nature portfolio

Peer Review File

Guide RNA structure design enables combinatorial CRISPRa programs for biosynthetic profiling

Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
Guide RNA structure design enables combinatorial CRISPRa programs for biosynthetic profiling

CRISPR-based gene expression has a great potential to control biosynthetic pathways. However, so far, for CRISPRa a limited set of scRNAs have been characterised. Here, the authors first use a computational approach to characterize the folding of scRNA's. Comparison with experimental testing let's them conclude that the calculated folding barrier is a good predictor to identify effective scRNAs. Next, they focus on a set of 3 orthogonal scRNAs and produce truncated scRNAs in order to tune activation levels. From this, they create a library of 64 scRNAs: 3 different binding sites, each targeted by scRNA of different lengths to give basal, low, medium and high expression levels. The authors then apply this library to control tetrahydrobiopterin biosynthesis as well as lacto-N-tetraose. The combinatorial expression analysis allows to identify bottlenecks and improve yields.
The paper is well written, the figures are very clear, and the supporting information is extensive. I appreciate that the authors are also discussing the limitations of their approach. The work is topical and an important contribution to the characterization and more wide-spread use of CRISPRa-based gene expression control. I therefore support publication. I have some minor questions and feedback:
• For Fig. 3, you conclude that 1) total expression is limited by host expression capacity and that 2) RFP has a deleterious effect. Even though likely to be true, I wonder how you can be sure about conclusion 1, given that RFP is always expressed when all three promoters are activated. It seems a confounding factor and it would be interesting if the overall level reachable is higher, if RFP would be replaced with another reporter.
• Given the discovery that RFP seems to cause more burden or toxicity than the other fluorescent reports, it is a pity that all the characterization has been carried out with RFP. Of course, I don't recommend repeating all the analyses with another fluorescent protein, but I wonder if some of the observed properties of J6 could be attributed to the RFP burden/toxicity. For example, that the spacer of length 19 performs better than the one of length 20 (Fig. 3). Also, the distinct behaviour and poorer prediction of J6 in Fig. S7 might be caused by the choice of reporter.
• In most figures you used a plate reader to quantify fluorescence, while in Fig. 4 you used flow cytometry. What was the rationale behind this choice? Would this figure look different if you normalize the fluorescence by OD?
• Related to this: Did you observe any differences in growth rates between different constructs (different reports, different scRNAs, different truncations) or were they the same everywhere?
• Please specify what carbon source (and at what concentration) was used in the EZ medium.
• It is good that the sequences are provided, but sequences in a pdf file is really not the best way of sharing this information. Please use a more suitable format, such as gb files.
• I did not see a statement on data and code availability.
• I highly encourage the authors to share their library of 64 plasmids on Addgene. This will boost the useof this method by others.

Reviewer #2:
Remarks to the Author:
In this manuscript, Fontana and colleagues systematically develop methods for the construction of efficient multiplex titratable CRISPR gene activation systems from establishing basic design principles to demonstrating proof of concept in manipulating a biosynthetic pathway in living cells. Their manuscript begins with a brief study of the activity of 14 randomized CRISPRa target sites that drive activation of a synthetic promoter. Through a study of thermodynamic parameters, they identify what they refer to as 'barrier energy' as a strong predictor of guide activity. They then show that the activity of three different efficient guides can be reproducibly titrated through truncation of the spacer sequence. Furthermore, they show that multiple CRISPRa targets can be activated simultaneously with

predictable activity by using synthetic promoters with different activation sequences through the construction of 64 strains expressing high, medium, or low activity guides for each of three target fluorescent proteins. Having established the feasibility of this system, the authors then apply this to profiling two biosynthetic pathways, showing how this fine programmable control of gene expression can be used to optimize compound production and identify pathway bottlenecks.

Overall, my feeling was that this paper represents a solid and useful piece of engineering, with high potential for applications in both basic research and industry. The manuscript is well-written and easy to understand, and the conclusions as I understand them appear to be well supported. As my expertise is primarily in predicting CRISPR guide efficiency, I will restrict my review largely to this portion of the manuscript.

To my knowledge, the authors' suggested folding barrier parameter is new in the context of CRISPR guide efficiency prediction, and based on figure 2d appears to be an excellent predictor of guide activity in the authors' CRISPRa system. I do think it would be helpful to contrast this a bit more explicitly with previous approaches though, particularly deltaG_B (Alkan et al., Genome Biology 2018), possibly on lines 196 – 208 or in the discussion, as this has been shown to be an important parameter for predicting genome editing efficiency (see supplementary note S6 and Figure S12 in Xiang et al, Nature Comms 2021) – though it has also been shown not to be a very important feature for bacterial CRISPRi (Yu et al., Genome Biology 2024). It took a while for me to understand how the authors' approach differed from the deltaG_U parameter in the deltaG_B calculation, and I feel it would be beneficial to make this explicit. It would also be useful to cite the original paper describing the findpath algorithm used to identify folding intermediates (Flamm et al., RNA 2000), possibly with a brief description or intuition for what this algorithm does.

Regarding the computational analysis, the authors mention custom scripts but do not make source code available. While the description on e.g. lines 570 – 594 appears comprehensive, it would be very helpful if the authors would make their scripts publicly available on github or similar with example input files. There are often small details (e.g. exact parameter settings) that can make reproducing computational work from text descriptions difficult, and having source code available would be very helpful in assuring that others can reproduce the results reported here. This is particularly important since the calculation of these kinetic parameters is central to their claims.

Some other more minor points:

Line 214: It has also recently been observed that predictions for eukaryotic genome editing efficiency also have very low accuracy for bacterial CRISPRi (Yu at al. Genome Biology 2024) – this could also be noted in the discussion on lines 490 to 504, as it suggests the transfer of design rules from eukaryotic genome editing applications to other technologies/systems may be problematic in general.

Lines 476 to 478: While I agree with the authors that their results are impressive, to my understanding they are derived from a fairly small number of measurements (~15) and this should be noted here (particularly when projecting a "near zero" failure rate). Some suggestions for how to further test this in the future would be welcome.

Refs:
Alkan et al. Genome Biology 2018:
https://genomebiology.biomedcentral.com/articles/10.1186/s13059-018-1534-x
Xiang et al. Nature Comms 2021:
https://www.nature.com/articles/s41467-021-23576-0#Sec26
Yu et al. Genome Biology 2024: https://link.springer.com/article/10.1186/s13059-023-03153-y
Flamm et al. RNA 2000: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1369916/

Reviewer #3:
Remarks to the Author:
The manuscript reports on the improvement of CRISPRa in E. coli. The authors improved the design method for guide RNAs. Although guide RNAs designed by conventional methods sometimes did not show the predicted activity due to misfolding, most of those designed by the newly developed method showed activity as predicted. CRISPRa has been well developed. The contribution of this manuscript is to improve gRNA design. Thus, this study may fit specialized journals focusing on nucleic acids would be more appropriate. The authors asserted the study's relevance to chemical production. However, the section was not well designed. Pteridine and human milk oligosaccharide, lacto-N-tetraose (LNT) were used as examples. The benefits of the method are overestimated because strains that produce trace amounts of the targets are used as parents. No titers are even provided for pteridine. Regarding LNT, strains producing at the 10-100 mM level have been constructed. But this study used the microM level production strain. In Line 452-453, the authors compared yields rather than titers, misleading the readers. The titers reported here are too low to compare with those in other papers. But CRISPRa was designed to improve titers, not yields in this study, making the comparison of yields rather puzzling.

Point-by-point response to the reviewers' comments, reproduced verbatim

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The reviewer raises interesting points and we present additional data that provide further illumination on these questions in new Supplementary Figures 8a and 10b. Our view is that each set of output genes has a slightly different limit on total expression, depending on the burden of the specific genes. Consistent with this view, reductions in J6-RFP output are only observed in the highest-expression strain (high-high-high) (original Figure 4b and new Supplementary Figure 8). Output sets including RFP may have lower expression limits than other output sets, meaning that changing RFP to another reporter could indeed generate more total expression.

We elaborate these points in the legend of Supplementary Figure 8a as follows:

The expression of high RFP results in the indicated expression defect in GFP (left group) and BFP (second group from left) outputs, but the defect is much smaller or zero when observing effects of high GFP or BFP expression on RFP output (middle and second-from-left groupings, respectively). RFP's relative resilience to expression defect suggests that it has dominant expression burden relative to the other outputs in this set. Only when all three outputs are highly expressed (left bar of rightmost grouping) is a substantial defect in RFP expression observed, suggesting that total combined expression could also play a role at high levels of these outputs.

As we now clarify in the results section, for individual characterization of promoters, we used RFP in all cases in an attempt to minimize differences in output inherent to different reporter proteins. Still, only J3 showed the spacer length 19 > spacer length 20 phenomenon, and only J6 exhibited the extra truncation sensitivity (Figure 3c and Supplementary Figure 7). We believe this means that these effects are not solely attributable to the use of RFP as the reporter (Figure 4b and new Supplementary Figure 10b). Overall, the relative outputs generated by the spacer truncations are consistent across promoters irrespective of the reporter protein. The difficulty in predicting the global impact of any given expressed protein is an important motivation for investigating intermediate expression levels when developing multi-gene expression programs (Discussion).

2. In most figures you used a plate reader to quantify fluorescence, while in Fig. 4 you used flow cytometry. What was the rationale behind this choice? Would this figure look different if you normalize the fluorescence by OD?

As now explained in the Methods, the rationale for using flow cytometry to measure RFP, GFP and BFP expression was practical: the flow cytometer allows for clearer separation and quantification of the three colors than the plate reader (Biotek Synergy HTX) used for the other experiments. Representative flow cytometry data for 9 selected fluorescent expression programs and an empty vector control are now presented in new Supplementary Figure 17. New Supplementary Figure 10b presents fluorescence normalized by OD for 19 selected strains measured with a monochromator-equipped plate reader (Biotek Synergy H1). For the strains tested, plate reader measurements showed high correlation to the original flow cytometry data when similarly normalized (r = 0.9822 for GFP, r = 0.913 for BFP, and r = 0.9884 for RFP) (new Supplementary Figure 10).

3. Related to this: Did you observe any differences in growth rates between different constructs (different reports, different scRNAs, different truncations) or were they the same everywhere?

We did new experiments to investigate the growth of multiple strains from the triple-fluorescent reporter library, and observed indistinguishable growth rates across the strains tested. This information is now included in a new Supplementary Figure 10c-d as growth data for 20 of the library members. Eight of the chosen strains express high levels of RFP and 2 of the strains express medium levels of RFP. We do not observe noticeable differences in growth within this set compared to the 2 chosen strains that express low levels of RFP or the 7 strains chosen with off-target (OT) guides for J6-RFP. As now written in the caption for Supplementary Figure

10c, we conclude that RFP expression burden in these conditions is not large enough to affect growth rate.

4. Please specify what carbon source (and at what concentration) was used in the EZ medium.

We have added explicit notes of carbon sources used and concentrations in each experiment in the Methods section. We used 2 g/L glucose in all EZ medium, with exception of the LNT production experiments, in which additional sugar was added as a substrate (10 g/L glucose) and additional lactose (2 g/L) as a feedstock.

5. It is good that the sequences are provided, but sequences in a pdf file is really not the best way of sharing this information. Please use a more suitable format, such as gb files.

We have now provided .gb files of selected plasmids as supplementary files, in addition to the sequence tables.

6. I did not see a statement on data and code availability.

We thank the reviewer for noting this omission, and we have updated data and code availability.

The new sections read:

> "Data supporting the findings of this work are available within the paper and its Supplementary Information files. A reporting summary for this article is available as a Supplementary Information file. The data sets generated and analyzed in this study are available from the corresponding author upon request. The source data for Figs. 2-6 and Supplementary Figs. 1, 3-5, and 7-16 are provided as a Source Data file."

And

> "Custom Python code to analyze input RNAs and generate the energetic parameters described in this work is available on GitHub (https://github.com/carothersresearch/gRNA_screen_docker) and can be run directly in that environment using a Codespace or locally using a Docker image."

7. I highly encourage the authors to share their library of 64 plasmids on Addgene. This will boost the useof this method by others.

We agree! The 64-plasmid library is under the deposition process to Addgene together with the pathway plasmids used in this study. This Addgene set is the same set of plasmids for which we are now providing the .gb sequence files.

Reviewer #2 (Remarks to the Author):

In this manuscript, Fontana and colleagues systematically develop methods for the construction of efficient multiplex titratable CRISPR gene activation systems from establishing basic design principles to demonstrating proof of concept in manipulating a biosynthetic pathway in living cells. Their manuscript begins with a brief study of the activity of 14 randomized CRISPRa target sites that drive activation of a synthetic promoter. Through a study of thermodynamic parameters, they identify what they refer to as 'barrier energy' as a strong predictor of guide

activity. They then show that the activity of three different efficient guides can be reproducibly titrated through truncation of the spacer sequence. Furthermore, they show that multiple CRISPRa targets can be activated simultaneously with predictable activity by using synthetic promoters with different activation sequences through the construction of 64 strains expressing high, medium, or low activity guides for each of three target fluorescent proteins. Having established the feasibility of this system, the authors then apply this to profiling two biosynthetic pathways, showing how this fine programmable control of gene expression can be used to optimize compound production and identify pathway bottlenecks.

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We thank the reviewer for all of the helpful suggestions and we now provide additional elaboration along all of these points in the Results and Discussion sections. As the reviewer noted, we found that Folding Barrier, which characterizes the rate at which scRNAs can convert between the MFE structure and the active structure, has the most predictive power among all of the parameters we examined. The Alkan et al. ΔG_H parameter is similar to our Binding Energy, which had poor predictive power of bacterial CRISPRa, and their ΔG_U was similar to our Folding Energy, but only considered the energy of unfolding the spacer sequence RNA. Folding Energy, on the other hand, considers the full scRNA sequence and thus includes the energy necessary to fold the scRNA from an inactive structure (e.g. when the spacer sequence binds to a complementary sequence internal to the Cas9 handle, as in Supplementary Figure 6) into the active structure. We believe that Folding Energy has better predictive power than Binding Energy because it accounts for the impact of all of the guide RNA sequence in determining the likelihood of folding into the active structure.

We have updated our Discussion section to include additional references and explanations to provide context by focusing on how the parameters in our work differ from parameters as in

Alkan et al., 2018. The revised Discussion section now has a paragraph that reads (changes in bold):

> The Folding Barrier metric outperformed current state-of-the-art gRNA design tools in its ability to predict CRISPRa activity[21,31]. There are many possible explanations for the inability of existing models to apply to bacterial CRISPRa systems. **It remains an open question whether guide RNA design rules derived from one function in one system, most commonly genome editing in eukaryotes, can be transferred to other functions and systems such as CRISPR gene regulation in prokaryotes.** First, many of these models account for genome structure, which will vary greatly between eukaryotes and prokaryotes[80,81]. Second, in regression models trained on large gene editing datasets, it is difficult to decouple gRNA efficiency from feedback on gene expression as part of the overall gene regulatory network, and therefore the predictions of these models may not be readily transferable between organisms. **Third, the models underlying these gRNA design tools** were trained on unmodified gRNAs and do not capture potential folding effects of extended RNA elements included in scRNAs for bacterial CRISPRa. These models could likely be improved by incorporating biophysical parameters in their predictions. Finally, considerations of nucleic acid interactions **in gRNA design** models tend to focus on the thermodynamics of spacer-DNA interactions, and neglect other important aspects of gRNA folding[30]. **For instance, a number of studies that model the thermodynamics of gRNA-Cas9-DNA complex formation employ parameters describing the impact of structure within the spacer sequence (e.g. ΔG_U) and of spacer-target hybridization (e.g. ΔG_H)[30,82,83]. Here, the conceptually similar parameter Binding Energy does not predict bacterial CRISPRa as well as Folding Energy and Net Binding Energy, which consider the spacer sequence in the context of the full scRNA sequence and structure (Supplementary Fig. S6).** Developing models that combine solely sequence-based kinetic folding parameters with heuristics from large-scale functional screening should further improve our ability to design modified guide RNAs for bacterial CRISPRa.

We also included more detail about defective scRNAs in the Binding Energy paragraph of the Results section, lines 204-207. The paragraph now includes:

> “These failures might be explained by interactions between the spacer and the dCas9-binding handle, which are not accounted for in Binding Energy but are included in Folding Energy and Folding Barrier due to consideration of the entire scRNA sequence.”

In addition to the following citations, we included Flamm et al. in places where findpath and ViennaRNA are mentioned, particularly this line in the Methods:

> “Folding Barrier was calculated by using the folding trajectories identified by Findpath[28] to predict the barrier height for the direct refolding pathway from the MFE structure to the active structure.”

All of the suggested references are now included in the manuscript as references 28, 30 (was 29 in the original submission), 82, and 83, respectively.

28. Flamm et al. RNA 2000: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1369916/

30. Yu et al. Genome Biology 2024: https://link.springer.com/article/10.1186/s13059-023-03153-y

82. Alkan et al. Genome Biology 2018: https://genomebiology.biomedcentral.com/articles/10.1186/s13059-018-1534-x

83. Xiang et al. Nature Comms 2021:

https://www.nature.com/articles/s41467-021-23576-0#Sec26

2. Regarding the computational analysis, the authors mention custom scripts but do not make source code available. While the description on e.g. lines 570 – 594 appears comprehensive, it would be very helpful if the authors would make their scripts publicly available on github or similar with example input files. There are often small details (e.g. exact parameter settings) that can make reproducing computational work from text descriptions difficult, and having source code available would be very helpful in assuring that others can reproduce the results reported here. This is particularly important since the calculation of these kinetic parameters is central to their claims.

The source code is now provided on GitHub (https://github.com/carothersresearch/gRNA_screen_docker); it can be run directly in that environment using a Codespace or locally using a Docker image.

Some other more minor points:

3. Line 214: It has also recently been observed that predictions for eukaryotic genome editing efficiency also have very low accuracy for bacterial CRISPRi (Yu at al. Genome Biology 2024) – this could also be noted in the discussion on lines 490 to 504, as it suggests the transfer of design rules from eukaryotic genome editing applications to other technologies/systems may be problematic in general.

As suggested, and as above, we have now included this point in the Discussion on lines 496-499. The revised section includes:

> "It remains an open question whether guide RNA design rules derived from one function in one system, most commonly genome editing in eukaryotes, can be transferred to other functions and systems such as CRISPR gene regulation in prokaryotes."

4. Lines 476 to 478: While I agree with the authors that their results are impressive, to my understanding they are derived from a fairly small number of measurements (~15) and this should be noted here (particularly when projecting a "near zero" failure rate). Some suggestions for how to further test this in the future would be welcome.

We apologize for the confusion, and we edited the discussion on lines 480-482 to make this clearer. The predictions were actually made from 39 total measurements ("the original J3 sequence, the 14 randomly selected targets described above, and 24 additional scRNAs designed to have Folding Barriers ranging from 5 to 35 kcal/mol"). The revised section now reads:

> "We found that a single kinetic parameter, Folding Barrier, can accurately predict bacterial CRISPRa across a broad range of expression levels, with a failure rate of zero for the set of 39 scRNA designs tested."

Reviewer #3 (Remarks to the Author):

The manuscript reports on the improvement of CRISPRa in E. coli. The authors improved the design method for guide RNAs. Although guide RNAs designed by conventional methods sometimes did not show the predicted activity due to misfolding, most of those designed by the newly developed method showed activity as predicted. CRISPRa has been well developed. The contribution of this manuscript is to improve gRNA design.

1. Thus, this study may fit specialized journals focusing on nucleic acids would be more appropriate.

We respectfully disagree. As written in our cover letter accompanying the original submission, by developing a novel approach to computational guide RNA design, we were able to use rules learned in our previous work (Fontana & Dong *et al.*, 2020 *Nat. Comm.* PMID: 32238808, Alba Burbano & Cardiff *et al.*, 2023 *Proc. Natl. Acad. Sci.* PMID: 37463216) to create orthogonal CRISPR activation systems for bacteria. This work represents a crucial step toward the goal of engineering genome-wide CRISPRa/i programs because we can now design functional multi-guide programs without trial-and-error experimentation. Through its modular system of synthetic promoters, this work demonstrates the immediate utility of multi-guide programs for biosynthetic profiling and metabolic pathway optimization. And, the manuscript describes state-of-the-art tools and workflows compatible with model- and data-driven design-build-test-learn cycles, creating entirely new routes for engineering larger and more complex programs. Based on these broad implications and the wide applicability of our tools, we believe this work is appropriate for the audience of *Nature Communications.*

2. The authors asserted the study's relevance to chemical production. However, the section was not well designed. Pteridine and human milk oligosaccharide, lacto-N-tetraose (LNT) were used as examples. The benefits of the method are overestimated because strains that produce trace amounts of the targets are used as parents. No titers are even provided for pteridine.

We respectfully disagree with the reviewer's premise. As now written about extensively in the Discussion section (also see below), it is extremely common in early stage metabolic engineering to start from strains that can produce trace levels of the target. A major challenge for the field is to effectively and efficiently optimize production from such starting points. We selected these particular pathways as experimental testbeds to investigate the utility of our new tools for biosynthetic profiling as would be common in early-stage metabolic engineering and optimization campaigns. When starting these campaigns, there is no way to know the levels of enzyme expression that will give the highest levels of production. Our tools and approaches provide a new route for efficiently searching expression design spaces. Underscoring the importance of these kinds of tools and approaches, we see that the highest levels of expression (High-High-High in our language) do not correspond to the highest levels of production in either the biopterin or LNT biosynthetic systems.

As suggested by the reviewer, we converted pteridine fluorescence into titers (concentration in production culture) and updated our figures and source data (updated Fig. 5 and new Supplementary Fig. 16). This update does not alter the key conclusion, as noted above, that the highest pteridine titers did not come from the highest levels of expression. We observed production levels up to 200 mg/L of pteridine within the set. We note that, in the literature, biopterin production titers from the same metabolic pathway in yeast yielded 1-5 mg/L (Ehrenworth *et al.*, 2015 *ACS Synth. Biol.* PMID: 26214239), significantly lower than the titers achieved here.

3. Regarding LNT, strains producing at the 10-100 mM level have been constructed. But this study used the microM level production strain. In Line 452-453, the authors compared yields rather than titers, misleading the readers. The titers reported here are too low to compare with those in other papers. But CRISPRa was designed to improve titers, not yields in this study, making the comparison of yields rather puzzling.

As now written in the Results and Discussion sections, the test-tube titers achieved here (2.5 mM) are similar to test-tube titers achieved elsewhere (4.2 mM) even though that study used 5-fold more lactose feedstock. Thus, our optimized strains obtain 3-fold higher yield compared to that previous work at the test-tube scale typical of early-stage strain development (see Discussion paragraphs below). We also note that improvements in yield explain the underlying mechanism that led to higher LNT titer in our system when the bottleneck identified through biosynthetic profiling was resolved.

The Results section has now been updated in the following paragraph (changes in bold):

> To increase β-1,3-galactosyltransferase activity, we replaced WbgO with the GalT enzyme from *Chromobacterium violaceum* (*Cv*GalT), an enzyme with faster turnover.[73] We placed *Cv*GalT under J6 control in the LNT pathway plasmid and paired it with the previously highest-producing scRNA library strain (medium-*lacY*, high-*lgtA*, high-*Cv*GalT). Compared to the corresponding WbgO strain, the *Cv*GalT strain produced a 5- to 10-fold increase in supernatant LNT titer, while LNT II accumulation decreased 5- to 20-fold, with the precise effect depending on the feedstock concentration (Figure 6e). These paired effects reflect the higher ability of *Cv*GalT to bind and convert LNT II before it is exported to accumulate in the supernatant[74]. The highest supernatant titer achieved **from the *Cv*GalT-containing** system increased to 2.52 mM LNT (1.78 g/L)**, compared to 0.576 mM (0.407 g/L) from the WbgO-containing system. This improvement reflects a 4.4-fold increase in mol/mol yield on lactose from 0.099 to 0.432.** Relieving the bottleneck identified by our biosynthetic profiling approach therefore resulted in **significantly more LNT production by improving the efficiency of the β-1,3-galactosyltransferase reaction.**

The discussion has been extensively re-written to explain the role of biosynthetic profiling in a metabolic engineering campaign and to more explicitly compare the production titers obtained in our manuscript with other work in the literature. The high titers given by the reviewer were obtained in scaled-up, typically fed-batch, bioreactor conditions (Liao et al. 2023 [PMID: 37467490], Zhu et al. 2021 [PMID: 34436880], Sugita and Koketsu 2022 [PMID: 35426313] for

example), which is significantly different from the small-scale, test-tube expression profiling demonstrated in our manuscript. We do not believe that direct comparisons of titer are as important as the demonstration of new tools and approaches for achieving these titers. In this study we aim not to maximize titer, but to explore the production landscape across intermediate levels of pathway expression as a starting point for later-stage process development. Nonetheless, we note that our test tube titers of 1.78 g/L LNT from 2 g/L of lactose compare very favorably to the 2.96 g/L of LNT from 5-fold higher concentrations of lactose (10 g/L) reported elsewhere for test-tube production (Sugita and Koketsu 2022). We also believe it is useful to discuss yield as part of this comparison due to the 5-fold differences in feedstock levels.

The Discussion section has now been updated as follows (changes in bold):

> Optimal multi-gene pathway expression could be influenced by many factors, possibly including total burden, enzyme imbalance, or toxic enzyme or metabolite effects. The difficulty in predicting these systems-level interactions means that finding global production optima often requires exploring large design spaces[85]. Toward this end, we successfully developed a scRNA library that can implement all combinations of four truncation-defined expression levels across three chosen genes, totaling 64 possible expression programs. For each of the pathways we examined, we found the optimal production to occur at non-maximal expression levels in at least one channel of expression (*rfp*, *sr*, and *lacY* in Figures 4, 5, and 6, respectively). Production from these pathways therefore maps ruggedly to the underlying design space of enzyme expression, and systematically profiling these effects revealed high-producing strains and also pathway bottlenecks potentially sensitive to optimization. Pursuing bottleneck optimization in the LNT pathway with an improved enzyme variant pushed **test-tube-scale** titers into g/L magnitude (1.78 g/L). **At the scale of test tubes typical of early-stage strain development, Sugita and Koketsu reported 2.96 g/L LNT[74], a similar but higher titer than observed here. Notably, the previous study used 10 g/L lactose feedstock (0.143 mol/mol yield on lactose) compared to only 2 g/L in the present work (0.432 mol/mol), representing a 3-fold higher yield from the combinatorial CRISPRa system.**
>
> **Well-tuned multi-gene expression programs identified though biosynthetic profiling provide starting points for later-stage optimization through genome engineering and process development[25]. A major challenge for the field is to effectively and efficiently optimize production from such starting points. Although beyond the scope of the current study, groups applying such efforts have often achieved 1-5 g/L LNT production titers in shake flasks and 5-50 g/L production in fed-batch bioreactors[86]. As an illustration, 8-fold increases in LNT titer (from 3.11 g/L to 25.4 g/L) and >2-fold increases in LNT yield on lactose (from 0.301 mol/mol to 0.773 mol/mol) were seen when scaling up a strain from 25 mL shake flask cultures to 1 L fed-batch bioreactor conditions, respectively[87]. We expect that similar increases in titer could be achieved by cultures of our optimized strain scaled up to similar fed-batch conditions.**

Broadly speaking, biosynthetic profiling using *trans*-acting scRNAs can greatly reduce the time needed to tune multi-gene programs, compared to traditional *cis*-acting tools like promoter, RBS, or ribozyme libraries[88,89]. We expect that the combinatorial scRNA library described here will provide a straightforward approach to identifying production maxima and optimizing burdensome pathways or toxic intermediate accumulation**, ahead of later-stage optimization**. In the future, this approach could be extended to non-model hosts with metabolic and physiological capabilities suitable for next-generation bioproduction applications[90–92].

Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
The authors have addressed all my questions and comments in a satisfactory manner. I support publication.

Reviewer #2:
Remarks to the Author:
The authors have thoughtfully responded to my previous review, and thoroughly address the two main comments I had: 1) further discussing previously used energy parameters in the literature, and 2) make their source code available. I have no further comments.

Reviewer #3:
Remarks to the Author:
I appreciate the authors' detailed responses. While I appreciate the significant improvements made in CRISPRa design and the elucidation of metabolic bottlenecks in the biosynthesis pathways examined, I still maintain my initial perspective. Only three genes are overexpressed for LNT production. The authors used the CRISPRa method to identify the bottleneck in these three steps. However, in real production strains, more than three genes need to be overexpressed. In the LNT production, more than three genes have to be expressed to increase UDP-GlcNAc and UDP-Gal pools in addition to the three steps from lactose (e.g., 10.1021/acs.jafc.2c02423, 10.1021/acs.jafc.3c02997). As for biopterin, the authors only focused on the three steps from GTP. Additionally, in most biological production systems (e.g., Artemisinic acid (Amyris), 1,4-butanediol (Genomatica)), multiple genes must be expressed. While the authors' CRISPRa method can effectively identify bottlenecks in systems with only three genes are overexpressed, it would be challenging to utilize this complex method in systems where dozens of genes are expressed. Thus, it fits better in a journal specializing in nucleic acids. Once again, I appreciate the thoroughness of the authors' response and the efforts made to address concerns raised during the review process.

REVIEWER COMMENTS

Point-by-point responses to Reviewer #3's comments are provided in blue text.

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We emphasize that the major advance of our work is the CRISPR sgRNA-promoter design method, the systematic characterization, and demonstration of immediate practical utility in multiple metabolic pathways. From this foundation, it is straightforward to construct additional promoters and target additional genes. This paper's design advance, particularly avoiding trial-and-error experimentation in gRNA design, will help precipitate the very improvements toward large pathway development that the reviewer is hoping for and considers challenging.

We have revised the manuscript to highlight these points. The major revisions include two new paragraphs in the Discussion (lines 552-573) and new Supplementary Figure 18 (lines 368-397 in Supplement). This new content explains in detail how our approach can be readily extended to construct larger programs with additional target genes.

We further emphasize that our existing three gene system has already enabled important new insights into metabolic pathway engineering, as described in detail in the manuscript. Tellingly, we have already received multiple requests for access to the genetic constructs and software developed here, pointing to overall interest in the system. We look forward to others' application of CRISPR control to new pathways, as well as the expansion of this approach to larger pathways. We suggest that taken together, our work represents a substantial advance that is appropriate for the broad readership of *Nature Communications*.