nature portfolio

Peer Review File

A glycolytic intermediate gatekeeps global mRNA decay in Escherichia coli

Corresponding Author: Dr Sue Lin-Chao

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
In this manuscript submitted to Nature Communications, Plágaro and colleagues seek to understand how glycolysis impacts RNA degradation in Escherichia coli. In short, the authors provide evidence that 3-phosphoglycerate and fructose-1,6-bisphosphate inhibit the RNA degradation, but not RNA binding activity of PNPase in vitro (Fig. 1), that the accumulation of fructose-1,6-bisphosphate within an E. coli cell can reduce growth and inhibit decay of a known PNPase substrate (Fig. 2), and introduction of a mutation into a PNPase-dependent mutant that causes increased fructose-1,6-bisphosphate levels results in decreased decay of some transcripts, but increased turnover of others (Fig. 3). Finally, the authors provide evidence that PNPase may either directly or indirectly interact with certain glycolytic enzymes such as PfkA, FbaA, and GapA.

Overall, the manuscript is well written, quite comprehensive, and generally support the authors extraordinary conclusions that glycolytic intermediates inhibit the ability of PNPase to degrade certain RNA substrates. But, it is my opinion that the authors should provide additional information, temper some of their statements, or provide additional data to support these conclusions. First, the authors should provide all of their transcriptomic results in the supplemental section (comment #1). Secondly, the authors should clarify that the interactions that they observe between PNPase and certain glycolytic enzymes detected through the two-hybrid assays or pull-downs could be indirect through another protein(s) (comment #2), as their results do not rule out that possibility. Finally, the authors should provide additional detail with regards to the micrococcal nuclease treatment of cell lysates prior to CLIP-MS, specifically, how the authors validated that the samples were RNA-free (comment #7). I have long opined that micrococcal nuclease treatment should be ineffective in removing RNA protected by proteins within a complex.

Major Criticisms:

1. The authors analyzed degradation profiles for 3490 transcripts in the E. coli K12-MG1655 genome, yet Table S1 only contains results for those representing those that the authors indicate show decreased or increased degradation. In my opinion, the authors should present the data for all transcripts analyzed in that or another supplementary table in order to make the complete results more accessible to a larger audience, which may lack the technical skills to download the raw data and/or analyze it.

2. L224-320. The results from the two-hybrid and co-immunoprecipitation experiments do not rule out the possibility that the interactions observed between PNPase and glycolytic enzymes such as PfkA, FbaA, or GapA are indirect, e.g., via RNase E. As other proteins can bridge the interactions in these assays. Along those lines, it is a bit surprising to me that these experiments were done with strains containing wild type rne rather than strains in which the region encoding the C-terminal domain of RNase E or just the PNPase binding site was deleted. I know from experience that these mutations are compatible with the two-hybrid strain. Regardless, I would suggest that the authors at least more clearly highlight here the caveat that these identified interactions between PNPase and glycolytic enzymes could be indirect potentially through interactions with RNase E, the rest of the RNA degradosome, or other proteins. In my opinion that is not adequately conveyed. I don't feel that the western blots of the membrane fractions sufficiently refute this possibility, if that was an intent behind them.

Minor comments:

3. Fig. 2A. Results of T-tests assessing the statistical significance of differences in doubling times and F16dp concentrations would bolster the conclusions made from these results.

4. L203-217. The authors compare their list of differentially expressed genes between a rnr rnb and rnr rnb fbp strains to those that co-IPed with a PNPase D492G mutant and refer to a manuscript submitted elsewhere. Either here or it the discussion it could be useful to compare genes identified as differentially expressed to those co-IPed with PNPase in a prior publication (PMC4748814), if feasible.

5. Figure 4A. "adenilate" should be "adenylate"

6. L236 Change "proteins by quadruplicate" to "proteins in quadruplicate"

7. L304-5. Did you verify by Qubit or some other assay that the sample was void of RNA after micrococcal nuclease treatment? RNAs coated by proteins should be inaccessible to the active site of micrococcal nuclease and should remain.

8. L579 correct "PNPAse" to "PNPase"

9. Fig. 6b. Some the bands in the western blot for PNPase are nonspecific. The authors should indicate what is a nonspecific band and which band should correspond to PNPase.

10. L678 "electronic" should be "electrostatic"

11. In general, hours should be abbreviated "h" and minutes should be "min". In some instances, the authors do not abbreviate.

12. L824 "after" should be "at"

13. L1229 "Ncytoplasm" should be "cytoplasm"

Reviewer #2

(Remarks to the Author)
Review of the manuscript “A glycolytic intermediate gatekeeps global mRNA decay in Escherichia coli”

This study uncovers a direct regulatory link between glycolysis and global mRNA decay in E. coli. This link is mediated by the enzyme polynucleotide phosphorylase (PNPase). PNPase is a highly conserved 3’-5’ exoribonuclease involved in RNA degradation and processing, often functioning within multiprotein RNA degrading complexes that include glycolytic enzymes. Key findings include the glycolytic intermediate fructose 1,6 bisphosphate (F16dp) binds to E. coli PNPase and inhibits its RNA degrading activity in vitro. Increasing intracellular F16dp levels in vivo slows bacterial growth and globally stabilizes mRNAs, especially in a strain where PNPase is essential. PNPase physically and transiently interacts with multiple glycolytic enzymes, as shown by bacterial two-hybrid assays and in vivo crosslinking/immunoprecipitation. These interactions between PNPase and other proteins suggest the formation of metabolons, where metabolic enzymes and RNA decay factors are physically associated. The authors propose that RNA phosphorolysis, an energy saving RNA degradation pathway, is modulated by glycolytic state, allowing the cell to coordinate metabolism with post transcriptional gene regulation. The overall significance of this work demonstrates that central metabolites of glycolysis F16dp and 3PG directly modulate PNPase, a major RNA degrading enzyme, revealing a mechanism by which bacterial cells couple metabolic status to global RNA stability. Because PNPase is essential in some species and conserved across life, this regulatory mechanism may be widespread among bacteria relevant to health, the environment, and biotechnology.

Major comments:
1. Fig 1B, the 5 nt-long substrate is clearly absent from PNPase incubation with high concentration of F16dp and 3PG. However, there is a ladder of RNA bands (independent from the metabolite used) in the upper part of the gel immediately below the 30 nt band. Please discuss.
2. Fig 1D and extended data Fig 2, an additional docking experiment but with 3PG metabolite could help to make a complete figure and to strengthen the data.
3. Fig 2A, as mentioned by the authors (lines 149-151) glucose might be important for growth defect or growth rate too. It looks like the richer the carbon source, the more dt difference. This could be related to tRNA processing kinetics (Fig 2D), which becomes the more important as growth rate increases. How would the strains behave on a much richer medium like LB our BHI?
4. Fig 2D, there are 2 bands for the processing intermediate leuX. What has been used for measuring the hal-life?
5. Using a fbp mutant is interesting to force the system to accumulate F16dp and observe phenotypes. However, have the authors tried to add F16dp directly into the growth medium to inactivate PNPase?
6. Fig 3C, while the KEGG analysis did not reveal any specific pathways, I am curious about common functions (mRNAs, sRNAs, tRNAs, rRNAs). Please discuss.
7. Small RNAs not only recruit the RNA degradosome to degrade target mRNAs, but they are also themselves degraded

through the RNA degradosome and PNPase (Andrade 2012). It could be an important point to address whether a well characterized system, such as SgrS turnover or when inducing the degradation of ptsG mRNA for example (especially that ptsG encode a glucose phosphotransferase), would be affected by F16dp.
8. PNPase is quite conserved and is also found in human. Have the authors tried to demonstrate any potential parallels with F16dp and the human PNPase (e.g. molecular docking)? In any case, it would be important to discuss this in the discussion section.
9. Fig 4, I realize that the choice of PfkA and GapA because of the most interacting partners, but they do not interact with PNPase as strongly than TpiA, GpmA, PykA, the last one with the strongest interaction. This series of experiments started with the idea of close proximity between PNPase (lines 224-227). The authors should clarify this. Also, I would expect to see PNPase and RNAse E included as a control in this experiment given the known interaction with each other.
10. Fig 6 does not clearly indicates which of these enzymes interact with each of three FLAG-tagged baits. Furthermore at this point of the manuscript, this section would benefit from explicitly showing how these enzymes relate to PNPase
11. in term of interaction/closeness to these enzymes. Please clarify this.

Minor comments:
Please add a schematic of the metabolic pathway and mutation mentioned in lines 153-156.

An interesting experiment to perform with the BACTH experiment in Fig4 could be to investigate the interaction between PNPase and RNAse E. Given that PNPase conformation changes according to F16dp concentration, one could presume the PNPase/RNAse E interaction is affected.

Fig 5, the pfkA-, fbaA- or gapA may be misleading. I would write pfkA-3XFLAG or fbaA-3XFLAG or gapA-3XFLAG everywhere.

Reviewer #3

(Remarks to the Author)
The manuscript by Plagaro et al. describes the interplay between Escherichia coli metabolism and RNA degradation. In this work, the authors investigate the influence of several metabolites, mainly fructose-1,6-diphosphate (F16dP), on the activity of polynucleotide phosphorylase (PNPase). They show that, in vitro, these metabolites are capable of inhibiting PNPase activity, despite having no apparent effect on the enzyme's ability to bind substrate RNA. The authors also present a series of cellular experiments indicating that F16dP influence intracellular RNA levels. In addition, they report interactions between PNPase and several enzymes involved in bacterial metabolic pathways. Overall, the manuscript addresses an interesting and long-standing hypothesis regarding the connection between RNA metabolism and central carbon metabolism in bacteria. The study therefore has the potential to provide valuable insights into the regulatory interplay between metabolic state and RNA degradation. However, several points require clarification or further discussion before the manuscript can be considered for publication.
1. It is somewhat difficult to follow the experimental logic without prior knowledge of bacterial carbon metabolism. The introduction would benefit from a brief overview of the relevant metabolic pathways so that readers can better understand the relationships between the investigated enzymes, metabolites and the physiological conditions used in the study. In particular, the authors could briefly describe the metabolic context of the carbon sources mentioned in the manuscript (e.g., glucose, glycerol, citrate, and acetate).
2. The authors report that the investigated metabolites affect PNPase catalytic activity but not RNA binding. Given this observation, the rationale behind modelling F16dP, Mg^{2+}, and RNA separately and subsequently overlaying the models is not entirely clear. A clearer explanation of the modelling strategy and its limitations would help the reader interpret these results. If the authors propose that the metabolite binds in the catalytic site, it would be informative to test this hypothesis experimentally in order to strenghten the proposed model, for example by introducing mutations in residues predicted to participate in metabolite binding and examining whether these mutations affect metabolite binding or enzymatic inhibition.
3. I am not fully convinced by the interpretation of the experiments performed in the PNP-essential fbp deletion strain. The authors show that accumulation of F16dP slightly slows bacterial growth and leads to increased RNA stability, and they interpret this as evidence for inhibition of PNPase activity. However, it is not entirely clear that the presented data uniquely support this interpretation.
a. It is well established that RNA degradation in Escherichia coli is closely linked to cellular metabolism. For example, the degradosome itself contains a metabolic enzyme (enolase). Deletion of a metabolic enzyme may therefore influence multiple cellular pathways. Consequently, the observed increase in RNA stability could potentially arise from broader metabolic perturbations, rather than solely from inhibition of PNPase activity. In addition, the RNA-seq results indicate that 5% of transcripts show increased degradation, suggesting that the cellular response to the deletion may be more complex than simple inhibition of PNPase.
b. The authors also show that leuX, previously reported to be regulated by PNPase, displays an increased half-life. However, the Northern blot shown in Fig. 2d is somewhat difficult to interpret. In the study cited as reference 23, three leuX intermediates were observed, not all of which were strictly dependent on PNPase. In the current manuscript the RNA bands appear only partially resolved, making it difficult to determine which band was quantified for the half-life estimation. It would be helpful if the authors clarified how the signal was quantified and how they ensured that only the relevant band was included in the analysis. Information about the reproducibility of these measurements and the associated error estimates would also strengthen this result.
c. The authors identified 275 transcripts as PNPase substrates, of which 89 showed prolonged half-life in the mutant strain. Were the remaining transcripts unchanged? If accumulation of F16dP indeed inhibits PNPase activity, one might expect stabilization of a larger fraction of PNPase substrates. It would be helpful if the authors discussed possible explanations for

this observation.
4. In light of the points raised above, several statements in the discussion appear somewhat stronger than the experimental evidence currently supports and could benefit from more cautious wording. For example, the statement: "… our data show that the single deletion of a metabolic enzyme in a PNP-essential background leads to an increase in F16dP, which is sufficient to globally prolong mRNA half-life through metabolic inhibition of PNPase in the PNP-essential fbp-deletion strain" appears convincingly supported only up to the first comma. Similarly, "Additional deletion of fbp in the PNP-essential strain led to a 1.5-fold increase in F16dP levels, which correlated with compromised growth and global RNA stabilization, including stabilization of direct substrates of PNPase. These results strongly support the F16dP-mediated inhibition of PNPase in this strain" - this may be somewhat premature based on the available data. While the observations are consistent with this hypothesis, they do not yet demonstrate a direct causal relationship.
5. It would be helpful for the authors to clarify where the affinity tags were fused to PNPase. The FLAG tag appears to be located at the N-terminus, but this should be explicitly stated. In Supplementary Fig. 1, two bands of similar intensity are visible for the purified PNPase variants. The origin of these bands is not immediately clear. Since Polynucleotide phosphorylase is known to form trimers, it would be useful to discuss whether the tag might influence oligomerization. Structurally, the N-terminus lies relatively close to the protomer-protomer interface, and therefore it would be helpful to clarify whether the authors tested whether the tagged protein forms trimers with the same efficiency as the WT enzyme.
6. A related point concerns the bacterial two-hybrid experiments. T25 and T18 proteins used in this assay are substantially larger than the FLAG tag, and it would therefore be useful to clarify whether PNPase retains its native oligomeric state when fused to these proteins. The interaction signal for PNPase self-association appears weaker than for some of the interactions reported with metabolic enzymes, could the authors provide an explanation for this observation?
7. The manuscript would benefit from a broader discussion of how RNA degradation and carbon metabolism may be coordinated spatially within the bacterial cell. For example, do the authors envision that PNPase interacts with metabolic enzymes while simultaneously participating in RNA degradation? Would such interactions occur sequentially, or within larger multiprotein assemblies? Is the relevant PNPase population the degradosome-associated enzyme or the free cytoplasmic pool? Even if the present data do not directly address these questions, discussing these possibilities would help place the findings in a clearer biological context.
8. In Fig. 6B it is not entirely clear whether the samples visible on the Western blot correspond to crosslinked complexes or to material obtained after reversal of crosslinking. It would also be helpful if molecular weight markers were clearly shown. In addition, PNPase appears to migrate at approximately 100 kDa, and more than one band is visible. This migration does not correspond exactly to the expected molecular weight of the PNPase monomer (~80 kDa), nor to the expected size of a crosslinked complex with the bait proteins (~40 kDa). Could the authors provide a brief explanation of how these bands were interpreted?

Minor coments:
1. Figure 1 – please indicate in the legend that RNA is shown in green.
2. Line 53 – the manuscript refers to "ribonuclease E (Rne)", while later "RNase E" is used. Please ensure consistent nomenclature.
3. The AlphaFold3 structure should not be described as "our model", as it was generated by computational prediction rather than derived directly from experimental data
4. "Our in vivo crosslinking results indicate that glycolytic proteins form transient complexes" - What observations lead the authors to conclude that these complexes are transient?
5. The phrase "5S rRNA, determined by bromophenol staining" is somewhat unclear. Bromophenol blue is typically used as a tracking dye rather than a nucleic acid stain. The authors may wish to clarify the staining method used for RNA visualization.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Summary:

In this manuscript submitted to Nature Communications, Plágaro and colleagues seek to understand how glycolysis impacts RNA degradation in Escherichia coli. There is a growing body of evidence indicating that metabolism and metabolites modulate RNA decay in bacteria.
In short, the authors provide evidence that 3-phosphoglycerate and fructose-1,6-bisphosphate, two intermediates of glycolysis, inhibit the RNA degradation, but not RNA binding activity of PNPase in vitro (Fig. 1), that the accumulation of fructose-1,6-bisphosphate within an E. coli cell can reduce growth and inhibit decay of a known PNPase substrate (Fig. 2), and introduction of a mutation into their PNPase-dependent mutant that causes increased fructose-1,6-bisphosphate levels results in decreased decay of some transcripts, but increased turnover of others (Fig. 3). Finally, the authors provide evidence that PNPase may either directly or indirectly interact with certain glycolytic enzymes such as PfkA, FbaA, and GapA (Figs. 4, 5, and 6).

Overall, the manuscript is well written, quite comprehensive, and support the authors extraordinary conclusions that certain glycolytic intermediates inhibit the ability of PNPase to degrade certain RNA substrates. As the authors noted, this modulation of RNA decay by glycolytic intermediates may also be important in numerous other bacteria particularly those in which PNPase may be the sole exoribonuclease and in humans, which also contain a mitochondrial targeted PNPase.

The authors satisfactorily address my prior criticisms, which improved the manuscript. Some minor editing issues listed below should be addressed.

Major Criticisms:

None.

Minor comments:

L31. I think the authors meant "exploited" and not "explored"

L155. Suggested change "presence of these metabolites suggests that they bind the catalytic site."

L161. Replace the comma with a semicolon or period

L180 Change to "on the growth rate"

L197. Replace comma with a semicolon or period.

L232. the "2" in "log2" should be subscripted.

L367. Replace the comma with a semicolon or period.

L390. Replace the comma with a semicolon or period.

L1061. the "2" in "log2" should be subscripted.

L1421 and L1423. the "2" in "log2" should be subscripted.

Extended data figure 5 panels. the "2" in "log2" should be subscripted.

Reviewer #2

(Remarks to the Author)
The revised version of this manuscript has thoroughly addressed all the points I raised in my review. I find the new version of the manuscript satisfactory and support its publication.

Reviewer #3

(Remarks to the Author)
The authors have provided valuable additional data and revised the text, which has strengthened the manuscript. They have also clarified several of my initial concerns. However, in my opinion, a few critical points have not yet been sufficiently addressed.
Ad 2 - I am having difficulty following the logic regarding the rationale for this experiment. The mutant used to demonstrate metabolite binding in the active site is not marked in Figure 1d as an interacting residue in the AlphaFold model (nor is D486 mentioned in the text). It also does not show up in the molecular dynamics simulations (Extended Data Figure 3). If I understand the setup correctly, the authors should perform the assay using a PNPase mutant targeting the residues that the model actually highlights as the primary interactors (e.g. S437, S438, S439, H403) to demonstrate lack of thermal shift. Given the data presented, it remains unclear why the results obtained for the D492 mutant indicate a total lack of interaction with any of the metabolites.
Additionally, there appears to be an error in the text: “residue D492 is predicted to correspond to the phosphate-binding site, which mutation abolishes catalysis.” - this residue corresponds to the magnesium-binding site, not the phosphate-binding site.
Ad 3 - The revised text states: “…simultaneously disrupting its protective role through conformational changes that prevent PNPase interaction with other proteins such as RNase E, RhlB or Hfq.” Do the authors structural models actually support major conformational changes upon metabolite binding? Furthermore, to the best of my knowledge, PNPase's protective role has only been established in conjunction with Hfq.
Ad 6 - The authors state: “Therefore, whether these tags interfere with the formation of the PNPase trimer, and whether PNPase retains its native oligomeric state is irrelevant to our major conclusion.” - if the authors are using a PNPase protomer as bait (because the two large fused protein domains prevent native trimerization) it fundamentally changes the relevance of the experiment. A protomer exposes completely different surface areas than a trimer and lacks native functionality. Consequently, any interacting proteins captured by a monomeric bait may be biologically irrelevant to the functional trimer. The fact that the structural impact of these fusions on the bacterial two-hybrid assay remains unknown represents a major limitation. Given that in vivo crosslinking followed by immunoprecipitation demonstrates cellular proximity but does not definitively prove a direct biochemistry-level interaction, the authors definitive conclusion in the abstract

(“Bacterial two-hybrid and in vivo crosslinking followed by immunoprecipitation demonstrate a physical and transient interaction...”) is considerably overstated. In light of these experimental limitations, the claim of a direct physical interaction is not sufficiently supported by the data provided.
Line 805 - this should be pI instead of pH

Version 2:

Reviewer comments:

Reviewer #3

(Remarks to the Author)
The authors have addressed all of my concerns and provided necessary explanations. One thing that remains puzzling for me is the result of the thermal shift assay. In the F16DP model, D492 supports nicely the binding of the metabolite and here I would agree that this is a strong interaction, however, it is surprising that in the presence of the other strong interactions removal of this one amino acid results in abolishment of metabolite binding. However, in the 3PG model the residue D492 is over 4A away from 3PG, what suggests lack of interaction and even if any, then very weak and I highly doubt that mutation of this residue would result in total abolishment of metabolite binding, considering the fact that there are many other, much closer and stronger contacts between the metabolite and the protein. Therefore, the AlphaFold model might be wrong.

We are grateful for the thoughtful reviews, critical comments and suggested experiments. We express our sincere gratitude to the referees in the “Acknowledgements” section of the revised manuscript.

To respond to their suggestions, we have included new Extended Data Fig. 1 presenting an overview of central carbon metabolism in *E. coli* (extended introduction suggested by Reviewer 3). We have incorporated additional information into Supplementary Table 1 (all detectable transcripts and those that coIP-ed with PNPase in a prior publication, suggested by Reviewer 1). We have performed additional experiments which have been included in Fig. 1d (additional docking suggested by Reviewer 2), Extended Data Fig. 3b, c and e (additional molecular dynamics simulation suggested by Reviewer 2), Extended Data Fig. 3f (additional nanoDSF with mutant PNPase suggested by Reviewer 3), Extended Data Fig. 4a (growth curve in LB suggested by Reviewer 2), Extended Data Fig. 7a (additional positive control for BACTH assay suggested by Reviewer 2) and Extended Data Fig. 7b (BACTH in newly constructed strain in response to Reviewer 1). The numbering and descriptions of the Extended Data Figures have been updated accordingly throughout the figures and the main text.

We have replaced bar graphs in Fig. 2c with box plots that include information about the distribution of the underlying data. Similarly, under the “Data availability” section we added a statement indicating Source Data are provided with this paper.

We think that the additional experiments and the revisions we made have strengthened our conclusions and improved the clarity of our manuscript. We believe we have addressed all criticisms, comments and suggestions raised by the referees, for which we provide our point-by-point responses below. We hope that our revised manuscript is now acceptable for publication in Nature Communications.

Sincerely,
Sue Lin-Chao

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

In this manuscript submitted to Nature Communications, Plágaro and colleagues seek to understand how glycolysis impacts RNA degradation in Escherichia coli. In short, the authors provide evidence that 3-phosphoglycerate and fructose-1,6-bisphosphate inhibit the RNA degradation, but not RNA binding activity of PNPase in vitro (Fig. 1), that the accumulation of fructose-1,6-bisphosphate within an E. coli cell can reduce growth and inhibit decay of a known PNPase substrate (Fig. 2), and introduction of a mutation into a PNPase-dependent mutant that causes increased fructose-1,6-bisphosphate levels results in decreased decay of some transcripts, but increased turnover of others (Fig. 3). Finally, the authors provide evidence that PNPase may either directly or indirectly interact with certain glycolytic enzymes such as PfkA, FbaA, and GapA.

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Major Criticisms:

1. The authors analyzed degradation profiles for 3490 transcripts in the E. coli K12-MG1655 genome, yet Table S1 only contains results for those representing those that the authors indicate show decreased or increased degradation. In my opinion, the authors should present the data for all transcripts analyzed in that or another supplementary table in order to make the complete

results more accessible to a larger audience, which may lack the technical skills to download the raw data and/or analyze it.

Done, thank you for the comment.

We have added information of the 3490 detectable transcripts into Supplementary Table 1. In addition, in response to the related Point 4 by this reviewer, we have also added the information regarding transcripts identified as differentially degraded compared to those that co-IPed with PNPase in a prior publication (PMC4748814). Moreover, following Point 6 by Reviewer 2, we added information about the common function (mRNA, tRNA, rRNA or sRNA) of each transcript. Accordingly, the table description (Line 1353) now reads “Supplementary Table 1 contains information of the 3490 detectable transcripts in *E. coli* PNP-essential *fbp*-deletion and the isogenic parental strains, including gene name, database identifier, common function, differential degradation, maSigPro degradation group, RNA half-life, log2 fold-change in RNA-half-life and binding by PNPase D492G or PNPase inhibited by tungstate in a prior publication [36]. NA indicates not applicable."

2. L224-320. The results from the two-hybrid and co-immunoprecipitation experiments do not rule out the possibility that the interactions observed between PNPase and glycolytic enzymes such as PfkA, FbaA, or GapA are indirect, e.g., via RNase E. As other proteins can bridge the interactions in these assays. Along those lines, it is a bit surprising to me that these experiments were done with strains containing wild type rne rather than strains in which the region encoding the C-terminal domain of RNase E or just the PNPase binding site was deleted. I know from experience that these mutations are compatible with the two-hybrid strain. Regardless, I would suggest that the authors at least more clearly highlight here the caveat that these identified interactions between PNPase and glycolytic enzymes could be indirect potentially through interactions with RNase E, the rest of the RNA degradosome, or other proteins. In my opinion that is not adequately conveyed. I don't feel that the western blots of the membrane fractions sufficiently refute this possibility, if that was an intent behind them.

We thank the reviewer for this critical comment and agree with the points. Done.

We have performed additional BACTH assay in an *E. coli* DHP1 derivative strain in which the *rne* region encoding the C-terminal domain of RNase E (amino acids 500 to 1061) was deleted (*i.e.*, *E. coli* DHP1_Rned500). Our results show that PfkA, FbaA, TpiA, GapA, GpmA or PykA interact with PNPase in *E. coli* DHP1_Rned500, indicating that these BACTH interactions are independent from the C-terminal domain of RNase E. These results are included into Extended Data Fig. 7b

(Line 1436). However, these interactions could be indirect in *E. coli* through other unknown proteins.

Along these lines, crosslinking and immunoprecipitation experiments do not rule out indirect interactions, consequently, the revised text in the discussion (Line 483 and Line 493) now reads “Although these results do not rule out the possibility that these interactions are indirect...” as well as “BACTH identified multiple glycolytic enzymes (PfkA, FbaA, TpiA, GapA, GpmA and PykA) physically interacting with PNPase independently of RNase E. This protein-protein interaction, whether direct or indirect (*i.e.*, potentially mediated by other protein), is further supported by in vivo crosslinking and immunoprecipitation...”

Minor comments:

3. Fig. 2A. Results of T-tests assessing the statistical significance of differences in doubling times and F16dp concentrations would bolster the conclusions made from these results.

Done. We thank the referee for this suggestion that helped us strengthen our conclusions.

The results of all the T-tests have been incorporated into Fig. 2. Similarly, results of the T-tests for the new experiment suggested in Point 3 by Reviewer 2 are included in Extended Data Fig. 4a. The respective figure legends (Line 754 and Line 1417) read as "Significant differences in mean values, as determined by a two-sided, unpaired, parametric t-test, are indicated by asterisks (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, NS = not significant)."

4. L203-217. The authors compare their list of differentially expressed genes between a rnr rnb and rnr rnb fbp strains to those that co-IPed with a PNPase D492G mutant and refer to a manuscript submitted elsewhere. Either here or it the discussion it could be useful to compare genes identified as differentially expressed to those co-IPed with PNPase in a prior publication (PMC4748814), if feasible.

Done.

Accordingly, the revised results section (Line 254) now reads “These results are further supported by analysis of a previously published dataset, comprising 277 transcripts targeted by chromosomally encoded PNPase inhibited by tungstate [36]. Of these transcripts, 72 were DDTs identified in the PNP-essential *fbp*-deletion strain, of which 71 showed increased RNA half-lives and 1 showed decreased RNA half-life (Supplementary Table 1). Of the remaining transcripts, the

stability of 128 remained unchanged and 77 transcripts were undetectable. Although KEGG analysis of these 72 PNPase-bound DDTs did not reveal specific pathway enrichment, they included 70 mRNAs and 2 sRNAs (encoded by *glmZ* and *sroH*). Only 3 DDTs were identified bound to ectopically expressed PNPase D492G and chromosomally encoded PNPase inhibited by tungstate. In summary, of the total 166 differentially degraded PNPase targets, 157 showed prolonged RNA half-lives and 9 showed reduced RNA half-lives in the PNP-essential *fbp*-deletion strain. The stability of 258 PNPase substrates remained unchanged." This information has been added into Supplementary Table 1 as described above (Point 1 by this reviewer). The specific pathways in which PNPase targets participate is discussed later (Line 443), please also see Point 6 by Reviewer 2 and Point 3 by Reviewer 3.

5. Figure 4A. "adenilate" should be "adenylate"

Done. Thank you for this comment. "Adenylate" is now spelled correctly in Fig. 4 (Line 766).

6. L236 Change "proteins by quadruplicate" to "proteins in quadruplicate"

Done. We are grateful for this correction and have revised the manuscript (Line 288) accordingly.

7. L304-5. Did you verify by Qubit or some other assay that the sample was void of RNA after micrococcal nuclease treatment? RNAs coated by proteins should be inaccessible to the active site of micrococcal nuclease and should remain.

Yes, we verified that RNA was effectively removed after micrococcal nuclease treatment as described below. We also agree that some RNA may remain, we consequently tempered our statement and the revised result section (Line 384) now reads as "We treated the cell lysates with micrococcal nuclease to remove most nucleic acids before fractionation and immunoprecipitation."

Prior to immunoprecipitation, RNA degradation was assessed by agarose gel electrophoresis, where no detectable rRNA or mRNA bands were observed (**Fig. R1**a, lane number 1). Following nuclease treatment for 40 min, no RNA signal could be detected (**Fig. R1**a, lane number 11). There was no appreciable protein degradation before or after nuclease treatment (**Fig. R1**b). In addition, attempts to isolate RNA from treated lysates using a standard hot phenol extraction protocol did not yield detectable RNA. Together, these results indicate that RNA was effectively removed under our experimental conditions and that any residual RNA, if present, was below the detection limit of these assays.

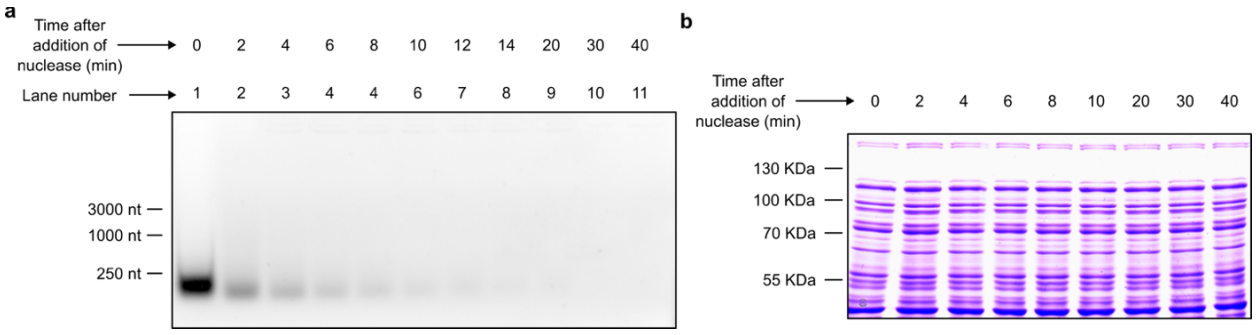

Fig. R1: Effects of micrococcal nuclease treatment on residual low molecular weight RNA and total proteins present in cell lysates. **a**, Analysis of equal aliquots of cell extracts treated with micrococcal nuclease for the indicated amount of time in formaldehyde-free agarose gel. This experiment shows the gradual degradation (disappearance) of low-molecular weight RNA during nuclease treatment. **b**, Analysis of equal aliquots of cell extracts treated with micrococcal nuclease for the indicated amount of time in Coomassie-stained, reducing and denaturing, polyacrylamide gel.

8. L579 correct "PNPAse" to "PNPase"

Done. We thank the reviewer for this correction that we include in the revised text (Line 729).

9. Fig. 6b. Some the bands in the western blot for PNPase are nonspecific. The authors should indicate what is a nonspecific band and which band should correspond to PNPase.

Done. We thank the referee for this suggestion. In the revised Fig. 6b (Line 789) and Extended Data Fig. 9b (Line 1457) the additional polypeptide detected in both the wild-type and the Δ*pnp* strains has been labelled as “non-specific signal”. The other polypeptide, detected exclusively in the wild type strains but not in the Δ*pnp* strains, has been labeled as “PNPase.”

10. L678 "electronic" should be "electrostatic"

Done. We are thankful for this indication and wording has been revised accordingly (Line 850).

11. In general, hours should be abbreviated "h" and minutes should be "min". In some instances, the authors do not abbreviate.

Done. We thank the referee for this comment, all mentions of minutes and hours have accordingly been abbreviated in the revised manuscript.

12. L824 "after" should be "at"

Done. We thank the reviewer for this comment and have revised the text accordingly (Line 1007).

13. L1229 "Ncytoplasm" should be "cytoplasm"

Done. We are grateful for this correction and the manuscript was revised accordingly (Line 1461).

Reviewer #2 (Remarks to the Author):

Review of the manuscript “A glycolytic intermediate gatekeeps global mRNA decay in Escherichia coli”

This study uncovers a direct regulatory link between glycolysis and global mRNA decay in E. coli. This link is mediated by the enzyme polynucleotide phosphorylase (PNPase). PNPase is a highly conserved 3’-5’ exoribonuclease involved in RNA degradation and processing, often functioning within multiprotein RNA degrading complexes that include glycolytic enzymes. Key findings include the glycolytic intermediate fructose 1,6 bisphosphate (F16dp) binds to E. coli PNPase and inhibits its RNA degrading activity in vitro. Increasing intracellular F16dp levels in vivo slows bacterial growth and globally stabilizes mRNAs, especially in a strain where PNPase is essential. PNPase physically and transiently interacts with multiple glycolytic enzymes, as shown by bacterial two-hybrid assays and in vivo crosslinking/immunoprecipitation. These interactions between PNPase and other proteins suggest the formation of metabolons, where metabolic enzymes and RNA decay factors are physically associated. The authors propose that RNA phosphorolysis, an energy saving RNA degradation pathway, is modulated by glycolytic state, allowing the cell to coordinate metabolism with post transcriptional gene regulation. The overall significance of this work demonstrates that central metabolites of glycolysis F16dp and 3PG directly modulate PNPase, a major RNA degrading enzyme, revealing a mechanism by which bacterial cells couple metabolic status to global RNA stability. Because PNPase is essential in some species and conserved across life, this regulatory mechanism may be widespread among bacteria relevant to health, the environment, and biotechnology.

Major comments:

1. Fig 1B, the 5 nt-long substrate is clearly absent from PNPase incubation with high concentration of F16dp and 3PG. However, there is a ladder of RNA bands (independent from the metabolite used) in the upper part of the gel immediately below the 30 nt band. Please discuss.

Done. Thank you for this comment.

The revised discussion (Line 417) now reads “In these assays, we observed a ladder of RNA bands similar to that previously reported for PNPase from different organisms, including *E. coli* [27]. The appearance of this ladder is independent of the 5’-FAM-labelled oligonucleotide sequence and length, as well as the presence of inhibitory metabolites, and is likely due to PNPase stalling, as previously discussed [27].”

2. Fig 1D and extended data Fig 2, an additional docking experiment but with 3PG metabolite could help to make a complete figure and to strengthen the data.

Done. We are grateful for this suggestion that has strengthened our data.

We have performed additional docking of PNPase with 3PG using AlphaFold 3, these results have been incorporated into Fig. 1d (Line 719). Accordingly, the revised results section (Line 98) now states “The binding site was then predicted by docking three molecules of F16dP or 3PG and homotrimeric PNPase using AlphaFold 3, which showed one F16dP or 3PG molecule located at the known active site.” The corresponding MD simulations have been incorporated into Extended Data Fig. 3b, c and e (Line 1390). Accordingly, the results section (Line 128) now reads “We explored whether the F16dP-bound and 3PG-bound PNPase models were stable by comparison to unbound PNPase through molecular dynamics simulation with GROMACS. As shown in Extended Data Fig. 3b, the root mean square deviation (RMSD) of F16dP-bound or 3PG-bound PNPase relative to the initial conformation (2.97 ± 0.16 Å or 2.77 ± 0.18 Å for the last 2 ns of simulation, respectively), was indistinguishable from that of unbound PNPase (2.76 ± 0.23 Å). Furthermore, the similarity in the root mean square fluctuation (RMSF) of the F16dP-bound, 3PG-bound and the unbound protein (Extended Data Fig. 3c) suggested that the ligand did not affect protein flexibility. Moreover, F16dP and 3PG interacted with the same residues at the initial and final time points of the molecular dynamics simulation (Extended Data Fig. 3d-e), suggesting that the models of F16dP-bound PNPase and 3PG-bound PNPase are stable."

3. Fig 2A, as mentioned by the authors (lines 149-151) glucose might be important for growth defect or growth rate too. It looks like the richer the carbon source, the more dt difference. This could be related to tRNA processing kinetics (Fig 2D), which becomes the more important as growth rate increases. How would the strains behave on a much richer medium like LB our BHI?

We agree with the point raised. Done.

When cultured in LB, the growth of the PNP-essential strain (dt of 24 min) was statistically indistinguishable from that of the isogenic wild type (dt of 24 min). These results have been

incorporated into Extended Data Fig. 4a (Line 1413). Accordingly, the results section (Line 171) now indicates “A similar effect was observed in a rich medium primarily composed of amino acids. Namely, growth of the PNP-essential strain on LB (dt of 24 min) did not significantly differ from that of the isogenic wild type (dt of 24 min) (Extended Data Fig. 4a).”

4. Fig 2D, there are 2 bands for the processing intermediate leuX. What has been used for measuring the hal-life?

We used both visible processing intermediates bands. We added arrowheads to the Fig. 2 (Line 740) and the revised legend (Line 748) accordingly indicates “Northern blot analysis showing the half-life of the processing intermediates of *leuX* transcript, indicated by arrowheads.”

5. Using a fbp mutant is interesting to force the system to accumulate F16dp and observe phenotypes. However, have the authors tried to add F16dp directly into the growth medium to inactivate PNPase?

Thank you for this comment.

Given that *E. coli* does not uptake F16dP for growth (doi: 10.1002/jcp.1030360103), the metabolite would not enter the cell when added into to the growth medium. The revised manuscript (Line 180) now clearly explains that “To investigate the impact of increased F16dP in the growth rate of the PNP-essential strain, given that *E. coli* does not uptake F16dP for growth [33], we used a genetic engineering approach.”

6. Fig 3C, while the KEGG analysis did not reveal any specific pathways, I am curious about common functions (mRNAs, sRNAs, tRNAs, rRNAs). Please discuss.

Done.

Following suggestions from other reviewers (Point 4 by Reviewer 1 and Point 3 by Reviewer 3) we included RNA targets previously identified bound to chromosomally encoded PNPase in the presence of the inhibitor tungstate, as well as targets that showed decreased RNA half-life.

The revised results section (Line 233, Line 247 and Line 254) now reads “The 1321 DDTs included 1296 mRNAs, 11 sRNAs, 11 tRNAs and 3 rRNAs” as well as “While KEGG analysis of the 97 PNPase-bound DDTs did not reveal specific pathway enrichment, they included 96 mRNAs and 1 tRNA (encoded by *lysZ*)” and “These results are further supported by analysis of a previously published dataset, comprising 277 transcripts targeted by chromosomally encoded PNPase inhibited by tungstate [36]. Of these transcripts, 72 were DDTs identified in the PNP-essential fbp-

deletion strain, of which 71 showed increased RNA half-lives and 1 showed decreased RNA half-life (Supplementary Table 1). Of the remaining transcripts, the stability of 128 remained unchanged and 77 transcripts were undetectable. Although KEGG analysis of these 72 PNPase-bound DDTs did not reveal specific pathway enrichment, they included 70 mRNAs and 2 sRNAs (encoded by *glmZ* and *sroH*)." The specific pathways in which PNPase targets participate is discussed later (Line 443).

7. Small RNAs not only recruit the RNA degradosome to degrade target mRNAs, but they are also themselves degraded through the RNA degradosome and PNPase (Andrade 2012). It could be an important point to address whether a well characterized system, such as SgrS turnover or when inducing the degradation of ptsG mRNA for example (especially that ptsG encode a glucose phosphotransferase), would be affected by F16dp.

We agree this is an interesting point. Done.

Following this and the related Point 6 by this reviewer we extended our discussion (Line 449) as follows "While the direct substrates of PNPase did not show specific pathway enrichment, they included the sRNAs GlmZ and SroH, which contribute to cell envelope synthesis and the response to envelope stress, respectively. GlmZ post-transcriptionally regulates translation of *glmS* mRNA, which encodes an enzyme in the pathway for cell envelope biosynthesis [46]. In comparison, deletion of the small RNA SroH in *E. coli* MG1655 increases sensitivity to cell envelope stress [47]. In contrast, the SgrS small RNA, which triggers degradation of the *ptsG* mRNA under conditions of phosphosugar accumulation [48], was undetectable in our RNA-seq data. Consistently, the stability of the *ptsG* mRNA remained unchanged (half-life of 3.4 min), comparable to that previously reported in *E. coli* growing in LB supplemented with glucose (3.3 min). This indicates that phosphosugar stress responses are not activated under our experimental conditions."

8. PNPase is quite conserved and is also found in human. Have the authors tried to demonstrate any potential parallels with F16dp and the human PNPase (e.g. molecular docking)? In any case, it would be important to discuss this in the discussion section.

This is a very interesting question.

Indeed, we purified human PNPase in collaboration with two of the acknowledged researches (Dr. Hanna S. Yuan and Dr. Yi-Ching Li) and determined its activity by measuring the degradation of the same RNA substrate under the same conditions described in this manuscript in the presence of varying concentrations of F16dP (**Fig. R2**a). These results suggest that the activity of human

PNPase is inhibited by F16dP *in vitro* (**Fig. R2**b). Although these results are exciting, they are preliminary and the assay conditions require further optimization to enable reliable measurement of F16dP IC_{50}. Moreover, since human PNPase is located within the mitochondria, where metabolism and metabolite concentration differ from that of *E. coli*, we intentionally omitted these results during drafting of the manuscript because we felt they were beyond the scope of our story.

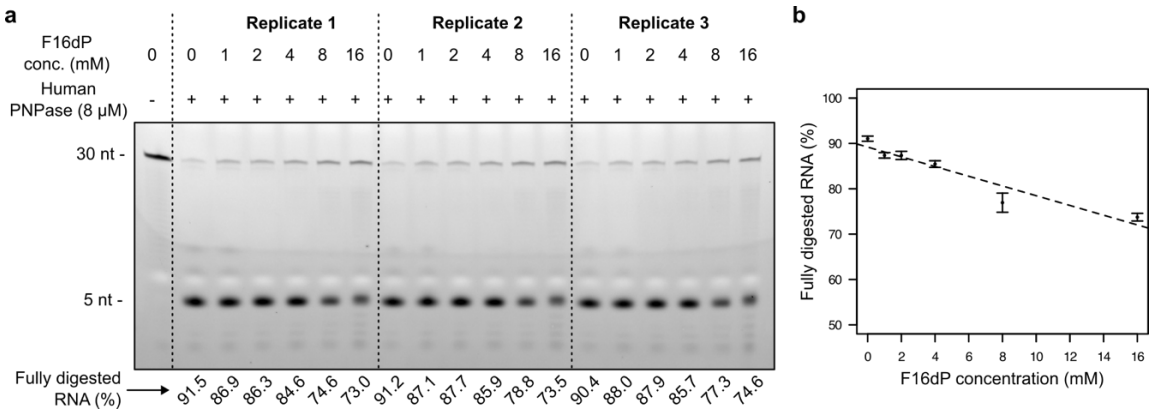

Fig. R2: Ribonuclease activity of human PNPase in the presence of F16dP**. a,** Denaturing polyacrylamide gels illustrating *in vitro* ribonuclease assays that show the effect of F16dP on the degradation of the 5'-FAM-U30 RNA substrate by human PNPase. The amount of fully digested RNA (5 nt-long) relative to the total RNA loaded is indicated. **b**, Percentage of fully digested oligonucleotide, relative to the total amount loaded, by human PNPase at different concentrations of F16dP. Values are shown as the mean ± SD of three independent replicates.

Nevertheless, the revised discussion (Line 544) now covers this question as follows "It was recently reported that glucose deprivation stabilizes mitochondrial RNAs of human embryonic kidney cells [63]. While metabolism and metabolites within the mitochondrion differ from *E. coli*, PNPase is also found in human mitochondria, an organelle descendant of a bacterial endosymbiont [64]. Our study discovers that *E. coli* PNPase in inhibited by glycolytic metabolites, which provides a hint to further investigate the symptoms of human patients with disease-linked PNPase variants or with metabolic disorders that may impact mitochondrial PNPase activity."

9. Fig 4, I realize that the choice of PfkA and GapA because of the most interacting partners, but they do not interact with PNPase as strongly than TpiA, GpmA, PykA, the last one with the strongest interaction. This series of experiments started with the idea of close proximity between PNPase (lines 224-227). The authors should clarify this.

We agree with the point raised. Done.

We recognize that our original wording was misleading. We clarify that since expression of all T25 and T18 fusion proteins was not equal (Extended Data Fig. 6), the β-galactosidase activity resulting from their interaction would not be proportional to the interaction strength. We clarify this in the revised manuscript. Accordingly, the new results section (Line 281 and Line 333) reads “for the BACTH assays, we used β-galactosidase activity as a reporter for protein-protein interaction” as well as “given that expression of all T25 and T18 fusion proteins was not equal, the β-galactosidase activity resulting from their interaction would not correlate with interaction strength. We therefore used the number of interacting partners as a criterion for...”. Accordingly, revised Fig. 4 (Line 766) indicates that we determined “relative β-galactosidase activity” instead of “relative strength of protein-protein interaction.”

Also, I would expect to see PNPase and RNAse E included as a control in this experiment given the known interaction with each other.

Done.

We included a control BACTH experiment with all pairwise combinations between T25 and T18 fusion to PNPase or RNase E, as show in Extended Data Fig. 7a (Line 1736). Now the results section (Line 313) accordingly reads “We supported these results by performing additional BACTH assays to confirm the known interaction between PNPase and RNase E (Extended Data Fig. 7a).”

10. Fig 6 does not clearly indicates which of these enzymes interact with each of three FLAG-tagged baits.

We thank the reviewer for drawing our attention to this critical point. Done.

These enzymes were identified by CLIP-MS using *E. coli gapA*-3xFLAG. To solve the issue, in the revised Fig 6a (Line 789) “GapA” has been replaced by “GapA-3xFLAG”. The original text (Line 345) already indicated “previous studies identified more proteins interacting with GapA, as determined by affinity purification of endogenously SPA-tagged proteins [39,40], we subsequently used *E. coli* gapA-3xFLAG to study the composition of glycolytic multi-protein complexes by CLIP-MS.” And the original figure legend (Line 791) already indicated “Proteins identified in *E. coli gapA*-3xFLAG by CLIP-MS.” So, we did not make additional changes.

Furthermore at this point of the manuscript, this section would benefit from explicitly showing how these enzymes relate to PNPase

Done.

The revised results section (Line 371) explicitly describes how these enzymes relate to PNPase as follows “Among the 42 metabolic and 31 non-metabolic enzymes that we identified, 25 and 16, respectively, are encoded by transcripts whose stability was previously shown to be PNPase-dependent [24] (denoted by asterisks in Fig. 6a).”

11. in term of interaction/closeness to these enzymes. Please clarify this.

Done. Thank you for the comment.

The revised results section (Line 378) reads “Additionally, our in vivo crosslinking results show that GapA associates with four of the glycolytic enzymes that interact with PNPase in BACTH assays (FbaA, TpiA, GapA and GpmA).”

Minor comments:

Please add a schematic of the metabolic pathway and mutation mentioned in lines 153-156.

Done. We are grateful for this recommendation.

We have included a schematic representation in Fig. 2b (Line 740) and, accordingly, the figure legend (Line 743) now reads “Interruption of glycolytic flux by deletion of *fbp* is indicated in this schematic representation of glycolysis, truncated from Fig. 1a.”

An interesting experiment to perform with the BACTH experiment in Fig4 could be to investigate the interaction between PNPase and RNAse E. Given that PNPase conformation changes according to F16dp concentration, one could presume the PNPase/RNAse E interaction is affected.

As described above, following a previous suggestion from this reviewer (Point 9), we performed a control BACTH assay that confirmed the known interaction between PNPase and RNase E (see Extended Data Fig. 7a). While it would be interesting to see how increased F16dP impacts the BACTH interaction between RNase E and PNPase, given that *E. coli* does not uptake F16dP (doi: 10.1002/jcp.1030360103) we feel this is more suitable for future studies.

We nevertheless thank the reviewer for raising this point, as we discuss this idea further with another reviewer (Point 3a by Reviewer 3).

Fig 5, the pfkA-, fbaA- or gapA may be misleading. I would write pfkA-3XFLAG or fbaA-3XFLAG or gapA-3XFLAG everywhere.

Done. We are grateful for this critical comment.

We have revised Fig. 5a (Line 778) and the corresponding figure legend (Line 781) now reads "Chromosome nucleotide sequence and translated amino acid sequence of *E. coli pfka*-3xFLAG, *fbaA*-3xFLAG or *gapA*-3xFLAG".

Reviewer #3 (Remarks to the Author):

The manuscript by Plagaro et al. describes the interplay between Escherichia coli metabolism and RNA degradation. In this work, the authors investigate the influence of several metabolites, mainly fructose-1,6-diphosphate (F16dP), on the activity of polynucleotide phosphorylase (PNPase). They show that, in vitro, these metabolites are capable of inhibiting PNPase activity, despite having no apparent effect on the enzyme's ability to bind substrate RNA. The authors also present a series of cellular experiments indicating that F16dP influence intracellular RNA levels. In addition, they report interactions between PNPase and several enzymes involved in bacterial metabolic pathways. Overall, the manuscript addresses an interesting and long-standing hypothesis regarding the connection between RNA metabolism and central carbon metabolism in bacteria. The study therefore has the potential to provide valuable insights into the regulatory interplay between metabolic state and RNA degradation. However, several points require clarification or further discussion before the manuscript can be considered for publication.

1. It is somewhat difficult to follow the experimental logic without prior knowledge of bacterial carbon metabolism. The introduction would benefit from a brief overview of the relevant metabolic pathways so that readers can better understand the relationships between the investigated enzymes, metabolites and the physiological conditions used in the study. In particular, the authors could briefly describe the metabolic context of the carbon sources mentioned in the manuscript (e.g., glucose, glycerol, citrate, and acetate).

Thank you for this suggestion. Done.

We include new Extended Data Fig. 1 with the figure legend (Line 1364) as follows "Schematic overview of central carbon metabolism, entry point of different carbon sources and the diversion of precursor metabolites to biosynthetic pathways, adapted from the Ecocyc database [25]. Blue, orange and green indicate reactions within glycolysis, the pentose phosphate pathway and the tricarboxylic acid cycle, respectively. Specific periplasmic transporters for glycerol, glucose,

acetate or citrate are indicated". Accordingly, the revised introduction (Line 56) now reads "These three pathways constitute central carbon metabolism, a network of biochemical reactions that generate the metabolite precursors necessary for biomass production from different carbon sources [25], such as glucose, glycerol, citrate or acetate (Extended Data Fig. 1)."

2. The authors report that the investigated metabolites affect PNPase catalytic activity but not RNA binding. Given this observation, the rationale behind modelling F16dP, Mg^{2+}, and RNA separately and subsequently overlaying the models is not entirely clear. A clearer explanation of the modelling strategy and its limitations would help the reader interpret these results.

We agree with the points raised. Done.

The modellings are shown separately within Extended Data Fig 3a of the revised manuscript (Line 1390). Moreover, the revised manuscript (Line 113) explains the rationale behind the modelling as follows "Finally, we supported the accuracy of the models by three additional AlphaFold 3 dockings of magnesium, phosphate or RNA (U_{30}) with homotrimeric PNPase."

If the authors propose that the metabolite binds in the catalytic site, it would be informative to test this hypothesis experimentally in order to strenghten the proposed model, for example by introducing mutations in residues predicted to participate in metabolite binding and examining whether these mutations affect metabolite binding or enzymatic inhibition.

Thank you for this suggestion. Done.

Additionally, we measured the thermal stability of the catalytic site mutant PNPase D492G. These results have been incorporated into Extended Data Fig. 3f. Accordingly, the revised results section (Line 147) now reads "To test if metabolites bind at the catalytic site, we performed the same experiments under the same conditions except using the catalytic site mutant PNPase_D492G. As shown in Extended Data Fig. 3f, the T_i of PNPase_D492G in a binding buffer (62.4 ºC) remained unchanged in the presence of F6P (62.4 ºC), F16dP (62.4 ºC) or 3PG (62.4 ºC). Although the lower T_i of the D492G mutant compared to the wild type enzyme indicates that this mutation reduces PNPase thermal stability, the unchanged thermal stability of PNPase_D492G in the presence of metabolites suggests that metabolites bind in the catalytic site."

3. I am not fully convinced by the interpretation of the experiments performed in the PNP-essential fbp deletion strain. The authors show that accumulation of F16dP slightly slows bacterial growth and leads to increased RNA stability, and they interpret this as evidence for inhibition of PNPase activity. However, it is not entirely clear that the presented data uniquely support this interpretation.

a. It is well established that RNA degradation in Escherichia coli is closely linked to cellular metabolism. For example, the degradosome itself contains a metabolic enzyme (enolase). Deletion of a metabolic enzyme may therefore influence multiple cellular pathways. Consequently, the observed increase in RNA stability could potentially arise from broader metabolic perturbations, rather than solely from inhibition of PNPase activity. In addition, the RNA-seq results indicate that 5% of transcripts show increased degradation, suggesting that the cellular response to the deletion may be more complex than simple inhibition of PNPase.

We agree with the idea that response to the deletion may be more complex than simple inhibition of PNPase.

Together in response to the related Point 3c by this reviewer, Point 4 by Reviewer 1 and Point 6 by Reviewer 2, these are covered in the revised discussion (Line 443) as follows “Additional deletion of *fbp* in the PNP-essential strain led to a 1.5-fold increase in F16dP levels, which correlated with compromised growth and global RNA stabilization. The stabilization of 157 direct substrates of PNPase contrasted with destabilization of 9 substrates. This complex response is consistent with the protective or degradative modes of PNPase in vivo [36]. Specifically, increased F16dP may impact PNPase ribonuclease activity by occluding the active site, while simultaneously disrupting its protective role through conformational changes that prevent PNPase interaction with other proteins such as RNase E, RhlB or Hfq. While the direct substrates of PNPase did not show specific pathway enrichment, they included the sRNAs GlmZ and SroH, which contribute to cell envelope synthesis and the response to envelope stress, respectively. GlmZ post-transcriptionally regulates translation of *glmS* mRNA, which encodes an enzyme in the pathway for cell envelope biosynthesis [46]. In comparison, deletion of the small RNA SroH in *E. coli* MG1655 increases sensitivity to cell envelope stress [47]. In contrast, the SgrS small RNA, which triggers degradation of the *ptsG* mRNA under conditions of phosphosugar accumulation [48], was undetectable in our RNA-seq data. Consistently, the stability of the *ptsG* mRNA remained unchanged (half-life of 3.4 min), comparable to that previously reported in *E. coli* growing in LB supplemented with glucose (3.3 min). This indicates that phosphosugar stress responses are not activated under our experimental conditions. Under PNPase inhibition conditions (i.e., increased F16dP), the stability of 258 PNPase substrates remained unchanged, indicating that their degradation may be rate-limited by other ribonucleases (such as RNase E, which has been shown to have cleavage sites in 89 of these transcripts [49]).

Collectively, our study shows that F16dP inhibits the ribonuclease activity of PNPase in vitro and that increased F16dP compromises the growth of a PNP-essential strain. However, the deletion of *fbp* could cause broader metabolic perturbations and other inhibitors such as citrate [9] or ATP [26] may contribute. While we do not demonstrate a direct causal relationship between increased F16dP and PNPase inhibition, our findings associate PNPase-dependent post-transcriptional regulation with glucose utilization and glycolytic flux."

b. The authors also show that leuX, previously reported to be regulated by PNPase, displays an increased half-life. However, the Northern blot shown in Fig. 2d is somewhat difficult to interpret. In the study cited as reference 23, three leuX intermediates were observed, not all of which were strictly dependent on PNPase. In the current manuscript the RNA bands appear only partially resolved, making it difficult to determine which band was quantified for the half-life estimation. It would be helpful if the authors clarified how the signal was quantified and how they ensured that only the relevant band was included in the analysis. Information about the reproducibility of these measurements and the associated error estimates would also strengthen this result.

We agree with the point. Done.

The revised Fig. 2d (Line 740) clearly indicates the intermediates that were used for determination of RNA half-life with arrowheads. Accordingly, the revised legend for Fig. 2 (Line 748) reads "Northern blot analysis showing the half-life of the processing intermediates of *leuX* transcript indicated by arrowheads". Moreover, the revised methods section (Line 1016) now indicates "The amount of visible processing intermediates of *leuX* at each time point was determined relative to abundance before rifampicin treatment and normalized to the signal of the 5S rRNA". Information about the reproducibility is indicated in the revised figure and legend, with half-life values presented as the mean ± standard deviation of three independent biological replicates, along with the statistical significance of the observed differences.

The presence of two intermediate bands in our data, compared to a single band reported in the cited study (doi: 10.1093/nar/gkp997), is explained by differences in gel resolution. In our experiments, RNA was resolved using long, high-resolution gels, allowing separation of closely spaced intermediates. In contrast, in the cited study, shorter gels with lower resolving power likely resulted in co-migration of these species into a single band.

c. The authors identified 275 transcripts as PNPase substrates, of which 89 showed prolonged half-life in the mutant strain. Were the remaining transcripts unchanged? If accumulation of F16dP

indeed inhibits PNPase activity, one might expect stabilization of a larger fraction of PNPase substrates. It would be helpful if the authors discussed possible explanations for this observation.

Done.

We acknowledge our oversight and the revised results section (Line 242) now reads "Of these PNPase-bound substrates, 97 were DDTs, of which 89 showed prolonged RNA half-lives and 8 showed reduced RNA half-lives in the PNP-essential *fbp*-deletion strain (Supplementary Table 1). Of the remaining transcripts, the stability of 138 remained unchanged and 40 transcripts were undetectable (Supplementary Table 1)." In the discussion (Line 459), we explain this observation as follows "Under PNPase inhibition conditions (*i.e.*, increased F16dP), the stability of 138 PNPase substrates remained unchanged, indicating that their degradation may be rate-limited by other ribonucleases (such as RNase E, which has been shown to have cleavage sites in 59 of these transcripts [49])."

4. In light of the points raised above, several statements in the discussion appear somewhat stronger than the experimental evidence currently supports and could benefit from more cautious wording. For example, the statement: “... our data show that the single deletion of a metabolic enzyme in a PNP-essential background leads to an increase in F16dP, which is sufficient to globally prolong mRNA half-life through metabolic inhibition of PNPase in the PNP-essential fbp-deletion strain” appears convincingly supported only up to the first comma. Similarly, “Additional deletion of fbp in the PNP-essential strain led to a 1.5-fold increase in F16dP levels, which correlated with compromised growth and global RNA stabilization, including stabilization of direct substrates of PNPase. These results strongly support the F16dP-mediated inhibition of PNPase in this strain” - this may be somewhat premature based on the available data. While the observations are consistent with this hypothesis, they do not yet demonstrate a direct causal relationship.

We agree with the points. Done.

We have tempered these statements and the revised manuscript (Line 266 and Line 464) now reads “Collectively, our data show that the single deletion of a metabolic enzyme in a PNP-essential background leads to an increase in F16dP, which correlates with global prolongation of mRNA half-life, possibly through metabolic inhibition of PNPase in the PNP-essential *fbp*-deletion strain” as well as “Collectively, our study shows that F16dP inhibits the ribonuclease activity of PNPase *in vitro* and that increased F16dP compromises the growth of a PNP-essential strain. However, the deletion of *fbp* could cause broader metabolic perturbations and other inhibitors

such as citrate [9] or ATP [26] may contribute. While we do not demonstrate a direct causal relationship, our findings associate PNPase-dependent post-transcriptional regulation with glucose utilization and glycolytic flux."

5. It would be helpful for the authors to clarify where the affinity tags were fused to PNPase. The FLAG tag appears to be located at the N-terminus, but this should be explicitly stated.

Done.

The revised manuscript (Line 77) now indicates that "We determined the activity of N-terminally FLAG-tagged, purified *E. coli* PNPase". Accordingly, the methods section (Line 814) now reads "To generate pBAD FLAG-Pnp, in which the expression of N-terminally FLAG-tagged PNPase is under the control of an arabinose-inducible promoter..."

In Supplementary Fig. 1, two bands of similar intensity are visible for the purified PNPase variants. The origin of these bands is not immediately clear.

Thank you for this critical comment. To clarify this, we added arrows to Extended Data Fig 2b to indicate the expected size of PNPase trimer, dimer and monomer. Moreover, the revised figure legend (Line 1381) reads as "Since the PNPase samples originated from single size-exclusion chromatography fractions, the two bands migrating far above the expected dimer size likely correspond to different trimeric conformations".

Since Polynucleotide phosphorylase is known to form trimers, it would be useful to discuss whether the tag might influence oligomerization. Structurally, the N-terminus lies relatively close to the protomer-protomer interface, and therefore it would be helpful to clarify whether the authors tested whether the tagged protein forms trimers with the same efficiency as the WT enzyme.

During preparation of related unpublished results, chromatographically purified C-terminally 12xHis-tagged PNPase was run under native conditions along N-terminally FLAG-tagged PNPase. Both proteins resolved as two very close bands of the size expected for the PNPase trimer (**Fig. R3**a), while no bands were observable in the size range expected for the dimer or monomer. These results indicate that PNPase can be purified as a trimer independently of tag type (*i.e.*, FLAG or 12xHis) and location (*i.e.*, N-terminal or C-terminal). Additionally, purified FLAG-PNPase resolved as a single band of approximately 90 KDa when run under denaturing and reducing conditions (**Fig. R3**b), demonstrating that purified PNPase was intact (*i.e.*, full-length).

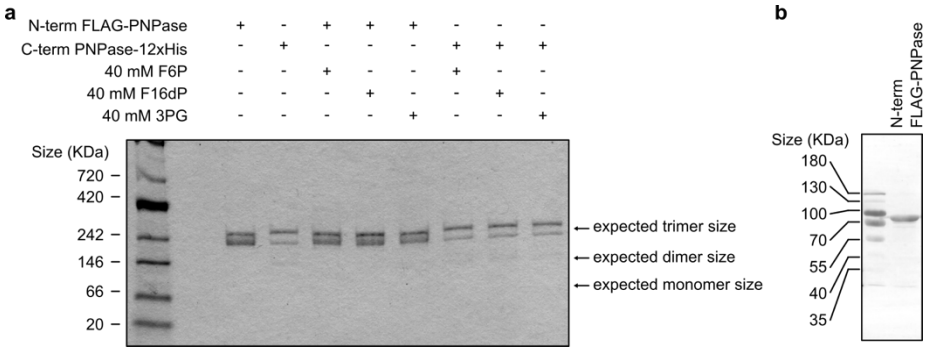

Fig. R3: Electrophoretic analysis of different PNPase variants. **a**, Native polyacrylamide gel electrophoresis of the indicated PNPase variants. The expected size of the PNPase homotrimer, homodimer or monomer is indicated. **b**, Denaturing polyacrylamide gel electrophoresis showing the size of purified FLAG-PNPase under reducing conditions.

We only used the trimeric form of purified PNPase for our experiments, accordingly, the revised legend for Extended Data Fig. 2 (Line 1379) now reads as "a, Size exclusion chromatography of PNPase variants in a Superose 6 column. Red lines indicate each fraction corresponding to the PNPase trimer that was used for further experiments". Accordingly, the methods section about PNPase purification (Line 901) now reads "To confirm the size of the purified proteins, the fraction containing the trimeric form of either PNPase...", and the section about ribonuclease assays (Line 919) now reads "The reaction buffer contained [...], 2 nM of trimeric PNPase..."

Whether the tag impacts efficiency of trimer formation is beyond the scope of this paper and we hope these clarification and changes clear your concern.

6. A related point concerns the bacterial two-hybrid experiments. T25 and T18 proteins used in this assay are substantially larger than the FLAG tag, and it would therefore be useful to clarify whether PNPase retains its native oligomeric state when fused to these proteins.

Thank you for raising this point.

Because of the size of the T25 and T18 proteins, the oligomeric state of fusion proteins will likely be impacted, however, as explained in the publication (doi: 10.1073/pnas.95.10.5752) that originally described BACTH assays "two putative interacting proteins are genetically fused to two complementary fragments, T25 and T18, that constitute the catalytic domain of *Bordetella pertussis* adenylate cyclase. Association of the two-hybrid proteins results in functional complementation between T25 and T18 fragments and leads to cAMP synthesis." Therefore, whether these tags interfere with the formation of the PNPase trimer, and whether PNPase retains

its native oligomeric state is irrelevant to our major conclusion. While it would be interesting to see if the complexes between glycolytic enzymes and PNPase retain their native oligomeric state, we feel this is more fit for future experiments.

The interaction signal for PNPase self-association appears weaker than for some of the interactions reported with metabolic enzymes, could the authors provide an explanation for this observation?

Done.

Similar to what we answered to a point raised by another reviewer (Reviewer 2 Point 9), we recognize that our original wording was misleading. Since expression of all T25 and T18 fusion proteins was not equal (Extended Data Fig. 6), the β-galactosidase activity resulting from their interaction would not be proportional to the interaction strength. We clarify this in the revised manuscript. Accordingly, the new results section (Line 281 and Line 333) reads “for the BACTH assays, we used β-galactosidase activity as a reporter for protein-protein interaction” as well as “given that expression of all T25 and T18 fusion proteins was not equal, the β-galactosidase activity resulting from their interaction would not correlate with interaction strength. We therefore used the number of interacting partners as a criterion for...”. Accordingly, revised Fig. 4 indicates that we determined “relative β-galactosidase activity” instead of “relative strength of protein-protein interaction.”

7. The manuscript would benefit from a broader discussion of how RNA degradation and carbon metabolism may be coordinated spatially within the bacterial cell. For example, do the authors envision that PNPase interacts with metabolic enzymes while simultaneously participating in RNA degradation? Would such interactions occur sequentially, or within larger multiprotein assemblies? Is the relevant PNPase population the degradosome-associated enzyme or the free cytoplasmic pool? Even if the present data do not directly address these questions, discussing these possibilities would help place the findings in a clearer biological context.

We thank the reviewer for this insightful comment. Done.

We add this point to the revised discussion (Line 504) as follows “Glycolytic activity has been detected in both the membrane and cytoplasm fractions of *E. coli* cells [52]. Given that PNPase associates with RNase E, RhlB and enolase in the membrane-bound degradosome, we anticipate PNPase could be targeted through metabolon association in this environment. However, this does not rule out the possibility that the activity of PNPase is also impacted by high concentrations of

metabolites within the cytoplasm, where PNPase can form protein complexes independent of RNase E."

8. In Fig. 6B it is not entirely clear whether the samples visible on the Western blot correspond to crosslinked complexes or to material obtained after reversal of crosslinking.

We thank the reviewer for this critical comment. Done.

We indicate that the samples were analyzed after reversal of crosslinking in the revised text (Line 388) as follows "As shown in Fig. 6b, western blotting after reversal of crosslinking with anti-FLAG antibodies of cytoplasm fractions ..." This is additionally clarified by indicating the presence (+) of the reducing agent 2-mercaptoethanol (BME) in the revised Fig. 6b and revised Extended Data Fig. 9b.

It would also be helpful if molecular weight markers were clearly shown.

Done.

We have added additional molecular weight markers to the revised Fig. 6b and revised Extended Data Fig. 9b.

In addition, PNPase appears to migrate at approximately 100 kDa, and more than one band is visible. This migration does not correspond exactly to the expected molecular weight of the PNPase monomer (~80 kDa), nor to the expected size of a crosslinked complex with the bait proteins (~40 kDa). Could the authors provide a brief explanation of how these bands were interpreted?

Along these lines, and following the related Point 9 by Reviewer 1, in the revised Fig. 6b (Line 789) and Extended Data Fig. 9b (Line 1457) the additional polypeptide detected in both the wild-type and the *Δpnp* strains has been labelled as "non-specific signal". The other polypeptide, detected exclusively in the wild type strains but not in the *Δpnp* strains, has been labeled as "PNPase."

To clarify the difference in the expected size of PNPase (80 kDa) and the observed size (90-100 kDa) the revised results (Line 394) reads as "Importantly, PNPase was detected in the immunoprecipitation samples from the pfkA-3xFLAG strain (b-II, upper panel, lane 4) at the size previously described [42]". The figure legend (Line 803) has been revised accordingly "The difference between PNPase expected migration size (80 KDa) and the observed size (90 KDa) is

most likely due to the acidic isoelectric point of the enzyme (*i.e.*, pH 5.0), which makes acidic proteins migrate higher than expected compared to the marker."

Minor coments:

1. Figure 1 – please indicate in the legend that RNA is shown in green.

Done.

As described above, following a previous suggestion from this reviewer (Point 2), we show the docking of PNPase with RNA in Extended Data Fig. 3a of the revised manuscript. The revised figure legend (Line 1394) reads as "Magnified views show docking for U_{30} RNA (in green) in the central channel..."

2. Line 53 – the manuscript refers to "ribonuclease E (Rne)", while later "RNase E" is used. Please ensure consistent nomenclature.

Thank you for this comment. Done.

Ribonuclease E is consistently abbreviated as "RNase E" in the revised manuscript.

3. The AlphaFold3 structure should not be described as "our model", as it was generated by computational prediction rather than derived directly from experimental data

Thank you for this indication. Done.

The revised manuscript (Line 101 and Line 113) reads "The AlphaFold 3 models suggest that, through interaction with residues in PNPase active site, F16dP and 3PG occlude the active site and prevent access to the RNA 3'-end. We noted that the AlphaFold 3 models of F16dP-bound and 3PG-bound PNPase..." as well as "...in agreement with the AlphaFold 3 model. Finally, we supported the accuracy of the models by three additional AlphaFold 3 dockings of magnesium, phosphate or RNA (U_{30}) with homotrimeric PNPase."

4. "Our in vivo crosslinking results indicate that glycolytic proteins form transient complexes" - What observations lead the authors to conclude that these complexes are transient?

We thank the reviewer for this critical comment.

We adapted the historical wording used by the author that defined metabolons, who described them undistinguishably as "transient", "loose", "weak" or "labile" (doi: 10.1016/S0074-

7696(08)60529-X, 10.1146/annurev.bi.56.070187.000513). We recognize that the definition of these terms has changed during the years and “labile” may be more suitable. Since we do not know the exact behavior of glycolytic metabolons, in the revised manuscript we firstly refer to them as “transient/labile” (Line 331) as follows “Based on the BACTH protein-protein interactions we asked whether we could identify transient/labile glycolytic protein-protein interactions by in vivo crosslinking and immunoprecipitation followed by mass spectrometry (CLIP-MS) as described previously”. These terms are used together after this (Line 375 and Line 481) as follows “Our in vivo crosslinking results indicate that glycolytic proteins form transient or labile complexes” and “Additionally, we show that transient/labile multi-protein complexes stabilized by in vivo protein crosslinking contain eight consecutive glycolytic enzymes. Although these results do not rule out the possibility that these interactions are indirect, they provide the best support for the hypothesis that glycolytic metabolons are formed by the transient or labile association between sequential metabolic enzymes in *E. coli*.”

5. The phrase “5S rRNA, determined by bromophenol staining” is somewhat unclear. Bromophenol blue is typically used as a tracking dye rather than a nucleic acid stain. The authors may wish to clarify the staining method used for RNA visualization.

Done. The original wording was wrong, thank you for the correction.

In the revised manuscript (Line 1018) we correct and clarify this and now the text reads as “The signal of the 5S rRNA was determined by staining of total RNA with a staining solution containing 0.3% methylene blue and 0.3 M sodium acetate (pH 5.2) for 1 min, followed by membrane washing in water for 5 min with shaking, and scanning in an Epson Perfection 4990 scanner.”

Dear referees,

We are grateful for the opportunity to submit a second revision of our manuscript. We appreciate the reviewers' constructive feedback and have addressed the remaining critical points. In this revision, we have incorporated all editorial suggestions from Reviewer 1. Furthermore, in response to Reviewer 3, we have added rotated views of AlphaFold3 models and molecular dynamics simulations to Fig. 1d and Extended Data Fig. 3d–e to clearly show PNPase residues predicted to interact with metabolites. We have also revised the discussion regarding the protective role of PNPase, and tempered our statement in the abstract regarding the interaction between PNPase and glycolytic enzymes. We hope that these revisions adequately address the concerns raised by the reviewers and accurately reflect our results/findings.

Sincerely yours

Sue Lin-Chao

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

Summary:

In this manuscript submitted to Nature Communications, Plágaro and colleagues seek to understand how glycolysis impacts RNA degradation in Escherichia coli. There is a growing body of evidence indicating that metabolism and metabolites modulate RNA decay in bacteria.

In short, the authors provide evidence that 3-phosphoglycerate and fructose-1,6-bisphosphate, two intermediates of glycolysis, inhibit the RNA degradation, but not RNA binding activity of PNPase in vitro (Fig. 1), that the accumulation of fructose-1,6-bisphosphate within an E. coli cell can reduce growth and inhibit decay of a known PNPase substrate (Fig. 2), and introduction of a mutation into their PNPase-dependent mutant that causes increased fructose-1,6-bisphosphate levels results in decreased decay of some transcripts, but increased turnover of others (Fig. 3).

Finally, the authors provide evidence that PNPase may either directly or indirectly interact with certain glycolytic enzymes such as PfkA, FbaA, and GapA (Figs. 4, 5, and 6).

Overall, the manuscript is well written, quite comprehensive, and support the authors extraordinary conclusions that certain glycolytic intermediates inhibit the ability of PNPase to degrade certain RNA substrates. As the authors noted, this modulation of RNA decay by glycolytic intermediates may also be important in numerous other bacteria particularly those in which PNPase may be the sole exoribonuclease and in humans, which also contain a mitochondrial targeted PNPase.

The authors satisfactorily address my prior criticisms, which improved the manuscript. Some minor editing issues listed below should be addressed.

We thank the reviewer for this positive comment and additional corrections.

Major Criticisms:

None.

Minor comments:

L31. I think the authors meant "exploited" and not "explored"

Done, thank you for this correction.

L155. Suggested change "presence of these metabolites suggests that they bind the catalytic site."

Done.

L161. Replace the comma with a semicolon or period

Done.

L180 Change to "on the growth rate"

Done.

L197. Replace comma with a semicolon or period.

Done.

L232. the "2" in "log2" should be subscripted.

Done.

L367. Replace the comma with a semicolon or period.

Done.

L390. Replace the comma with a semicolon or period.

Done.

L1061. the "2" in "log2" should be subscripted.

Done.

L1421 and L1423. the "2" in "log2" should be subscripted.

Done.

Extended data figure 5 panels. the "2" in "log2" should be subscripted.

Done.

Reviewer #2 (Remarks to the Author):

The revised version of this manuscript has thoroughly addressed all the points I raised in my review. I find the new version of the manuscript satisfactory and support its publication.

Thank you for this comment.

Reviewer #3 (Remarks to the Author):

The authors have provided valuable additional data and revised the text, which has strengthened the manuscript. They have also clarified several of my initial concerns.

We thank the reviewer for this comment.

However, in my opinion, a few critical points have not yet been sufficiently addressed.

Ad 2 - I am having difficulty following the logic regarding the rationale for this experiment. The mutant used to demonstrate metabolite binding in the active site is not marked in Figure 1d as an interacting residue in the AlphaFold model (nor is D486 mentioned in the text). It also does not show up in the molecular dynamics simulations (Extended Data Figure 3). If I understand the setup correctly, the authors should perform the assay using a PNPase mutant targeting the

residues that the model actually highlights as the primary interactors (e.g. S437, S438, S439, H403) to demonstrate lack of thermal shift. Given the data presented, it remains unclear why the results obtained for the D492 mutant indicate a total lack of interaction with any of the metabolites.

We apologize for the uncareful figure preparation that led to confusion.

Although docking with AlphaFold 3 showed 3PG or F16dP interacting with PNPase residues, H403, S437, S438, S439, D486 and D492, and F16dP additionally interacting with R93, as shown in the unprocessed models in **Fig. R4**, residues D486 and D492 were mistakenly not indicated in Fig. 1 and Extended Fig. 3 due to the angle shown in the processed screenshots. We have revised Fig. 1d and Extended Data Fig. 3d-e to include a 90º longitudinal rotation that clearly shows D486 and D492. Accordingly, the revised Fig. 1 legend (Line 749) now reads “Magnified views show docking for 3PG or F16dP in the active site, and 90º longitudinal rotation view, respectively,” and the revised Extended Data Fig. 3 legend (Line 1410) reads as “d, Structure of F16dP bound to PNPase active site at the beginning (time = 0 ns) and the end (time = 5 ns) of molecular dynamics simulation, and 90º longitudinal rotation view. e, Structure of 3PG bound to PNPase active site at the beginning (time = 0 ns) and the end (time = 5 ns) of molecular dynamics simulation, and 90º longitudinal rotation view.” Accordingly, the revised text (Line 99) now reads "The binding site was then predicted by docking three molecules of F16dP or 3PG and homotrimeric PNPase using AlphaFold 3, which showed one F16dP or 3PG molecule in all PNPase monomers interacting with six residues: H403, S437, S438, S439, D486 and D492, F16dP was additionally predicted to interact with R93 (Fig. 1d). Except for R93, the interacting residues are within the PH2 domain. Of these, three (S437, S438 and S439) pertain to the phosphate binding site, and two (D486 and D492) pertain to the magnesium binding site."

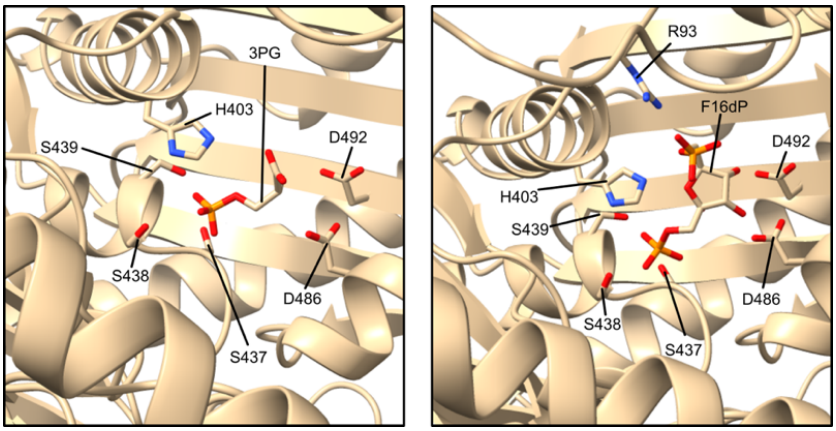

Fig. R4: Unprocessed AlphaFold 3 model of 3PG or F16dP docking to PNPase active site, respectively. Residues predicted to be involved in binding are indicated.

Regarding the rationale for the additional nanoDSF experiment (Extended Data Fig 3f), among the residues in the active predicted by AlphaFold3 to interact with metabolites, the mutation D492G is already known to abolish catalysis without an impact on PNPase quaternary structure (doi: 10.1016/S0022-2836(02)00645-9). Accordingly, the revised manuscript (Line 155) reads "Of the residues within the active site predicted by AlphaFold to bind metabolites (*i.e.*, S437, S438, S439, D486, D492), the mutation D492G is well-characterized and know to affect the catalytic center severely and specifically, without impacting the quaternary structure of the protein [28]. Therefore, to test if metabolites bind at the catalytic site, we repeated these experiments using the catalytic site mutant PNPase D492G."

Additionally, there appears to be an error in the text: “residue D492 is predicted to correspond to the phosphate-binding site, which mutation abolishes catalysis.” - this residue corresponds to the magnesium-binding site, not the phosphate-binding site.

Thank you for pointing out this error, the revised manuscript (Line 114) reads as "residue D492 is predicted to correspond to the magnesium-binding site, which mutation abolishes catalysis."

Ad 3 - The revised text states: “…simultaneously disrupting its protective role through conformational changes that prevent PNPase interaction with other proteins such as RNase E, RhlB or Hfq.” Do the authors structural models actually support major conformational changes upon metabolite binding? Furthermore, to the best of my knowledge, PNPase's protective role has only been established in conjunction with Hfq.

We agree that PNPase's protective role has only been established in conjunction with Hfq.

Therefore, the revised discussion (Line 458) reads as “This complex response is consistent with the protective or degradative modes of PNPase in vivo [36]. Specifically, increased F16dP may impact PNPase ribonuclease activity by occluding the active site, while simultaneously disrupting PNPase protective role, which has been established in conjunction with the RNA chaperone Hfq [36]. This metabolite-induced disruption would destabilize their common substrates. Interestingly, two of the destabilized PNPase substrates (*cydB* and *dmsA*) have been identified as Hfq substrates [46], however, this mechanism remains to be studied.”

Ad 6 - The authors state: “Therefore, whether these tags interfere with the formation of the PNPase trimer, and whether PNPase retains its native oligomeric state is irrelevant to our major conclusion.” - if the authors are using a PNPase protomer as bait (because the two large fused protein domains prevent native trimerization) it fundamentally changes the relevance of the

experiment. A protomer exposes completely different surface areas than a trimer and lacks native functionality. Consequently, any interacting proteins captured by a monomeric bait may be biologically irrelevant to the functional trimer. The fact that the structural impact of these fusions on the bacterial two-hybrid assay remains unknown represents a major limitation. Given that in vivo crosslinking followed by immunoprecipitation demonstrates cellular proximity but does not definitively prove a direct biochemistry-level interaction, the authors definitive conclusion in the abstract (“Bacterial two-hybrid and in vivo crosslinking followed by immunoprecipitation demonstrate a physical and transient interaction...”) is considerably overstated. In light of these experimental limitations, the claim of a direct physical interaction is not sufficiently supported by the data provided.

Thank you for the further detailed explanation. We agree with the fact that the structural impact of the fusions on the bacterial two-hybrid assay remains unknown and represents a major limitation to conclude “Bacterial two-hybrid and *in vivo* crosslinking followed by immunoprecipitation demonstrate a physical and transient interaction...”. In light of this experimental limitation, we restate the abstract (Line 26) as "Bacterial two-hybrid and *in vivo* crosslinking followed by immunoprecipitation suggest potential interaction between PNPase and multiple glycolytic enzymes."

Line 805 - this should be pI instead of pH

Done. Thank you for this correction.

We are grateful that all of the concerns previously raised by Reviewer 3 have been addressed. We agree with the remaining point raised by Reviewer 3; in response, we have revised our text to more accurately reflect our genetic results. We provide our point-by-point responses below.

Reviewer #3 (Remarks to the Author):

The authors have addressed all of my concerns and provided necessary explanations. One thing that remains puzzling for me is the result of the thermal shift assay. In the F16DP model, D492 supports nicely the binding of the metabolite and here I would agree that this is a strong interaction, however, it is surprising that in the presence of the other strong interactions removal of this one amino acid results in abolishment of metabolite binding. However, in the 3PG model the residue D492 is over 4A away from 3PG, what suggests lack of interaction and even if any, then very weak and I highly doubt that mutation of this residue would result in total abolishment of metabolite binding, considering the fact that there are many other, much closer and stronger contacts between the metabolite and the protein. Therefore, the AlphaFold model might be wrong.

To clearly acknowledge the model-based nature of the structural insights, the revised manuscript now reads "Together, these model-based static structures suggest that metabolites inhibit PNPase activity by occluding the enzyme active site through binding." Moreover, to properly address the limitations of the AlphaFold models the revised discussion section now reads "However, in the model-based AlphaFold structures, F16dP was predicted to closely interact with the active site residue D492, while 3PG was more than 4 Å away from this residue, suggesting a weaker or negligible interaction. Therefore, the absence of a thermal shift for PNPase_D492G in the presence of 3PG indicates that the AlphaFold models may not accurately reflect the binding mode." We hope that these changes accurately reflect our experimental results and address the final point raised by Reviewer 3.