nature portfolio

Peer Review File

CRISPRi screen identifies FprB as a synergistic target for gallium therapy in *Pseudomonas aeruginosa*

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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)
Authors applied a genome-wide CRISPR interference approach (CRISPRi-seq), to identify potential synergistic targets with gallium. They identified a highly conserved gene fprB, encoding a ferredoxin-NADP+ reductase. Using a null mutant, they also confirmed the loss of fprB, and observed that lowers the minimum inhibitory concentration of gallium from 320 μM to 10 μM and shifted gallium's mode of action from bacteriostatic to bactericidal. However, the novelty of this article is very minimal. They have used a known CRISPR technique, and the identification of target is also not novel. In addition, the fprB mutant they identified reduced the MIC, however, if it is the target the MIC should be increased more than 320 μM. So, it confirms the target is something else, however it may be a secondary target. The study is also not expanded to confirm or support the results. The protocols are also not written clearly to reproduce.

Reviewer #2

(Remarks to the Author)
The manuscript by Zhang and coworkers represents an interesting and useful advance in the generation of genetic methods to determine the importance of P. aeruginosa genes under different conditions, and they used their method to identity a synergistic target for gallium in the growth inhibition of this bacterium.
The experiments conducted are adequate and their interpretation of the results/conclusion seem valid; nevertheless, I have some specific concerns and suggestions for the authors:
1) Abstract, L 38 "dramatically" is subjective and its better to use fold change in MIC or other more quantitative expression.
2) The authors should acknowledge that other had found significantly lower Ga MIC towards the same strains, mainly because Ga activity is potentiated at low iron concentrations, in minimal medium such as minimal succinate one, and since in this study Mueller Hinton (a rich medium was used), likely it contains high iron concentrations. See:
Kaneko Y, Thoendel M, Olakanmi O, Britigan BE, Singh PK. The transition metal gallium disrupts Pseudomonas aeruginosa iron metabolism and has antimicrobial and antibiofilm activity. J Clin Invest. 2007 Apr;117(4):877-88. doi: 10.1172/JCI30783. Epub 2007 Mar 15. PMID: 17364024; PMCID: PMC1810576.
García-Contreras R, Lira-Silva E, Jasso-Chávez R, Hernández-González IL, Maeda T, Hashimoto T, Boogerd FC, Sheng L, Wood TK, Moreno-Sánchez R. Isolation and characterization of gallium resistant Pseudomonas aeruginosa mutants. Int J Med Microbiol. 2013 Dec;303(8):574-82. doi: 10.1016/j.ijmm.2013.07.009. Epub 2013 Aug 6. PMID: 23992635.
3) Although the authors explained the role of the HitAB system in gallium sensitivity and that mutations inactivating those genes increase gallium tolerance, since this system is likely the main gallium transporter, they do not mention that perhaps another way gallium can be internalized to cells is by the siderophore pyochelin, see
Frangipani E, Bonchi C, Minandri F, Imperi F, Visca P. Pyochelin potentiates the inhibitory activity of gallium on Pseudomonas aeruginosa. Antimicrob Agents Chemother. 2014 Sep;58(9):5572-5. doi: 10.1128/AAC.03154-14. Epub 2014 Jun 23. PMID: 24957826; PMCID: PMC4135843.

I wonder if they identified genes related to pyochelin synthesis and transport as important for gallium tolerance in the screening.
4) Fig 1 C and 1 D, pyocyanin concentration is very easy to determine by spectrophotometry, please do it and present concentration changes together or rather than picture, also swarming halo diameter can be easily determined.
5) While the role of FprB in counteracting the oxidative stress promoted by gallium seems likely, simple experiments such as adding ROS scavengers or commercial purified catalase and showing this decrease the inhibitory effects of gallium in the

fprB mutant may strength the conclusion.
6) Interestingly, FprB may be not only a suitable synergistic target for Gallium but perhaps also for other compounds that promote ROS and the authors demonstrated this with H2O2 and plumbagin, but it may be also suitable to test if it has synergy with antibiotics that promote ROS.
7) What would be the gallium sensitivity of a hitAB fprB mutant?
8) Can a frpB mutant be complemented with frpA?
9) Please note that the first work ever reporting that mutations of hitAB conferred resistance to gallium by decreasing its internalization was

García-Contreras R, Lira-Silva E, Jasso-Chávez R, Hernández-González IL, Maeda T, Hashimoto T, Boogerd FC, Sheng L, Wood TK, Moreno-Sánchez R. Isolation and characterization of gallium resistant Pseudomonas aeruginosa mutants. Int J Med Microbiol. 2013 Dec;303(8):574-82. doi: 10.1016/j.ijmm.2013.07.009. Epub 2013 Aug 6. PMID: 23992635.

You seem to give the credit to other publications.

10) L 108 “major transporters of iron” do you mean iron or gallium?
11) Do you think that enhancing the bacterial oxidative stress response would be a way to decreases gallium inhibition in frpB mutants or in combinations of FrpB inhibitors with Gallium as it seems to be for gallium alone, see:
Tovar-García A, Angarita-Zapata V, Cazares A, Jasso-Chávez R, Belmont-Díaz J, Sanchez-Torres V, López-Jacome LE, Coria-Jiménez R, Maeda T, García-Contreras R. Characterization of gallium resistance induced in a Pseudomonas aeruginosa cystic fibrosis isolate. Arch Microbiol. 2020 Apr;202(3):617-622. doi: 10.1007/s00203-019-01777-y. Epub 2019 Nov 26. PMID: 31773196.
12) You have to be aware that sub optimal Ga concentrations can enhance P. aeruginosa virulence, see:
García-Contreras R, Pérez-Eretza B, Lira-Silva E, Jasso-Chávez R, Coria-Jiménez R, Rangel-Vega A, Maeda T, Wood TK. Gallium induces the production of virulence factors in Pseudomonas aeruginosa. Pathog Dis. 2014 Feb;70(1):95-8. doi: 10.1111/2049-632X.12105. Epub 2013 Nov 14. PMID: 24151196.
Do you think that would be minimized by targeting FrpB simultaneously with Ga?

Reviewer #3

(Remarks to the Author)
This manuscript by Zhang et al. describes development of an optimized CRISPRi-seq approach for comprehensive identification of genes that impact fitness of Pseudomonas under different conditions. They validate the approach by identification of essential genes in Pseudomonas and comparing the gene set to prior studies that use Tn-Seq, finding reasonable overlap and suggesting that some additional genes may be identified by CRISPRi. The CRISPRi system used was validated using several genes with known phenotypes and it performed as expected, including those genes suggested to be essential. They then used CRISPRi-seq to determine those Pseudomonas genes that impact Ga(NO3)3 resistances/susceptibility. The results suggest that genes involved in transport of iron and gallium impact resistance and redox status impact susceptibility through the interactions between iron and gallium pathways, along with NADP/NADPH ratios. The identification of FprB as impacting susceptibility to Ga(NO3)3 and mechanistic analyses suggesting susceptibility is due to triggering the Fenton reaction are overall well supported and compelling new information. There are some issues with presentation, writing, particularly grammar and clarity, and figures throughout, as well as a few overstatements of data obtained, but in general, this manuscript is valuable to the field and describes development of an improved tool that has numerous uses. The data obtained also support the potential of FprB as an adjunct to Pseudomonas and, potentially other bacterial species, therapeutic development.

Specific issues that should be addressed are:
1) There are numerous grammatical and typographical errors throughout the manuscript. It could use careful editing to improve readability.
2) The term ‘vulnerability’ is used throughout the manuscript and, although this term has been used somewhat in the field, it should be clearly defined the first time it is used for readers that may not be familiar with the usage in this context. I note the first use is in the Introduction of this manuscript.
3) Line 212. The term ‘best’ is not defined. How ‘best’ is determined should be clearly stated.
4) Line 213. PAMs have not been defined. This acronym should be defined.
5) Line 277-278. The ‘standard’ for these terms is not very clear and the reasons for choosing these cutoffs is not clear. The choices seem somewhat arbitrary. Do they have biological significance? These issues should be more clearly addressed and outlined within the methods and/or the discussion.
6) Lines 456-458. None of the accession numbers for Addgene return anything. This fact suggest that these materials are actually NOT available. This MUST be fixed prior to acceptance of this manuscript.
7) Lines 459-462. These data have not yet been released. They should be released prior to or at the same time as publication. Please confirm the release dates.
8) Lines 481-482. These statements are not supported by statistical analyses. It is unclear that any of the differences observed are due to anything other than random variation between the experimental protocols. The statements should be removed or qualified to make them more accurate based on the presented data.
9) Line 490. The statement that ‘no notable growth impairment’ is present is not accurate. The appear to be differences in the lag during early growth in all cases. The curves should be analyzed in more detail or these statements should be modified to make them more accurately reflect the data presented.
10) Line 562. The statement ‘clear discrepancy’ is not really accurate. Differences exist, possibly for the reasons mentioned,

but the current statement is over-done.
11) Fig. 3g. It is unclear why different concentrations of Ga(NO3)3 were used for the different panels. Either the data for the same concentrations should be presented or some explanation for why these concentrations were used should be provided.
12) Fig. 3e and 3g. The explanations for these panels have been switched. This should be corrected.
13) Fig. 4c. No error bars or statistics are provided for these data. Either this should be corrected or the conclusions from these data should be modified to make it clear that the differences may not be significant.
14) Fig. 5f. There are no cyan spots shown on the figure, but they are referenced as being cyan for down-regulated genes. This issue should be corrected in the figure.
15) Fig. 6c. It is unclear how many replicates these data represent. This should be corrected both in the graph and the figure legend.
16) Lines 775-776. The impact of FprB in vivo is modest. Therefore, suggesting that there is 'great potential' for FprB is premature. This statement should be modified to suggest that 'further study of the potential of targeting FprB is warranted'.
17) Extended Fig. 1. Panels b and c use different colors for different concentrations of Dox. The colors should be consistent between these panels.
18) Extended Fig. 5. Panel d does not indicate the number of replicates. This should be indicated both in the graph and in the figure legend.
19) Extended Fig. 6. The figure does not indicate the number of replicates. This should be indicated both in the graph and in the figure legend. The single asterisk is not defined in the figure legend. This should be corrected.
20) Extended Fig. 7. Panel c is a great deal too small. It should be enlarged so that the text is easily readable. Panel e it is unclear what concentration of Ga(NO3)3 was used in these experiments. Please clarify this information. Panel h should have a more careful analysis of different portions of the curve between the graphs. There appear to be differences during early log phase that may be significant.
21) There is no explanation or discussion for why FprA would not impact Ga(NO3)3 sensitivity. It is possible that its essential nature masks the impact of Ga(NO3)3, but this issue seems to be completely overlooked. More careful analysis of overlap in FprA and FprB functon(s) may be warranted and this glaring issue should at least be discussed.

Reviewer #4

(Remarks to the Author)
Zhang et al report the development of a genome-wide CRISPRi screen in the bacterial pathogen Pseudomonas aeruginosa and its application to find sensitizers of a Gallium nitrate treatment. They discover that the downregulation or deletion of the FeS-containing reductase FprB leads to a drastic increase of P. aeruginosa sensitivity towards Gallium ions, and report that an increased oxidative stress is associated with it. The increased sensitivity is also demonstrated in biofilms and in a mouse lung infection model. The manuscript contains a wealth of data that look convincing and of high quality. It is written in a clear, scholarly manner.
The technical advance achieved through the CRISPRi method seems to be of relevance for a broader community of P. aeruginosa researchers to me. The application to find synthetic lethality mechanisms to Gallium treatment is more exotic, but interesting from a scientific point of view. The authors provide a good body of microbiological and biochemical data, but open questions regarding the mechanism of synthetic lethality remain. The authors state that FprB deletion leads to increased Fe2+ levels and subsequently increased ROS/hydrogen peroxide, but it is unclear why this occurs only in a Ga-dependent manner. The molecular role of Ga ions with respect to the FprB-dependent increase of Fe2+ remains obscure. The in vivo models are mentioned very briefly at the end. The data look good, but the required dose is very high (I calculated ca. 160 mg/kg).
In spite of these limitations, I find the study rich and of high quality, and therefore recommend its publication after a revision.
Comments
- line 58: The reference (Shi et al) is not well-suited to support the content of the sentence.
- line 59: virulence, not virulent
- line 79: I cannot find the reference Zhu et al in the reference section
- lines 113-139: Almost a full page of the introduction is spent to describe the upcoming study. I find this redundant and recommend to shorten this to a few sentences.
- Figure 2: d: y-axis label is missing. I printed the figure on paper, and some letters are very hard to read due to their small size. I recommend increasing the font.
- Figure 2c: The overlap of growth-affecting genes in this studies and other studies is quite small. Moreover, the majority of genes (403 out of 740) found here were not found in other studies. Can the author comment on reasons behind this limited reproducibility between studies? Along this line, what is the reproducibility between gene sets 1 and 2?
- line 578: I suggest adding a definition for 'vulnerability' here. I found it in the Extended Figure after searching a while, but explaining it here would facilitate reading.
- The abbreviation 'COG' is not explained
- Figure 3: The captions for e and g seem confused.
- Figure 4e: It can be read when augmenting it on a screen to 200% or more, but it is too small to be read on paper. Please enlarge the figure here or place it as an Extended Figure.
- Figure 4c (and elsewhere): I assume the unit is CFU per ml? Please add.
- Figure 5a: The light blue panel of 0 Ga in the fprB mutant disappeared when printed. Please choose a different color.
- Figure 5f: The nor and nos genes are regulated strongly, but the significance is borderline. This needs to be mentioned in the text. I wonder whether they should be discussed so prominently in view of this. This part ('potentially exacerbating ROS leakage, line 743') would be more convincing if the authors measured NO levels.
- Line 728: I do not agree with the statement '…loss function of mgtA leads to accumulation of intracellular Fe (Marshall et al. 2009), which explains the observation of Fig. 5a' – There is NO accumulation of iron in the fprB mutant in the absence of Ga.

Also the statement in line 874 needs to be modified. It is only true in the presence of Ga, and not due to the absence of FprB alone.
- Extended Data Fig 4b: Add axis labels above the columns
- Extended Data Fig 7c: Impossible to read. I have not found more data in the supplement. Can the authors provide the alignment in a readable format?
- Ref Liu, Xue…. is incomplete.

Reviewer #5

(Remarks to the Author)
In this paper, Zhang et al. employed a chemical-genetic approach to identify potential synergistic targets with gallium, a drug that failed a phase II clinical trial for treating chronic Pseudomonas aeruginosa infections due to limited plasma and sputum concentrations. The authors constructed a CRISPRi-based genome-wide pooled mutant library and analyzed the fitness effects of these mutants using CRISPRi-seq when exposed to gallium. They identified fprB, which encodes a ferredoxin-NADP+ reductase, as a key synergistic target, with its deletion significantly sensitizing cells to gallium. They extend the work to find out that FprB 40 modulates oxidative stress induced by gallium, via control of the iron homeostasis and reactive oxygen species accumulation

Major comments
1. Rationale for choosing DOX as the inducible system to trigger gene silencing. The authors compared the tet-inducible system that uses DOX with the arabinose-inducible system for inducing dCas9 expression. However the rationale of this decision is not clear (potential applicability for in vivo infection models). Figure 1a and b suggests that DOX has a wider dynamic range of expression levels. However, the screen is performed at only one Dox concentration.

2. Novelty of CRISPRi system in Pseudomonas. CRISPRi systems using anhydrotetracycline (aTc) have also been developed in Pseudomonas (Tan et al. 2018). How does the DOX-based system compare to the aTc-based system? 2. DOX has been known the have slow-release kinetics where there is a temporal delay in the resumption of the tet-responsive promoter activity (A-Mohammadi et al. 1997; Robertson et al. 2002). Did the authors test or observe any delay in resumption of CRISPRi mutant growth after removal of the inducer (DOX)?

3. Number of generations vs time. Some of the data refer to number of generations and some to hs of incubation, which makes comparison difficult, for example fig. 1 and fig. 2.

4. Genomic coverage during treatment, including essential genes is not discussed. The CRISPRi library in PA14 was pre-induced with Dox for 14-generations prior to the Ga(NO3)3 treatment. Based on Fig 2e, mutants belonging to the “vulnerable and quick-responsive’ category are already strongly depleted after 14 generations. Did the authors addressed mutant relative abundance before gallium treatment to ensure genomic coverage?

5. The concentration of Ga(NO3)3 used for the CRISPRi-seq led to 70%-80% of growth reduction. What was the rational behind the severe growth reduction to 70-80%? As shown in Fig. 3b Ga(NO3)3 causes a drop in optical density from 0.8 to 0.2. How competitive fitness is affected when the cell density of treatment and control are so different? Could this difference be a confounding factor? Fig. 3C shows a volcano plot, with very few mutants showing decreased fitness, which is surprising given the high concentrations used. Could the lower cell density result in less competition, compared to no treatment. This confounding could decrease the sensisitivy of the competition assay.

6. Did the authors include any non-genome-targeting sgRNAs in the library? This is an important control to have to account for any effect of the CRISPRi system. Expression of dCas9 is known to have several deleterious effect in the cells.

7. Lines 542–544: “To improve the robustness of the CRISPRi study, we included two sgRNAs for each target, with the best sgRNAs identified by the RNA design pipeline assigned to set 1, and the second best to set 2.” Which strand was targeted? The efficiency of the CRISPRi systems is known to be greatly influenced by the targeted strand. How were the best sgRNAs determined? While this information may be detailed in the cited paper, it is important to briefly summarize it here for the reader. Was the selection based on factors such as mismatches, distance from the TSS, or proximity to the ATG?

8. Does the second set of sgRNAs have reduced specificity? If so, how do the authors ensure that their CRISPRi system does not introduce off-target effects? Even minor nonspecific interactions between dCas9 and unintended chromosomal regions could compromise the, or at least obscure the results. Such effects could mislead the analysis, making certain genes falsely appear as pleiotropic contributors to fitness.

9. The authors claim to have ‘refined’ the list of essential genes. Did the authors mean to have identified the ‘comprehensive’ list of essential genes? This study reports the largest number of essential genes identified in Pseudomonas (740). However, the authors used a log2FC threshold of -1 as the cutoff. Could this threshold be too lenient to define essential genes? Might some of these genes instead be related to general fitness rather than being truly essential?"

10. The growth defect for hitA is more pronounced than for hitB (Fig. 3e). How did the authors evaluate the fitness of the hitA exclusively? Given that hitA and hitB are part of the same operon, targeting hitA with an sgRNA would inadvertently repress hitB. Could the observed growth defect for hitA reflect a pleiotropic effect from simultaneously repressing both genes?

11. Line 775. “In contrast, the wild-type and complemented strain were not similarly affected”. Did the mean that the wild-type and complemented strain were actually similarly affected?"

12. Is gallium and synergy with F

Minor comments

1. Line 85-87. “The lack of an effective strategy for cloning sgRNAs has further hindered the application of genome-wide CRISPRi in P. aeruginosa”. However, systems like Mobile-CRISPRi are well-established and address this challenge (Qu et al. 2019; Yu et al. 2024).
2. There appears to be a noticeable decrease in pyocyanin levels even in the absence of Dox compared to the wild type (Fig. 1c). Could this indicate that the system is leaky?
3. Line 469-470, this sentence could use clarity.
4. Line 471. “To achieve tunable CRISPRi in P. aeruginosa, we developed a tet-inducible system for this bacterium.” However, this tet-inducible system has previously been used for CRISPRi system (Liu et al. 2020).
5. The authors used a tet-inducible reporter system to assess their CRISPRi system. It is unclear from the text whether the LuxABCDE luminescence reporter system is integrated into the genome or is plasmid-borne. A brief description would help the readers.
6. Line 518-519. “In strains PAO1 and PA14, growth was ‘significantly’ inhibited with 3.13 ng/mL Dox compared to the no-inducer control”. Was a statistical test performed to assess the significance?
7. Line 532, remove “by”
8. Line 542, the authors used previously developed sgRNA design platform. Default parameters?
9. Fig 3C is not cited in the text. Additionally, the legends in Fig 3C (increased and decreased fitness) are confusing, as they seem to refer to mutants as well.
10. Line 718. A brief description of the RNA-seq condition would be helpful.

References:
A-Mohammadi, S., Alvarez-Vallina, L., Ashworth, L.J., and Hawkins, R.E. 1997. Delay in resumption of the activity of tetracycline-regulatable promoter following removal of tetracycline analogues. Gene Ther 4(9): 993–997. doi:10.1038/sj.gt.3300491.
Liu, X., Kimmey, J.M., Matarazzo, L., Bakker, V. de, Maele, L.V., Sirard, J.-C., Nizet, V., and Veening, J.-W. 2020. Exploration of bacterial bottlenecks and Streptococcus pneumoniae pathogenesis by CRISPRi-Seq. Cell Host & Microbe 0(0). Elsevier. doi:10.1016/j.chom.2020.10.001.
Qu, J., Prasad, N.K., Yu, M.A., Chen, S., Lyden, A., Herrera, N., Silvis, M.R., Crawford, E., Looney, M.R., Peters, J.M., and Rosenberg, O.S. 2019. Modulating Pathogenesis with Mobile-CRISPRi. Journal of Bacteriology 201(22): 10.1128/jb.00304-19. American Society for Microbiology. doi:10.1128/jb.00304-19.
Robertson, A., Perea, J., Tolmachova, T., Thomas, P.K., and Huxley, C. 2002. Effects of mouse strain, position of integration and tetracycline analogue on the tetracycline conditional system in transgenic mice. Gene 282(1–2): 65–74. doi:10.1016/s0378-1119(01)00793-4.
Tan, S.Z., Reisch, C.R., and Prather, K.L.J. 2018. A robust CRISPRi gene repression system in Pseudomonas. J. Bacteriol. 200(7): e00575-17. doi:10.1128/JB.00575-17.
Yu, M.A., Banta, A.B., Ward, R.D., Prasad, N.K., Kwon, M.S., Rosenberg, O.S., and Peters, J.M. 2024. Investigating Pseudomonas aeruginosa Gene Function During Pathogenesis Using Mobile-CRISPRi. In Pseudomonas aeruginosa. Edited by G. Bertoni and S. Ferrara. Springer US, New York, NY. pp. 13–32. doi:10.1007/978-1-0716-3473-8_2.

Reviewer #6

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Version 1:

Reviewer comments:

Reviewer #2

(Remarks to the Author)
Thanks for adressing all my comments so well!

Reviewer #3

(Remarks to the Author)
This revised manuscript by Zhang et al. addresses the majority of concerns within the manuscript, though there remain a few issues that have not been fully addressed. The response to review provided by the authors is quite extensive and detailed.

In most cases, the comments are fully addressed and addressed well. It is clear that they made an attempt to address everything, but they have overlooked a few minor to moderate issues with how they have addressed the comments. Despite these issues, the manuscript remains an important contribution to the field.

Specific issues that should be addressed are:
1) In response R2.3 a list of detailed explanations are provided for why pyochelin-mediated gallium uptake does not impact gallium sensitivity in their study. Despite having these explanations, they do not briefly summarize these possibilities in the text of the manuscript and they should have done exactly that, included the explanations in the discussion.
2) The issue with the availability of materials from AddGene remains. Although the authors attempt to provide evidence that the materials are available, as of my review, the materials could still not be accessed. Confirmation of availability should be completed prior to acceptance and/or publication.
3) In R3.20 analysis of temporal growth dynamics was done in response to review. However, no mention of the fact that fprA did display slight and significant complementation of the fprB mutation within the 20 hr regions of the growth curve. This indicates that there is functional overlap between fprA and fprB, requiring revision of the text accordingly. This is particularly imporant in the Figure Legend for Extended Fig. 7 and lines 801-803 in the manuscript. It appears that FprA does impact Ga(NO3)3 sensitivity, though only slightly (P=0.0134).
4) In R3.21 the text should be revised from "rescue" to "fully rescue" and "their non-redundant" should be "only partially redundant".
5) In R4.7 the authors address the question in the response quite well, but they do not address the question within the text of the manuscript. Please modify the text of the manuscript within the discussion to address this question, using a more brie summary than the one included in this response.
6) With regard the revision of the section in Lines 518-519 of the original manuscript, the authors propose that removing the word “significantly” addresses the issue. However, it does not. If there is not significant growth inhibition, then there is no real growth inhibition. A statistical test should be done and if it is not significant, this sentence should be removed or changed to "growth was not significantly inhibited by 3.13 ng/ml".

Reviewer #4

(Remarks to the Author)
The authors have provided a thorough and careful revision of the manuscript. My suggestions were addressed adequately. I have two minor remaining points:
- Extended data figure 6B: The description and/or in the panel is incorrect, because it suggests that one condition is Ga+NADPH and the other is NADPH. Please rephrase.
- I am not an Addgene user, but it seems that the Addgene entries are still not accessible. I suggest withholding publication of the paper until this is the case.

Reviewer #5

(Remarks to the Author)
The authors have worked diligently and addressed most comments and concerns. Three aspects were partially addressed (Q5)
1) Related to R5.Q.2.1. The authors claim that the rationale of choosing the DOX-inducible system relies on the potential applicability for in vivo infection models. Upon request of clarifications the authors, responded there was “a higher relevance of tetracycline-regulated systems in model organisms and the ability to regulate gene expression in a more controlled and predictable manner, which is essential for in vivo applications”. However, this answer does not satisfy the rationale of the choice. The authors should explain or demonstrate why the DOX-inducible system is more predictable and more controlled as they say

2) Related to R5.Q.2.1. Superiority of the DOX-inducible system over the previously published aTc (Tan et al 2018). The authors claim that their system is more tightly regulated overcoming the leakiness of the previous promoter. While they show phenotypic assays where indeed the phenotypes are reduced compared with the wild type, the results do not support the author’s arguments. The authors could only support their claim with direct comparisons of both aTC and DOX-inducible systems with gene expression analysis by qPCR.

3) Related to R5. Q.4. Genomic coverage of the CRISPRi library during treatment exposure: the authors acknowledge that they did not provide any details on this issue before, and that mutant depletion of highly vulnerable mutants could occur during competition previous to the drug exposure. They support the timing at which they have performed the experiment showing that reduced time of induction (7 generations) does not sensitize the mutants (Extended Data Fig. 4a). For the most important claim (14 generations) they mention that “>90% of sgRNAs retained non-zero read counts across all four replicates, indicating sufficient coverage for reliable downstream analysis”. This claim is supported by a supplementary table S5 with raw counts for the essential gene screening. However, having non-zero read counts does not guarantee a reliable downstream analysis as the authors suggest. The authors should compile the data provided in the suppl table S5 showing where indeed they have genomic coverage in the library (% genes/operons represented by CRISPRi mutants over the whole genome). While the results obtained (target gene pursued) in this particular work will not change, this analysis will be useful toward enhancing the sensitivity of CRISPRi-seq screens.

Reviewer #6

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

We would like to sincerely thank the reviewers for their constructive and supportive comments, which have been invaluable in improving our manuscript. We believe that the revisions made in response to these suggestions have significantly strengthened the scientific quality, clarity, and overall presentation of the work. Below, we provide detailed, point-by-point responses to all reviewer comments, along with explanations of the corresponding changes implemented in the revised manuscript.

Reviewer #1:

Authors applied a genome-wide CRISPR interference approach (CRISPRi-seq), to identify potential synergistic targets with gallium. They identified a highly conserved gene fprB, encoding a ferredoxin-NADP+ reductase. Using a null mutant, they also confirmed the loss of fprB, and observed that lowers the minimum inhibitory concentration of gallium from 320 μM to 10 μM and shifted gallium's mode of action from bacteriostatic to bactericidal. However, the novelty of this article is very minimal. They have used a known CRISPR technique, and the identification of target is also not novel. In addition, the fprB mutant they identified reduced the MIC, however, if it is the target the MIC should be increased more than 320 μM. So, it confirms the target is something else, however it may be a secondary target. The study is also not expanded to confirm or support the results. The protocols are also not written clearly to reproduce.

Response:

We sincerely appreciate the reviewer's evaluation of our work. Below, we address the concerns raised by reviewer #1:

Point 1: Novelty of the CRISPRi-seq approach and identification of *fprB*

1.1. Novelty of the CRISPRi-seq implementation in *P. aeruginosa*

While CRISPRi-seq has been applied in other bacterial systems, to the best of our knowledge, our study represents the initial successful establishment of a genome-wide CRISPRi-seq platform in *P. aeruginosa* (A CRISPRi library covering >5000 genes). As highlighted in the Introduction, previous attempts in *P. aeruginosa* faced challenges such as efficient sgRNA cloning strategy for genome-wide library. Our work addresses these limitations through:

- A tightly regulated tetracycline-inducible system with minimal basal activity (Fig. 1a, Extended Data Fig. 1a-b).
- A *ccdB*-based counter-selection strategy that achieved high sgRNA cloning efficiency (Fig. 1h-i), overcoming previous technical barriers.
- Validation across diverse strains, including clinical isolates (Fig. 1g).

This methodological advancement, as noted by Reviewer 2 ("an improved tool that has numerous uses") and Reviewer 3 ("technical advance of relevance to the broader community"), provides a critical resource for *P. aeruginosa* research.

1.2. Innovation in identifying FprB as a synergistic target for gallium therapy

While ferredoxin-NADP reductases are known in bacterial redox systems, our discovery of FprB's role in gallium susceptibility is entirely novel. Key findings include:

- A "32-fold reduction in MIC" (320 μM to 10 μM) measured in MHB medium and "shift from bacteriostatic to bactericidal activity" in the Δ*fprB* mutant (Fig. 4a-c, Extended Data Fig. 5a).

- Mechanistic linkage of FprB to “iron homeostasis and ROS modulation” (Fig. 5a-e), a pathway not previously full elucidated with gallium efficacy.
- Conservation of FprB across 980/981 *P. aeruginosa* genomes (Fig. 6a-b), underscoring its broad relevance.

As Reviewer 4 noted, this work provides "compelling new information" on gallium’s interaction with bacterial metabolism, advancing beyond prior studies focused solely on iron uptake systems (e.g., hitAB).

1.3. MIC reduction and target validation

The reviewer’s concern about MIC reduction reflects a misunderstanding of gallium’s mechanism. Gallium is not a direct inhibitor of FprB but disrupts iron-dependent pathways broadly. Our data show that FprB loss exacerbates gallium-induced oxidative stress by:
- Increasing intracellular Fe^{2+} (Fig. 5a), which amplifies Fenton reactions in the presence of gallium-generated H_2O_2 (Fig. 5bc).
- Elevating ROS levels only under gallium treatment (Fig. 5bc), explaining the conditional lethality.

This aligns with Reviewer 3’s interpretation that "susceptibility is due to triggering the Fenton reaction" and Reviewer 2’s emphasis on "redox status impacts susceptibility." Thus, FprB is not a "secondary target" but a genetic vulnerability that enhances gallium’s primary effects, a novel concept in antimicrobial synergy.

Point 2: Protocol clarity and reproducibility

We have expanded the Methods section to include:
- Detailed sgRNA library construction parameters.
- Step-by-step conjugation protocols.
- Full CRISPRi-seq data analysis pipelines.

Other necessary methods were all included as well.

All plasmids and libraries are deposited at Addgene, and raw sequencing data are publicly accessible. Reviewer 2 acknowledged the "wealth of data that look convincing and of high quality," and Reviewer 3 highlighted the "clear, scholarly" presentation, affirming reproducibility.

In summary, we thank the reviewer for prompting us to clarify these points. The majority of reviewers recognized the significance of our CRISPRi-seq platform (Reviewer 2: "valuable to the field"; Reviewer 3: "rich and of high quality") and the mechanistic insights into gallium synergy (Reviewer 4: "compelling new information"). We are confident that these advancements will accelerate research on *P. aeruginosa* and antimicrobial development. Thank you again for your feedback, which has strengthened our manuscript.

Reviewer #2:

The manuscript by Zhang and coworkers represents an interesting and useful advance in the generation of genetic methods to determine the importance of P. aeruginosa genes under different conditions, and they used their method to identity a synergistic target for gallium in the growth inhibition of this bacterium.

The experiments conducted are adequate and their interpretation of the results/conclusion seem valid; nevertheless, I have some specific concerns and suggestions for the authors:

Thank you very much for your thoughtful and constructive feedback on our manuscript. We are grateful for your positive assessment of our work and are pleased that you consider our study an interesting and useful advance. We also appreciate your specific concerns and suggestions, which have helped us further improve the clarity and rigor of the manuscript. We have carefully addressed each of your points, as detailed below.

Q2.1

1) Abstract, L 38 “dramatically” is subjective and its better to use fold change in MIC or other more quantitative expression.

R2.1: We thank the reviewer for the helpful suggestion. The subjective term "dramatically" has been replaced with a more precise and quantitative description: The revised sentence now reads: “In addition, we identified a highly conserved gene, fprB, encoding a ferredoxin-$NADP^+$ reductase, whose deletion sensitized P. aeruginosa to gallium, lowering its MIC by 32-fold and shifting mode of action from bacteriostatic to bactericidal.”

This change is highlighted in the revised manuscript, line 35-37.

Q2.2

2) The authors should acknowledge that other had found significantly lower Ga MIC towards the same strains, mainly because Ga activity is potentiated at low iron concentrations, in minimal medium such as minimal succinate one, and since in this study Mueller Hinton (a rich medium was used), likely it contains high iron concentrations. See:

Kaneko Y, Thoendel M, Olakanmi O, Britigan BE, Singh PK. The transition metal gallium disrupts Pseudomonas aeruginosa iron metabolism and has antimicrobial and antibiofilm activity. J Clin Invest. 2007 Apr;117(4):877-88. doi: 10.1172/JCI30783. Epub 2007 Mar 15. PMID: 17364024; PMCID: PMC1810576.

García-Contreras R, Lira-Silva E, Jasso-Chávez R, Hernández-González IL, Maeda T, Hashimoto T, Boogerd FC, Sheng L, Wood TK, Moreno-Sánchez R. Isolation and characterization of gallium resistant Pseudomonas aeruginosa mutants. Int J Med Microbiol. 2013 Dec;303(8):574-82. doi: 10.1016/j.ijmm.2013.07.009. Epub 2013 Aug 6. PMID: 23992635.

R2.2: We thank the reviewer for bringing attention to the important role of medium composition, specifically iron concentrations, in determining the MIC of gallium. We also appreciate the references provided, which are highly relevant to our study. As the reviewer pointed out, gallium's activity is potentiated at low iron concentrations, as seen in minimal media such as succinate medium, whereas Mueller-Hinton broth (MHB), a rich medium, likely contains higher iron levels.

We agree that this should be acknowledged in the manuscript to provide a more comprehensive interpretation of our results. We have added the following clarifications in the revised manuscript:
Line 690: We now specify that the MIC was measured in MHB medium to better define the testing conditions.
Lines 896-901: "Notably, the deletion of fprB led to a substantial reduction in the minimum inhibitory concentration (MIC) of gallium from 320 μM to 10 μM in Mueller-Hinton broth, a nutrient-rich medium widely used as the standard for antibiotic susceptibility testing. It is worth noting that lower MIC values for gallium have been reported in low-iron media (Kaneko et al. 2007; García-Contreras et al. 2013), suggesting that iron availability plays a critical role in modulating gallium toxicity."

Q2.3
3) Although the authors explained the role of the HitAB system in gallium sensitivity and that mutations inactivating those genes increase gallium tolerance, since this system is likely the main gallium transporter, they do not mention that perhaps another way gallium can be internalized to cells is by the siderophore pyochelin, see
Frangipani E, Bonchi C, Minandri F, Imperi F, Visca P. Pyochelin potentiates the inhibitory activity of gallium on Pseudomonas aeruginosa. Antimicrob Agents Chemother. 2014 Sep;58(9):5572-5. doi: 10.1128/AAC.03154-14. Epub 2014 Jun 23. PMID: 24957826; PMCID: PMC4135843.

I wonder if they identified genes related to pyochelin synthesis and transport as important for gallium tolerance in the screening.

R2.3: We appreciate the reviewer pointing out the potential role of pyochelin-mediated gallium uptake. We indeed included genes related to pyochelin biosynthesis (operons pchDCBA/pchEFGHI) and transport (fptA/fptX) in our gallium CRISPRi-seq screening. However, none of these genes exhibited significant phenotypes related to gallium sensitivity or tolerance (see Supplementary Table S7).
We propose the following explanations for this result:

1) Low expression of pyochelin-related genes: Transcriptional profiling (Supplementary Table S9) indicated minimal expression of pyochelin biosynthesis and transport genes in LB medium, which was used to preculture the CRISPRi library prior to gallium exposure. This observation is consistent with known Fur-mediated repression of pyochelin genes under iron-replete conditions. Since CRISPRi efficacy is contingent on baseline gene expression, the low transcriptional activity of these genes likely hindered the detection of any significant phenotypic changes.
2) Context-dependent role of pyochelin in gallium uptake: In the study by Frangipani et al. (2014), the authors observed a modest 1-fold increase in MIC for pyochelin mutants (Δ*pchD*, Δ*fptX*, and Δ*fptAX*) specifically in iron-depleted DCAA medium after 24 hours. This suggests that pyochelin-dependent gallium uptake may be more relevant under conditions of iron limitation, which were not present in our experimental setup.
3) Pyochelin as a public good: Pyochelin is a public good in *P. aeruginosa* populations, meaning that mutants with defects in pyochelin biosynthesis can still acquire pyochelin secreted by the

wild-type or other competent cells within the population. This could have masked the effects of pyochelin-related mutations during our CRISPRi-seq screening, as mutants could still access pyochelin produced by other cells.

Q2.4

4) Fig 1 C and 1 D, pyocyanin concentration is very easy to determine by spectrophotometry, please do it and present concentration changes together or rather than picture, also swarming halo diameter can be easily determined.

R2.4: Thank you for your constructive suggestion. We agree that quantifying pyocyanin concentration and swarming halo diameter provides more precise and valuable data. In response, we have added the quantification of swarming halo diameters in the revised **Figure 1d&e** and presented pyocyanin concentration changes in **Figure 1c** to complement the visual representation and provide a clearer, quantitative comparison.

Q2.5

5) While the role of FprB in counteracting the oxidative stress promoted by gallium seems likely, simple experiments such as adding ROS scavengers or commercial purified catalase and showing this decrease the inhibitory effects of gallium in the fprB mutant may strength the conclusion.

R2.5: We thank the reviewer for the valuable suggestion regarding the role of FprB in counteracting oxidative stress. Following the suggestions, we conducted experiments using DMSO and commercial purified catalase to evaluate their effects on alleviating the growth defect caused by gallium in the Δ*fprB* mutant. The results of these experiments are now presented in **Extended Figure S6** of the revised manuscript.

We observed that the addition of DMSO provided partial relief of the growth defect caused by gallium treatment, suggesting that ROS scavengers like DMSO can mitigate the oxidative stress induced by gallium in the Δ*fprB* mutant, indicating a potential role for FprB in modulating the oxidative stress response (**Extended Figure S6C**).

In contrast, the addition of catalase did not relieve the growth defect, which we attribute to the inability of catalase, an extracellular enzyme, to effectively penetrate the bacterial membrane. This finding suggests that the oxidative stress induced by gallium in *P. aeruginosa* likely involves multiple reactive species, and the response to this stress may be more complex than what can be mitigated by exogenous catalase alone. We have further discussed these observations in the revised manuscript:

Line 753-755 “Conversely, exogenous supplementation of the hydroxyl radical scavenger dimethyl sulfoxide (DMSO) partially restored gallium-impaired growth in the ΔfprB background (Extended Data Fig. 6c).”

Q2.6

6) Interestingly, FprB may be not only a suitable synergistic target for Gallium but perhaps also for other compounds that promote ROS and the authors demonstrated this with H2O2 and plumbagin, but it may be also suitable to test if it has synergy with antibiotics that promote ROS.

R2.6: We thank the reviewer for this valuable suggestion. To explore FprB's potential broader role in ROS-mediated synergies, we systematically evaluated the susceptibility of the Δ*fprB* mutant to three antibiotics with distinct mechanisms: colistin (membrane disruption), ciprofloxacin (DNA

gyrase inhibition), and apramycin (protein synthesis inhibition). Notably, none exhibited enhanced sensitivity in the mutant strain compared to wild-type (**Extended Data Figure 6d**). Furthermore, we specifically tested for gallium-antibiotic synergy by checkerboard assays, and no synergistic interactions were observed (fractional inhibitory concentration index > 0.5 in all cases) (**Extended Data Figure 6e**)

We hypothesize two explanations:

1) ROS potentiation by FprB deficiency may be pathway-specific. While FprB's ferredoxin-NADP+ reductase activity critically modulates redox homeostasis, its deletion might predominantly sensitize cells to direct oxidative stressors (e.g., H_2O_2, plumbagin) rather than antibiotics whose ROS effects are secondary to primary target inhibition.

2) Antibiotic efficacy is governed by multifactorial determinants. Even for antibiotics reported to induce ROS (e.g., ciprofloxacin), their bactericidal activity primarily depends on target-binding kinetics, which may dominate over ROS-mediated effects under our experimental conditions.

This study focused on gallium-specific synergies, but we fully agree that exploring FprB's interplay with ROS-inducing antimicrobials warrants future investigation. Thus, we have included these considerations in the revised manuscript (lines 755-762), as yellow highlighted.

"To test if FprB can serve as a synergistic target for antibiotics that induce ROS, we treated the ΔfprB mutant with colistin (membrane disruptor), ciprofloxacin (DNA gyrase inhibitor), and apramycin (protein synthesis inhibitor). However, no increased synergistic or even additive effect was observed (Extended Data Fig. 6d), indicating ROS potentiation by FprB deficiency may be pathway-specific, which likely requires direct oxidative stressors rather than antibiotic-induced ROS under these conditions. In addition, checkerboard assays demonstrated the absence of synergistic interactions between gallium and colistin, ciprofloxacin, or apramycin (Extended Data Fig. 6e)."

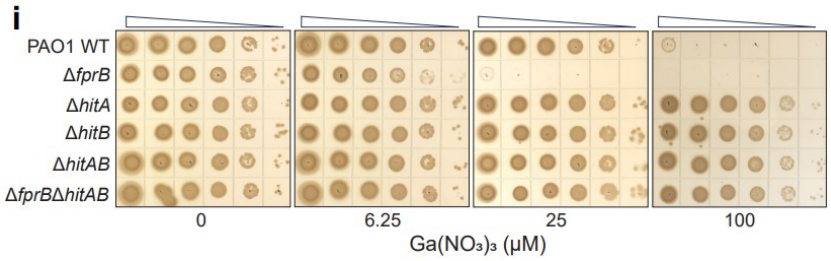
Q2.7

7) What would be the gallium sensitivity of a hitAB fprB mutant?

R2.7: We appreciate the reviewer's insightful question. To investigate this, we generated the Δ*hitA*Δ*hitB*Δ*fprB* triple deletion mutant and assessed its sensitivity to gallium. The results, presented in the revised **Figure 3i**, show that the increased gallium sensitivity associated with the loss of FprB is dependent on the presence of HitAB. This finding is consistent with the well-established role of HitAB in gallium transport. Additionally, it provides further evidence that the enhanced gallium sensitivity observed upon FprB loss requires active internalization of gallium into *P. aeruginosa* cells.

We have incorporated this result in the revised manuscript (line 681- 683) as highlighted :

"Strikingly, the effect of fprB on enhancing P. aeruginosa sensitivity to gallium was abolished by deletion of hitAB (Fig. 3i), demonstrating that HitAB is the primary gallium transporter under these conditions and the gallium sensitivity induced by fprB deletion depends on active gallium uptake."



Revised Fig 3i

Q2.8

8) Can a frpB mutant be complemented with frpA?

R2.8: We appreciate the reviewer for raising this question. To assess the functional redundancy between FprA and FprB, we performed genetic complementation by introducing fprA into the Δ*fprB* mutant. As shown in **Extended Data Figure 7e**, expression of FprA failed to rescue the enhanced gallium sensitivity phenotype observed in the Δ*fprB* strain. This result indicates that FprA cannot compensate for the loss of FprB, suggesting limited functional interchangeability between these paralogs.

This observation aligns with two findings:

1) The CRISPRi-seq screening classified *fprA* as an essential gene, while *fprB* was identified as dispensable, highlighting distinct roles for these two genes.
2) FprB specifically modulates gallium-associated oxidative stress and iron homeostasis, functions that are not shared by FprA.

Taken together, these results demonstrate that the mechanism by which FprB contributes to gallium sensitivity operates independently of FprA under the conditions tested.

Q2.9

9) Please note that the first work ever reporting that mutations of hitAB conferred resistance to gallium by decreasing its internalization was

García-Contreras R, Lira-Silva E, Jasso-Chávez R, Hernández-González IL, Maeda T, Hashimoto T, Boogerd FC, Sheng L, Wood TK, Moreno-Sánchez R. Isolation and characterization of gallium resistant Pseudomonas aeruginosa mutants. Int J Med Microbiol. 2013 Dec;303(8):574-82. doi: 10.1016/j.ijmm.2013.07.009. Epub 2013 Aug 6. PMID: 23992635.

You seem to give the credit to other publications.

R2.9: Thank you for pointing out this important reference. We acknowledge that García-Contreras et al. (2013) were indeed the first to report that mutations in *hitAB* confer resistance to gallium by reducing its internalization. We regret any confusion caused by the oversight in our manuscript and have now updated the text to properly credit their pioneering work. In the revised manuscript, we have clarified that our findings build upon this previous research, expanding on the role of HitAB in gallium resistance.

We appreciate the reviewer's attention to this detail, and the citation has been added in the appropriate section of the manuscript (line102 and line 893).

Q2.10

10) L 108 “major transporters of iron” do you mean iron or gallium?

R2.10: Thank you for your comment. In this sentence, our intention was to describe the biological role of HitAB as major transporters of iron. However, we also recognize that HitA and HitB are involved in gallium uptake, as demonstrated in previous studies. To clarify this point, we have revised the text as follows (line 100-102):

" Notably, HitA and HitB, which form the major transporters of Fe3+, have emerged as key factors in gallium tolerance due to their role in Ga3+ uptake (García-Contreras et al. 2013; Goss et al. 2018)."

Q2.11

11) Do you think that enhancing the bacterial oxidative stress response would be a way to decreases gallium inhibition in frpB mutants or in combinations of FrpB inhibitors with Gallium as it seems to be for gallium alone, see:

Tovar-García A, Angarita-Zapata V, Cazares A, Jasso-Chávez R, Belmont-Díaz J, Sanchez-Torres V, López-Jacome LE, Coria-Jiménez R, Maeda T, García-Contreras R. Characterization of gallium resistance induced in a Pseudomonas aeruginosa cystic fibrosis isolate. Arch Microbiol. 2020 Apr;202(3):617-622. doi: 10.1007/s00203-019-01777-y. Epub 2019 Nov 26. PMID: 31773196.

R2.11: We appreciate the reviewer’s insightful question regarding the potential role of oxidative stress response enhancement in mitigating gallium toxicity in Δ*fprB* mutant. As highlighted by Tovar-García et al. (2020), increased oxidative stress defenses—such as upregulation of NADPH, glutathione, or superoxide dismutase (SOD)—have been associated with gallium resistance in *P. aeruginosa*.

To investigate whether enhancing redox homeostasis could alleviate gallium toxicity in Δ*fprB*, we supplemented the mutant with exogenous NADPH (1 mM). While this treatment partially improved gallium tolerance in Δ*fprB*, the rescued survival was significantly less than the wild-type strain (**Extended Data Fig. 6b and Figure 3i**). Notably, the minimum inhibitory concentration (MIC) of gallium for Δ*fprB* remained unchanged, suggesting that increasing antioxidant capacity alone is unlikely to fully restore gallium resistance. This observation aligns with the idea that FprB deficiency disrupts both iron homeostasis and redox balance, leading to a more complex sensitivity phenotype that cannot be rescued solely by enhancing the antioxidant response. However, this finding reinforces the potential of FprB as a key target for synergy in gallium-based antimicrobial strategies. We have expanded the discussion to incorporate these insights and provide a more comprehensive interpretation of FprB’s role as a therapeutic target in gallium therapy (Lines 915–921).

“Increased oxidative stress responses, such as upregulation of NADPH, glutathione, or superoxide dismutase (SOD), have been reported to enhance P. aeruginosa tolerance to gallium (Tovar-García et al. 2020). In our study, supplementation with 1 mM NADPH slightly improved the survival of the ΔfprB mutant but did not restore its MIC (Extended Data Fig. 6b), suggesting that FprB deficiency disrupts both iron homeostasis and redox balance. This complex sensitivity phenotype cannot be fully rescued by enhancing the antioxidant response alone.”

Q2.12

12) You have to be aware that sub optimal Ga concentrations can enhance P. aeruginosa virulence, see:

García-Contreras R, Pérez-Eretza B, Lira-Silva E, Jasso-Chávez R, Coria-Jiménez R, Rangel-Vega A, Maeda T, Wood TK. Gallium induces the production of virulence factors in Pseudomonas aeruginosa. Pathog Dis. 2014 Feb;70(1):95-8. doi: 10.1111/2049-632X.12105. Epub 2013 Nov 14. PMID: 24151196.

Do you think that would be minimized by targeting FrpB simultaneously with Ga?

R2.12: We sincerely thank the reviewer for raising this important point regarding the potential virulence-enhancing effects of suboptimal gallium concentrations. While García-Contreras et al. demonstrated that sub-MIC gallium exposure can upregulate virulence factors in *P. aeruginosa* in vitro, their study did not explore whether this effect translates to enhanced pathogenicity in vivo, a gap that our current work cannot definitively address.

We believe that targeting FprB simultaneously with gallium could help mitigate any potential virulence enhancement caused by suboptimal gallium concentrations. This is due to the reduced gallium threshold observed in Δ*fprB* mutants, which allows for effective therapeutic outcomes at concentrations lower than those reported to induce virulence factor expression (e.g., MIC=10 μM in our study versus 25 μM in García-Contreras' work).

This consideration further emphasizes the value of FprB as a potential synergy target for gallium therapy. We are grateful to the reviewer for highlighting this issue, and we have incorporated this perspective into the revised discussion to provide a more comprehensive interpretation of FprB's role in gallium-based treatments. Lines 944-949:

"Furthermore, suboptimal concentrations of gallium (25 μM) have been reported to enhance the virulence of P. aeruginosa in vitro, raising concerns about the potential side effects of gallium therapy (García-Contreras et al. 2014). We propose that simultaneously targeting FprB with gallium therapy could mitigate this issue. The reduced gallium MIC (10 μM) observed in ΔfprB mutants enables effective therapeutic outcomes at concentrations lower than those required to induce virulence factor expression."

Reviewer #3:

This manuscript by Zhang et al. describes development of an optimized CRISPRi-seq approach for comprehensive identification of genes that impact fitness of Pseudomonas under different conditions. They validate the approach by identification of essential genes in Pseudomonas and comparing the gene set to prior studies that use Tn-Seq, finding reasonable overlap and suggesting that some additional genes may be identified by CRISPRi. The CRISPRi system used was validated using several genes with known phenotypes and it performed as expected, including those genes suggested to be essential. They then used CRISPRi-seq to determine those Pseudomonas genes that impact Ga(NO3)3 resistances/susceptibility. The results suggest that genes involved in transport of iron and gallium impact resistance and redox status impact susceptibility through the interactions between iron and gallium pathways, along with NADP/NADPH ratios. The identification of FprB as impacting susceptibility to Ga(NO3)3 and mechanistic analyses suggesting susceptibility is due to triggering the Fenton reaction are overall well supported and compelling new information. There are some issues with presentation, writing, particularly grammar and clarity, and figures throughout, as well as a few overstatements of data obtained, but in general, this manuscript is valuable to the field and describes development of an improved tool that has numerous uses. The data obtained also support the potential of FprB as an adjunct to Pseudomonas and, potentially other bacterial species, therapeutic development.

We sincerely appreciate the reviewer's recognition of the value of our work and the potential applications of the CRISPRi-seq approach we developed. We are grateful for the thoughtful feedback and have made careful revisions to address the issues related to presentation, grammar, clarity, and figures. Additionally, we have revised the manuscript to ensure that any overstatements of the data are corrected for accuracy and clarity. We believe these revisions enhance the quality of the manuscript, and we would like to thank the reviewer once again for the constructive feedback, which has significantly strengthened our work.

Specific issues that should be addressed are:
Q3.1
1) There are numerous grammatical and typographical errors throughout the manuscript. It could use careful editing to improve readability.
R3.1: We sincerely appreciate your constructive feedback. In response, we have carefully reviewed the manuscript and made all necessary corrections to address the grammatical and typographical errors. We believe these revisions have significantly improved the overall readability and clarity of the manuscript.

Q3.2
2) The term 'vulnerability' is used throughout the manuscript and, although this term has been used somewhat in the field, it should be clearly defined the first time it is used for readers that may not be familiar with the usage in this context. I note the first use is in the Introduction of this manuscript.
R3.2: Thank you for this helpful suggestion. We agree that the term "vulnerability" should be clearly defined to ensure accessibility for readers less familiar with its usage in this context. In our study,

we use vulnerability to describe the degree to which gene silencing impairs bacterial fitness, while responsiveness captures the timing and dynamics of this fitness loss during CRISPRi-seq screening.

To address this, we have now introduced the conceptual definitions of both terms at their first mention in the Introduction (Lines 109–111):

"In this context, vulnerability refers to the extent to which gene silencing impairs bacterial fitness, while responsiveness captures the timing and dynamics of fitness loss following gene knockdown."

Here we provide the conceptual definition in the introduction section.

To provide further clarity, we have also included a more detailed operational definition in the Results section (Lines 605–610), specifying the criteria used to classify vulnerability and responsiveness based on depletion phenotypes and temporal patterns of fitness reduction:

"Here, vulnerability is defined as a strong depletion phenotype (specifically genes with $\log_2FC < -4$ at any time point) indicating a more severe fitness defect than the essentiality threshold ($\log_2FC < -1$). Responsiveness reflects the timing of the fitness defect appearance, with "quick-responsive" genes showing substantial depletion ($\log_2FC < -2$ for vulnerable; $\log_2FC < -1$ for invulnerable) by generation 7, while "slow-responsive" genes reach similar levels only at later time points (generation 14 or 21)."

Q3.3

3) Line 212. The term 'best' is not defined. How 'best' is determined should be clearly stated.

R3.3: We thank the reviewer for highlighting this ambiguity. In the revised manuscript, we have clarified how "best" sgRNAs were determined (lines 193-200) as follows:

"All sgRNAs were designed to target the coding strand of the genes. Briefly, all possible candidate sgRNAs for each gene are scored on the basis of (1) distance of sgRNA target binding site to target start codon (on-target score), and (2) number and within-spacer position of mismatches with all other potential binding sites in the genome, i.e., all other Protospacer Adjacent Motifs (PAM) sites (off-target score). These scores are weighted, prioritizing candidates with a minimal maximum expected off-target effect. Given this metric, the two best-ranking sgRNAs were selected for this study and assigned to set 1 (5981 sgRNAs) and 2 (5971 sgRNAs), respectively."

Q3.4

4) Line 213. PAMs have not been defined. This acronym should be defined.

R3.4: Thanks. This has been fixed as highlighted in line 197 of the revised manuscript. "all other Protospacer Adjacent Motifs (PAM) sites "

Q3.5

5) Line 277-278. The 'standard' for these terms is not very clear and the reasons for choosing these cutoffs is not clear. The choices seem somewhat arbitrary. Do they have biological significance? These issues should be more clearly addressed and outlined within the methods and/or the discussion.

R3.5: We thank the reviewer for this insightful comment. We agree that further clarification is warranted regarding the rationale behind the thresholds used to define gene categories (e.g., $\log_2FC \leq -1$ for essentiality, as well as subsequent stratifications of vulnerability and invulnerability). These thresholds were chosen based on both biological reasoning and empirical patterns observed in our dataset. In response, we have revised the Methods and Discussion sections to provide a clearer

explanation.

Specifically, we used a baseline cutoff of $\log_2FC \leq -1$ to define essential genes, such depletion typically corresponds to a substantial fitness cost, reflecting impaired growth or viability under screening conditions. To increase transparency and provide flexibility for interpretation, we also included essential gene lists derived from more stringent thresholds ($\log_2FC \leq -2$, -3, and -4), yielding 389, 255, and 151 essential genes, respectively. These results are now presented in the revised manuscript (**Extended Data Fig. 2b**), offering a broader view of gene essentiality.

To better capture intermediate phenotypes and minimize false negatives, we further categorized genes as "vulnerable" or "invulnerable." These additional categories reflect the nuanced behavior of gene knockdown phenotypes, which can vary depending on induction timing and sgRNA efficacy. Within these groups, we distinguished "quick" and "slow" responders based on the 7-generation $\log_2FC$ value. A $\log_2FC < -2$ (for vulnerable genes) or < -1 (for invulnerable genes) at this early timepoint indicates a more immediate fitness cost, whereas slower depletion patterns suggest functional redundancy, delayed phenotypic manifestation, metabolic buffering or slower turnover of functional gene products.

While these thresholds are, to some extent, operational, we aimed to anchor them in biologically meaningful differences. To support this framework, we selected representative genes from each category and conducted growth curve assays under varying doxycycline concentrations, mimicking cumulative repression over time (**Extended Data Fig. 3**). These results partially validated our stratification: in general, "vulnerable" genes—particularly quick responders (clusters 1 and 2)—tend to show stronger growth inhibition compared to "invulnerable" ones (clusters 3 and 4). Notably, "vulnerable, quick-responsive" genes tend to exhibit complete growth arrest under high inducer conditions, whereas "vulnerable, slow-responsive" genes tend to show partial inhibition, even at high inducer levels.

We think this approach prioritizes both sensitivity and interpretability in categorizing gene essentiality over time, and we believe it offers a valuable framework for exploring gene function dynamics in CRISPRi-based fitness profiling.

To address the reviewer's concern, we have added explanatory text to both the Methods and Discussion sections to clarify the basis and biological significance of these cutoffs.

Line 263-267 for the methods: "For vulnerable/invulnerable categorization, we adopted a two-tiered classification system: (1) initial grouping based on log2FC thresholds (Extended Data Fig. 2c), which was optimized to maximize biological relevance while minimizing false positives; and (2) temporal stratification into quick/slow responders using 7-generation log2FC cutoffs (-2 for vulnerable, -1 for invulnerable), reflecting distinct kinetic profiles of gene repression impact on fitness."

Line 859-864 for the discussion: Subdividing essential genes into "vulnerable" and "invulnerable" categories—and further into "quick" versus "slow" responders—captures the temporal dynamics of bacterial cell fitness response to gene silencing. A rapid, pronounced drop implies acute dependence on the targeted gene, whereas a delayed depletion may reflect metabolic buffering or slower turnover of functional gene products. Conversely, genes showing moderate yet consistent depletion were classified as invulnerable, highlighting subtler contributions to cellular fitness. Such categorization of essential genes provides valuable insights into their potential as therapeutic targets (Hawkins et al. 2020; Bosch et al. 2021). Nevertheless, it is important to recognize that sgRNA efficiency may confound these classifications, and its contribution requires

further systematic investigation in P. aeruginosa.

Q3.6

6) Lines 456-458. None of the accession numbers for Addgene return anything. This fact suggest that these materials are actually NOT available. This MUST be fixed prior to acceptance of this manuscript.

R3.6: We fully agree with the reviewer on the importance of making research materials accessible to the scientific community. We have consistently prioritized sharing resources in our previous publications, and we view the dissemination of tools and platforms—such as the CRISPRi system described in this manuscript—as equally important as the scientific findings themselves.

We have indeed deposited the materials to Addgene, but several logistical and technical challenges have delayed their public release. Specifically:

1) Strain compatibility with Addgene requirements: Our pCRISPRi-ccdB plasmid requires a specific *E. coli* strain for propagation. Addgene does not accept auxotrophic strains, so we had to reengineer a compatible *E. coli* host, which required additional time and validation.
2) Shipping restrictions from China: Due to increased scrutiny from U.S. customs, Addgene notified us that live strain shipments from China were subject to indefinite delays (please see attached email correspondence with Addgene). We waited for several months but were unable to resolve the issue through regular channels.
3) Alternative solution via collaboration: To overcome this, we enlisted the help of a colleague in Belgium. The plasmids and necessary E. coli strains have been successfully shipped to Belgium and then transferred to Addgene. **At the time of submitting this revised manuscript, we had received notification from Addgene confirming the successful delivery of our package, ensuring that the materials will be readily available during the review process** (see Addgene registration details and the notice of thearrival of the materials at addgene below).
4) Interim availability: While Addgene finalizes the upload, we are already sharing these materials upon request and have already provided them to other after prepinting on BioRxiv during this revision (Michiels lab from Belgium already get them). **We are fully committed to ensuring that these tools are readily accessible during the interim.**

We kindly ask for the reviewer's understanding regarding these logistical hurdles. We assure you that all materials will be made publicly available on Addgene as soon as their internal review is completed.

addgene

Inventory for Deposit 85599

Plasmid ID	Plasmid	Article	Authors
234855	Pooled sgRNA Libraries Set 1 for Pseudomonas aeruginosa PA14	Genome-wide CRISPRi-seq Identified Ferredoxin-NADP Reductase FprB as a Synergistic Target for Gallium Therapy in Pseudomonas aeruginosa.	Zhang et al
234856	Pooled sgRNA Libraries Set 2 for Pseudomonas aeruginosa PA14	Genome-wide CRISPRi-seq Identified Ferredoxin-NADP Reductase FprB as a Synergistic Target for Gallium Therapy in Pseudomonas aeruginosa.	Zhang et al

Thank you again for emphasizing this important point.

addgene

Inventory for Deposit 84804

Plasmid ID	Plasmid	Article	Authors
226432	pCRISPRi-ccdB	Genome-wide CRISPRi-seq Identified Ferredoxin-NADP Reductase FprB as a Synergistic Target for Gallium Therapy in Pseudomonas aeruginosa.	Zhang et al

Angela Holmes (Addgene) 2025-05-02 03:50
抄送 Xue Liu、Yu Zhang 章宇

详情

Please reply above this line

You are registered as a CC on this support request.
Reply to this email to add a comment to the request.

Angela Holmes (Addgene)
May 1, 2025, 3:50 PM EDT

Dear Xue,

I wanted to let you know that the shipment with your samples arrived this afternoon. I imagine Hannah or another member of our Scientific Support team will be in touch with you when they begin processing your samples.

Thank you so much for working through all the logistical challenges with us!

Best,
~Angela

Q3.7

7) Lines 459-462. These data have not yet been released. They should be released prior to or at the same time as publication. Please confirm the release dates.

R3.7: We appreciate the reviewer's comment regarding data availability. We have now ensured that all sequencing data associated with this study have been deposited and are publicly accessible via the NCBI BioProject database under the accession number PRJNA1063516.
https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1063516/

Q3.8

8) Lines 481-482. These statements are not supported by statistical analyses. It is unclear that any of the differences observed are due to anything other than random variation between the experimental protocols. The statements should be removed or qualified to make them more accurate based on the presented data.

R3.8: Thank you for this suggestion. In response to the reviewer's comment, we have removed the unsupported comparative statements between the Tet-inducible and arabinose-inducible systems. The revised text now reads:
Line 505-509: "The maximum induction led to approximately a 108-fold increase in expression at 20 hours post-induction, without any discernible growth delay, confirming the system's efficiency

and titratability. In parallel, the well-characterized arabinose-inducible system in P. aeruginosa (McMackin et al. 2021) showed a dynamic range of approximately 29-fold induction under the same conditions (Fig. 1a)."

Q3.9

9) Line 490. The statement that 'no notable growth impairment' is present is not accurate. The appear to be differences in the lag during early growth in all cases. The curves should be analyzed in more detail or these statements should be modified to make them more accurately reflect the data presented.

R3.9: Thank you for your suggestion. We have modified the statement to make them more accurately reflect the data presented:

Line 516-519: "While no differences in overall growth yield were observed, slight variations in early growth kinetics were observed across different levels of Dox induction (Extended Data Fig. 1c), indicating a modest impact on lag phase."

Q3.10

10) Line 562. The statement 'clear discrepancy' is not really accurate. Differences exist, possibly for the reasons mentioned, but the current statement is over-done.

R3.10: We agree with your suggestion and have revised "clear discrepancy" to "differences " to make the statement more accurate. The revised text reads like:

Line 587-588 "However, differences exist among the Tn-seq studies, potentially due to variations in culture conditions or cut-offs for essential gene definition."

Q3.11

11) Fig. 3g. It is unclear why different concentrations of Ga(NO3)3 were used for the different panels. Either the data for the same concentrations should be presented or some explanation for why these concentrations were used should be provided.

R3.11: Thank you for raising this important point. We agree that the use of different $Ga(NO_3)_3$ concentrations in **Fig. 3g** requires clarification. During the CRISPRi screening, a sub-inhibitory concentration of $Ga(NO_3)_3$ was used to avoid complete growth inhibition, which would otherwise lead to global depletion of the library and obscure gene-specific effects. In Fig. 3g, we evaluated individual gene knockdowns across a range of $Ga(NO_3)_3$ concentrations to capture the most informative phenotypes. Different genes exhibited varying levels of sensitivity or tolerance, and therefore the concentration that best recapitulated the screening phenotype was chosen for each gene. This approach allowed us to illustrate the most representative and biologically meaningful responses consistent with our genome-wide data. We have included this explanation in the revised manuscript in the figure legend "Representative phenotypes under selected concentrations of $Ga(NO_3)_3$, chosen to best illustrate differential sensitivity, are shown."

Q3.12

12) Fig. 3e and 3g. The explanations for these panels have been switched. This should be corrected.

R3.12: Thank you for pointing this out. We have corrected the labeling and corresponding explanations for Fig. 3e and 3g in the revised manuscript to ensure consistency and accuracy.

Q3.13

13) Fig. 4c. No error bars or statistics are provided for these data. Either this should be corrected or the conclusions from these data should be modified to make it clear that the differences may not be significant.

R3.13: We appreciate the reviewer's suggestion to improve the clarity and rigor of our data presentation. In Fig. 4c, error bars are included; however, they appear minimal due to $\log_{10}$ transformation of the data, which compresses visual differences. To address this, we have revised the figure legend to clarify the presence of error bars.

Q3.14

14) Fig. 5f. There are no cyan spots shown on the figure, but they are referenced as being cyan for down-regulated genes. This issue should be corrected in the figure.

R3.14: Thank you for pointing out this oversight. We have revised Fig. 5f to ensure consistency between the figure and its legend. Specifically, we have now used cyan and red to represent down- and up-regulated genes, respectively, as originally intended.

Q3.15

15) Fig. 6c. It is unclear how many replicates these data represent. This should be corrected both in the graph and the figure legend.

R3.15: Thank you for your comment. We have performed three independent replicates and have updated the figure legend to reflect this. Additionally, we revised the bar plot to include individual data points, providing a clearer representation of data variability.

Q3.16

16) Lines 775-776. The impact of FprB in vivo is modest. Therefore, suggesting that there is 'great potential' for FprB is premature. This statement should be modified to suggest that 'further study of the potential of targeting FprB is warranted'.

R3.16: Thank you for your thoughtful suggestion. We agree that the statement regarding the "great potential" of FprB is premature. Following your recommendation, we have modified the statement to read:

Line 827-828 "These results underscore the need for further investigation into FprB as a therapeutic target."

Q3.17

17) Extended Fig. 1. Panels b and c use different colors for different concentrations of Dox. The colors should be consistent between these panels.

R3.17: Thank you for pointing this out. We agree that consistent color coding improves clarity and visual interpretation. In the revised manuscript, we have updated **Extended Fig. 1b** and **1c** to ensure that the colors representing different doxycycline concentrations are uniform across both panels.

Q3.18

18) Extended Fig. 5. Panel d does not indicate the number of replicates. This should be indicated

both in the graph and in the figure legend.

R3.18: Thank you for your suggestion. In the revised figure, we have shown the individual data points for each group in Panel d, making the number of replicates visually clear.

Q3.19

19) Extended Fig. 6. The figure does not indicate the number of replicates. This should be indicated both in the graph and in the figure legend. The single asterisk is not defined in the figure legend. This should be corrected.

R3.19: Thank you for your careful review. We have updated the figure to include the number of replicates, both in the graph and the figure legend. Additionally, we have clarified the meaning of the single asterisk in the figure legend.

Q3.20

20) Extended Fig. 7. Panel c is a great deal too small. It should be enlarged so that the text is easily readable. Panel e it is unclear what concentration of Ga(NO3)3 was used in these experiments. Please clarify this information. Panel h should have a more careful analysis of different portions of the curve between the graphs. There appear to be differences during early log phase that may be significant.

R3.20: Thank you for your thoughtful comments. In response:

Panel c:We now provide the underlying data in **Supplementary Table S10** (Excel file) to allow detailed examination by interested readers.

Panel e: We apologize for the confusion. To clarify, no $Ga(NO_3)_3$ was used in the experiments shown in Panel e. These data are intended to demonstrate that fprA is an essential gene in both P. aeruginosa PA14 and PAO1 strains under standard conditions. We have revised the figure legend accordingly to make this clear.

Panel h: To address this, we quantified the area under the curve (AUC) for both the 0–12 hour window (early log phase) and the full 0–20 hour growth curve. These analyses are now included as **Extended Fig. 7f**, allowing a more careful comparison of temporal growth dynamics.

We appreciate the reviewer's time and attention to detail, which helped us improve both the clarity and scientific rigor of our data presentation.

Q3.21

21) There is no explanation or discussion for why FprA would not impact Ga(NO3)3 sensitivity. It is possible that its essential nature masks the impact of Ga(NO3)3, but this issue seems to be completely overlooked. More careful analysis of overlap in FprA and FprB functon(s) may be warranted and this glaring issue should at least be discussed.

R3.21: Thank you for your insightful comment. We agree that the lack of discussion on why FprA does not impact $Ga(NO_3)_3$ sensitivity is an oversight. In the revised manuscript, we have included a discussion addressing this point, including the possibility that its essential nature may mask the

impact of $Ga(NO_3)_3$.

The added text:

Line 921-928: “Notably, FprA and FprB, the two ferredoxin-$NADP^+$ reductases in P. aeruginosa, exhibit functional divergence in their response to gallium stress. While FprB emerged as a critical modulator of $Ga(NO_3)_3$ sensitivity in this study, FprA displayed no discernible role under these conditions. The inability of FprA overexpression to rescue ΔfprB gallium hypersensitivity underscores their non-redundant roles. FprA is essential for normal growth of P. aeruginosa, indicating that this protein may underpin fundamental redox homeostasis processes that are indispensable for viability, potentially masking its involvement in gallium-specific resistance mechanisms.”

Reviewer #4:

Zhang et al report the development of a genome-wide CRISPRi screen in the bacterial pathogen Pseudomonas aeruginosa and its application to find sensitizers of a Gallium nitrate treatment. They discover that the downregulation or deletion of the FeS-containing reductase FprB leads to a drastic increase of P. aeruginosa sensitivity towards Gallium ions, and report that an increased oxidative stress is associated with it. The increased sensitivity is also demonstrated in biofilms and in a mouse lung infection model. The manuscript contains a wealth of data that look convincing and of high quality. It is written in a clear, scholarly manner.

The technical advance achieved through the CRISPRi method seems to be of relevance for a broader community of P. aeruginosa researchers to me. The application to find synthetic lethality mechanisms to Gallium treatment is more exotic, but interesting from a scientific point of view. The authors provide a good body of microbiological and biochemical data, but open questions regarding the mechanism of synthetic lethality remain. The authors state that FprB deletion leads to increased Fe2+ levels and subsequently increased ROS/hydrogen peroxide, but it is unclear why this occurs only in a Ga-dependent manner. The molecular role of Ga ions with respect to the FprB-dependent increase of Fe2+ remains obscure.

The in vivo models are mentioned very briefly at the end. The data look good, but the required dose is very high (I calculated ca. 160 mg/kg).

In spite of these limitations, I find the study rich and of high quality, and therefore recommend its publication after a revision.

R4.1:

We sincerely thank the reviewer for the thorough evaluation and positive assessment of our work. We are particularly grateful for the recognition of the technical advance represented by our genome-wide CRISPRi screen and its potential relevance to the broader *P. aeruginosa* research community. We agree that the molecular interplay between gallium (Ga^{3+}) and FprB-mediated Fe^{2+}/ROS homeostasis warrants further investigation.

We acknowledge the reviewer's calculation of the gallium dose (160 mg/kg) and have clarified in the revised Methods (lines 461-463) that this regimen aligns with the dosages used in other studies (Goss et al. 2018, PMID: 30257953). However, we want to emphasize that FprB co-targeting reduces the effective gallium dose by 32-fold in vitro, suggesting potential for dose optimization in future preclinical studies.

Comments

- line 58: The reference (Shi et al) is not well-suited to support the content of the sentence.

R4.2: We thank the reviewer for pointing out this citation issue. We have replaced the original reference (Shi et al.) with a more appropriate citation: a recent comprehensive review on ESKAPE pathogens that specifically addresses antimicrobial resistance, epidemiological trends, clinical impacts, and therapeutic strategies (PMID: 38831030) as highlighted in line 53 in the revised manuscript. This change better aligns with the context of our discussion on *P. aeruginosa* as a critical ESKAPE pathogen.

- line 59: virulence, not virulent

R4.3: We have corrected “virulent” to “virulence” in the revised manuscript.

- line 79: I cannot find the reference Zhu et al in the reference section
R4.4: We appreciated the reviewers’ feedback, and have updated the reference list and made sure this reference can be found in the refence section in the revised version.

- lines 113-139: Almost a full page of the introduction is spent to describe the upcoming study. I find this redundant and recommend to shorten this to a few sentences.
R4.5: We agree this paragraph contains too much description of the upcoming study and have shorten it in the revised manuscript: line 107-116.

- Figure 2: d: y-axis label is missing. I printed the figure on paper, and some letters are very hard to read due to their small size. I recommend increasing the font.
R4.6: We thank the reviewers’ careful check, and we have added the label for the y-axis, and increased the font to make sure the letters can be readable.

Q4.7
- Figure 2c: The overlap of growth-affecting genes in this studies and other studies is quite small. Moreover, the majority of genes (403 out of 740) found here were not found in other studies. Can the author comment on reasons behind this limited reproducibility between studies? Along this line, what is the reproducibility between gene sets 1 and 2?
R4.7: Thank you for this insightful comment. Our aim in this study was to generate a comprehensive, rather than merely “refined,” list of essential and fitness-contributing genes in *P. aeruginosa* through genome-wide CRISPRi-seq profiling. We have revised the manuscript text accordingly to reflect this intent.

The relatively small overlap with previous studies, and the large number of unique genes identified can be attributed to several important factors:
1) Differences in experimental conditions: Our growth conditions differ from those used in Tn-seq. CRISPRi-seq allows conditional gene silencing under more controlled and tunable expression systems, which may uncover conditionally essential or stress-sensitive genes missed by previous Tn-seq approaches. This might also explain the limited overlap between the 3 Tn-seq studies.
2) Broader inclusion criteria: We initially applied a relatively lenient threshold ($\log_2FC < -1$) to capture not only strictly essential genes but also genes that substantially contribute to bacterial fitness under our specific conditions. Many of these genes may not be essential in all contexts, but their inclusion provides a broader resource for functional prioritization and follow-up analysis.
3) Threshold stringency analysis: We have now performed additional analysis using more stringent cutoffs ($\log_2FC < -2, -3, -4$), as shown in the revised Extended Data Fig. 2a. As expected, increasing the stringency led to smaller essential gene sets (389, 255, and 151 genes, respectively), while also increasing their overlap with prior Tn-seq studies.
4) Statistical refinement: To increase the rigor of our gene calling, we re-analyzed the data using DESeq2, incorporating both $\log_2FC < -1$ and adjusted p-value < 0.05 as selection criteria. This refinement reduced the total number of essential/fitness-contributing genes from 740 to 618 and improved confidence in the resulting gene set.

About the reproducibility between Set 1 and Set 2:

We appreciate the reviewer's suggestion to comment on the reproducibility between Set 1 and Set 2. This comparison has now been added in **Extended Data Fig. 2**. While both sets identified overlapping clusters of fitness-associated genes, there are notable differences in their performance. We investigated the underlying causes of these discrepancies in detail:

1) Off-target effects: To rule out sgRNA off-targeting as a confounding factor, we examined the maximum predicted off-target scores for both sets (**Extended Fig. 1f**). As shown, both sets had consistently low off-target potential, which is expected since our sgRNA design pipeline excluded guides with high predicted off-target risk.

2) sgRNA efficiency and position: A key difference lies in the positioning of sgRNAs relative to the target gene start codon. Set 1 sgRNAs tend to target sites closer to the start codon, which correlates with stronger predicted repression efficiency. We analyzed the distribution of essential genes identified by each set across the different vulnerability/responsiveness clusters (**Extended Fig. 2d**). Overall, Set 1 identified more essential genes, especially those in Cluster 1 and 2 (defined as vulnerable), which are more stringently essential and show better overlap with Tn-seq datasets. These results suggest that Set 1 sgRNAs generally perform better in achieving effective gene knockdown, in consistence with the sgRNA design principles.

3) Exceptions and limitations: Notably, a small number of "real" essential genes—defined as genes consistently identified by CRISPRi and at least three Tn-seq studies—were only detected using Set 2. In these cases, Set 2 sgRNAs likely outperformed their Set 1 counterparts in repression efficiency. This indicates that while proximity to the start codon is an important factor, other sequence-intrinsic features—such as PAM strength, GC content, and sgRNA secondary structure—also influence CRISPRi efficacy. These factors merit systematic exploration in future sgRNA design optimization for *P. aeruginosa*.

In summary, our dataset was designed to be inclusive and comprehensive, capturing a wide spectrum of fitness-related genes rather than only a core set of essentials. We fully agree that distinguishing true essentials from context-dependent fitness genes is important, and our data—now analyzed with improved statistical rigor and multiple stringency thresholds—can serve as a foundation for future functional validation and prioritization.

Q4.8

- line 578: I suggest adding a definition for 'vulnerability' here. I found it in the Extended Figure after searching a while, but explaining it here would facilitate reading.

R4.8 We thank the reviewer for this helpful suggestion. As recommended, we have added the definition for both vulnerability and responsiveness at the relevant part of the manuscript.

Line 605-610 "Here, vulnerability is defined as a strong depletion phenotype (specifically genes with $\log_2FC < -4$ at any time point) indicating a more severe fitness defect than the essentiality threshold ($\log_2FC < -1$). Responsiveness reflects the timing of the fitness defect appearance, with "quick-responsive" genes showing substantial depletion ($\log_2FC < -2$ for vulnerable; $\log_2FC < -1$ for invulnerable) by generation 7, while "slow-responsive" genes reach similar levels only at later time points (generation 14 or 21)."

We have also introduced these conceptual definitions briefly in the Introduction section to provide readers with an early overview.

Line 109-111: "In this context, vulnerability refers to the extent to which gene silencing impairs bacterial fitness, while responsiveness captures the timing and dynamics of fitness loss following gene knockdown."

- The abbreviation 'COG' is not explained
R4.9: We have revised the text and included the explanation for COG (Clusters of Orthologous Groups). Please refer to line 619.

- Figure 3: The captions for e and g seem confused.
R4.10: We apologized for the confusion. Here, "CRISPRi hitA" refers to a mutant in which an sgRNA targeting the *hitA* gene was used in the assay. To improve clarity, we have revised the labeling to sgRNA-*hitA* (CRISPRi).

- Figure 4e: It can be read when augmenting it on a screen to 200% or more, but it is too small to be read on paper. Please enlarge the figure here or place it as an Extended Figure.
R4.11: We apologized for readability of the panel e, and have enlarged the figure to made sure it is readable after printing on paper.

- Figure 4c (and elsewhere): I assume the unit is CFU per ml? Please add.
R4.12: Yes. It is CFU per ml, and we added per ml in the unit in the revised figure.

- Figure 5a: The light blue panel of 0 Ga in the fprB mutant disappeared when printed. Please choose a different color.
R4.13: We have replaced the color and checked its visibility on printed paper.

- Figure 5f: The nor and nos genes are regulated strongly, but the significance is borderline. This needs to be mentioned in the text. I wonder whether they should be discussed so prominently in view of this. This part ('potentially exacerbating ROS leakage, line 743') would be more convincing if the authors measured NO levels.
R4.14: We thank the reviewer for this insightful comment. In response, we have: 1) Acknowledged the statistical limitation by revising the text (lines 767-769) to clarify that while nor and nos genes exhibit substantial fold-changes ($|\log2FC| > 2$), their adjusted p-values approach the significance threshold ($Padj \approx 0.05$), indicating a trend-level association that warrants further validation. 2) we provided experimental validation: To address the reviewer's suggestion, we measured intracellular NO levels in the Δ*fprB* mutant and wild-type strains. Consistent with the transcriptional downregulation of *norBC*, *nosZ*, and *nosR*, the Δ*fprB* mutant exhibited significantly elevated NO levels compared to wild-type (**Fig. 5h**). This phenotype was further exacerbated under gallium treatment (**Fig. 5h**), supporting our hypothesis that FprB deficiency potentiates nitrosative stress.

- Line 728: I do not agree with the statement '…loss function of mgtA leads to accumulation of intracellular Fe (Marshall et al. 2009), which explains the observation of Fig. 5a' – There is NO accumulation of iron in the fprB mutant in the absence of Ga. Also the statement in line 874 needs to be modified. It is only true in the presence of Ga, and not due to the absence of FprB alone.

R4.15: We agree with the reviewer's comments regarding mgtA and have removed the inappropriate statement related to its role in iron accumulation.
We also fully acknowledge the reviewer's concern about the statement in line 846 and have revised the text to clarify that the observed increase in intracellular iron and ferrous iron (Fe^{2+}) levels is a combined effect of FprB deficiency and gallium treatment. The revised text now reads: "Mechanistic investigation showed the ΔfprB mutant displayed elevated intracellular iron and ferrous iron (Fe2+) levels upon gallium treatment (Fig. 5a), which may react with the gallium-induced ROS," as highlighted at line 906-909 in the revised manuscript.

- Extended Data Fig 4b: Add axis labels above the columns
R4.16: Thank you for your suggestion. We have addressed this by adding axis labels above the columns in the revised (now renamed as) Extended Data Fig. 4c.

- Extended Data Fig 7c: Impossible to read. I have not found more data in the supplement. Can the authors provide the alignment in a readable format?
R4.17: We have added an extra Excel file to provide this information. Supplementary Table S10 FprB alignment.

- Ref Liu, Xue…. is incomplete.
R4.18: We apologized for this and have included the complete information.

Reviewer #5&6:

In this paper, Zhang et al. employed a chemical-genetic approach to identify potential synergistic targets with gallium, a drug that failed a phase II clinical trial for treating chronic Pseudomonas aeruginosa infections due to limited plasma and sputum concentrations. The authors constructed a CRISPRi-based genome-wide pooled mutant library and analyzed the fitness effects of these mutants using CRISPRi-seq when exposed to gallium. They identified fprB, which encodes a ferredoxin-NADP+ reductase, as a key synergistic target, with its deletion significantly sensitizing cells to gallium. They extend the work to find out that FprB modulates oxidative stress induced by gallium, via control of the iron homeostasis and reactive oxygen species accumulation

We sincerely appreciate the thorough assessment and insightful summary of our findings provided by Reviewer #5 and 6. We acknowledge the reviewer's reference to the limited plasma and sputum concentrations of gallium observed in the Phase II clinical trial for chronic *P. aeruginosa* infections. Our findings highlight FprB as a promising synergistic target, which may enhance gallium's therapeutic efficacy and expand its clinical potential. Below, we address the reviewer's specific concerns in detail.

Major comments

Q5.1

1. Rationale for choosing DOX as the inducible system to trigger gene silencing. The authors compared the tet-inducible system that uses DOX with the arabinose-inducible system for inducing dCas9 expression. However the rationale of this decision is not clear (potential applicability for in vivo infection models). Figure 1a and b suggests that DOX has a wider dynamic range of expression levels. However, the screen is performed at only one Dox concentration.

R5.1: We appreciate the reviewer's suggestions regarding the rationale for choosing the DOX-inducible system for this study.

As the reviewer highlighted, the decision to use the DOX-inducible system was primarily driven by its potential applicability for in vivo infection models. Specifically, we chose the DOX system over IPTG or arabinose-based systems due to the higher relevance of tetracycline-regulated systems in model organisms and the ability to regulate gene expression in a more controlled and predictable manner, which is essential for in vivo applications. To clarify this point, we have added the following sentence to the revised manuscript:

Line 497-498: " This inducer system was refined over IPTG- or arabinose-inducible systems due to its greater potential for application for in vivo infection studies, "

Additionally, we aimed to optimize the tet-inducible system for *P. aeruginosa* as a valuable tool for the scientific community. While we only used one DOX concentration in the screening of this study, we believe that future work will explore the dynamic range of this system more thoroughly.

We hope this clarification improves the rationale behind our choice of the DOX-inducible system and highlights the advantages it offers for in vivo infection studies.

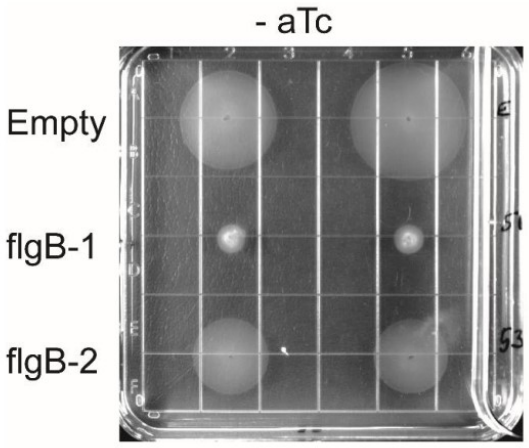
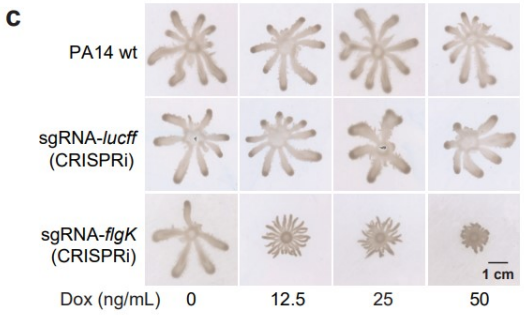
Q5.2.1

2. Novelty of CRISPRi system in Pseudomonas. CRISPRi systems using anhydrotetracycline (aTc)

have also been developed in Pseudomonas (Tan et al. 2018). How does the DOX-based system compare to the aTc-based system?

R5.2.1: We thank the reviewer for this insightful question. While the aTc-inducible CRISPRi system reported by Tan et al. (2018) represented an advance, their system displayed substantial basal leakiness in the absence of inducer, which limits its applicability for studying essential genes. As stated in their study: "**in the absence of the inducer anhydrotetracycline (aTc), the target gene remained repressed, indicating leaky expression from the Ptet promoter in *P. aeruginosa***"—a conclusion supported by Western blot data in both *P. aeruginosa* and *P. putida* (Tan et al., Fig. S1–S2).

In contrast, we have optimized a doxycycline (Dox)-inducible CRISPRi system in *P. aeruginosa*, building on a system we previously validated in *S. pneumoniae* (CHM, 2021, PMID: 33120116). Our modified system exhibits markedly reduced basal expression in the absence of Dox, as demonstrated in **Figure 1c,d,e** of our manuscript. Moreover, it enables dose-dependent gene repression with improved tunability and minimal background activity. These improvements address key limitations of the previous aTc-based system and enhance the utility of CRISPRi for functional studies, particularly those involving essential genes in *P. aeruginosa*.

Tan et al. 2018	This study
	

sgRNA	PAM site	Target Sequence
flgB-1	GCGA	GTCGAAACTGATGCTCATGG
flgB-2	GTGT	GGCTGCGAACCTGTTTGC

Q5.2.2

2. DOX has been known the have slow-release kinetics where there is a temporal delay in the resumption of the tet-responsive promoter activity (A-Mohammadi et al. 1997; Robertson et al. 2002). Did the authors test or observe any delay in resumption of CRISPRi mutant growth after removal of the inducer (DOX)?

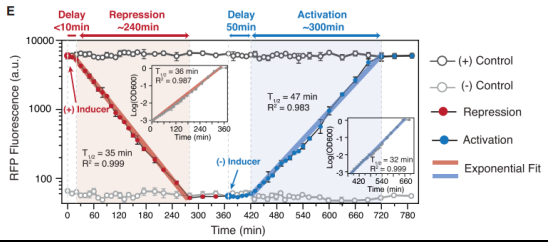
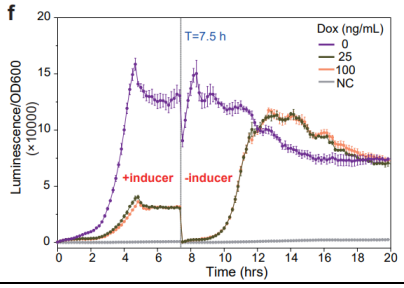
R5.2.2: We appreciate the reviewer's comment regarding the potential delay in the resumption of tet-responsive promoter activity upon Dox removal. In response, we conducted a restoration assay using a luciferase reporter strain to assess the dynamics of gene expression recovery following the withdrawal of Dox.

Our results show that within approximately 1.5 hours after Dox removal, expression of the

previously repressed luxABCDE operon resumes and becomes detectable via luciferase activity. This observation confirms that while there is a measurable delay in de-repression, the system remains responsive within a biologically reasonable timeframe.

For comparison, Qi et al. (2013, Cell) reported a ~50-minute delay in the IPTG-inducible CRISPRi system, though this estimate does not account for downstream processes such as protein folding and maturation. Thus, while the Dox-inducible system may exhibit slightly slower kinetics, it remains functionally suitable for applications where rapid reversibility is not the primary requirement.

We have added these data and relevant discussion to the revised manuscript (Fig 1f, line 530-533).

IPTG-inducible system by Q et al. 2013	Our Dox system
	

Q5.3

3. Number of generations vs time. Some of the data refer to number of generations and some to hs of incubation, which makes comparison difficult, for example fig. 1 and fig. 2.

R5.3: We thank the reviewer for pointing this out. To improve clarity and facilitate comparison between figures, we have added the corresponding incubation times to the legend of Fig. 2. This additional information should help readers more easily relate the data presented in terms of both time and number of generations.

Q5.4

4. Genomic coverage during treatment, including essential genes is not discussed. The CRISPRi library in PA14 was pre-induced with Dox for 14-generations prior to the Ga(NO3)3 treatment. Based on Fig 2e, mutants belonging to the "vulnerable and quick-responsive' category are already strongly depleted after 14 generations. Did the authors addressed mutant relative abundance before gallium treatment to ensure genomic coverage?

R5.4: We appreciate the reviewer's thoughtful question regarding genomic coverage and essential gene representation prior to gallium treatment.

In the study, we actually evaluated the performance of the CRISPRi library under two pre-induction conditions: 7 generations and 14 generations of Dox exposure before $Ga(NO_3)_3$ treatment. With 7 generations of pre-induction, we observed limited phenotypic resolution and a low number of high-confidence hits (**Extended Data Fig. 4a**), likely due to insufficient time for effective repression of fitness-related genes. In contrast, the 14-generation pre-induction condition yielded a more robust depletion profile and improved screening sensitivity. We have included the 7-generations preinduction results in the revised manuscript, and have added the following description:

"A parallel screen with only 7 generations of pre-induction yielded fewer candidate hits with low confidence (Extended Data Fig. 4a). Therefore, we focused our analysis on 14 generation pre-induction dataset, which provided higher-resolution phenotypic profiles." Line 637-639.

We acknowledge that extended pre-induction can result in the depletion of fast-responding or highly vulnerable mutants prior to treatment. However, this trade-off was necessary to reveal phenotypes that require sustained gene repression to become apparent. To address potential concerns regarding library representation, we examined sgRNA distribution following both 7 and 14 generations of pre-induction using data from our essential gene screen, which was conducted under the same induction conditions. Illumina sequencing was performed at a depth of 1000–2000× coverage, and we confirmed that genomic representation remained robust. Specifically, >90% of sgRNAs retained non-zero read counts across all four replicates, indicating sufficient coverage for reliable downstream analysis (supplementary table S5 raw counts for the essential gene screening).

Q5.5.1

5. The concentration of Ga(NO3)3 used for the CRISPRi-seq led to 70%-80% of growth reduction. What was the rational behind the severe growth reduction to 70-80%? As shown in Fig. 3b Ga(NO3)3 causes a drop in optical density from 0.8 to 0.2. How competitive fitness is affected when the cell density of treatment and control are so different? Could this difference be a confounding factor?

R5.5.1: Thank you for raising this important concern. The rationale for using a $Ga(NO_3)_3$ concentration that resulted in ~70–80% growth inhibition was to apply a strong yet non-lethal stress level that sufficiently perturbs cellular physiology without completely halting growth. This level of inhibition allows us to probe stress-responsive pathways and identify genes contributing to conditional fitness or tolerance mechanisms, which might be missed under milder stress.

We acknowledge that the lower OD_{600} (~0.2 vs. ~0.8 in control) reflects a significant difference in cell density, which can introduce potential confounding in relative barcode abundance. To minimize such bias, we ensured the following:

1) Sufficient cell counts and read depth were maintained across all samples to allow robust fitness calculations.

2) Biological replicates were performed to account for variability.

3) We applied dual cutoffs (adjusted p-value and log_2FC) using DESeq2 to control for statistical noise and growth-dependent artifacts.

4) Independent validations (e.g., individual CRISPRi strains or gene deletions) were conducted for top hits to confirm phenotypes.

Importantly, our approach follows precedent: similar or stronger growth-inhibition levels (~60–80%) have been adopted in other CRISPRi-seq stress screens (e.g., PMID: 35637331 for clarithromycin), which demonstrated enhanced sensitivity in detecting relevant gene targets under pressure.

That said, we agree that differences in growth kinetics may influence barcode competition dynamics. We have now added a note in the Discussion to explicitly acknowledge this limitation and the precautions we took to mitigate its impact. Line 881-887.

Q5.5.2

Fig. 3C shows a volcano plot, with very few mutants showing decreased fitness, which is surprising given the high concentrations used. Could the lower cell density result in less competition, compared to no treatment. This confounding could decrease the sensisitivy of the competition assay.

R5.5.2: We acknowledge the observation that the relatively small number of mutants showing decreased fitness in Fig. 3C may seem surprising given the high $Ga(NO_3)_3$ concentration used. One potential explanation is that the reduced bacterial growth under gallium stress could result in decreased competition between strains, particularly in cultures with lower cell density. This could limit the sensitivity of the competition assay, potentially masking fitness differences among mutants with moderate sensitivities.

Additionally, it is important to consider that gallium stress may induce selective pressure that primarily affects specific genes or pathways, rather than broadly impairing cellular functions. This could contribute to the observed skew in the volcano plot, where fewer mutants show negative fitness effects. To address this, we performed independent validations of top candidates, including individual knockdowns, which were able to confirm the relevance of the gallium-induced phenotypes (Fig. 3e–g). Nonetheless, we recognize the reduced competition under growth-inhibitory conditions as a potential confounding factor and have now included this consideration in the discussion of the revised manuscript. Line 881-887.

6. Did the authors include any non-genome-targeting sgRNAs in the library? This is an important control to have to account for any effect of the CRISPRi system. Expression of dCas9 is known to have several deleterious effect in the cells.

R5.6: We thank the reviewer for highlighting this important point. To control for potential non-specific effects of the CRISPRi system, we successfully incorporated 16 non-genome-targeting sgRNAs into our library (sequences listed in the Supplementary Table S4). We further redid the essential gene screening. These nontargeting controls were monitored across all screening conditions (7, 14, and 21 generations of induction), and consistently showed no measurable fitness defects in the essential gene screens. The data supporting this are now included in the revised **Supplementary Table S5**.

Q5.7

7. Lines 542–544: “To improve the robustness of the CRISPRi study, we included two sgRNAs for each target, with the best sgRNAs identified by the RNA design pipeline assigned to set 1, and the second best to set 2.” Which strand was targeted? The efficiency of the CRISPRi systems is known to be greatly influenced by the targeted strand. How were the best sgRNAs determined? While this information may be detailed in the cited paper, it is important to briefly summarize it here for the reader. Was the selection based on factors such as mismatches, distance from the TSS, or proximity to the ATG?

R5.7: We appreciate the reviewer's suggestion on clarifying the sgRNA design details and agree this is important to include. We have adapted the manuscript as such (Line 191-202):

“The sgRNAs were designed by our previously published R script for automatic sgRNA selection (https://github.com/veeninglab/CRISPRi-seq) using the option "HawkinsEcoMedian" for the reprAct_penalties input parameter, and otherwise default parameters (de Bakker et al. 2022). All sgRNAs were designed to target the coding strand of the genes. Briefly, all possible candidate sgRNAs for each gene are scored on the basis of (1) distance of sgRNA target binding site to target

start codon (on-target score), and (2) number and within-spacer position of mismatches with all other potential binding sites in the genome, i.e., all other Protospacer Adjacent Motifs (PAM) sites (off-target score). These scores are weighted, prioritizing candidates with a minimal maximum expected off-target effect. Given this metric, the two best-ranking sgRNAs were selected for this study and assigned to set 1 (5981 sgRNAs) and 2 (5971 sgRNAs), respectively. The 20-nt spacer sequences for all designed sgRNAs and their targets, as well as the 16 non-targeting (NT) control sgRNAs, are provided in Supplementary Table S4."

Q5.8

8. Does the second set of sgRNAs have reduced specificity? If so, how do the authors ensure that their CRISPRi system does not introduce off-target effects? Even minor nonspecific interactions between dCas9 and unintended chromosomal regions could compromise the, or at least obscure the results. Such effects could mislead the analysis, making certain genes falsely appear as pleiotropic contributors to fitness.

R5.8: We appreciate the reviewer's concern regarding the specificity of the second set of sgRNAs and the potential for off-target effects, and recognize we should have addressed this more clearly in the manuscript.

sgRNA design was based our automated pipeline (de Bakker et al. 2022). Briefly, all possible candidate sgRNAs for each gene are scored on the basis of (1) distance of sgRNA target binding site to target start codon (on-target score), and (2) number and within-spacer position of mismatches with all other potential binding sites in the genome, i.e., all other PAM sites (off-target score). Given this metric, the two best-ranking sgRNAs were selected for this study and assigned to sgRNA set 1 and 2, respectively. As such, sgRNAs in set 1 have lower predicted off-target activity than those in set two, on average. However, since nearly all genes possess multiple PAMs, two highly specific sgRNA binding sites are present for virtually every gene. We have visualized this in the revised **Extended Data Fig. 1f:** expected maximum off-target effects are practically the same across all genes for which two sgRNAs could be designed. The two sets of sgRNAs are therefore expected to have very similar specificities. Since our libraries are predicted to have essentially equally low off-target specificity, we feel our findings are strengthened by this approach.

We have included this Extended Data Fig. 1f: in the revised manuscript, and changed the methods section accordingly. We feel that having addressed this issue in more detail improved the manuscript and we are therefore grateful the reviewer pointed out this valid concern.

Q5.9

9. The authors claim to have 'refined' the list of essential genes. Did the authors mean to have identified the 'comprehensive' list of essential genes? This study reports the largest number of essential genes identified in Pseudomonas (740). However, the authors used a log2FC threshold of -1 as the cutoff. Could this threshold be too lenient to define essential genes? Might some of these genes instead be related to general fitness rather than being truly essential?"

R5.9: Thank you for this thoughtful comment. In this study, our aim was to generate a comprehensive—rather than merely refined—list of essential and fitness-contributing genes in *P. aeruginosa* through genome-wide CRISPRi-seq profiling. We agree that the term "comprehensive" more accurately captures the scope of our work, and we have revised the manuscript accordingly to

reflect this focus. In addition, we have moderated the corresponding descriptions to avoid overstatement. The section title has been updated to: “Genome-wide CRISPRi-seq allows identification of essential genes in P. aeruginosa PA14.”

To maximize coverage, we initially applied a relatively inclusive threshold ($log_2FC < –1$) to capture not only universally essential genes but also those that significantly contribute to bacterial fitness under our screening conditions. We acknowledge that this may include conditionally essential or stress-responsive genes that are not strictly essential across all contexts. This broader inclusion was intentional, as it enables functional prioritization and facilitates exploration of context-dependent essentiality.

To strengthen analytical rigor, we reanalyzed the data using DESeq2, combining the $log_2FC < –1$ threshold with an adjusted p-value < 0.05. This refinement reduced the number of identified genes from 740 to 618, improving statistical robustness and confidence in the resulting gene set.

To provide additional transparency and interpretative flexibility, we further evaluated essential gene sets under more stringent thresholds ($log_2FC \leq –2$, –3, and –4), which yielded 389, 255, and 151 genes, respectively. These results, now included in **Extended Data Fig. 2b**, illustrate the trade-off between sensitivity and specificity and allow users to tailor gene selection based on desired stringency.

We fully recognize the importance of distinguishing general fitness genes from truly essential ones. While further validation through orthogonal assays will be required to refine this list, we believe our dataset provides a comprehensive, flexible, and high-resolution resource for essential gene discovery in *P. aeruginosa*.

Q5.10

10. The growth defect for hitA is more pronounced than for hitB (Fig. 3e). How did the authors evaluate the fitness of the hitA exclusively? Given that hitA and hitB are part of the same operon, targeting hitA with an sgRNA would inadvertently repress hitB. Could the observed growth defect for hitA reflect a pleiotropic effect from simultaneously repressing both genes?

R5.10: We appreciate and agree with the reviewer’s insightful comment regarding the potential pleiotropic effect of repressing both *hitA* and *hitB* due to their operonic organization. To specifically assess the contribution of *hitA*, we constructed individual *hitA* and *hitB* deletion mutants, as well as a double mutant, and evaluated their respective fitness phenotypes. These additional analyses enabled us to distinguish the individual roles of *hitA* and *hitB* in gallium susceptibility, as shown in **Figure 3i**. Our results confirmed that disrupting *hitA* or *hitB* individually reduces *P. aeruginosa* susceptibility to gallium treatment.

We have now clarified this point in the revised manuscript (Text lines 659-661) and appreciate the reviewer’s suggestion to strengthen the interpretation of our findings.

“The sgRNA targeting hitA likely also represses hitB due to the polar effects of the CRISPRi system, which may account for the more pronounced phenotype observed upon hitA repression.”

11. Line 775. “In contrast, the wild-type and complemented strain were not similarly affected”. Did the mean that the wild-type and complemented strain were actually similarly affected?"

R5.11: We apologized for the confusion. We have revised the text to clarify the expression “Biofilm formation in the wild-type and complemented strain was not affected”. Line 823

12. Is gallium and synergy with FprB worth prioritizing over other drug-target combinations? Many drugs are FDA-approved, but their use is discontinued due to adverse effects. Is FprB a druggable target?

R5.12 : We agree that gallium, while mechanistically informative, is not a novel or clinically ideal compound due to known toxicity and pharmacokinetic limitations. Our inclusion of gallium is not intended as a therapeutic recommendation but as a probe to reveal stress-sensitive nodes in P. aeruginosa metal homeostasis—specifically, the functional vulnerability of FprB.

We acknowledge that FprB is not a canonical drug target, and there are no currently available small-molecule inhibitors. However, our genetic and phenotypic data support the idea that FprB plays a protective role under metal stress, and gallium treatment unmasked this function. Importantly, FprB is an NADPH-dependent enzyme with a defined active site, and our mutational analysis indicates that its enzymatic activity is required for the observed gallium resistance phenotype. This suggests a plausible avenue for future inhibitor design.

We agree with the reviewer that it would be premature to overstate the translational potential of targeting FprB at this stage. To reflect this, we revised the manuscript (Line 826–827) to say: "These results underscore the need for further investigation into FprB as a therapeutic target."

Additionally, we removed the phrase "great potential target" to avoid overstatement and have retitled this section as "Targeting FprB may enhance the efficacy of gallium-based therapies against P. aeruginosa."

Ultimately, our intention is to highlight FprB as a mechanistically interesting node that may guide future antibacterial strategies, especially when combined with metal-based oxidative stress-inducing agents. We appreciate the opportunity to clarify this point.

Minor comments

1. Line 85-87. "The lack of an effective strategy for cloning sgRNAs has further hindered the application of genome-wide CRISPRi in P. aeruginosa". However, systems like Mobile-CRISPRi are well-established and address this challenge (Qu et al. 2019; Yu et al. 2024).

R: We appreciate the reviewer's insightful comment and fully acknowledge that systems such as Mobile-CRISPRi (Qu et al., 2019; Yu et al., 2024) have significantly advanced genome-wide CRISPRi applications in *P. aeruginosa*, particularly by addressing key sgRNA cloning challenges. Indeed, our CRISPRi-seq system was developed building upon these important advances, and we highly appreciate their contributions to the field.

Our original statement was intended to emphasize the remaining technical hurdles in constructing efficient and scalable sgRNA libraries, especially for high-throughput applications. To better reflect this nuance, we have revised the manuscript (Lines 78–81) as follows:

"While systems like Mobile-CRISPRi (Yu et al., 2024) have addressed some sgRNA cloning challenges in P. aeruginosa, efficient and scalable sgRNA cloning remains a limitation for the broader application of genome-wide CRISPRi in this organism."

2. There appears to be a noticeable decrease in pyocyanin levels even in the absence of Dox compared to the wild type (Fig. 1c). Could this indicate that the system is leaky?

R: We thank the reviewer for this observation. Following a suggestion raised by Reviewer #2 (Q2.4), we conducted additional quantitative assays for both pyocyanin production and swarming motility (now included in revised Fig. 1c–e). These results show no significant difference between the wild-type strain and the non-induced CRISPRi strain, suggesting that there is no appreciable basal repression of the target gene under non-induced conditions.

That said, we acknowledge the reviewer's concern. As shown in **Fig. 1a**, the luciferase reporter exhibits a low level of signal even without induction, which may indicate minimal leakiness of the system. We do not claim that the tet-inducible system is completely leak-free. However, the level of basal expression is sufficiently low to allow for functional studies, including those targeting highly sensitive essential genes.

3. Line 469-470, this sentence could use clarity.
R: The sentence has been clarified:
"CRISPR interference (CRISPRi) utilizes a nuclease-deficient Cas9 protein (dCas9) in combination with a single guide RNA (sgRNA) to bind to a specific DNA sequence. This binding forms a complex that blocks the transcriptional machinery, leading to reduced or silenced expression of the target gene." Line 491-494.

4. Line 471. "To achieve tunable CRISPRi in P. aeruginosa, we developed a tet-inducible system for this bacterium." However, this tet-inducible system has previously been used for CRISPRi system (Liu et al. 2020).
R: We apologize for the inaccurate expression and have revised it to: "To achieve tunable CRISPRi in P. aeruginosa, we engineered an optimized tetracycline-inducible (tet-inducible) genetic circuit encompassing Ptet and a repressor gene tetR, codon-adapted for P. aeruginosa". line 494-495.

We acknowledge that similar tet-inducible systems have been previously applied for CRISPRi in *Streptococcus pneumoniae* (Liu et al., 2020), and we appreciate the reviewer's attention to this point.

5. The authors used a tet-inducible reporter system to assess their CRISPRi system. It is unclear from the text whether the LuxABCDE luminescence reporter system is integrated into the genome or is plasmid-borne. A brief description would help the readers.
R: The system was integrated into the genome by Tn7 transposition. We have included this information in the revised manuscript line 147-149
"The Ptet-lux-Tn7T-Gm plasmid can be introduced into P. aeruginosa via tri-parental conjugation (Peters et al. 2019), resulting in Tn7 transposition of the Ptet-luxABCDE fragment and its integration into the chromosome downstream of glmS."

6. Line 518-519. "In strains PAO1 and PA14, growth was 'significantly' inhibited with 3.13 ng/mL Dox compared to the no-inducer control". Was a statistical test performed to assess the significance?
R: We have removed the 'significantly'.

7. Line 532, remove "by"
R: Thanks. Done.

8. Line 542, the authors used previously developed sgRNA design platform. Default parameters?
R: We appreciate the reviewer's comment. The parameters used in the sgRNA design platform are now specified in the Methods section (Lines 191–202) for clarity. Additionally, we have revised the text to provide better guidance to the readers:
Line 191-193: "The sgRNAs were designed by our previously published R script for automatic sgRNA selection (https://github.com/veeninglab/CRISPRi-seq) using the option "HawkinsEcoMedian" for the reprAct_penalties input parameter, and otherwise default parameters (de Bakker et al. 2022). "

9. Fig 3C is not cited in the text. Additionally, the legends in Fig 3C (increased and decreased fitness) are confusing, as they seem to refer to mutants as well.
R: Thank you for pointing this out. **Fig. 3C** was indeed cited at Line 636. We appreciate the reviewer's suggestion to improve the presentation of Fig. 3C. To address the confusion, we have revised the figure to clarify the labels.

10. Line 718. A brief description of the RNA-seq condition would be helpful.
R: Thank you for the suggestion. To clarify, we have added a brief description of the RNA-seq conditions. Specifically, RNA-seq analysis was performed on wild-type and ΔfprB mutant strains grown in LB medium to an OD600 of ~0.5-0.7. This information is now included in the Methods section for better clarity. Line 414-426.

We also sincerely appreciate the reviewers' careful and detailed evaluation, which has greatly improved the quality of our manuscript.

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CRISPRi. In Pseudomonas aeruginosa. Edited by G. Bertoni and S. Ferrara. Springer US, New York, NY. pp. 13–32. doi:10.1007/978-1-0716-3473-8_2.

Reviewer #6 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.
We appreciate the reviewers for their time and efforts to help us to improve our manuscript.

We sincerely thank the editors and reviewers for their continued support and thoughtful comments during the revision process. We are pleased that the manuscript is now accepted in principle and have carefully addressed the final points raised. Below, we provide a point-by-point response outlining the additional clarifications and minor revisions made in this version.

Point-to-point response

Reviewer #2 (Remarks to the Author):

Thanks for adressing all my comments so well!

Response:

We sincerely thank the reviewer for the kind words and encouragement. We are glad that the revisions have addressed your concerns, and we greatly appreciate your constructive feedback throughout the review process, which has significantly helped improve the quality of this manuscript.

Reviewer #3 (Remarks to the Author):

This revised manuscript by Zhang et al. addresses the majority of concerns within the manuscript, though there remain a few issues that have not been fully addressed. The response to review provided by the authors is quite extensive and detailed. In most cases, the comments are fully addressed and addressed well. It is clear that they made an attempt to address everything, but they have overlooked a few minor to moderate issues with how they have addressed the comments. Despite these issues, the manuscript remains an important contribution to the field.

Response:

We sincerely thank the reviewer for the continued engagement and thoughtful evaluation of our revised manuscript. We appreciate your recognition of the efforts we made to address the previous concerns and your kind words regarding the overall contribution of our study.

We acknowledge that there may still be areas where our responses or revisions could have been clearer or more thorough. We have carefully revisited the relevant points and, where necessary, further clarified or refined the manuscript to address the remaining issues as below.

Specific issues that should be addressed are:

1) In response R2.3 a list of detailed explanations are provided for why pyochelin-mediated gallium uptake does not impact gallium sensitivity in their study. Despite having these explanations, they do not briefly summarize these possibilities in the text of the manuscript and they should have done exactly that, included the explanations in the discussion.

Response 3.1:

We appreciate the reviewer's suggestion. To address this, we have added a concise summary of the possible reasons for the lack of pyochelin-related phenotypes directly into the Discussion section as below.

"Interestingly, none of the genes involved in pyochelin biosynthesis (e.g., pchDCBA,

pchEFGHI) or transport (fptA, fptX) exhibited significant phenotypes in our gallium CRISPRi-seq screen. This may be explained by several factors: First, these genes showed minimal expression in our iron-replete conditions (Supplementary Data 9), consistent with Fur-mediated repression and likely limiting the efficacy of CRISPRi knockdown. Second, prior studies suggest that pyochelin-mediated gallium uptake plays a more prominent role under iron-limiting conditions, which were not part of our experimental setup. Third, as a diffusible siderophore, pyochelin functions as a public good, allowing even biosynthesis-deficient mutants to benefit from siderophores secreted by other cells, potentially masking fitness effects in pooled screens."

2) The issue with the availability of materials from AddGene remains. Although the authors attempt to provide evidence that the materials are available, as of my review, the materials could still not be accessed. Confirmation of availability should be completed prior to acceptance and/or publication.

Response 3.2:

We appreciate the reviewer's concern regarding material availability. As noted, the process of depositing biological materials to Addgene was particularly challenging due to the lack of an official channel for shipping bacterial strains from China. Despite this, we made every effort to overcome logistical and regulatory barriers, and we are pleased to confirm that the key reagents are now publicly available:

- The pooled sgRNA library is accessible via Addgene:

https://www.addgene.org/pooled-library/liu-crispri-p-aeruginosa/

- The doxycycline-inducible CRISPRi plasmid (ptet-lux-Tn7T-Gm) is also available:

https://www.addgene.org/226434/

Regarding pCRISPRi-ccdB, the deposition remains technically infeasible due to Addgene's current policies. This plasmid requires a custom auxotrophic strain for maintenance and cannot be propagated in standard hosts. Addgene does not accept auxotrophic strain. For this reason, we have made the plasmid available upon request from the corresponding author and have already distributed it to collaborators (e.g., in Belgium) during the revision process, demonstrating our commitment to accessibility.

We respectfully note that many publications in Nature Communications and related journals comply with data and material availability policies by offering distribution upon request, rather than through Addgene. In this context, we have gone above and beyond by depositing key tools to Addgene and providing documentation of their availability. We hope the reviewer will agree that our approach aligns with both the spirit and the letter of Nature Portfolio's policies and supports transparency and sharing within the scientific community.

3) In R3.20 analysis of temporal growth dynamics was done in response to review. However, no mention of the fact that fprA did display slight and significant complementation of the fprB mutation within the 20 hr regions of the growth curve.

This indicates that there is functional overlap between fprA and fprB, requiring revision of the text accordingly. This is particularly imporant in the Figure Legend for Extended Fig. 7 and lines 801-803 in the manuscript. It appears that FprA does impact Ga(NO3)3 sensitivity, though only slightly (P=0.0134).

Response 3.3:

We thank the reviewer for this careful observation. We acknowledge that *fprA* complementation produced a statistically significant, though modest, increase in gallium tolerance at the 20-hour timepoint. While the biological effect was limited, this result suggests partial functional overlap between FprA and FprB.

To address this, we have revised the text accordingly:

Figure legend (Extended Data Fig. 7e, f):

"Complementation of fprA under the fprB promoter in the ΔfprB mutant led to a slight increase in gallium tolerance at 20 hours (P = 0.0134), with limited biological impact."

In the main text:

"Introducing the fprA coding sequence under the control of the original fprB promoter into the ΔfprB mutant led to a slight but statistically significant increase in gallium tolerance at later timepoints, though the biological effect was limited (Supplementary Fig. 7e, f). These findings suggest that although FprA and FprB are both ferredoxin-$NADP^+$ reductases, their functions are only partially overlapping."

4) In R3.21 the text should be revised from "rescue" to "fully rescue" and "their non-redundant" should be "only partially redundant".

Response 3.4:

Done. The text has been revised to:

"The inability of FprA overexpression to fully rescue the gallium hypersensitivity of the ΔfprB mutant underscores their only partially redundant roles."

5) In R4.7 the authors address the question in the response quite well, but they do not address the question within the text of the manuscript. Please modify the text of the manuscript within the discussion to address this question, using a more brie summary than the one included in this response.

Response 3.5:

We thank the reviewer for pointing this out. A concise summary of the rationale behind our broader essential gene set and its relationship to previous Tn-seq studies has now been added to the Discussion, as suggested:

"Compared to previous Tn-seq studies, our CRISPRi-seq screen identified a broader set of essential and fitness-contributing genes. This divergence likely reflects differences in experimental conditions, including medium composition and stress exposure, as well as the tunable and conditional nature of CRISPRi-based repression. In addition, we used relatively inclusive thresholds to capture both core essential and context-dependent fitness genes, thereby generating a more comprehensive resource. Reanalysis with stricter cutoffs showed greater convergence with prior studies, supporting the robustness of the core gene set. These results demonstrate that

CRISPRi-seq complements transposon-based approaches by uncovering additional layers of genetic vulnerability."

6) With regard the revision of the section in Lines 518-519 of the original manuscript, the authors propose that removing the word "significantly" addresses the issue. However, it does not. If there is not significant growth inhibition, then there is no real growth inhibition. A statistical test should be done and if it is not significant, this sentence should be removed or changed to "growth was not significantly inhibited by 3.13 ng/ml".

Response 3.6:

We appreciate the reviewer's thoughtful comment. To assess whether doxycycline affects bacterial growth, we performed statistical analysis of the growth curves shown in Supplementary Fig. 1c (PA14::sgRNA-lucff and PAO1::sgRNA-lucff), using doxycycline concentrations ranging from 0 to 100 ng/mL. Growth was quantified by calculating the area under the curve (AUC) from three biological replicates per condition. We employed a Kruskal–Wallis test followed by Dunn's multiple comparisons.

In strain PA14, no significant differences in growth were observed at any doxycycline concentration. In strain PAO1, while some concentrations (e.g., 12.5 and 25 ng/mL) yielded statistically significant differences, these reflected slightly increased, rather than decreased, growth. No concentration tested resulted in significant growth inhibition in either strain. To reflect these findings accurately and conservatively, we have revised the manuscript to state: "No significant growth inhibition was observed across the tested doxycycline concentrations (Supplementary Fig. 1c)."

Reviewer #4 (Remarks to the Author):

The authors have provided a thorough and careful revision of the manuscript. My suggestions were addressed adequately. I have two minor remaining points:

Response:

We sincerely thank Reviewer #4 for their positive assessment and constructive feedback.

- Extended data figure 6B: The description and/or in the panel is incorrect, because it suggests that one condition is Ga+NADPH and the other is NADPH. Please rephrase.

Response:

We thank the reviewer for this helpful clarification. To avoid ambiguity, we have revised the figure legend for Supplementary Fig. 6b as follows:

"Plate spot assay of the Δ*fprB* strain comparing growth in the presence of 6.25 μM $Ga(NO_3)_3$ with or without 1 mM NADPH. Bacterial cultures were adjusted to an initial OD_{600} of 0.003 to assess the effect of NADPH supplementation on $Ga(NO_3)_3$-induced growth inhibition."

- I am not an Addgene user, but it seems that the Addgene entries are still not accessible. I suggest withholding publication of the paper until this is the case.

Response:
As detailed in our previous response (R3.2), the key resources from our study are now publicly available and searchable via Addgene:
Pooled sgRNA library: https://www.addgene.org/pooled-library/liu-crispri-p-aeruginosa/
Doxycycline-inducible CRISPRi plasmid (ptet-lux-Tn7T-Gm):
https://www.addgene.org/226434/

Reviewer #5 (Remarks to the Author):
The authors have worked diligently and addressed most comments and concerns. Three aspects were partially addressed (Q5)
1) Related to R5.Q.2.1. The authors claim that the rationale of choosing the DOX-inducible system relies on the potential applicability for in vivo infection models. Upon request of clarifications the authors, responded there was "a higher relevance of tetracycline-regulated systems in model organisms and the ability to regulate gene expression in a more controlled and predictable manner, which is essential for in vivo applications". However, this answer does not satisfy the rationale of the choice. The authors should explain or demonstrate why the DOX-inducible system is more predictable and more controlled as they say
Response 5.1:
We thank the reviewer for this insightful comment and apologize for not clearly stating our rationale earlier. The doxycycline (DOX)-inducible system is widely used in vivo due to its superior stability, oral bioavailability, and broad tissue distribution. It enables predictable and tunable gene expression via drinking water or feed, with minimal variability (Redelsperger et al., PMID: 27423155).

In our previous studies Cell Host Microbe 2021(PMID: 33120116), Cell Host Microbe 2024(PMID: 38417443), we successfully used DOX to induce CRISPRi in murine infection models, achieving consistent gene silencing. The availability of commercial DOX formulations and its well-characterized pharmacokinetics further support its suitability for in vivo CRISPRi applications. We have revised the manuscript text to include this rationale. "This inducer system was refined over IPTG- or arabinose-inducible systems due to its greater potential for application for in vivo infection studies. Doxycycline offers stable, tunable induction with consistent performance, as demonstrated in our previous murine CRISPRi models."

2) Related to R5.Q.2.1. Superiority of the DOX-inducible system over the previously published aTc (Tan et al 2018). The authors claim that their system is more tightly regulated overcoming the leakiness of the previous promoter. While they show phenotypic assays where indeed the phenotypes are reduced compared with the wild type, the results do not support the author's arguments. The authors could only support their claim with direct comparisons of both aTC and DOX-inducible systems with gene expression analysis by qPCR.
Response 5.2:
We appreciate the reviewer's comment and understand the importance of direct

comparisons when assessing regulatory stringency. However, we would like to clarify that we do not have access to the aTc-inducible system used in Tan et al. (2018), and thus a head-to-head experimental comparison in our hands is not feasible at this stage.

Nevertheless, we base our argument on both the published data from Tan et al. and our own functional assays. In the Tan et al. study, swarming assays targeting flgB clearly showed that gene expression remained repressed even in the absence of aTc, leading the authors themselves to conclude that:

"in the absence of the inducer anhydrotetracycline (aTc), the target gene remained repressed, indicating leaky expression from the Ptet promoter in P. aeruginosa."

In contrast, our Dox-inducible CRISPRi system shows minimal repression of gene expression in the absence of Dox. As shown in Fig. 1c–e, targeting key phenotypic genes involved in swarming motility and pyocyanin production, we observed negligible inhibitory effects under non-induced conditions—indicating low basal dCas9 expression and tight regulation. Additionally, we benchmarked our system against the widely used arabinose-inducible system (known for its well control expression) and found that our Dox-inducible system performed comparably or even slightly better in terms of dynamic range and inducibility (Fig. 1a).

Taken together, although we cannot provide a direct quantitative comparison with the Tan et al system via qPCR, the combination of published evidence and our functional data supports the conclusion that our Dox-inducible CRISPRi system is tightly regulated and suitable for genome-wide screening applications in *P. aeruginosa*.

3) Related to R5. Q.4. Genomic coverage of the CRISPRi library during treatment exposure: the authors acknowledge that they did not provide any details on this issue before, and that mutant depletion of highly vulnerable mutants could occur during competition previous to the drug exposure. They support the timing at which they have performed the experiment showing that reduced time of induction (7 generations) does not sensitize the mutants (Extended Data Fig. 4a). For the most important claim (14 generations) they mention that ">90% of sgRNAs retained non-zero read counts across all four replicates, indicating sufficient coverage for reliable downstream analysis". This claim is supported by a supplementary table S5 with raw counts for the essential gene screening. However, having non-zero read counts does not guarantee a reliable downstream analysis as the authors suggest. The authors should compile the data provided in the suppl table S5 showing where indeed they have genomic coverage in the library (% genes/operons represented by CRISPRi mutants over the whole genome). While the results obtained (target gene pursued) in this particular work will not change, this analysis will be useful toward enhancing the sensitivity of CRISPRi-seq screens.

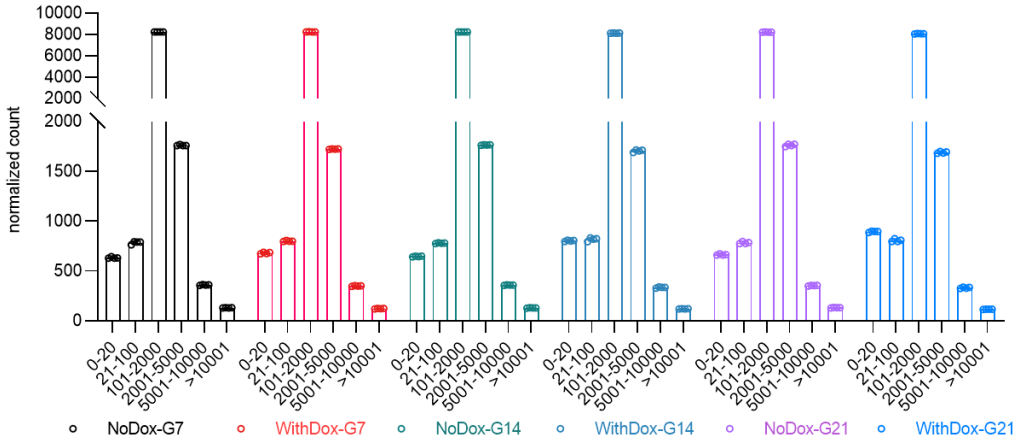
Response 5.3:

We thank the reviewer for raising this important point regarding library coverage and potential dropout in CRISPRi-seq screening. We agree that the presence of non-zero read counts alone does not guarantee reliable representation for downstream analysis.

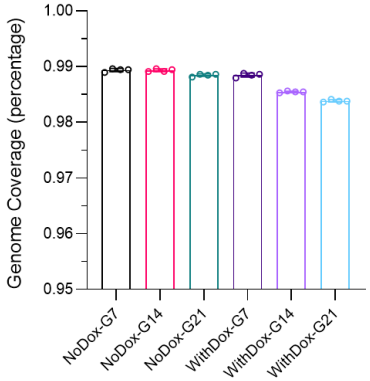
To address this, we performed an additional analysis of the read count data in

Supplementary Data 5 (Revision Fig 1). We applied a more stringent threshold, considering only sgRNAs with >20 normalized read counts across all four replicates as reliably covered. Using this criterion, over 98% of genes and operons retained sufficient coverage after 14 generations of induction (Revision Fig 2). These results suggest the library retained high complexity.

We acknowledge that the choice of a threshold (e.g., 20 normalized reads) for defining coverage is somewhat arbitrary and depends heavily on sequencing depth and experimental context. Rather than imposing a fixed cutoff, we have chosen to provide the complete raw count data in Supplementary Data 5. This allows readers to apply their own thresholds based on their analytical standards and the sequencing depth achieved in similar experiments.



Revision Fig 1: Distribution of raw sgRNA read counts from CRISPRi-seq screening as shown in Supplementary Data 5.
Bar plots showing the distribution of raw read counts for sgRNAs across all four replicates at 14 generations of induction. Data correspond to Supplementary Data 5. These plots illustrate the overall complexity and coverage of the CRISPRi library under screening conditions.



Revision Fig. 2 Genome-wide coverage of the CRISPRi library across induction.
Bar plots showing the proportion of genes with normalized raw counts $\geqslant$20 in each replicate before induction and after 7, 14, and 21 generations of doxycycline induction. This threshold was used to define reliably covered targets. The results indicate high retention and stability of sgRNA representation across induction generations.

Reviewer #6 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Response:

We sincerely thank Reviewer #6 for participating in the review process and for contributing to this thoughtful and constructive evaluation. We also appreciate Nature Communications' initiative to engage and recognize Early Career Researchers in peer review, which we believe is essential for fostering the next generation of scientific leaders.