PEER REVIEW FILE

<u>**Reviewers' comments:**</u>

Reviewer #1 (Remarks to the Author):

Review of the manuscript (NCOMMS-17-08792) "The transcript cleavage factor paralogue TFS4 is a potent RNA polymerase inhibitor" by Fouqueau et al.

The manuscript by Werner and co-workers investigates the functional properties and biological role of a novel transcription factor TFS4 from S. solfataricus, a paralog of eukaryotic transcript cleavage factor TFIIS. Authors show that in contrast to S. solfataricus TFS1, an archaeal bona fide functional homolog of TFIIS that induces endonucleolytic activity of RNA polymerase (RNAP) and stimulates productive transcription, TFS4 is an effective transcriptional inhibitor that acts by destabilizing pre-initiation and initiation complexes and altering their catalytic properties. Authors demonstrated that TFS4 acts competitively with TFS1 with which it shares substantial sequence homology, and most likely the binding site in the secondary channel of RNAP. Unexpectedly, it was found that the inhibitory activity of TFS4 can also be suppressed by basal transcription initiation factor TFE which stabilizes the pre-initiation complex (PIC) and facilitates the open complex formation through interactions with RNAP clamp domain and upstream DNA. These results point to a possible allosteric link between the structural changes in the mobile elements of the secondary channel induced by TFS4 and the distant large-scale movements of the clamp domain that lead to disruption of RNAP/basal factor-DNA interactions.

Unlike TFS1 which contains an elongated loop in the C-terminal Zn-binding ribbon (C-ZR) domain with two acidic residues at the tip responsible for catalysis of RNA hydrolysis, TFS4 has a shorter loop in the C-ZR with three basic residues that are essential for the inhibitory activity. Loop swapping and residue substitution experiments revealed that the stimulatory or inhibitory activities of each factor are defined by the nature of the loop and its tip motif. This is the most striking result of this work. Importantly, under normal growth conditions, TFS4 expression in S. solfataricus cells is very low. However, it is strongly induced upon viral infection causing cell growth retardation. Thus, TFS4 may represent the first known example of a general transcriptional repressor in archaea that may play an important role in cellular antiviral defense strategy.

The findings are novel and provide an important contribution to the field that warrants publication in Nature Communications. The paper will be interesting for molecular biologists and microbiologists studying the organization of transcriptional machinery and its regulation in

archaea and the mechanisms of microbial host response to viral infections. However, some of the data presented are incomplete; the manuscript contains several experimental discrepancies and inconsistencies that should be resolved. Some of the claims are not directly supported by experimental evidence. Additional experiments may be required to strengthen the paper and make it acceptable for publication. Below are my critiques, comments, and suggestions.

Major critique

1. The first problem of this work is the lack of direct evidence that would support authors' assertion that TFS4 inhibition has two components: a destabilization of RNAP-nucleic acid complexes at high factor/RNAP rations, and a catalytic inhibition based on interference with the active site of RNAPs that can be observed at low factor concentrations. The first claim is confirmed by direct data based on EMSAs and to some extent by the results of abortive initiation assays using the minimal archaeal PIC complex prepared on SSV1 T6 promoter DNA (Fig. 3, panels a, b, e, f). Additional DNAse or permanganate footprinting data would help strengthen the conclusion. However, the second claim is supported only by an indirect data. First, authors show that TFS4 inhibits productive transcription in promoter-directed runoff transcription assay at factor concentrations substoichiometric to RNAP (Fig. 4a). It appears that >50% inhibition is observed at the TFS4/RNAP molar ratio of 1:10. At the same time, the decrease of the runoff formation is not accompanied by accumulation of any RNAs shorter than 71 nt. Similar inhibitory effect was observed during the extension of RNA primer using a DNA/RNA scaffold template in transcription elongation assay (Fig. 4c). Unfortunately, in both assays, the autoradiograms only show transcripts >40 nt and >60 nt, respectively. Thus, it appears that TFS4 inhibits transcription in "all or nothing" fashion. Since TFS4 also inhibits the multiround abortive synthesis (although at 2-fold excess over RNAP), and even destabilizes the elongation complex (Fig 4e), it is possible that the factor acts during the PIC or the open complex formation, or at a subsequent step prior to substrate polymerization. To prove that TFS4 indeed inhibits the catalytic step, authors should analyze whether Vmax or apparent Km values are affected, using a stepwise RNA extension assay with different concentrations of NTPs. Alternatively, could TFS4 act as a termination factor? Experiments with immobilized TEC using His-tagged RNAP would help address this question.

2. My second concern is the demonstration of the biological role of TFS4. Fig. 6 clearly shows that STIV virus infection dramatically increases the level of TFS4 expression and slows down/inhibits cell growth. However, the direct role of TFS4 overexpression in this process has not been demonstrated. Instead, authors show the inhibitory effect of a hybrid factor TFS1-tip4 in the growth of a related organism S. acidocaldarius. While these observations are consistent with the proposed role TFS4, it would be nice to have more direct data to support this view. Will it be possible to construct an FTS4-deletion strain of S. solfataricus and analyze its growth curves with and without SITV infection? Alternatively, could TFS4 be overexpressed in S. solfataricus using a compatible plasmid vector under inducing and non-inducing conditions (like

those shown in Fig. 6d and 6e)?

Minor comments.

1. Introduction, p. 4, 2nd para, 2nd sentence. Figures 1 and S1 do not show any information on RPB9 activity. Fig. 1 shows the evolutionary conservation of TFIIS-homolog and -paralog proteins, whereas Fig. S1 shows the sequence alignment of TFS4 and phylogenetic distribution of TFS paralogues in Sulfolobales.

2. Introduction, p. 4, 2nd para, 3rd sentence. Reference 23 is incorrect: Langer and Zillig 1993 did not report on the function of A12.2 in RNAPI and C11 in RNAPIII.

3. Introduction, p. 4, 2nd para, 6th and 7th sentence. Add a reference. Authors should also cite Laptenko et al., EMBO J, 2003, where the structural model for the bacterial Gre-RNAP complex has been presented, and the role of the GreA's tip acidic residues in catalysis has been demonstrated.

4. Introduction, p.5, 1st para, 2nd sentence. A typo: …three lysine residues.

5. Introduction, p.5, 1st para, last sentence. The statement is unclear; please rephrase.

6. Results, p.6, 1st para, 3rd sentence. Fig. 1 does not show the sequence or the functional properties of TFS2. The sequence alignment of S. solfataricus TFS2 and TFS3 homologs should be added to Fig. 1 or Fig. S1.

7. Results, p.6, 1st para, 6th and 7th sentence. Do authors mean to say that TFS2 and TFS3 are not RNAP-binding factors? Maybe the choice of TFS4 (over TFS2 and TFS3) for this study should be explained in more details. Also, the loop (tip) of TFS4 is 8 residues shorter than that of TFS1, so it may not reach the catalytic site of RNAP.

8. Results, p.7, 2nd para, 4th sentence, and Fig. 2b. Why were the 50 nt and 49 nt cleavage products not extended? The reaction mix contained the same three substrates: ATP, GTP, and UTP? I would expect extension reaction to be more efficient than RNA cleavage.

9. Results, p.8, 1st para, 4th sentence and Fig. 3a. The efficiency of PIC formation is rather low. Was heparin added to the reaction prior to electrophoresis by EMSA?

10. Results, p.8, 1st para, 7th sentence, and Fig. 3b. What was the concentration of TFS4 in the assay shown in Fig 3b?

11. Results, p.9, 2nd sentence from the bottom and Fig. 4a. TFS4 inhibits runoff synthesis by ~50% even when added at substoichiometric concentrations to RNAP (0.1:1). Note that RNAP was present in ~2-6 fold excess over DNA. Could this inhibitory effect be because TFS4 is specific (has a high binding affinity) to PIC and has a low affinity to free RNAP? Also, the lower part of the gel autoradiogram is not visible. Could the synthesis of smaller (5-40 nt) RNA products be affected by TFS4?

12. Results, p.10, 1st para, last sentence and Fig. 4d and 4e. The efficiency of TEC formation is very low: only a minor fraction of the DNA/RNA scaffold forms a stable complex with RNAP (a slow-migrating band of TEC on the gel in panels d and e). It is difficult to assess and compare the effects of TFS1 and TFS4 on the stability of TEC. The evidence for inhibition of elongation complex formation by TFS4 is not very convincing. What is the reason for such a low yield of TEC?

13. Discussion, p.15, 2nd and 3rd sentence. Similar to the effect of mutations of acidic tip in TFIIS and TFS1, mutation of the tip residues in E. coli GreA (including the catalytic Asp and Glu) also convert Gre factors into strong inhibitors of transcription elongation that lead to a dominant lethal phenotype (see Laptenko et al., EMBO J, 2003).

14. Discussion, p.15, 4th sentence from the bottom. The inhibition of RNAP catalysis by TFS4 was not shown directly (see Major critique 1), it's only a conjecture.

15. Discussion, p.16, 1st para, last sentence. The expression of TFS4 during the stationary phase of uninfected S. solfataricus was not shown.

16. Methods, p. 18 and 19, Abortive and promoter-directed transcription assays, and elongation assays. Reaction conditions are not described clearly. What is the size of promoter DNA fragment used in the assays? The final concentrations of DNA and RNAP in all reactions should be indicated.

17. Fig. 3. Panels g and h. The indication for the presence/absence of factors in the reaction appears to be incorrect: lane 2 is supposed to be a positive control with both TFB and TBP present (compare lanes 2 and 3 in panels e and f).

18. Fig. 7c. The depiction of the secondary channel (funnel) in the schematic representation of Sso RNAP is incorrect. The funnel should be placed on the downstream side of the protein facing the two Mg ion, not the 5'-end of the nascent RNA.

19. Supplementary Fig. S4. Incorrect legend. TFEα/β should be indicated as present (+) only at the right side of panels a and b.

Reviewer #2 (Remarks to the Author):

Review of Fouqueau et al. for Nature Communications

Fouqueau et al. describe the discovery and characterization of an interesting new transcription factor present in the archaea Sulfolobus sulfatarius that is evolutionarily but not functionally related to the well-known transcript cleavage/fidelity factor TFIIS (TFS1 in arachaea). This new factor, called TFS4, was discovered bioinformatically along with TFS2 and TFS3, which remain uncharacterized. TFS4 appears to have an N-terminal RNAP-binding domain like TFS1, but the C-terminal domain that interacts in the secondary channel has a different structure and contains 4 Lys residues near its tip whereas two acidic residues are located near the tip in TFS1 (and in TFIIS and in the structurally unrelated bacterial Gre factors). These acidic residues are responsible for chelating a second Mg ion in the RNAP active site that is required for transcript cleavage. The authors establish that TFS4 is a transcription inhibitor and show that, although not expressed in typical growth conditions, it is induced by infection with a Sulfolobus virus. The authors convincingly establish that TFS4 inhibits nucleotide addition in RNAP and also appears to destabilize both elongation complexes and preinitiation complexes formed with TBP, TFB, and TFE (although TFE may be indirectly competitive with TFS4). Although a complete picture of the in vivo function of TFS4 does not emerge from the findings, this is a remarkably well-rounded characterization of a transcription factor for the manuscript that first describes its discovery. The work will be of general interest in the transcription field and to researchers in general interested in gene regulation (this type of inhibitor is not restricted to archaea as bacterial examples related to Gre factors have been found, and it would be unsurprising if eukaryotic examples remain to be discovered). I believe the manuscript is largely suitable for publication and have just a few larger questions and a list of comments for the authors to consider in a final revision.

Major points

1. The authors suggest that the inhibitory effect of TFS4 results from an allosteric effect of TFS4 on RNAP or ECs, that to say binding of TFS4 in the secondary channel alters the conformation of RNAP in a way that inhibits PIC formation and transcript elongation and promotes EC dissociation. This is certainly one model to be considered and I understand why the authors may favor it based on effects on PIC formation and EC dissociation. However, binding of proteins in

the secondary channel can also be expected to inhibit NTP binding directly by blocking the path of NTP entry to the active site and somewhat less obviously can potentially inhibit DNA binding on the downstream side of the active site. Thus, an alternative model for TFS4 action could be simple direct competition for substrate NTP or DNA, yet the authors do not mention this possibility let alone exclude it experimentally. At a minimum, the alternative model should be described. It would be desirable to also include simple experiments to test the direct competition models. Do low concentrations of TFS4 raise the apparent Kntp for substrates during elongation? Do changes in NTP and TFS4 concentration give competitive effects? Would higher concentrations of DNA lessen the inhibitory effect of TFS4 on EC dissociation?

2. The effects of virus infection on TFS4 expression are interesting and consistent with the authors descriptions, but the results stop short of directly defining the role of TFS4 in phage infection. Is there some reason TFS4 expression can't be blocked in Sulfolobus? (eg, a knockout mutant or some sort of antisense RNA interference expt?). I don't know the system well enough to know how hard this would be, but it would be very interesting to know if the virus infection is altered in a TFS4 knockout. If that's a doable experiment, then I'd recommend trying to include it. If it's not a doable experiment, then I'd recommend setting up the last section of the results by explaining that it would be the ideal experiment but is not feasible in Sulfolobus and therefore an alternative approach was used (to help readers understand why the approach used was selected).

3. Can the authors say anything about the relative strengths of TFS1 and TFS4 binding. Do they compete with roughly the same affinity or does one bind much tighter than the other. It would be particularly interesting of TFS4 bound much tighter than TFS1 because TFS1 must bind weakly enough to allow NTP binding and tighter binding of TFS4 would make it an effective block to NTP entry. The authors perform competition experiments so this seems like an entirely reasonable question to address in this manuscript. The data may already exist or a simple extension of experiments already performed would address this quite interesting question.

Minor points

1. Abstract, third line and several other places in the manuscript. "While" is best reserved to mean "contemporaneously" -"Although" is a better word choice here.

2. Abstract line 8. Although I agree an "allosteric" model is a reasonable explanation, since the experiments to not provide unambiguous discrimination against a direct competition model it might be safest to be more conservative in the abstract.

3. p3 li5. The "secondary channel" is generally accepted nomenclature for bacterial RNAP (and perhaps for archaeal RNAP – I'm uncertain in that case), but for eukaryotic RNAPII the terms

"pore" and "funnel" are the generally accepted nomenclature for the outer and inner parts of the secondary channel. It might help target a broader audience to explain both sets of terms here.

4. p3 li9 should be a comma after phase

5. p3 li11 It is more accurate from a cellular perspective to say that DNA moves backwards through RNAP than that RNAP moves backwards on DNA since the DNA is generally more mobile than the RNAP. Either frame of reference is correct, however.

6. p3 third line from bottom "urgent" implies a need to act quickly. Perhaps "strong" would be a better choice.

7. p4 li17 Generally, cleavage factors are thought to stabilize binding of the second Mg rather than alter the position of binding and it might be better to substitute stabilize for allow here.

8. p4 li22 The first sentence of this para is a little awkward. Perhaps something like "Here we describe the discovery of a fascinating multiplication of tfs-like genes in archaea and present the characterization of two of them, TFS1 and TFS4, that display opposite stimulatory and inhibitory effects on transcription.

9. p4 third line from the bottom. This itemized list is not parallel; (i) destabilizes, (ii) inhibits, (iii) no verb, (iv) no verb. Rephrase to create a parallel construction.

10. p5 li3 typo "tree" should be "three"

11. p6 li16 Is it possible that His residues substitute for the missing Cys residues in the ZR domains?

12. p7 li7 Fig. 2a does not show the template referred to in this citation. It would be very helpful to readers to depict the template structure/sequence in key segments and a brief experimental schematic.

13. p8 li15 It is unclear to me why the inhibition of PIC formation necessarily means an allosteric effect. Couldn't parts of TFS4 be located where they directly compete with DNA?

14. p9 li11 The difference in salt concentrations for high vs low is not particularly large. Is Cl even the relevant counterion for in vivo. For many organisms Cl is excluded and can compete for protein-phosphate ionic interactions.

15. p10 li4 Wasn't an effect of TFS4 on inhibition of elongation shown in Fig. 2?

16. p11 last line. This is a point where it's particularly unclear why the simple steric inhibition of NTP binding isn't considered as an explanation for inhibitory effects on elongation.

17. p12 li14 use of two "which" in the independent clause is confusing.

18. p15 li7 There also are data on a Gre mutant in which substitution of one or two of the acidic residues turns it into an inhibitor. I apologize for not looking up the citation, but I think it's work from Arkady Mustaev, and would be worth citing here.

19. p15 li11 Again here, it's unclear why the direct competition for NTP binding isn't considered.

20. Fig. 4 Why is the formation of ECs so inefficient? What percentages of DNA and RNAP are formed into EC? Since it's hard to see appearance of DNA released from EC upon TFS4 binding, the conclusion that TFS4 disrupts EC is not particularly well supported by the experiment. Isn't it possible that DNA remains bound and TFS4 binding just causes the EC to smear out during electrophoresis?

21. Fig. 5d and 5e Is the x-axis correctly labeled? It seems like it should be TFS4/RNAP ratio. Also, maybe don't use gray for the triple A mutant – it's hard to distinguish from black on the figure.

1   **Detailed response to reviewer's comments**

2

3   Reviewer #1 (Remarks to the Author):

4

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6   potent RNA polymerase inhibitor" by Fouqueau et al.

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8   The manuscript by Werner and co-workers investigates the functional properties and biological role of

9   a novel transcription factor TFS4 from S. solfataricus, a paralog of eukaryotic transcript cleavage factor

10   TFIIS. Authors show that in contrast to S. solfataricus TFS1, an archaeal bona fide functional homolog

11   of TFIIS that induces endonucleolytic activity of RNA polymerase (RNAP) and stimulates productive

12   transcription, TFS4 is an effective transcriptional inhibitor that acts by destabilizing pre-initiation and

13   initiation complexes and altering their catalytic properties. Authors demonstrated that TFS4 acts

14   competitively with TFS1 with which it shares substantial sequence homology, and most likely the

15   binding site in the secondary channel of RNAP. Unexpectedly, it was found that the inhibitory activity of

16   TFS4 can also be suppressed by basal transcription initiation factor TFE which stabilizes the

17   preinitiation

18   complex (PIC) and facilitates the open complex formation through

19   interactions with RNAP clamp domain and upstream DNA. These results point to a possible allosteric

20   link between the structural changes in the mobile elements of the secondary channel induced by TFS4

21   and the distant large-scale movements of the clamp domain that lead to disruption of RNAP/basal

22   factor-DNA interactions.

23

24   Unlike TFS1 which contains an elongated loop in the C-terminal Zn-binding ribbon (C-ZR) domain with

25   two acidic residues at the tip responsible for catalysis of RNA hydrolysis, TFS4 has a shorter loop in

26   the

27   C-ZR with three basic residues that are essential for the inhibitory activity. Loop swapping and residue

28   substitution experiments revealed that the stimulatory or inhibitory activities of each factor are defined

29   by the nature of the loop and its tip motif. This is the most striking result of this work. Importantly,

30   under normal growth conditions, TFS4 expression in S. solfataricus cells is very low. However, it is

31   strongly induced upon viral infection causing cell growth retardation. Thus, TFS4 may represent the

32   first known example of a general transcriptional repressor in archaea that may play an important role in

33   cellular antiviral defense strategy.

34   The findings are novel and provide an important contribution to the field that warrants publication in

35

36

37 However, some of the data presented are

38 incomplete; the manuscript contains several experimental discrepancies and inconsistencies that

39 should be resolved. Some of the claims are not directly supported by experimental evidence.

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41 publication. Below are my critiques, comments, and suggestions.

42

43 Major critique

44 1. The first problem of this work is the lack of direct evidence that would support authors' assertion

45 that TFS4 inhibition has two components: a destabilization of RNAP-nucleic acid complexes at high

46 factor/RNAP rations, and a catalytic inhibition based on interference with the active site of RNAPs that

47 can be observed at low factor concentrations. The first claim is confirmed by direct data based on

48 EMSAs and to some extent by the results of abortive initiation assays using the minimal archaeal PIC

49 complex prepared on SSV1 T6 promoter DNA (Fig. 3, panels a, b, e, f). Additional DNAse or

50 permanganate footprinting data would help strengthen the conclusion.

51

52 We have followed the recommendation of reviewer-1 and carried out permanganate footprinting

53 experiments. The results are shown in supplementary figure 2. We use the same SSV1 T6 DNA

54 template as in the EMSA experiments shown in figure 3, where the noncomplementary T-residue in the

55 4-bp bubble (highlighted in red) at register -1 serves as positive control. In the presence of both TBP

56 and TFB additional permanganate reactivity can be detected at the -5, -7 and -12 positions (highlighted

57 with asterisks on the DNA sequence), which is further enhanced by the addition of TFE alpha/beta. The

58 inclusion of TFS4 abolishes the permanganate reactivity, in good agreement with the PIC destabilizing

59 activity of TFS4. The addition of TFE alpha/beta counteracts this TFS4 inhibition, also in perfect

60 agreement with the results of our EMSA assays shown in figure 3.

61

62 However, the second claim is supported only by an indirect data. First, authors show that TFS4 inhibits

63 productive transcription in promoter-directed runoff transcription assay at factor concentrations

64 substoichiometric to RNAP (Fig.4a). It appears that >50% inhibition is observed at the TFS4/RNAP

65 molar ratio of 1:10. At the same time, the decrease of the runoff formation is not accompanied by

66 accumulation of any RNAs shorter than 71 nt. Similar inhibitory effect was observed during the

67 extension of RNA primer using a DNA/RNA scaffold template in transcription elongation assay (Fig.

68 4c). Unfortunately, in both assays, the autoradiograms only show transcripts >40 nt and >60 nt,

69   respectively. Thus, it appears that TFS4 inhibits transcription in "all or nothing" fashion. Since TFS4

70   also inhibits the multiround abortive synthesis (although at 2-fold excess over RNAP), and even

71   destabilizes the elongation complex (Fig 4e), it is possible that the factor acts during the PIC or the

72   open complex formation, or at a subsequent step prior to substrate polymerization.

73

74   We completely agree with reviewer-1, TFS4 does not impair transcription processivity, which would

75   result in the accumulation of short, partial products that we never observed. In order to provide further

76   evidence for this we have included a 'longer' gel below this paragraph. These figures demonstrate that

77   no smaller (nor longer) transcripts are synthesized, and the minimal size of transcripts resolved on the

78   new gels is about 9 nt. We do not feel that it is necessary to include the 'extended' gel in the figure of

79   the article due to space constraints – but are happy to reconsider if the editor deems it critical. We

80   concede to reviewer-1's point of view that - in theory - a TFS4-based interference with transcription

81   initiation or -elongation complex stability and integrity would impair transcription activity. However, the

82   catalytic inhibition occurs at much *lower* TFS4 concentrations than are necessary to destabilise these

83   complexes, as shown in our direct binding EMSA experiments. The molecular basis for the catalytic

84   inhibition could be interference with substrate NTP binding and/or phosphodiester bond formation.

85
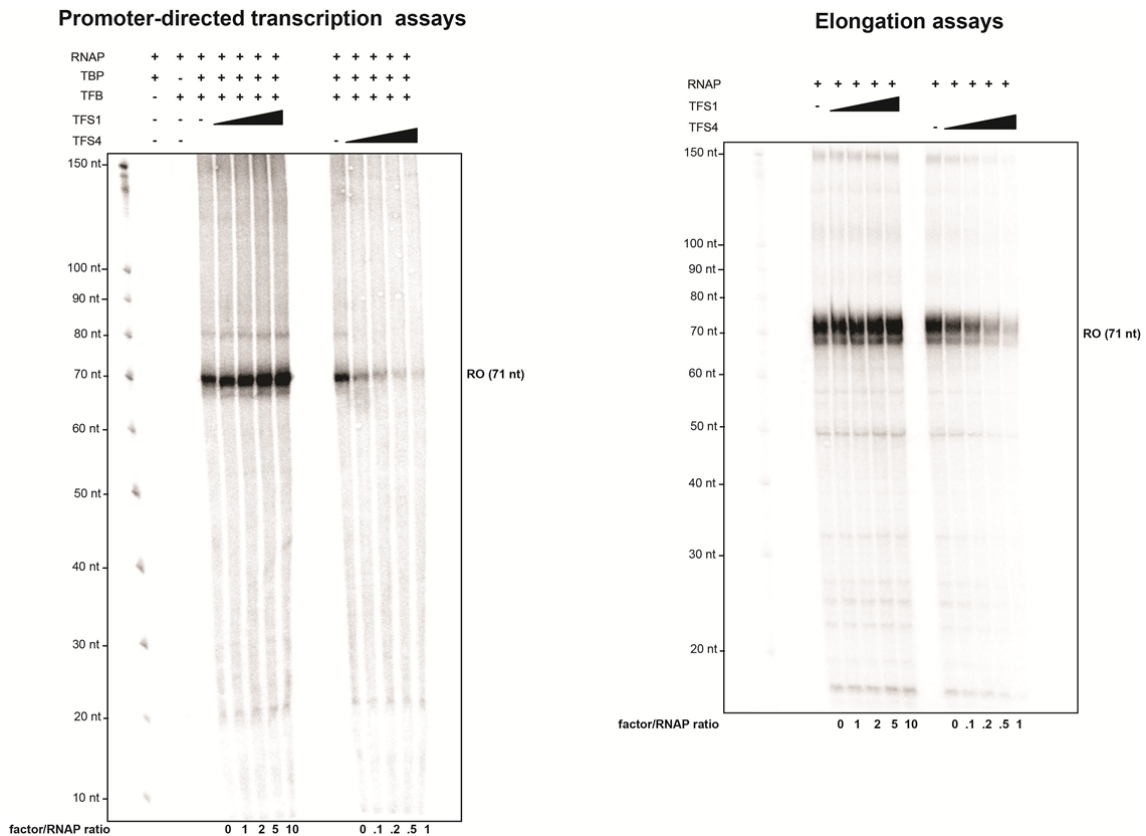
86

3

87

To prove that TFS4 indeed inhibits the catalytic step, authors should analyze whether Vmax or apparent Km values are affected, using a stepwise RNA extension assay with different concentrations of NTPs.

91

In order to elaborate on the underlying molecular mechanism of catalytic inhibition, we followed reviewer-1's advice and carried out single NTP addition experiments using abortive transcription assays over a range of NTP substrate concentrations, and using a range of TFS4 concentrations. We show the results as double reciprocal plot in a *Lineweaver-Burke* fashion in figure 3. The results clearly show that the trend lines corresponding to different TFS4 concentrations intersect the (1/v) Y-axis at the same point, while the intersections with the x-axis (1/[S]) differ at varying TFS4 concentrations. This is strongly *indicative of a competitive inhibition*, and in molecular terms likely reflects that TFS4 binding interferes with NTP substrate binding, or interferes with the access of NTP substrates to their binding site(s). This is in perfect agreement with the proposed binding site of TFS4 in the NTP entry pore, and with the binding modes of eukaryotic (TFIIS) and bacterial (Gre) cleavage factors in the secondary, NTP entry, channels of their cognate RNA polymerases. The results have been incorporated into figure 3h and are discussed on page 9:

'To further elaborate on the underlying mechanism of inhibition we tested whether the TFS4 inhibition varied with NTP concentrations. We carried out abortive initiation assays at a range of NTP substrate- and TFS4 concentrations, and the results are illustrated as double reciprocal plot in Figure 3g (primary data in supplementary Fig. 6a and b). The results show that TFS4 acts as competitive inhibitor interfering with NTP binding. At 250 nM TFS4 (5-fold excess over RNAP) the apparent K_m for NTP is increased ~30-fold from 27±3 µM to 870±81 µM.

110

Alternatively, could TFS4 act as a termination factor? Experiments with immobilized TEC using His-tagged RNAP would help address this question.

113

Transcription termination factors classically disrupt *elongation complexes* in a gene sequence-dependent context, either at the 3' of the gene to facilitate RNA3' formation or in the 5' region of a gene as mechanism of regulation (termination-antitermination). Since TFS4 also targets transcription *initiation complexes*, and since we have no evidence nor theoretical mechanism to suggest any sequence-dependent activity we do not favor to use the term *termination factor* for TFS4.

119

120 2. My second concern is the demonstration of the biological role of TFS4. Fig. 6 clearly shows that
121 STIV virus infection dramatically increases the level of TFS4 expression and slows down/inhibits cell
122 growth. However, the direct role of TFS4 overexpression in this process has not been demonstrated.
123 Instead, authors show the inhibitory effect of a hybrid factor TFS1-tip4 in the growth of a related
124 organism S. acidocaldarius.

125

126 The choice of *S. acidocaldarius* is not only a technical one – it is amenable to genetics and vector-
127 based overexpression is possible – but chiefly because this organism does not encode any TFS4
128 homologues and therefore provides the perfect 'clean' strain background for an 'ectopic'
129 overexpression experiment.

130

131 While these observations are consistent with the proposed role TFS4, it would be nice to have more
132 direct data to support this view. Will it be possible to construct an TFS4-deletion strain of S. solfataricus
133 and analyze its growth curves with and without SITV infection?

134

135 *S. solfataricus* P2 and its derivative strain 2-2-12 do not allow gene knockouts.

136

137 Alternatively, could TFS4 be overexpressed in S. solfataricus using a compatible plasmid vector under
138 inducing and noninducing conditions (like those shown in Fig. 6d and 6e)?

139

140 There are no reliable vector tools for the controlled overexpression of recombinant proteins in *S.*
141 *solfataricus* P2 and 2-2-12.

142

143 Minor comments.

144

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146 activity. Fig. 1 shows the evolutionary conservation of TFIIS-homolog and -paralog proteins, whereas
147 Fig. S1 shows the sequence alignment of TFS4 and phylogenetic distribution of TFS paralogues in
148 Sulfolobales.

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153 report on the function of A12.2 in RNAPI and C11 in RNAPIII.

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158 Laptenko et al., EMBO J, 2003, where the structural model for the bacterial Gre-RNAP complex has

159 been presented, and the role of the GreA's tip acidic residues in catalysis has been demonstrated.

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163 4. Introduction, p.5, 1st para, 2nd sentence. A typo: …three lysine residues.

164

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166

167 5. Introduction, p.5, 1st para, last sentence. The statement is unclear; please rephrase.

168

169 We rephrased the sentence to: 'Interestingly, infection of Sso with the *Sulfolobus* Turreted Icosahedral

170 Virus (STIV) induces TFS4 expression suggesting that TFS4 plays a role in the antiviral host response

171 and defence.'

172

173 6. Results, p.6, 1st para, 3rd sentence. Fig. 1 does not show the sequence or the functional properties

174 of TFS2. The sequence alignment of S. solfataricus TFS2 and TFS3 homologs should be added to Fig.

175 1 or Fig. S1.

176

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178

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180 RNAP-binding factors? Maybe the choice of TFS4 (over TFS2 and TFS3) for this study should be

181 explained in more details. Also, the loop (tip) of TFS4 is 8 residues shorter than that of TFS1, so it may

182 not reach the catalytic site of RNAP.

183

184 This study if focused on the TFS variant that is most closely related to the canonical archaeal transcript

185 cleavage factors *yet* has functionally diverged in intriguing ways. Studies with TFS2 and -3 are

186 underway and will be published elsewhere in due time. The fact that the computational homology

187 model suggests that the tip domain is shorter in TFS4 compared to TFS1 is interesting, but no stringent

188 conclusions concerning the depth of penetration into the RNAP active site can be drawn based on

189 sequence conservation and homology modelling alone. Thus it is not inconceivable that the TFS4 tip is

190 drawn deeper into the NTP entry channel (via electrostatic interactions with the three lysines) than the

191 TFS1 tip, which would support the notion that the insertion of TFS4 leads to conformational changes in

192 RNAP (like Gfh1), while the insertion of TFS1 less so, or not at all (like Gre).

193

194 8. Results, p.7, 2nd para, 4th sentence, and Fig. 2b. Why were the 50 nt and 49 nt cleavage products

195 not extended? The reaction mix contained the same three substrates: ATP, GTP, and UTP? I would

196 expect extension reaction to be more efficient than RNA cleavage.

197

198 The generated transcript size reflects the equilibrium between synthesis and cleavage. Under the given

199 reaction conditions ([GTP]=[UTP]=250 μM and [ATP]=1.25 μM) and TFS concentrations the cleavage

200 products prevail in the presence of cleavage factor TFS1. The schematic in Figure 2A has been

201 corrected with respect to the ATP concentration. The K_m of NTPs in archaeal RNAPs is approximately

202 30 micro molar, which at least in part explains why cleavage 'wins' over extension. But more

203 importantly, the negative control in this experiment is the TFS1 double alanine substitution mutant

204 (TFS1-AA) which is not able to induce transcript cleavage, and likewise the addition of TFS4 does not

205 induce cleavage – leaving the extended transcript the dominant transcript species. Figure 2 panel D

206 shows the reactivation of stalled complexes under conditions where all four NTPs are present. In the

207 absence of cleavage factors the Sso RNAP cannot resume elongation (see below), while the addition

208 of TFS1 (but not TFS1-AA or TFS4) efficiently reactivates the stalled complexes, which highlights the

209 importance of transcript cleavage factors in archaea. Just to be clear on this point, in the absence of

210 CTP the fully Watson-Crick base pairing-compliant RNA can only be extended to the A residue at

211 register +50. The major extension product in our assay is 51 nt long and arises from mis-incorporation

212 of a single non-complementary nucleotide at the 3' terminus. This is a well-known phenomenon that

213 occurs in archaeal RNAP (Fouqueau et al., NAR 2013) and RNA polymerase II (Jeon et al., JBC 1996),

214 and requires transcript cleavage factors for reactivation. We have introduced a sentence to clarify the

215 details into the revised manuscript:

216 *'Initiation of transcription by the addition of RNAP and the two essential basal transcription factors TBP*

217 *and TFB in the presence of ATP, GTP and UTP resulted in TECs stalling after synthesizing a 51 nt*

218 *transcript. This transcript is generated by the misincorporation of a single nucleotide at the 3' end of the*

219 *50 nt C-less transcript, a phenomenon observed in archaeal RNAP [8] as well as eukaryotic RNAPII [29].'*

220 … and at the bottom of the paragraph:

221   *'Without cleavage factor only a negligible fraction of the 51 nt transcript (containing the misincorporated*

222   *3'-end nucleotide) could be extended, in agreement with the cleavage assays results discussed above.'*

223

224   9. Results, p.8, 1st para, 4th sentence and Fig. 3a. The efficiency of PIC formation is rather low. Was

225   heparin added to the reaction prior to electrophoresis by EMSA?

226

227   We beg to differ with reviewer-1, the efficiency of PIC formation in our experiments is not low but

228   typical for archaeal PIC (Werner and Weinzierl, 2002 and 2005, Blombach et al., 2015, Sheppard et al.,

229   2016). The addition of heparin is very important in these experiments to suppress the TBP/TFB factor-

230   independent binding of RNAP to the promoter template.

231

232   10. Results, p.8, 1st para, 7th sentence, and Fig. 3b. What was the concentration of TFS4 in the assay

233   shown in Fig 3b?

234

235   The concentration of TFS4 is 100 nM. This information was added to the legend of Figure 3.

236

237   11. Results, p.9, 2nd sentence from the bottom and Fig. 4a. TFS4 inhibits runoff synthesis by ~50%

238   even when added at substoichiometric concentrations to RNAP (0.1:1). Note that RNAP was present in

239   ~2-6 fold excess over DNA. Could this inhibitory effect be because TFS4 is specific (has a high binding

240   affinity) to PIC and has a low affinity to free RNAP?

241

242   Despite several attempts, it has not been possible to measure the direct binding affinity of TFS1 or

243   TFS4 to RNAP in the binary complexes, Or to the PIC or to the TEC, and we have therefore been very

244   careful not to make any statement about absolute binding affinities of TFS1 and -4 throughout the

245   manuscript. We can only pass a semi-quantitative judgment on relative binding affinities based on

246   competition between TFS1 and -4 during PIC formation (figure 3b), which suggest that TFS4 binds with

247   higher affinity to the PIC than TFS1. Similarly, we have measured the TFS1 concentration-dependent

248   relief of TFS4 inhibition in transcription elongation assays (Supplementary figure 8), and found that the

249   apparent IC_{50} for TFS1 is 230 nM at a TFS4 concentration of 100 nM. This suggests that the affinity of

250   TFS4 to RNAP is higher than the affinity of TFS1 for RNAP, in good agreement with the EMSA PIC

251   competition experiments.

252

253   Also, the lower part of the gel autoradiogram is not visible. Could the synthesis of smaller (5-40 nt)

254   RNA products be affected by TFS4?

255

As stated above, TFS4 does *not* impair transcription processivity and does not result in the accumulation of partial RNA products. In order to provide further evidence for this we have included a 'longer' gel in this document (see above), but not in the revised manuscript file due to space constraints.

12. Results, p.10, 1st para, last sentence and Fig. 4d and 4e. The efficiency of TEC formation is very low: only a minor fraction of the DNA/RNA scaffold forms a stable complex with RNAP (a slow migrating band of TEC on the gel in panels d and e). It is difficult to assess and compare the effects of TFS1 and TFS4 on the stability of TEC. The evidence for inhibition of elongation complex formation by TFS4 is not very convincing. What is the reason for such a low yield of TEC?

We have followed reviewer-1s advice and repeated the TEC EMSA assays. The new optimized binding experiments show now very clearly (i) a superhift of the RNAP-DNA-RNA upon the addition of TFS1 reflecting RNAP-DNA-RNA-TFS1 complex formation, and (ii) that the addition of TFS4 disrupts the RNAP-DNA-RNA complex. In order to allow a direct comparison we have used the same factor:RNAP ratios for TFS1 and -4.

13. Discussion, p.15, 2nd and 3rd sentence. Similar to the effect of mutations of acidic tip in TFIIS and TFS1, mutation of the tip residues in E. coli GreA (including the catalytic Asp and Glu) also convert Gre factors into strong inhibitors of transcription elongation that lead to a dominant lethal phenotype (see Laptenko et al., EMBO J, 2003).

We have altered the following sentence in the discussion to include reviewer-1's suggestion: 'The catalytically inactive cleavage factor mutant TFS1-AA impairs processivity *in vivo* and *in vitro* in a fashion that is distinct from TFS4 (Fig. 4a), in direct comparison with the cognate human TFIIS-AA **and bacterial GreA-AA** mutant variants that also have inhibitory activities and a dominant negative lethal phenotype[39,40].

14. Discussion, p.15, 4th sentence from the bottom. The inhibition of RNAP catalysis by TFS4 was not shown directly (see Major critique 1), it's only a conjecture.

Our new experiments led additional credence to the hypothesis of catalytic inhibition. We modified the text based on our observations that TFS4 lowers the Km for NTP substrates to the following:

9

289 *'The TFS4 inhibition appears to have two components, a catalytic inhibition based on interference with*

290 *the access of NTP substrate to the active site of RNAPs.*

291

292 15. Discussion, p.16, 1st para, last sentence. The expression of TFS4 during the stationary phase of

293 uninfected *S. solfataricus* was not shown.

294

295 There is no evidence to suggest the expression of TFS4 mRNA or TFS4 protein under conditions *other*

296 *than* during STIV virus infection. We added a panel comparing the TFS4 immunoreactivity in biomass

297 from uninfected *S. solfataricus* P2 cultures isolated in the exponential and stationary growth phase,

298 respectively, to Supplementary Figure 12a. These results clearly show that TFS4 expression is not

299 detectable in either growth phase. We also rephrased the sentence to the following: "Starvation and

300 oxidative stress induce dramatic depletion of TFEβ. It is therefore possible that the inhibitory effect of

301 TFS4 on transcription initiation is more dominant under certain growth conditions".

302

303 16. Methods, p. 18 and 19, Abortive and promoter-directed transcription assays, and elongation

304 assays. Reaction conditions are not described clearly. What is the size of promoter DNA fragment used

305 in the assays? The final concentrations of DNA and RNAP in all reactions should be indicated.

306

307 We have amended these omissions. The transcription templates are described in the published

308 literature (Hirtreiter et al., NAR 2010 and Blombach et al., eLife 2015). DNA template sequences used

309 in this study are now listed in Supplementary Fig.12. The concentrations of RNAP and DNA is explicitly

310 stated in the methods section in the revised manuscript.

311

312 17. Fig. 3. Panels g and h. The indication for the presence/absence of factors in the reaction appears

313 to be incorrect: lane 2 is supposed to be a positive control with both TFB and TBP present (compare

314 lanes 2 and 3 in panels e and f).

315

316 We corrected the mistake.

317

318 18. Fig. 7c. The depiction of the secondary channel (funnel) in the schematic representation of Sso

319 RNAP is incorrect. The funnel should be placed on the downstream side of the protein facing the two

320 Mg ion, not the 5'-end of the nascent RNA.

321

322 We have altered the schematic drawing accordingly.

323

324   19. Supplementary Fig. S4. Incorrect legend. TFE/ should be indicated as present (+) only at the right

325   side of panels a and b.

326

327   We introduced the suggested change.

328

329

330

331 **Reviewer #2 (Remarks to the Author):**

332

333 Review of Fouqueau et al. for Nature Communications

334

335 Fouqueau et al. describe the discovery and characterization of an interesting new transcription factor

336 present in the archaea Sulfolobus sulfatarius that is evolutionarily but not functionally related to the

337 well-known transcript cleavage/fidelity factor TFIIS (TFS1 in arachaea). This new factor, called TFS4,

338 was discovered bioinformatically along with TFS2 and TFS3, which remain uncharacterized. TFS4

339 appears to have an N-terminal RNAP-binding domain like TFS1, but the C-terminal domain that

340 interacts in the secondary channel has a different structure and contains 4 Lys residues near its tip

341 whereas two acidic residues are located near the tip in TFS1 (and in TFIIS and in the structurally

342 unrelated bacterial Gre factors). These acidic residues are responsible for chelating a second Mg ion in

343 the RNAP active site that is required for transcript cleavage. The authors establish that TFS4 is a

344 transcription inhibitor and show that, although not expressed in typical growth conditions, it is induced

345 by infection with a Sulfolobus virus. The authors convincingly establish that TFS4 inhibits nucleotide

346 addition in RNAP and also appears to destabilize both elongation complexes and preinitiation

347 complexes formed with TBP, TFB, and TFE (although TFE may be indirectly competitive with TFS4).

348 Although a complete picture of the in vivo function of TFS4 does not emerge from the findings, this is a

349 remarkably well-rounded characterization of a transcription factor for the manuscript that first describes

350 its discovery. The work will be of general interest in the transcription field and to researchers in general

351 interested in gene regulation (this type of inhibitor is not restricted to archaea as bacterial examples

352 related to Gre factors have been found, and it would be unsurprising if eukaryotic examples remain to

353 be discovered). I believe the manuscript is largely suitable for publication and have just a few larger

354 questions and a list of comments for the authors to consider in a final revision.

355

356 Major points

357 1. The authors suggest that the inhibitory effect of TFS4 results from an allosteric effect of TFS4 on

358 RNAP or ECs, that to say binding of TFS4 in the secondary channel alters the conformation of RNAP

359 in a way that inhibits PIC formation and transcript elongation and promotes EC dissociation. This is

360 certainly one model to be considered and I understand why the authors may favor it based on effects

361 on PIC formation and EC dissociation. However, binding of proteins in the secondary channel can also

362 be expected to inhibit NTP binding directly by blocking the path of NTP entry to the active site and

363 somewhat less obviously can potentially inhibit DNA binding on the downstream side of the active site.

364 Thus, an alternative model for TFS4 action could be simple direct competition for substrate NTP or

365 DNA, yet the authors do not mention this possibility let alone exclude it experimentally. At a minimum,

366 the alternative model should be described. It would be desirable to also include simple experiments to

367 test the direct competition models. Do low concentrations of TFS4 raise the apparent Kntp for

368 substrates during elongation? Do changes in NTP and TFS4 concentration give competitive effects?

369

370 This is a fair point also raised by reviewer-1. We have followed up on it by carrying out additional

371 experiments to shed light on the mechanism of catalytic inhibition. In order to address the possibility of

372 a competition between TFS4- and substrate NTP binding we have carried out abortive initiation assays

373 using different NTP concentrations at a range of TFS4 concentrations. Our results indeed suggest that

374 the catalytic inhibition has a component characteristic of a *competitive inhibition*, and in molecular

375 terms likely reflects that TFS4 binding interferes with NTP substrate binding, or interferes with the

376 access of NTP substrates to their binding sites. This is in perfect agreement with the proposed binding

377 site of TFS4 in the NTP entry pore, and with the binding site of TFIIS in the RNAPII-TFIIS structure and

378 the bindings site of the bacterial transcript cleavage Gre factors, and regulators including Gfh1 and

379 DksA. The new results are incorporated into figure 3 and the body text of the manuscript on page 9:

380 *'To further elaborate on the underlying mechanism of inhibition we tested whether the TFS4 inhibition*

381 *varied with NTP concentrations. We carried out abortive initiation assays at a range of NTP substrate-*

382 *and TFS4 concentrations, and the results are illustrated as double reciprocal plot in Figure 3g (primary*

383 *data in supplementary Fig. 6a and b). The results show that TFS4 acts as competitive inhibitor*

384 *interfering with NTP binding. At 250 nM TFS4 (5-fold excess over RNAP) the apparent K_m for NTP is*

385 *increased ~30-fold from 27±3 µM to 870±81 µM.*

386

387 Would higher concentrations of DNA lessen the inhibitory effect of TFS4 on EC dissociation?

388 We did carry out the template competition experiments suggested above, but found that increased

389 DNA concentrations did not alter the inhibition by TFS4. However, since this is negative evidence and

390 does not contribute significantly to the work presented in this paper we did not include it in the revised

391 manuscript.

392

393 2. The effects of virus infection on TFS4 expression are interesting and consistent with the authors

394 descriptions, but the results stop short of directly defining the role of TFS4 in phage infection. Is there

395 some reason TFS4 expression can't be blocked in Sulfolobus? (eg, a knockout mutant or some sort of

396 antisense RNA interference expt?). I don't know the system well enough to know how hard this would

397 be, but it would be very interesting to know if the virus infection is altered in a TFS4 knockout. If that's

398 a doable experiment, then I'd recommend trying to include it. If it's not a doable experiment, then I'd

399 recommend setting up the last section of the results by explaining that it would be the ideal

400 experiment but is not feasible in Sulfolobus and therefore an alternative approach was used (to help

401 readers understand why the approach used was selected).

402

403 As pointed out in the response to reviewer-1, the deleting TFS4 in *S. solfataricus* P2 is experimentally

404 not feasible. We have followed reviewer-2's advice and included the following section in the discussion

405 section of the revised manuscript:

406 *'Because infection by STIV strongly induces TFS4 expression, we propose that TFS4 is part of a novel*

407 *host response mechanism in archaea to combat viral infection. However, whether this response is*

408 *specific to STIV or represents a more general host response to viral infection is unknown. Future*

409 *experiments including mutagenesis studies of TFS4 in a crenarchaeal species that serves as host for*

410 *virus infection and is amenable to genetic intervention will play an important role in elucidating the*

411 *biological role of global transcription inhibition by TFS4.'*

412

413 3. Can the authors say anything about the relative strengths of TFS1 and TFS4 binding. Do they

414 compete with roughly the same affinity or does one bind much tighter than the other. It would be

415 particularly interesting of TFS4 bound much tighter than TFS1 because TFS1 must bind weakly

416 enough to allow NTP binding and tighter binding of TFS4 would make it an effective block to NTP

417 entry. The authors perform competition experiments so this seems like an entirely reasonable question

418 to address in this manuscript. The data may already exist or a simple extension of experiments already

419 performed would address this quite interesting question.

420

421 See above, our attempts to characterize the accurate binding affinities of TFS1 and -4 to RNAP were

422 not successful, while it was possible to determine the *relative* binding affinities in competition

423 experiments using EMSAs and transcription assays. Firstly, figure 3b shows that even at a 10-fold

424 excess of TFS1 over TFS4 PIC formation is not yet fully restored, which suggest that the *affinity of*

425 *TFS4 to the PIC is higher than TFS1*. Secondly, we carried out competition experiments using the

426 elongation scaffold assays shown in supplementary figure 6. TFS4 at 100nM is able to inhibit RNAP

427 efficiently, while TFS1 is able to counteract 50% of this inhibition at a concentration of 230 nM, in other

428 words in ~2.3 fold excess, which indicates that the affinity of TFS4 is higher to the TEC compared to

429 TFS1.

14

430   We have included the following sentence in the manuscript on page 8:

431   *'Since a 10-fold excess of TFS1 over TFS4 barely restores the PIC signal in the EMSA the relative*

432   *affinity of TFS4 for the DNA-TBP-TFB-RNAP complex is higher than that of TFS1 (Fig. 3b).'*

433   And on page 11:

434   *'Considering that the RNAP binding sites of TFS1 and -4 likely are identical or at least overlap, we*

435   *wanted to test whether TFS1 was able to counteract TFS4 inhibition in transcription elongation assays.*

436   *Under our experimental conditions a two-fold excess of TFS1 over TFS4 is required to achieve 50%*

437   *relief of inhibition, which indicates that TFS4 has a higher relative affinity for RNAP in the TEC*

438   *compared to TFS1 (Supplementary Fig. 8a and b).'*

439

440   Minor points

441

442   1. Abstract, third line and several other places in the manuscript. "While" is best reserved to mean

443   "contemporaneously" -"Although" is a better word choice here.

444

445   We introduced the suggested change.

446

447   2. Abstract line 8. Although I agree an "allosteric" model is a reasonable explanation, since the

448   experiments to not provide unambiguous discrimination against a direct competition model it might

449   be safest to be more conservative in the abstract.

450

451   Our new data demonstrate that the catalytic inhibition is, at least in part, competitive in terms of NTP

452   binding. Our direct binding EMSA experiments show clearly that TFS4 counteracts transcription

453   complex stability of PIC and TEC. Since TFS4 enters the RNAP through the NTP channel and does not

454   compete with DNA template binding, the destabilising effect is *very likely* to be allosteric, and in our

455   opinion it is well justified to keep the term 'allosteric' in the abstract. If the editor feels strongly about

456   this point, we will naturally concur and moderate our language.

457

458   3. p3 li5. The "secondary channel" is generally accepted nomenclature for bacterial RNAP (and

459   perhaps for archaeal RNAP – I'm uncertain in that case), but for eukaryotic RNAPII the terms "pore"

460   and "funnel" are the generally accepted nomenclature for the outer and inner parts of the secondary

461   channel. It might help target a broader audience to explain both sets of terms here.

462

The field should strive towards a unified nomenclature of RNAP motifs and domains. Yet, there is no unambiguous nomenclature adapted for archaeal RNAP. For improved clarity, we settled on the use of the term *secondary channel*. A sentence was added in Introduction on page 3:

'The shape of the universally conserved RNAP core resembles a crab claw with a DNA binding channel facilitating interactions with the DNA template, and a secondary channel, also called the NTP entry channel or funnel and pore motifs, in eukaryotic RNAPs[2].'

4. p3 li9 should be a comma after phase

We introduced the suggested change.

5. p3 li11 It is more accurate from a cellular perspective to say that DNA moves backwards through RNAP than that RNAP moves backwards on DNA since the DNA is generally more mobile than the RNAP. Either frame of reference is correct, however.

This is a very interesting almost philosophical consideration, did the mountain go to the prophet or vice versa? We did not change the sentence since reviewer-2 concedes that both frames of reference are factually correct.

6. p3 third line from bottom "urgent" implies a need to act quickly. Perhaps "strong" would be a better choice.

We introduced the suggested change.

7. p4 li17 Generally, cleavage factors are thought to stabilize binding of the second Mg rather than alter the position of binding and it might be better to substitute stabilize for allow here.

We introduced the term 'stabilise'.

8. p4 li22 The first sentence of this para is a little awkward. Perhaps something like "Here we describe the discovery of a fascinating multiplication of tfs-like genes in archaea and present the characterization of two of them, TFS1 and TFS4, that display opposite stimulatory and inhibitory effects on transcription.

16

497   We introduced the suggested change.

498

499   9. p4 third line from the bottom. This itemized list is not parallel; (i) destabilizes, (ii) inhibits, (iii) no verb,

500   (iv) no verb. Rephrase to create a parallel construction.

501

502   We introduced the suggested change, which now reads:

503   *'TFS4 inhibits (i) PIC formation, (ii) abortive initiation, (iii) promoter-directed transcription, as well as (iv)*

504   *transcription elongation.'*

505

506   10. p5 li3 typo "tree" should be "three"

507

508   We introduced the suggested change.

509

510   11. p6 li16 Is it possible that His residues substitute for the missing Cys residues in the ZR domains?

511

512   Histidine residue have been shown to be able to substitute for cysteine residue in Zinc-ribbon (ZR)

513   domains, however TFS3 does not encode any suitably proximal histidine residues making the

514   presence of ZR domains unlikely.

515

516   12. p7 li7 Fig. 2a does not show the template referred to in this citation. It would be very helpful to

517   readers to depict the template structure/sequence in key segments and a brief experimental

518   schematic.

519

520   We introduced the suggested change and included the sequences of DNA templates used in this study

521   in supplementary figure 12.

522

523   13. p8 li15 It is unclear to me why the inhibition of PIC formation necessarily means an allosteric effect.

524   Couldn't parts of TFS4 be located where they directly compete with DNA?

525

526   Without a high resolution structure of the TFS4-RNAP complexes a steric occlusion mechanism

527   between TFS4 and the template DNA cannot be ruled out. However, all our results suggest that TFS4

528   binds in the secondary channel of RNAP, a position which is not likely compatible with an DNA

529   template competition mechanism.

530

531 14. p9 li11 The difference in salt concentrations for high vs low is not particularly large. Is Cl even the

532 relevant counterion for in vivo. For many organisms Cl is excluded and can compete for

533 proteinphosphate ionic interactions.

534

535 The accurate characterization of the intracellular ionic strength and ionic composition is very

536 challenging. To the best of our knowledge no data are available regarding the intracellular salt

537 concentrations in any *Sulfolobus* species. However, based on the growth medium composition, the

538 external chloride concentration is below 1 mM and the overall ionic strength is below 15 mM. Our *in*

539 *vitro* experiments are all carried out using *stringent conditions,* and the binding and reaction buffers are

540 optimized to minimize background interactions and to obtain optimal specificity; both salt

541 concentrations (150 and 250 mM) are likely above the *predicted* intracellular salt concentrations. The

542 fact remains that TFE alpha/beta function is sensitive to the relatively small differences in chloride

543 concentrations.

544

545 15. p10 li4 Wasn't an effect of TFS4 on inhibition of elongation shown in Fig. 2?

546

547 Figure 2 shows that TFS4 cannot reactivate arrested elongation complexes, while figure 4 shows that

548 TFS4 inhibits transcription elongation complexes.

549

550 16. p11 last line. This is a point where it's particularly unclear why the simple steric inhibition of NTP

551 binding isn't considered as an explanation for inhibitory effects on elongation.

552

553 See above, our kinetic experiments show that TFS4 inhibition indeed is dependent on the NTP

554 concentration, and we are discussing this new finding in the revised manuscript.

555

556 17. p12 li14 use of two "which" in the independent clause is confusing.

557

558 We altered the sentence to the following:

559 *'As a negative control, we monitored the protein levels of the RNAP subunit Rpo7 and the chromatin*

560 *protein Alba, which remained unchanged during the infection time course. This suggests that the*

561 *increase in TFS4 levels was specific to TFS4 and not due to a general decrepitude as a result of virus*

562 *infection (Fig. 6c).'*

563

564 18. p15 li7 There also are data on a Gre mutant in which substitution of one or two of the acidic

565   residues turns it into an inhibitor. I apologize for not looking up the citation, but I think it's work from

566   Arkady Mustaev, and would be worth citing here.

567

568   We have introduced the suggested reference (Laptenko et al., EMBO J, 2003), which describes that

569   the alanine substitution variants of GreA's acidic residues turn the factor into a inhibitor.

570

571   19. p15 li11 Again here, it's unclear why the direct competition for NTP binding isn't considered.

572

573   See above, our kinetic experiments show that TFS4 inhibition indeed is dependent on the NTP

574   concentration, and we are discussing this new finding in the revised manuscript.

575

576   20. Fig. 4 Why is the formation of ECs so inefficient? What percentages of DNA and RNAP are formed

577   into EC? Since it's hard to see appearance of DNA released from EC upon TFS4 binding, the

578   conclusion that TFS4 disrupts EC is not particularly well supported by the experiment. Isn't it possible

579   that DNA remains bound and TFS4 binding just causes the EC to smear out during electrophoresis?

580

581   Following both reviewer-1's and -2's recommendations we have repeated the TEC EMSA assays. The

582   new optimized binding experiments show very clearly (i) a superhift of the RNAP-DNA-RNA upon the

583   addition of TFS1 reflecting RNAP-DNA-RNA-TFS1 complex formation, and (ii) that the addition of

584   TFS4 disrupts the RNAP-DNA-RNA complex. For direct comparison we have used the same

585   factor:RNAP ratios for TFS1 and -4.

586

587   21. Fig. 5d and 5e Is the x-axis correctly labeled? It seems like it should be TFS4/RNAP ratio. Also,

588   maybe don't use gray for the triple A mutant – it's hard to distinguish from black on the figure.

589

590   We introduced the suggested change.

Reviewers' Comments:

Reviewer #1 (Remarks to the Author):

Authors have made corrections in the text and Figures and addressed all my questions and concerns. Authors also added new important data on the nature of TFS4 inhibitory activity which I think significantly improves the paper. It can now be published without further revision.

Reviewer #2 (Remarks to the Author):

Fouqueau et al have done an admirable job of revising their manuscript in response to suggestions from the reviewers, and the revised manuscript is largely suitable for publication.

In finalizing the manuscript, the authors may wish to consider slightly modifying and clarifying their explanation for effects of TFS4 on elongation. Reviewer 1 was concerned about the lack of shorter product accumulation in fig 4a and whether this was inconsistent with inhibition of catalysis. The competitive inhibition assay conducted by the authors supports their conclusion, but they may wish to additionally point out that inhibition of catalysis will not result in accumulation of shorter products if the NTP binding constant for the first round of nucleotide addition during initiation is much higher than the average NTP binding constant during elongation. This disparity in NTP binding affinities is typical for multisubunit RNA polymerases because the first round of nucleotide addition requires two NTPs instead of one, and the position of the template strand is not stabilized by an RNA-DNA hybrid. Additionally, the authors may wish to consider whether describing the appearance of shorter transcripts in fig 4b using the TFS4 mutant reflects "reduced transcription processivity". First, as processivity is loosely defined in the contemporary transcription field, it is often used to mean that RNAP has a greater or lesser tendency to release the DNA template. The appearance of the shorter transcripts could reflect release of the template but could also just reflect a position at which the TFS4 mutant is unusually good at inhibiting nucleotide addition, thus slowing the polymerase without releasing the template. More information would be needed to assess template release. Second, in polymer enzymology, processivity is measure of the ability of a chain to be extended by a processively bound enzyme rather than by a new enzyme molecule. Thus reduced processivity would not necessarily lead to an accumulation of shorter products just a change in how they are elongated. Because of this confusion, processivity may be a term best avoided in the transcription field.

These are minor points that the authors can consider, but overall the manuscript is in good shape for publication.

Reviewer #1 (Remarks to the Author):

Authors have made corrections in the text and Figures and addressed all my questions and concerns. Authors also added new important data on the nature of TFS4 inhibitory activity which I think significantly improves the paper. It can now be published without further revision.

Reviewer #2 (Remarks to the Author):

Fouqueau et al have done an admirable job of revising their manuscript in response to suggestions from the reviewers, and the revised manuscript is largely suitable for publication.

In finalizing the manuscript, the authors may wish to consider slightly modifying and clarifying their explanation for effects of TFS4 on elongation. Reviewer 1 was concerned about the lack of shorter product accumulation in fig 4a and whether this was inconsistent with inhibition of catalysis. The competitive inhibition assay conducted by the authors supports their conclusion, but they may wish to additionally point out that inhibition of catalysis will not result in accumulation of shorter products if the NTP binding constant for the first round of nucleotide addition during initiation is much higher than the average NTP binding constant during elongation. This disparity in NTP binding affinities is typical for multisubunit RNA polymerases because the first round of nucleotide addition requires two NTPs instead of one, and the position of the template strand is not stabilized by an RNA-DNA hybrid.

These are valid considerations, and we have incorporated this statement into the revised version of the manuscript:
'*The inhibition of catalysis would not result in accumulation of shorter products if the NTP binding constant for the first round of nucleotide addition during initiation is much higher than the average NTP binding constant during elongation. This disparity in NTP binding affinities is typical for multisubunit RNA polymerases because the first round of nucleotide addition requires two NTPs instead of one, and the position of the template strand is not stabilized by an RNA-DNA hybrid.*'

Additionally, the authors may wish to consider whether describing the appearance of shorter transcripts in fig 4b using the TFS4 mutant reflects "reduced transcription processivity".

This comment is likely based on a misunderstanding, **figure 4b** shows indeed an accumulation of partial products but this is caused by the TFS1-AA variant, and <u>not TFS4</u> at all, and hence the considerations below are not relevant.

First, as processivity is loosely defined in the contemporary transcription field, it is often used to mean that RNAP has a greater or lesser tendency to release the DNA template. The appearance of the shorter transcripts could reflect release of the template but could also just reflect a position at which the TFS4 mutant is unusually good at inhibiting nucleotide addition, thus slowing the polymerase without releasing the template. More information would be needed to assess template release. Second, in polymer enzymology, processivity is measure of the ability of a chain to be extended by a processively bound enzyme rather than by a new enzyme molecule. Thus, reduced processivity would not necessarily lead to an accumulation of shorter products just a change in how they are elongated. Because of this confusion, processivity may be a term best avoided in the transcription

field.

We do appreciate the complications arising from using the term processivity and have removed it from the original statement characterising the TFS1-AA variant, which now reads:
'Interestingly the TFS1-AA mutant leads to the accumulation of partial 45/46 nt transcripts (Fig. 4b)'
instead of *'Interestingly the TFS1-AA mutant reduced transcription processivity, which is reflected in the accumulation of partial 45/46 nt transcripts (Fig. 4b).'*

These are minor points that the authors can consider, but overall the manuscript is in good shape for publication.