Peer Review File

Manuscript Title: Structural basis of Rho-dependent transcription termination

Reviewer Comments & Author Rebuttals

Reviewer Reports on the Initial Version:

Referee expertise:

Referee #1: cryo-EM, transcription

Referee #2: bacterial transcription termination

Referee #3: cryo-EM, transcription

Referees' comments:

Referee #1 (Remarks to the Author):

This work by Molodtsov et al. describes several cryo-EM structures of Rho-dependent transcription termination complex. This work comes against a backdrop of two recent and highly controversial structural studies (published in Science and Molecular Cell) that sought to challenge the decades-old understanding of this process. The models put forth by those two studies failed to reconcile the vast biochemical, genetic, and structural findings of dozens of other group. Particularly concerning observation from these two studies was the state of Rho helicase, which was in open conformation and inverted orientation with respect to RNAP, which is contrary to the logical expectation, and which was not in a catalytically productive conformation.

The present study refreshingly clears the air around this confusion. The EM work provides a view of the Rho-TEC complex that agrees with and reconciles the textbook understanding that has been painstakingly built up from 40 years' worth of previous experiments. Importantly, the complexes generated here were tested for their ability to undergo Rho-dependent termination (something the two other papers did not do), confirming that the models correspond to relevant functional complexes. The study further provides unexpected and important new insights into the mechanism of Rho-dependent termination complex, including defining a new role of transcription elongation factor NusG as a stator for the Rho ATPase motor. Given its significance, the work should be published in Nature pending some minor revisions.

Comments

• Did authors see any sub-populations of alternate particle conformations in their dataset that might be of value in understand Rho/NusG mechanism?

• Figure S6 is very important for clarifying the state of the field and should be included in the main

text.

• The electron density in the PBS region of Rho is lower in all complexes and particularly in absence of PBS ligand. The authors remark that the lack of PBS ligand does not seem to have major influence on the conformation – given the resolution limitations, this statement should be better qualified.

• In Figure S1, it's interesting that dC5 and dC15 do not elicit a response compared to dC75 or λtR1. This implies either that RNA connectivity between PBSs is important or that perhaps the short RNAs weren't bound as stably due to a lack of cooperativity. Did the authors try to titrate to very high levels of dC5 or dC15 to see whether they might eventually be able to stimulate termination activity?

• In the RNA release scaffold assay for Rho-dependent termination as described in Figure S1A, the product in presence of λTR1 PBS-ligand shows one major band in case of complimentary DNA non template and template strands. However, the when using dC75 as PBS-ligand, laddering effect can be seen suggesting an imprecise termination stop. Considering that structurally TEC-Rho complex using both ligands look similar, is it possible to reconcile this difference?

• In the RNA extension scaffold assay for Rho-dependent termination as described in Figure S2A and S2B, the bands representing the termination in presence of λTR1 and dC75 ligand are different, with laddering in presence of dC75 ligand. Please comment.

• In Figure S2B, the λTR1 PBS-ligand appears to act as a better termination stop than dC75 in non-complementary DNA non template and template strands. Also, λTR1 PBS-ligand seems to lose potency to induce termination stop upon increasing the length of linker region. Please comment.

• It has been noted that Rho along with NusG can terminate the transcripts lacking rut sites or other sub-optimal transcripts (Peters et al, Genes Dev, 2012). How do authors reconcile this activity with structural arrangement where rut seems to help order the PBS region?

• In Figure S11 and associated text: considering that the resolution of Rho in general is low, it appears that features for both the nucleotide and the associated loop should be taken into account when assigning ATPase status. This could be more explicitly mentioned in the methods or text.

• Consistent with the last two statements of the second paragraph of page 6, it has been shown (Laswon et al, PNAS ,2016) that the addition of PBS ligands does indeed shift Rho toward a closed ring state and allow for more stable binding of the SBS substrate. Similarly, on pages 10-11, it could be noted that the ATPase cycle follows that suggested for Rho previously (Thomsen et al. Cell 2009) and ring helicases in general (Enemark, Nature, 2007)

• On page 10, 2nd paragraph, it should read as "thus is the biding site at which ATP hydrolysis is predicted to occur first." The typo in word binding should be corrected.

• On page 24, Material and methods section reads as "as described (2?)". This reference should be corrected.

Referee #2 (Remarks to the Author):

Molodtsov and colleagues provide a cryo-EM structural analysis of an E. coli transcription elongation complex with the termination factor Rho poised to disrupt the complex at a Rho-dependent terminator. The structure features a complete set of molecular components–RNA polymerase, the accessory factor NusG, RNA, and DNA–and strongly validates decades of biochemical and structural analysis of the mechanism of termination by Rho. In particular, it illustrates the binding of Rho to RNA polymerase in a configuration that enables the well-documented RNA helicase activity of Rho to disrupt the transcription complex. As the authors clearly establish, this structure is completely consistent with essentially all prior data about Rho function, including particularly the involvement of the universally conserved transcription factor NusG in termination. This structure is completely consistent with the notion that translation-coupled transcription and termination by Rho of transcription without translation are alternate and mutually exclusive states of the transcription complex.

The paper is particularly important because two recent publications present radically different structures of what are proposed to be pre-termination complexes of Rho with RNA polymerase (references 31 and 32 of Molodstov et al.). As Molodtsov et al. definitively argue, the structures of these papers are inconsistent with all expectation of the nature of a pre-termination complex, and inconsistent as well with the known function of NusG in termination and in translation-transcription coupling. And as Molodstov et al. further argue, neither of the two prior papers established that the complexes they analyzed are active in termination. In contrast, the complex assembled and analyzed by Molodstov et al. is shown clearly to be active in termination. Thus, it is important that the current paper, which almost certainly illustrates a correct and critical structure in termination, be prominently published.

Besides the issue just described, Molodstov et al. is a highly impressive work of science and a definitive analysis of an important process. The paper is rich in discovery. It strongly advances understanding the interaction of Rho with RNA, a subject of considerable previous structural analysis. This paper reveals the entire path of ~80 nucleotides of RNA interacting with Rho, including important base-specific interactions; previous important work had shown a more limited interaction of two base segments. The analysis produces a new consensus sequence for RNA that binds the primary site of Rho and activates the helicase activity. Previous work had produced a model of successive conformation changes around the Rho hexamer that drive helicase activity; Molodstov et al. relate these changes specifically to the Rho-NusG-RNA polymerase configuration structure so as to explain how Rho exerts force to pull RNA from the complex. Molodstov et al. show interaction of NusG bound to RNA polymerase with two particular monomers of the Rho hexamer so as to orient the whole process of helicase activity. The arguments of the paper are relentlessly persuasive.

The reviewer has no expertise in stuctural analysis and cannot comment on that methodology.

I have no substantive criticisms, and only a couple of very minor points.

1. There appears to be more NusG dependence for Rho function than in most assays; were conditions chosen to optimize this?
2. p. 5, line 9: Should the references be 22-23?
3. p. 7, 7 lines from bottom: maybe “channel” for “canne”

Referee #3 (Remarks to the Author):

The manuscript investigates how Rho, responsible for Rho-dependent transcription termination in bacteria, interacts with transcription elongation complex (TEC) and uses its ATPase activity to perform transcription termination. The authors used synthetic nucleic acid scaffolds that contain RNA transcripts of sequences shown to bind to Rho secondary binding site (SBS), and uses two RNA sequences added in trans, that contain sequences bound to Rho primary binding site (PBS). One sequence is derived from a natural promoter and one is a sequence mimic of dC75 (typical PBS-ligands are ~80 nt and C rich). The authors demonstrated that under these conditions, the complex can perform Rho-dependent transcription termination in a NusG, PBS ligand and ATP dependent fashion. The authors then captured the termination complexes in the presence of Mg-ADP-BeFx, previously shown to be able to trap Rho in a hydrolysis competent hexamer conformation. The authors obtained cryoEM structures of these complexes to ~4.1 – 4.3 Å resolution. The structures reveal how PBS-ligand and SBS-ligand bind to Rho and how Rho interacts with RNAP and NusG. Their structures contrast with two recent structural studies of the Rho-dependent complex and are in agreement with the mechanistic model derived from genetic and biochemical studies over the last few decades. Significantly, based on the interactions observed in the structures between Rho and PBS RNA, the authors derived detailed consensus PBS-ligand sequences. The results presented here are a significant advance in understanding how transcription termination occurs, reveal the consensus sequence and reconcile and provide molecular basis for decades of genetic and biochemical data. Importantly, it corrects the recently proposed models on Rho-dependent transcription termination and restores and provides molecular basis for the textbook model. The work is conducted with high standards and the manuscript is well written. I only have a few comments/things for the authors to clarify before it is accepted for publication.

1. Rho is a hexamer helicase belonging to the RecA family ATPase. Mechanistically Rho is more closely related to AAA+ ATPase, rather than F1-ATPase which contains alternating α and β subunits with only β subunits being active ATPase while every subunit in Rho is active. The opening paragraph should be re-written.

2. The authors used two separate RNA ligands, one binding to Secondary Binding Site (SBS) and another one to the primary binding site (PBS) for both in vitro and structural studies. Have the authors tried to use a continuous RNA ligand, which would have occurred under physiological

conditions, in their in vitro experiments ? What are the challenges ?

3. Given that the structural arrangement without PBS-ligand looks similar to that with PBS-ligand, the authors suggest that Rho assembly does not depend on PBS-ligand, which contradicts with the classical model (Fig. 1A) that suggests binding to PBS-ligand is the first step to assemble the termination complex, presumably because the affinity of PBS-ligands are much higher to Rho than SBS-ligands. Have the authors tested the relative binding with PBS- and SBS-ligand sequences they used? Could the assembly in the absence of PBS-ligand be due to the synthetic scaffold which contains the SBS ligand, and the in vitro conditions with high concentrations of Rho and NusG, thus allowing the complex to form? During actual transcription termination, PBS and SBS-ligand exist within one RNA molecule and indeed PBS-ligand sequence would emerge from the TEC first, recruiting Rho through high affinity binding and stabilising the Rho hexamer once all six sites are bound. Rho would thus be recruited to the TEC before being stabilised by NusG and binding to SBS-sequence. Please clarify and revise the interpretation of the structure of the complex in the absence of PBS-ligand. What happens when SBS-ligand is missing by incorporating random sequences ? I assume that it might still assembly although might not perform translocation ?

4. In Figure 4, it might be helpful to include a cartoon that relates the changes in Rho monomers to the RNA connecting to TEC as in Figure 1, similar to many cartoons used in describing AAA+ translocases. Based on the mechanisms, is there an optimal length between the SBS and PBS sequences ?

5. Can the authors comment on potential effects of NusA on the complex assembly and structures, as NusA is missing in this study while present in the other structural studies ?

6. Given the low resolution and indeed the electron density shown in Figure S11B, the authors can not be certain about the nucleotide states and hence the section describing the detailed hydrolysis cycle and mechanisms of translocation should be toned down. Based on the arrangement of Rho, the direction of RNA and the known translocase mechanisms of Rho, the authors can confidently deduce the direction of translocation of RNA, thus the consequences on the TEC, without detailed assignment of specific subunit's nucleotide states, which the data do not support.

7. Page 4 last paragraph: ADP.BeF does not necessarily represent the "ground" state. Indeed in previous Rho structures, having ADP.BeF allowed the authors to capture/mimic all nucleotide states due to the flexibility in its coordination (apo, ADP, ADP.BeFx – ATP bound or hydrolysis transition state).

8. Figure S1-2, lane labelling schemes should be standardised using +/- scheme.

9. Page 8, "Model building…. That 3' end of PBS ligand could be connected to the 5' end of the PBS ligand through an RNA connector having a minimal length of 10-11 nt". Do the authors mean the "3' end of PBS ligand could be connect to the 5' end of the SBS ligand" ? This should be marked on the figure.

10. Page 9 "In parallel structure determination experiments performed in the presence and absence

of NusG" - please show 2D or 3D classes.

11. Figure S2. Can the authors comment on (B), with n>7, the efficiency of termination seems to go down (comparing the top band intensity), can the structures explain these data ?

12. Figure S3E,4E,5E - local resolution map focusing on the Rho ring, looking at the hexamer plane itself should be shown to assess the quality of the nucleotide binding sites.

13. A pre-print of a termination complex of T. thermophilus has been deposited (https://www.biorxiv.org/content/10.1101/2022.09.02.506315v1.full). Please comment and compare the structures.

Author Rebuttals to Initial Comments:

RESPONSES TO REVIEWERS

Reviewer 1:

This work by Molodtsov et al. describes several cryo-EM structures of Rho-dependent transcription termination complex. This work comes against a backdrop of two recent and highly controversial structural studies (published in Science and Molecular Cell) that sought to challenge the decades-old understanding of this process. The models put forth by those two studies failed to reconcile the vast biochemical, genetic, and structural findings of dozens of other group. Particularly concerning observation from these two studies was the state of Rho helicase, which was in open conformation and inverted orientation with respect to RNAP, which is contrary to the logical expectation, and which was not in a catalytically productive conformation.

The present study refreshingly clears the air around this confusion. The EM work provides a view of the Rho-TEC complex that agrees with and reconciles the textbook understanding that has been painstakingly built up from 40 years' worth of previous experiments. Importantly, the complexes generated here were tested for their ability to undergo Rho-dependent termination (something the two other papers did not do), confirming that the models correspond to relevant functional complexes. The study further provides unexpected and important new insights into the mechanism of Rho-dependent termination complex, including defining a new role of transcription elongation factor NusG as a stator for the Rho ATPase motor. Given its significance, the work should be published in Nature pending some minor revisions.

We thank the reviewer for the favorable assessment.

Specific comments

• Did authors see any sub-populations of alternate particle conformations in their dataset that might be of value in understand Rho/NusG mechanism?

We did not. In particular, we looked for but did not find, subpopulations that possibly correspond to the structures reported in the 2021 *Science* and *Molecular Cell* papers (refs. 30 and 31 of the current version).

• Figure S6 is very important for clarifying the state of the field and should be included in the main text.

We agree with the reviewer that Fig. S6 is important. But space limitations preclude us from moving a display item from extended data to the main text.

• The electron density in the PBS region of Rho is lower in all complexes and particularly in absence of PBS ligand. The authors remark that the lack of PBS ligand does not seem to have major influence on the conformation – given the resolution limitations, this statement should be better qualified.

We address this point in the current version (page 5 paragraph 3): "The lower map quality, especially for Rho, in our structures of complex not having a PBS ligand precludes defining details of the PBS-ligand-dependent change in conformation or dynamics, but is consistent with a PBS-ligand-dependent decrease in dynamics."

• In Figure S1, it's interesting that dC5 and dC15 do not elicit a tR1. This implies either that RNA response compared to dC75 or connectivity between PBSs is important or that perhaps the short RNAs weren't bound as stably due to a lack of cooperativity. Did the authors try to titrate to very high levels of dC5 or dC15 to see whether they might eventually be able to stimulate termination activity?

We address this point in the current version (legend to Fig. S1): "The PBS ligands λtR1 *rut* and dC75 support efficient termination under these conditions (lanes 1 and 2 of each gel panel), but the shorter PBS ligands dC5 and dC15 do not (lanes 1 and 2 of each gel panel). It has been shown previously that dC15 and dC5 exhibit lower binding affinities for Rho than dC75, exhibiting half-maximally saturating concentrations 30-fold and 150-fold, respectively, the half-maximally saturating concentrations for dC75.[21] "

• In the RNA release scaffold assay for Rho-dependent termination as described in Figure S1A, the product in presence of λTR1 PBS-ligand shows one major band in case of complimentary DNA non template and template strands. However, the when using dC75 as PBS-ligand, laddering effect can be seen suggesting an imprecise termination stop. Considering that structurally TEC-Rho complex using both ligands look similar, is it possible to reconcile this difference?

We address this point in the current version (legend to Fig. S1): "The PBS ligand λtR1 rut supports efficient and immediate termination under these conditions; with λtR1 rut, in both the experiments of (a) and the experiments of (b), ~100% of RNA is released before RNA extension (lane 1 of each gel panel). The PBS ligand dC75 supports efficient but less immediate termination under these conditions; with dC75, in the experiments of (b), ~100% of RNA is released before RNA extension (lane 1 of each gel panel), but, in the experiments of (a), ~30% of RNA is released before RNA extension, and ~70% of RNA is released only after RNA extension by 1 nt or 2 nt (lane 2 of each gel panel). The results indicate that both λtR1 rut and dC75 are effective PBS ligands and that λtR1 *rut* is a more effective PBS ligand than dC75 (see Fig. S2)."

• In the RNA extension scaffold assay for Rho-dependent termination as described in Figure S2A and S2B, the bands representing the termination in presence of λTR1 and dC75 ligand are different, with laddering in presence of dC75 ligand. Please comment.

We address this point in the current version (legend to Fig. S2): "The PBS ligand λtR1 *rut* supports efficient and immediate termination under these conditions; with λtR1 *rut*, ~100% of RNA synthesis ceases before RNA extension (lane 1 of each gel panel). The PBS ligand dC75 supports efficient but less immediate termination under these conditions; with dC75, ~30% of RNA synthesis ceases before RNA extension, and ~70% of RNA synthesis ceases only after RNA extension by 1 nt or 2 nt (lane 2 of each gel panel). The results indicate that both λtR1 *rut* and dC75 are effective PBS ligands and that λtR1 *rut* is a more effective PBS ligand than dC75 (see Fig. S1)."

• In Figure S2B, the λTR1 PBS-ligand appears to act as a better termination stop than dC75 in non-complementary DNA non template and template strands. Also, λTR1 PBS-ligand seems to lose potency to induce termination stop upon increasing the length of linker region. Please comment.

We address the first part of this point in our response to the previous point (see above).

We address the second part of this point in the current version (legend to Fig. S2): "All six tested U-rich-RNA-segment lengths (n = 4, 5, 6, 7, 8, and 9 codons) support termination. In the experiments of (a), the six tested U-rich-RNA-segment lengths support termination comparably efficiently. In the experiments of (b), shorter lengths (n = 4, 5, and 6 codons) support termination more efficiently than longer lengths (n = 7, 8, and 9 codons)."

• It has been noted that Rho along with NusG can terminate the transcripts lacking rut sites or other sub-optimal transcripts (Peters et al, Genes Dev, 2012). How do authors reconcile this activity with structural arrangement where rut seems to help order the PBS region?

The relaxed sequence specificity of Rho and limited sequence requirements for PBS-ligand function ("previously essentially limited to the requirement for a ~50-80 nt sequence rich in C and poor in G[1-4,9]") imply that many RNA sequences can function as weakly active PBS ligands.

• In Figure S11 and associated text: considering that the resolution of Rho in general is low, it appears that features for both the nucleotide and the associated loop should be taken into account when assigning ATPase status. This could be more explicitly mentioned in the methods or text.

We explicitly mention both Mg-ADP-BeF_3 and associated protein segments in the current version (page 8 paragraph 3): " highest occupancy and order of Mg-ADP-BeF3 and associated protein segments".

• Consistent with the last two statements of the second paragraph of page 6, it has been shown (Laswon et al, PNAS ,2016) that the addition of PBS ligands does indeed shift Rho toward a closed ring state and allow for more stable binding of the SBS substrate. Similarly, on pages 10-11, it could be noted that the ATPase cycle follows that suggested for Rho previously (Thomsen et al. Cell 2009) and ring helicases in general (Enemark, Nature, 2007)

We cite Lawson et al. 2016 (ref. 21) at the mentioned location in the current version (page 5 paragraph 3): "We hypothesize, consistent with previous data[21], that the PBS ligand facilitates Rho-dependent termination allosterically, by inducing a conformational or dynamic state of Rho competent for ATP-dependent translocation."

We cite Thomsen et al, 2016 (ref. 22) at the mentioned location in the current version (page 9 paragraph 3): "Our structures, together with the ATPase cycle proposed for Rho[22], suggest the following mechanism for ATP-dependent translocation by Rho (Fig. 4e)."

• On page 10, 2nd paragraph, it should read as “thus is the biding site at which ATP hydrolysis is predicted to occur first.” The typo in word binding should be corrected.

The typo has been corrected.

• On page 24, Material and methods section reads as “as described (2?)”. This reference should be corrected.

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polymerase with two particular monomers of the Rho hexamer so as to orient the whole process of helicase activity. The arguments of the paper are relentlessly persuasive.

The reviewer has no expertise in structural analysis and cannot comment on that methodology.

I have no substantive criticisms, and only a couple of very minor points.

We thank the reviewer for the favorable assessment.

Specific comments:

1. There appears to be more NusG dependence for Rho function than in most assays; were conditions chosen to optimize this?

Yes. Conditions were chosen to optimize the NusG-dependence of Rho function.

2. p. 5, line 9: Should the references be 22-23?

The typo has been corrected.

3. p. 7, 7 lines from bottom: maybe “channel” for “canne”

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Reviewer 3:

The manuscript investigates how Rho, responsible for Rho-dependent transcription termination in bacteria, interacts with transcription elongation complex (TEC) and uses its ATPase activity to perform transcription termination. The authors used synthetic nucleic acid scaffolds that contain RNA transcripts of sequences shown to bind to Rho secondary binding site (SBS), and uses two RNA sequences added in trans, that contain sequences bound to Rho primary binding site (PBS). One sequence is derived from a

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Specific comments:

1. Rho is a hexamer helicase belonging to the RecA family ATPase. Mechanistically Rho is more closely related to AAA+ ATPase, rather than rather than F1-ATPase which contains alternating X and X subunits with only X subunits being active ATPase while every subunit in Rho is active. The opening paragraph should be re-written.

We deleted the paragraph in question when preparing the current version..

2. The authors used two separate RNA ligands, one binding to Secondary Binding Site (SBS) and another one to the primary binding site (PBS) for both in vitro and structural studies. Have the authors tried to use a continuous RNA ligand, which would have occurred under physiological conditions, in their in vitro experiments ? What are the challenges ?

We address this point in the current version (page 11, paragraph 3): "Key priorities for future work include determination of a structure of a Rho pre-termination complex containing a continuous RNA extending from the PBS ligand to the RNA 3' end (as opposed to the current complexes, in which the PBS ligand is provided *in trans*; Figs. 1b-3)."

We currently are preparing an NIH grant application, for submission on 11/07/2022, that proposes, as part of one Aim, determination of a structure of a Rho pre-termination complex containing a continuous RNA extending from the PBS ligand to the RNA 3' end.

3. Given that the structural arrangement without PBS-ligand looks similar to that with PBS-ligand, the authors suggest that Rho assembly does not depend on PBS-ligand, which contradicts with the classical model (Fig. 1A) that suggests binding to PBS-ligand is the first step to assemble the termination complex, presumably because the affinity of PBS-ligands are much higher to Rho than SBS-ligands. Have the authors tested the relative binding with PBS- and SBS-ligand sequences they used? Could the assembly in the absence of PBS-ligand be due to the synthetic scaffold which contains the SBS ligand, and the in vitro conditions with high concentrations of Rho and NusG, thus allowing the complex to form? During actual transcription termination, PBS and SBS-ligand exist within one RNA molecule and indeed PBS-ligand sequence would emerge from the TEC first, recruiting Rho through high affinity binding and stabilising

the Rho hexamer once all six sites are bound. Rho would thus be recruited to the TEC before being stabilised by NusG and binding to SBS-sequence. Please clarify and revise the interpretation of the structure of the complex in the absence of PBS-ligand. What happens when SBS-ligand is missing by incorporating random sequences ? I assume that it might still assembly although might not perform translocation ?

Under our conditions, assembly of Rho-NusG-TEC complex does not require a PBS ligand. Under our conditions, assembly requires only Rho, NusG, and a TEC that contains a U-rich RNA 5' extension able to serve as SBS ligand.

In the current version, we clarify our conclusion that the PBS ligand is not essential for assembly of the Rho-NusG-TEC complex, but is essential for subsequent steps in Rho-dependent termination by adding the qualifier "under our conditions" (page 5, paragraph 3): "This finding, together with our finding that the PBS ligand is required for Rho-dependent termination with our sequences and conditions (Figs. 1b, right, S1-S2), implies that, under our conditions, the PBS ligand is not essential for assembly of the Rho-NusG-TEC complex, but is essential for subsequent steps in Rho-dependent termination. "

4. In Figure 4, it might be helpful to include a cartoon that relates the changes in Rho monomers to the RNA connecting to TEC as in Figure 1, similar to many cartoons used in describing AAA+ translocases. Based on the mechanisms, is there an optimal length between the SBS and PBS sequences ?

We address the question of the length of the RNA segment between the PBS and SBS ligands in the current version (page 7, paragraph 4) "Model building based on our structures indicates that the 3' end of the PBS ligand can be connected to the 5' end of the SBS ligand through an RNA connector having a minimum length of 10-11 nt. Model building further suggests that there is freedom for bulging, looping, and/or secondary-structure formation in the RNA connector between the 3' end of the PBS ligand and the 5' end of the SBS ligand, and thus suggests the connector could accommodate longer lengths of RNA."

5. Can the authors comment on potential effects of NusA on the complex assembly and structures, as NusA is missing in this study while present in the other structural studies ?

We address this point in the current version (page 11, paragraph 3): "Key priorities for future work include...determination of a structure of a complex that includes the transcription elongation factor NusA"

We currently are preparing an NIH grant application, for submission on 11/07/2022, that proposes, as part of one Aim, determination of a structure of a Rho pre-termination complex containing NusA.

6. Given the low resolution and indeed the electron density shown in Figure S11B, the authors can not be certain about the nucleotide states and hence the section describing the detailed hydrolysis cycle and mechanisms of translocation should be toned down. Based on the arrangement of Rho, the direction of RNA and the known translocase mechanisms of Rho, the authors can confidently deduce the direction of translocation of RNA, thus the consequences on the TEC, without detailed assignment of specific subunit's nucleotide states, which the data do not support.

We deleted the paragraph on ATP binding site states when preparing the current version. In addition, we replaced "Our structures suggest" by "Our structures, together with the ATPase cycle proposed for Rho[22], suggest" at the start of the paragraph discussing the mechanism of ATP-dependent translocation by Rho when preparing the current version.

7. Page 4 last paragraph: ADP.BeF does not necessarily represent the "ground" state. Indeed in previous

Rho structures, having ADP.BeF allowed the authors to capture/mimic all nucleotide states due to the flexibility in its coordination (apo, ADP, ADP.BeFx – ATP bound or hydrolysis transition state).

We deleted the words "ground state" and the reference to "ground state" when preparing the current version..

8. Figure S1-2, lane labelling schemes should be standardised using +/- scheme.

We do not agree with the reviewer that a revised lane labelling scheme would be clearer.

9. Page 8, "Model building…. That 3' end of PBS ligand could be connected to the 5' end of the PBS ligand through an RNA connector having a minimal length of 10-11 nt". Do the authors mean the "3' end of PBS ligand could be connect to the 5' end of the SBS ligand" ? This should be marked on the figure.

The second "PBS" was a typo for "SBS." The typo has been corrected.

We show the connection points and the approximate path of the connecting RNA in Fig 4e of the current version..

10. Page 9 "In parallel structure determination experiments performed in the presence and absence of NusG" - please show 2D or 3D classes.

We deleted the sentence in question when preparing the current version..

11. Figure S2. Can the authors comment on (B), with n>7, the efficiency of termination seems to go down (comparing the top band intensity), can the structures explain these data ?

We address this point in the current version (legend to Fig. S2): "All six tested U-rich-RNA-segment lengths (n = 4, 5, 6, 7, 8, and 9 codons) support termination. In the experiments of (a), the six tested U-rich-RNA-segment lengths support termination comparably efficiently. In the experiments of (b), shorter lengths (n = 4, 5, and 6 codons) support termination more efficiently than longer lengths (n = 7, 8, and 9 codons)."

We do not know why shorter U-rich-RNA-segment lengths support termination more efficiently. One possibility is that shorter U-rich-RNA-segment lengths constrain Rho bound to the U-rich RNA segments to be closer to NusG and the TEC, thereby facilitating the formation of complexes.

12. Figure S3E,4E,5E - local resolution map focusing on the Rho ring, looking at the hexamer plane itself should be shown to assess the quality of the nucleotide binding sites.

We agree with the reviewer that additional local resolution maps could be useful. But space limitations preclude us from adding display items.

13. A pre-print of a termination complex of T. thermophilus has been deposited (https://www.biorxiv.org/content/10.1101/2022.09.02.506315v1.full). Please comment and compare the structures.

We address this point in the current version (page 10, paragraph 2): "Following submission of our manuscript, preprint reported a cryo-EM structure of a *Thermus thermophilus* Rho-TEC complex (without PBS ligand and without NusG) that exhibits a closed-ring Rho hexamer, RNA threaded through Rho, and the same orientation of Rho relative to the TEC as in our structures.[39] The preprint supports the

conclusion that these features are defining features of functional bacterial Rho pre-termination complexes."

Reviewer Reports on the First Revision:

Referees' comments:

Referee #1 (Remarks to the Author):

The authors have satisfactorily addressed all comments. Publication of the revised manuscript is strongly recommended.

Referee #2 (Remarks to the Author):

The authors have revised the manuscript carefully, and have provided detailed responses to the referees' comments. Certainly mine were completely satisfied. The manuscript is ready for publication.

Referee #3 (Remarks to the Author):

The authors have addressed/clarified issues raised and I am happy for the manuscript to be accepted for publication.