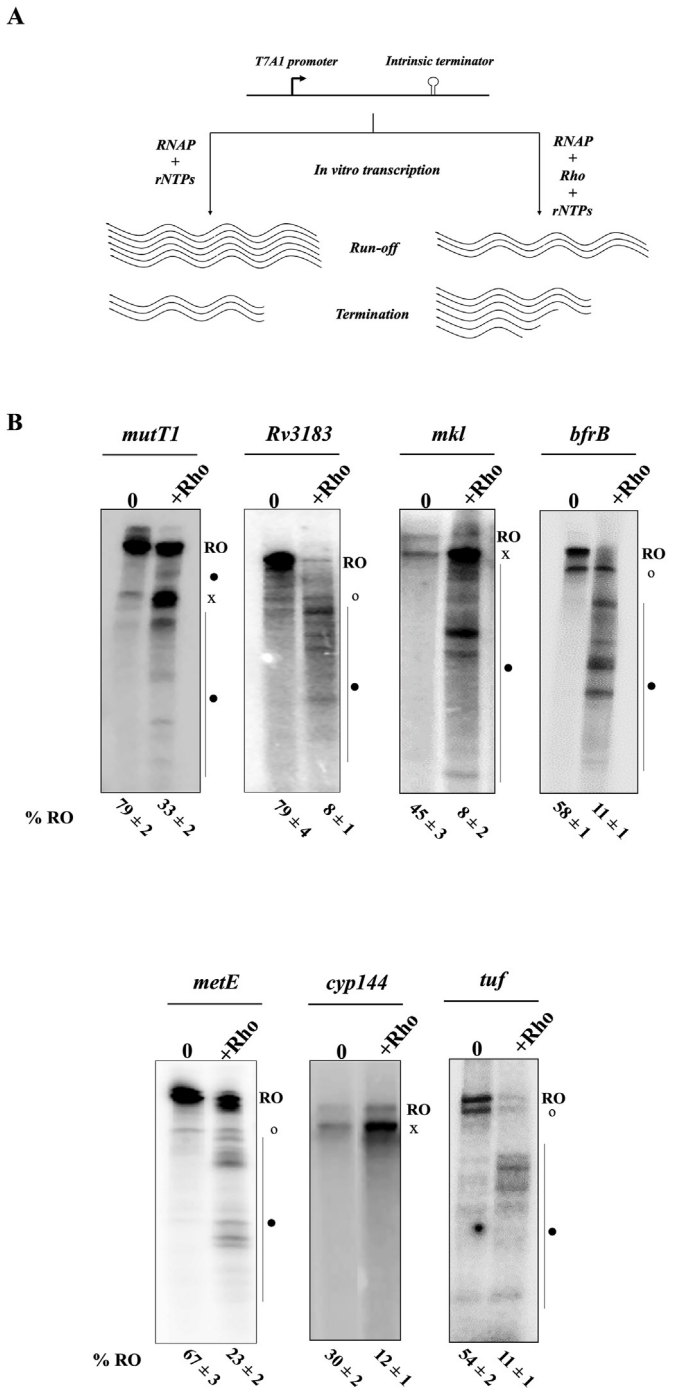


Fig. 5. Intrinsic terminator and Rho interact for transcription termination. (A) Schematic representation of *in vitro* transcription strategy (B) *In vitro* transcription assays were carried out using 10 nM linear DNA templates harboring intrinsic terminator (*mutT1, Rv3183, mkl, bfrB, metE, cyp144,* and *tuf*) downstream of T7A1 promoter in the presence and absence of MtbRho (100 nM) as described in Materials and methods. Transcripts were resolved on 6% 8 M urea-PAGE. RO corresponds to run-off transcripts. Intrinsic termination products are marked with open circles (o). RDT products are marked with closed circle (•). X represents the signal corresponding to the termination event by both intrinsic terminator and Rho together. Run-off percentage (% RO) were calculated ((Run-off)/(Run-off + Total termination))and represented with SD from three experimental replicates.

factors *in vivo*. Increase in intrinsic termination efficiency by NusA in *in vitro* reactions has been observed in *E. coli* [34]. Now, the role of NusA and/or NusG have been examined in *B. subtilis,* Mtb, and *Mycobacterium bovis* [14,15,27,35]. Suboptimal intrinsic terminators having lower number of U residues downstream of the stem serve as substrates for NusA and NusG in *B. subtilis* for efficient termination [15,27]. In contrast, Mtb NusG does not seem to enhance termination [35], while in *M. bovis*, it facilitates termination at non-canonical terminators [14]. As NusA and NusG are already known to modulate intrinsic termination contribution from other factors in improving the termination efficiency is not unanticipated. Indeed, in *B. subtilis* Rho null mutant, transcription read-through from inefficient intrinsic terminators of antisense transcripts was observed [36]. Rho participation described here is likely to be at a step after the formation of hairpin and pause based on prevailing understanding of Rho action [2,32,33,37]. Apart from the bonafide RDT sites, intrinsic terminator induced pausing of RNAP with or without the contribution of Nus factors can also facilitate Rho to act and terminate transcription at the intrinsic termination sites.

An additional role attributed to many stem-loop structures is in protecting the 3′ end of mRNA from the action of nucleases [8,28]. In one of the RNA 3′ ends mapping studies such structures were shown to protect from exonuclease action subsequent to RDT downstream [8]. While they suggested that the 3′ protected ends could serve as intrinsic terminators, the possibility was not examined [8,28]. In the other study, it was shown that *gal* mRNA is subjected to both intrinsic termination and RDT. The suboptimal intrinsic terminator of *gal* operon doubled up to protect the 3′ end from exonucleases, masking the detection of RDT downstream. Together the two mechanisms contribute to more effective termination at *gal* operon [28]. Although it was suggested that the two modes act independent of each other, from our data it is apparent that the signals are likely to be connected for better control of gene expression in some of the 3' UTRs.

Based on the present study, we considered different patterns of Rho action beyond the stop codons of the genes having weak intrinsic terminators (Fig. 6D) 1) intrinsic termination and RDT at distinct locations with RDT occurring first; 2) both at the same site; 3) RDT at the site of intrinsic termination and before; 4) RDT before, after, and at the intrinsic termination site; 5) RDT both at the site of intrinsic termination and downstream; 6) intrinsic termination and RDT at distinct locations with intrinsic termination occurring first. Among these possibilities, the data presented provide examples for the first four with mycobacterial intrinsic terminators and MtbRho. The 6th pattern was described by Wang et al. [28]. The 5th pattern is likely to be found if more terminators are analysed across the bacterial domain.

To conclude, the study of transcription termination has come a full circle. From the emerging theme it is apparent that even in the well-studied systems such as *E. coli* and *B. subtilis* intrinsic termination can be achieved with non-canonical structures. Although initially described in mycobacteria, it appears that non-canonical intrinsic terminators are found across bacterial kingdom. In many of these sites, a more efficient termination is brought about by the collaboration with Rho or other elongation factors like NusA and NusG. It remains to be seen whether Rho can combine with other elongation factors to ensure transcriptional read-through is curtailed. The association of NusA, NusG, and Rho with RNAP in recently elucidated structures [33,37], should facilitate in understanding their combined function in transcription elongation and termination.

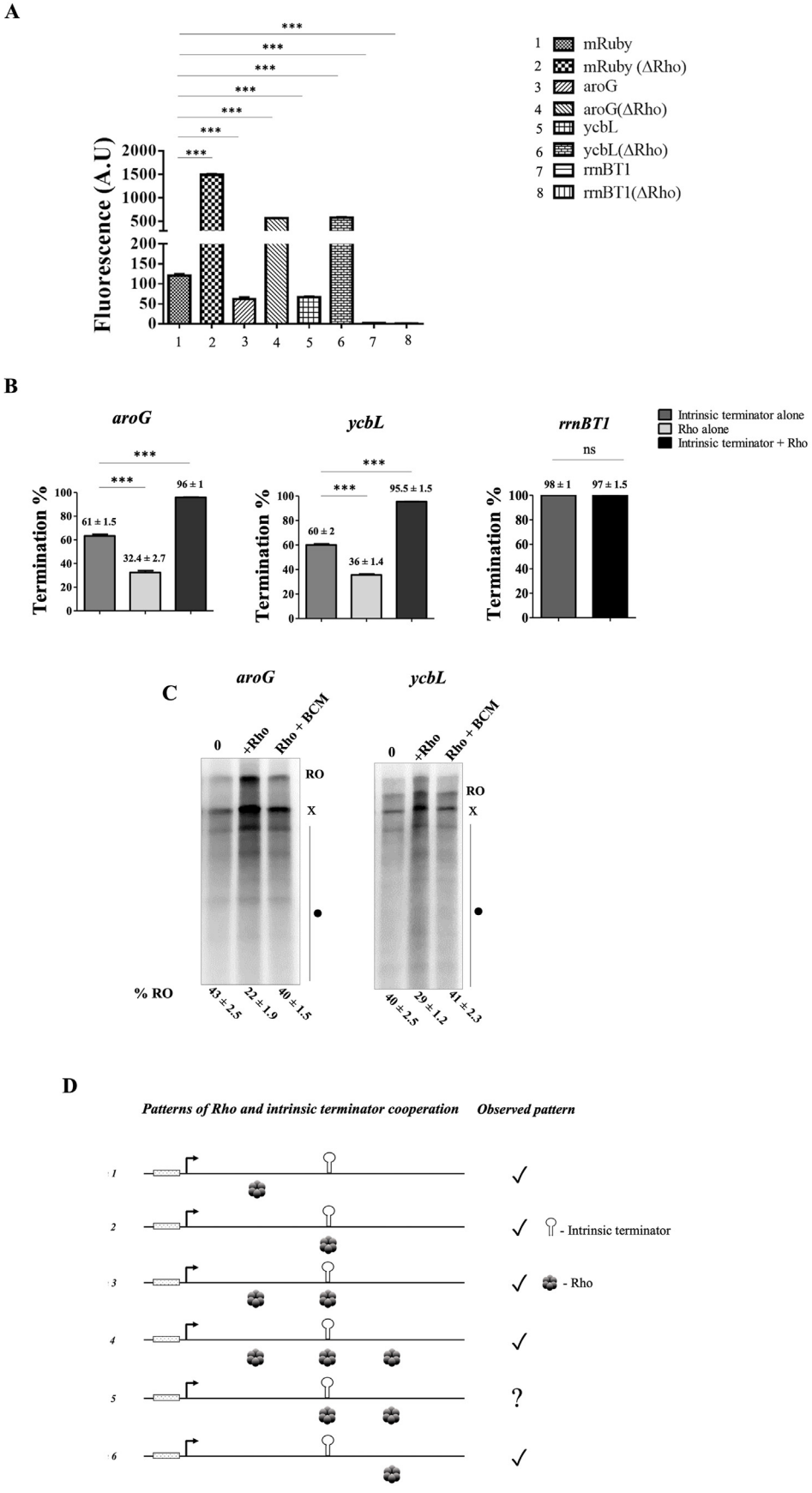


Fig. 6. Measurement of contribution of intrinsic terminator and Rho in *E. coli* (A) Comparison of mRuby expression level for *aroG*, *ycbL*, and *rrnBT1* intrinsic terminators, in the presence and absence of Rho, in *E. coli* AMO14 strain. (B) Estimation of termination efficiency and contribution of the two modes to transcription termination for *aroG*, *ycbL*, and *rrnBT1* genes using mRuby fluorescence. The mean ± SD calculation and statistical analysis performed here is same as described in Fig. 1E (C) *In vitro* transcription assays were carried out using 10 nM linear DNA templates harboring *aroG* or *ycbL* intrinsic terminator as described in Materials and Methods. RDT products are marked with closed circle (•). X represents termination product by both intrinsic terminator and Rho together. RO is run-off transcripts. Run-off percentage (%RO) were calculated as described above. (D) Schematic depiction of various Rho and intrinsic terminator interactions in transcription termination. Rho and intrinsic terminator positions in the figure depicted represent their site of action at the UTR. Tick mark (✓) highlights the Rho − intrinsic terminator cooperation pattern observed so far. Question mark indicates lack of examples for the pattern.

Declaration of competing interest

The authors declare no conflict of interest with the contents of this article.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.bbrc.2022.08.063.

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