nature portfolio

Peer Review File

Large-Scale CRISPR Screening in Primary Human 3D Gastric Organoids Enables Comprehensive Dissection of Gene-Drug Interactions

Corresponding Author: Dr Yuan-Hung Lo

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

Attachments originally included by the reviewers as part of their assessment can be found at the end of this file.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Review of the manuscript “Large-Scale CRISPR Screening in Primary Human 3D Gastric Organoids Enables Comprehensive Dissection of Gene-Drug Interactions”

The wide applicability of CRISPR screening to delineate anything from gene essentiality and synthetic lethality to drug interactions has been undertaken in the present manuscript to interrogate human-derived organoids. The authors performed knockout, transcription inhibition/activation, and single-cell CRISPR screens in p53/APC-mutant (DKO) gastric organoids to identify sensitizers of cisplatin. Their CRISPRi and CRISPRa screens identified a plethora of candidates, some of which were individually validated. One candidate – TAF6L – is described in more detail. The protein seems to cause a powerful cell cycle arrest that makes organoids vulnerable to the drug. Finally, Lo et al. performed a single-cell CRISPR screen with a smaller library identifying a novel metabolic pathway connected to the cisplatin mode of action.
The screening in organoids indeed comes with its own set of difficulties, clonal drift being probably the biggest challenge. Maintaining the library coverage at 1000x, the authors make sure that this issue is resolved, and their screen reproducibility seems to be good. However, as Lo et al. themselves note, many such screens have already been performed in organoids and have shown feasibility. What I find lacking in the present manuscript, is some consistency and a clear biological finding. The summary of my comments is below.

Major comments:

1. The authors first performed a knockout (KO) screen in DKO organoids by choosing a library targeting membrane proteins. Why is this? As all subsequent experiments use a library targeting DNA/RNA-binding proteins, this choice seems unreasonable. It does not allow for testing congruency between KO and CRISPRi screens. What is more, we never even get to see the quality of the KO screen. The proper way to do this is to show the discovery rate of essential genes and not just validation of randomly picked genes (Figure 1D and 1F).
2. The major issue with the authors’ interpretation of the screens comes from Figure S2C. The authors implicitly claim that the screens can identify synergisms, but this is false. The screens cannot measure the effect of the drug alone (control gRNA frequency would be the same with and without the drug, even in the case of severe viability effects of the drug), thus, any effect of the gRNA in the presence of the drug might be additive or synergistic, and the screen cannot differentiate between the two. The chance this interaction is synergistic is higher if the gRNA effect alone is low, but in the case of severe gRNA effects, additive interaction of gRNA+drug cannot be excluded. This is nicely overcome in single-cell screening, where the effect of the drug alone can indeed be deduced, so proper synergism is correctly calculated (Figure S5A); no such possibility exists for bulk screening. I emphasize the point because it warrants caution in interpreting any hit that shows viability effects in the absence of the drug – TAF6L – most importantly. The only way to show synergism is to separate the effects of the drug and the gRNA in the validation experiments – this is normally done through colony formation assays or viability assays. However, the authors normalize viability assays in ways that prevent this. Figure 6B suggests that TAF6L KD does not affect

viability, but I suspect this is the consequence of normalizing KD viability in the absence of the drug to 1, instead to control viability in the absence of the drug. The authors show that 84% of cells exit active cycling upon TAF6L KD (Figure 6E), which suggests quite a severe phenotype. In addition, TAF6L scores in 35% of all 2D cell lines (DepMap), having the strongest viability effects in lymphoma and gastric cancer cells. To what extent this contributes to the overall phenotype in the presence of cisplatin needs to be resolved.
3. While quality control for KO and CRISPRi screens can always be gauged through the discovery rate of essential genes, it is less clear how to assess the quality of CRISPRa screens. The authors show 10 sensitizing hits (Figure 3B and 3C), but the list (Table S6) shows that 12 control gRNAs scored as well, which is a substantial portion of all scoring gRNAs. What is more, the only persisting gRNAs in the screen are control gRNAs. Considering that the reproducibility of the CRISPRa screen was the lowest (Figure S2D), I am not convinced the quality of the screen is good. The authors also never chose to validate a single hit from the screen. Why?
4. In the CRISPRi screen, it would be good to see how many essential genes have been identified in the absence of cisplatin. While validating the candidates, five genes could not be validated due to their essentiality (Figure S4B). However, only two of these five scored in the viability (no-drug) screen. Was the gRNA used for validation taken from the screen or a new one was designed? If the former, how do authors explain such a low level of reproducibility?
5. Single-cell CRISPR screen represents the most novel aspect of this work. The screen seems to be of a high quality and indeed, the synergistic effects could be properly uncovered. It would be good to provide a little more data on the cisplatin treatment and GMDS downregulation upon different hits perturbation. First, can GMDS downregulation be confirmed by a western blot? And second, the authors claim they discovered “a crosstalk between fucosylation and cisplatin sensitivity” (lines 381-382). In fact, such a crosstalk is not discovered, since there is no determined relationship between GMDS and sensitivity. If the authors knockdown GMDS and do drug curves for cisplatin, they would be closer to establishing such a link.
6. Finally, some motivation for cisplatin as a drug of choice is warranted. The drug has been mostly replaced by oxaliplatin in gastric cancer treatment (PMID: 35130500).

Minor comments:

1. Correlation coefficients should be provided in Figure S2D.
2. Drug curves should be shown for Figure S4A, not the IC50 values only. In addition, the authors should clearly state in the methods section how the curves were calculated. Have they been normalized to no-drug condition for each gRNA or to no-drug condition of control gRNA? This is important, as I outline in major comment 2. The curves should be normalized to control gRNA no-drug condition so that any viability effect of the test gRNA can be deduced.
3. Some commenting on Figure 6E seems necessary. It is shown that cisplatin induces cell cycle arrest, decreasing a population of cycling cells by half. Furthermore, TAF6L KD has a similar, but stronger effect, decreasing the cycling population of cells by 84%. How do the authors explain the effects of the combination of cisplatin and TAF6L KD? It seems the KD rescues cisplatin’s effects, or vice versa. Why would this be detrimental to cell viability?

Reviewer #2

(Remarks to the Author)
The manuscript by Lo et al employs CRISPR technology to identify genes regulating cisplatin sensitivity. The manuscript present impressive technical innovations, as the authors employ CRISPR screens (involving cutting/knockout, interference/downregulation and activation/overexpression) in organoid cultures. This will be an important step towards expanding the use of CRISPR screens from 2D to 3D cultures. The authors employ rigorously-designed libraries and performed the screens in replicate. The data analyses are also solid. They find some known or expected hits, confirming that the screens in organoid cultures are feasible. They also identify new cisplatin regulators. While technological impressive, in my view the manuscript lacks in mechanistic findings.

Specific comments.

1. While the screens data is important for the field, the lack of mechanistic details is disappointing. The authors show that the GMDS gene involved in fucosylation and the TAF6L gene involved in recovery following cisplatin treatment are novel cisplatin sensitivity genes. However, how exactly they function is not investigated. At the very least, the authors should attempt DNA repair assays (quantification of DSBs and ICLs, impact on HR activation by RAD51 immunofluorescence, impact on FA pathway activation by FANCD2 ubiquitination, impact on replication fork stability) to test how these genes affect cisplatin-induced DNA repair.

2. There is no justification for using libraries targeting transmembrane proteins in Fig1 and DNA and RNA binding proteins in Fig3. Particularly for the DNA and RNA binding proteins library, the authors should present more details in the main text detailing the biological functions present in this library (DNA repair, transcription etc..). Are the majority of DNA repair GO genes present in this library? What other GO biological processes are hyper-represented in the library?

3. The choice of cisplatin exposure for the screens with this library is not justified (cisplatin treatment for 24h followed by 5 days of recovery, repeated for a total of 4 rounds). Why was this treatment scheme used? Is it clinically relevant? Were other cisplatin screens that the authors cited performed under similar conditions?

4. A western blot needs to be shown for the TP53 and APC knockouts presented in Fig S1A.

5. The ranks in the individual replicate screens should be presented for all the hits chosen for validation in Figure S4A. The authors state that they randomly chose 14 hits for validation and they validated 9 of them. S4A only shows the results for these 9. The identity and the validation results for the other 5 genes picked for validation should be presented. Moreover, it is unclear why the authors selected these 14 genes randomly, as opposed to choosing the top hits based on rankings.

6. Quantification of the growth defects presented as micrographs (Fig 1F, S4B) need to be presented, from multiple individual replicates with statistical analyses.
S4B.

7. Fig 3A should also include details on cisplatin concentration and treatment timeline employed.

Reviewer #3

(Remarks to the Author)
In the presented manuscript entitled "Large-Scale CRISPR Screening in Primary Human 3D Gastric Organoids Enables Comprehensive Dissection of Gene-Drug Interactions", Lo and colleagues present a proof-of-study centered on the intersection of CRISPR technology and 3D organoid models to uncover gene-drug interactions, with a particular focus on cisplatin sensitivity in gastric cancer. The authors leverage diverse CRISPR platforms (CRISPR knockout, interference, activation, and single-cell CRISPR screens) to systematically identify genes contributing to cisplatin sensitivity. The study explores the use of expression readouts to identify synergistic combinations of genetic perturbation and drug treatment at the individual gene level, highlights TAF6L as a novel cisplatin sensitization gene, and provides mechanistic insights into its role in cell recovery after DNA damage. It also emphasizes the importance of single-cell approaches in unraveling high-dimensional gene-drug interactions and transcriptomic convergence within DNA repair pathways.

The manuscript has several strengths, but there are critical areas requiring further development. Despite these limitations, the paper addresses a compelling approach with high translational potential. Revisions to improve its clarity, generalizability, and overall impact would strengthen the manuscript.

Strengths of the manuscript:
- Mechanistic Insights: The focus on TAF6L and its role in chromatin accessibility and cell proliferation following DNA damage provides a strong mechanistic foundation.
Single-Cell Resolution: The use of single-cell CRISPR screening enhances the resolution of gene-drug interactions, with the potential to uncover synergistic effects. However, the approach to examining additive effects vs synergism is overly simplistic and is unlikely to generalize broadly (see major comments).
- Translational Potential: The study is a strong example highlighting how exhaustive genetic screening can identify potential therapeutic targets for enhancing drug sensitivity. In this case, cisplatin sensitivity in gastric cancer treatment.

Major Comments:
- The novelty of the manuscript lies in the combination of many CRISPR modalities, as there are multiple examples of genetic screens on patient-derived tumor models with drug perturbation. However, the authors do not present much in the way of integrated analyses across modalities in instances where the same set of targets were probed at the bulk or single-cell levels. As an example, in Figure 4C, some genotypes have been perturbed by both modalities, but the correlation analysis appears to be only within a modality (grey in the heatmap).
- It is unclear why the authors selected DNA/RNA-binding protein-targeting libraries or membrane protein-targeting libraries instead of a genome-wide screen.
The study would be strengthened by broader validation. While the study demonstrates findings in TP53/APC DKO organoids, it is unclear if the response generalizes to other organoid models. Expanding validation to at least one additional model (patient-derived or more diverse organoid) will strengthen the work. In addition, the motivation to use the organoid model, which largely lacks the variability found in patient tumors, needs to be clarified.
- It is unclear what is driving the relationship between perturbation in 5B-C. The main grouping that appears upon treatment is the association of NER factors. Is this only due to viability phenotypes, or are there additional expression changes that characterize the grouping? Similarly, in 5B, are there pathways modulated in the absence of drug treatment by the genetic perturbation that make sense? Confidence that the associations are not random in 5B would strengthen the manuscript.
- The approach to arrive at an additive model presented in Figure 5 is overly simplistic. How well can the additive model be generalizable by summing up two pseudobulk conditions given a high dropout rate scRNA seq and the number of cells captured per condition? For the extreme of GMDS, the assumption appears to work, but it is unclear if this would work for all genes. In addition, phenotypes like incomplete penetrance would lead to adding over heterogeneous groups of cells even if the actual effect is additive, and there are frameworks to explicitly test for synergism. The manuscript would be strengthened by demonstrating that the results in Figure 5D (3. Additive model) are not driven by noise.
- A statement on data and code availability is lacking. There do not appear to be factors that would limit data sharing for the study. The authors should clarify if that is not the case.

Minor Suggestions:
- Overall, the choice of the drug is not well motivated. This is particularly important as the study does not highlight its application to the study of other drugs or large-scale chemical profiling.
- Improving figure labeling and legends is important for accessibility. Figure 4C, in particular, seems low quality, which could be due to the import/export upon submission.
- Minimal preprocessing information is included for the scRNA data. Details of computational methods still need to be

included.

Reviewer #4

(Remarks to the Author)
Large-Scale CRISPR Screening in Primary Human 3D Gastric Organoids Enables Comprehensive Dissection of Gene-Drug Interactions

Summary
Author present a large-scale CRISPR screen in gastric cancer organoids. They first show the validity and robustness of their screen, and inferring how each sgRNA affects cellular growth, with increasing or decreasing sgRNA abundance inferring growth advantages or disadvantages, respectively. They then design a custom panel of targeting genes which they suppress or activate in the subsequent CRISPR screens. They perform an activation or repression screen, both with and without cisplatin treatment. The analysis of the screen is well done with sufficient explanation of the reasoning behind each step. Drug sensitization is defined as a significant depletion in cell viability, i.e. lower number of gRNAs present in the dataset, compared to drug treatment in absence of the sensitization.
They end up with 41 novel cisplatin sensitization targets and focus on further validating one of them. However, this transition feels abrupt and requires a clearer connection. As TAF6L is not the top-ranked hit and showed substantial variability between replicates, the authors should provide a rationale for prioritizing this gene for further study. While it is reasonable to consider prior knowledge or biological relevance in selecting candidates, a brief explanation in the manuscript would help bridge the two parts of the study and ensure that the validation experiments are cohesively integrated into the overall narrative.

Major concerns

- Methods section requires more detail
The methods, particularly for bioinformatic analyses, are insufficiently described. Both the experimental and computational approaches need clearer documentation. Additionally, the authors should make their code publicly available (e.g., via GitHub) and provide detailed parameters within the manuscript. Specific issues include:
o ATAC-seq: The cut-off used to define differentially accessible peaks is not stated, nor are the parameters for peak calling. The reported number of differentially accessible peaks appears unusually high and should be clarified.
o scRNA-seq: The manuscript mentions that “a Random Forest classifier was trained” for differential gene expression analysis, but no further details are provided. As this is not a standard analysis for single-cell RNA-seq data, the authors should describe the process thoroughly and share the corresponding code.

- Biological and technical replicates
In both the CRISPR screens and several validation experiments, replicates are either absent, not shown, or not accounted for in the analysis. This pertains to experiments depicted in Figure 2D, Figure 2E, Figure 3E, Figure 3F and Figure 5D. Furthermore, for all western blot experiments, an uncut and unmodified image should be put in the supplement. Proper documentation and inclusion of replicates are essential to ensure the robustness of the findings.

- Data availability
At the time of review, there is no mention of sequencing data (raw or processed) being deposited in a public repository. Additionally, the computational code has not been made accessible to reviewers or the wider scientific community. This omission undermines reproducibility and should be addressed prior to publication.

Minor concerns
- Data visualization improvements
o Figure 4C and Figure 5G: Ensure font sizes are consistent and legible across all figures.
o Figure 5A: Remove hits that were not tested with Perturb-seq to avoid confusion.
o Figure 4G and Figure 4H: These figures are difficult to interpret. Consider replotting the data as enrichment over the normal genomic distribution (e.g., a lollipop plot) to better visualize regions of significant enrichment and aid in data interpretation.
o Figure 4F: The figure is unclear. Move the scale bar to the bottom for improved readability or try replotting for clarity.
o Figure S1C: In addition to the overall agreement between replicates, a correlation plot for their top hits should be shown. This approach allows for a more detailed assessment of variability and consistency for their top hits. Visualizing data per gene would help identify specific genes with high or low agreement, which may be obscured when examining the overall distribution.
- Cell-type specific effects in tumor cells vs. healthy cells present in the tumor microenvironment
Investigating how gene knockdown impacts different cell types (e.g., healthy vs. transformed cells) within the tumor microenvironment could strengthen the study. This would provide a deeper understanding of treatment vulnerabilities in gastric cancer. However, this is a suggestion rather than a requirement for publication.

Conclusion

Overall, this manuscript is well-written and presents significant findings that advance our understanding of gene-drug interactions in gastric cancer. While some improvements are necessary, particularly in the methods section, data availability, and figure clarity, the study is strong and deserving of publication.

Reviewer #5

(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 #1

(Remarks to the Author)
The authors have submitted a revised manuscript on their work on cisplatin resistancy using CRISPR screening in gastric organoids. In my view the revised manuscript does not come with enough substantial changes. Although the data around cisplatin sensitivity and GMDS are indeed interesting and now read better, I do not see that previously raised issues regarding all other aspects of the work have been addressed. Details are below.

Major comments:

1. There is no good motivation for the KO screen (Figure 1). If it was done to show that drop-out screening is possible in organoids, such work has already been performed. The screen quality is suboptimal – around 50% of essential genes are accurately identified. The authors speculate this might have to do with 3D vs. 2D culture differences, yet the cited work to support this claim (PMID: 32238925) has not identified any differences in core essential genes, but rather in novel vulnerabilities in 3D culture.
2. The authors have not included any comments about the CRISPRa screen, maintaining that the screen is of high quality. The two replicates have correlation of 0.25 for gRNAs and 0.16 for genes under the drug treatment. This means that each time a screen is performed largely a different set of hits is identified. I wonder why the quality of both KO and CRISPRa screens is subpar. The authors keep 1000x coverage after expansion but do not say what coverage is achieved on the day of infection. If the coverage at infection is 50x and cells are expanded to 1000x and maintained at 1000x, the real coverage is still 50x.
3. The conclusions drawn from the TAF6L story are unsupported. First, the FACS plot values and bar plot values in Figure 6E do not match. Even the individual measurements in the bar plot do not correspond to FACS values, so it is difficult to know what values to look at. Judging by the bar plot that includes triplicates, TAF6L overexpression does not recover cells from cell cycle arrest under cisplatin, although it rescues the viability (compare Figure 6D and 6E bar plot). Thus, it seems the effects have little to do with the cell cycle, contrary to what the authors claim in lines 415, 446-447, and 511. Although RNA-seq and ATAC-seq experiments are subsequently performed on TAF6L-knockdown and overexpression organoids, no clues emerge from these results. Overall, it remains unclear how TAF6L interacts with cisplatin.
4. I have asked the authors to provide direct evidence of GMDS protein downregulation under cisplatin and any of the NER genes gRNAs. This is their primary claim – that there is a synergism between NER disruption and cisplatin in downregulating GMDS. The authors responded by providing the western blot solely under cisplatin (Figure 5E), but not investigating the purported synergism. In addition, to validate the role of GMDS, the authors chose to overexpress it, which I find not an optimal solution. Namely, the GMDS dynamic is not preserved in OE organoids under cisplatin. The expression upon the drug treatment goes up, instead of down (Figure S6H), suggesting a substantially changed context in which experiments are conducted.

Reviewer #2

(Remarks to the Author)
In the revised manuscript, the authors satisfactorily addressed the reviewers' concerns. They provide a large amount of new data and a thoughtful evaluation and interpretation of their results. The new data support the conclusions put forward by the authors.

Reviewer #3

(Remarks to the Author)
The authors have addressed the concerns raised in my initial review. I appreciate the clarification of the rationale behind the library design choices and the implementation of a regression model, which strengthens the robustness of their approach. Additionally, the inclusion of preprocessing details and the statement on data and code availability enhances the transparency and reproducibility of the study. Overall, the revisions have improved the clarity and impact of the manuscript.

Reviewer #4

(Remarks to the Author)
The authors present a revised version of their manuscript entitled "Large-Scale CRISPR Screening in Primary Human 3D Gastric Organoids Enables Comprehensive Dissection of Gene-Drug Interactions". While some improvements have been made since the initial submission, several important issues raised in the previous round of review remain unaddressed and

continue to impact the overall assessment of the manuscript.

Major points:
• Data accessibility:
The authors have deposited the raw data to the GEO database, which is appreciated. However, the required access tokens for reviewers have not been provided. For the purposes of peer review, it is essential that reviewers can access and assess the deposited data. The authors should either include the access credentials in the next revision or make the dataset publicly available.
• Methods transparency and code availability:
Multiple reviewers, including myself, previously noted the lack of methodological detail and code availability. Although the authors have extended the Methods section, additional critical details are still lacking. In particular, the provided GitHub links point to example notebooks authored by others who performed similar analyses, rather than the actual code used in this study. For the sake of reproducibility and scientific integrity, it is imperative that the authors share the specific code used to generate the results reported in the manuscript. Without this, a thorough assessment of the analysis and its validity is not possible.
• Clarification on previous feedback:
I would like to apologize for a referencing error in my previous review. I referred to Figure 4, F-G, but intended to reference Figure 6, F-G. I kindly request that the authors consider re-plotting this data to show enrichment relative to a normal genomic distribution (e.g., via a lollipop plot) to enhance interpretability and highlight regions of significant enrichment. See attached figure for clarity.

• Reproducibility of CRISPR screens:
The reproducibility of CRISPRi and particularly CRISPRa replicate screens remains unclear. The apparent variability raises concerns about the robustness of the results, and it is difficult to determine whether repeating the experiment would yield consistent findings. This concern underscores the importance of a detailed analysis of replicate agreement (see next point).
• Analysis of replicate agreement:
A previously raised suggestion regarding Figure S1C has not been addressed. Specifically, it was recommended that the authors include a correlation analysis of the top hits between replicates. Such an analysis would allow for a more granular view of variability and would help identify genes with consistent versus inconsistent behavior across replicates. A focused plot showing correlation of the top 100 hits between replicates would significantly improve the evaluation of the screen's performance, especially given potential sources of variability (e.g., transduction efficiency, gRNA efficacy, sequencing depth, and batch effects).
• Supplemental data (tables) description:
The supplemental tables require clearer annotation and integration with the main text. At present, it is difficult to discern what the reported values represent or how they were derived. For example, terms such as "average phenotype of strongest 3" are unclear without further methodological context. Again, this ties back to the need for code availability and improved transparency.

In conclusion, while the authors have taken some steps to improve the manuscript, several substantial issues remain unresolved. I encourage the authors to thoroughly address the points above, particularly regarding code availability, replicate reproducibility, and data presentation. These revisions are essential before the manuscript can be considered for publication.

Reviewer #5

(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 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Concerning my comments, nothing new has been added in terms of data, the authors have just changed the phrasing in a few places. All in all, the raised issues regarding different aspects of the work have therefore not been addressed.

Reviewer #4

(Remarks to the Author)
I would like to thank the authors for carefully addressing my remaining concerns. They have taken all the necessary steps to ensure data sharing, code reproducibility, and have adequately responded to all outstanding questions. I fully support the publication of the manuscript.

Reviewer #5

(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.

Reviewer #1 (Remarks to the Author): expert in gastric cancer organoids and CRISPR screens

Review of the manuscript “Large-Scale CRISPR Screening in Primary Human 3D Gastric Organoids Enables Comprehensive Dissection of Gene-Drug Interactions”

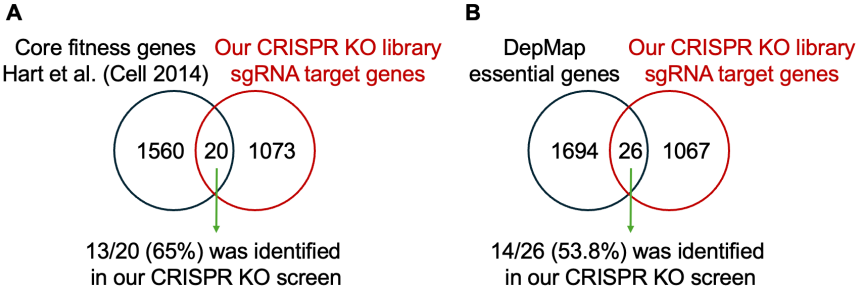
The wide applicability of CRISPR screening to delineate anything from gene essentiality and synthetic lethality to drug interactions has been undertaken in the present manuscript to interrogate human-derived organoids. The authors performed knockout, transcription inhibition/activation, and single-cell CRISPR screens in p53/APC-mutant (DKO) gastric organoids to identify sensitizers of cisplatin. Their CRISPRi and CRISPRa screens identified a plethora of candidates, some of which were individually validated. One candidate – TAF6L – is described in more detail. The protein seems to cause a powerful cell cycle arrest that makes organoids vulnerable to the drug. Finally, Lo et al. performed a single-cell CRISPR screen with a smaller library identifying a novel metabolic pathway connected to the cisplatin mode of action. The screening in organoids indeed comes with its own set of difficulties, clonal drift being probably the biggest challenge. Maintaining the library coverage at 1000x, the authors make sure that this issue is resolved, and their screen reproducibility seems to be good. However, as Lo et al. themselves note, many such screens have already been performed in organoids and have shown feasibility. What I find lacking in the present manuscript, is some consistency and a clear biological finding. The summary of my comments is below.

Major comments:

1. The authors first performed a knockout (KO) screen in DKO organoids by choosing a library targeting membrane proteins. Why is this? As all subsequent experiments use a library targeting DNA/RNA-binding proteins, this choice seems unreasonable. It does not allow for testing congruency between KO and CRISPRi screens. What is more, we never even get to see the quality of the KO screen. The proper way to do this is to show the discovery rate of essential genes and not just validation of randomly picked genes (Figure 1D and 1F).

RESPONSE: We thank Reviewer #1 for their comment. In our CRISPR KO screen **(Figure 1)**, we selected a membrane protein sgRNA library for a pilot experiment to test the feasibility of conducting a CRISPR screen in human organoids. This particular library was chosen because it had been extensively validated (a gift from Dr. Michael C. Bassik at Stanford) and was readily available for use in the lab. We aimed to ensure that our pilot experiment provided sufficient sgRNA library information to evaluate the feasibility of CRISPR screening and validate the hits identified. These included the identification of several essential genes **(see Reviewer Panel 1 below)** and the well-known tumor suppressor gene *LRIG1* **(Figure 1D)**.

Our KO sgRNA library targets 1,093 genes, including 20 genes identified as core fitness genes by Hart et al. (Cell, 2014, PMID: 26627737) through whole-genome CRISPR KO screens in six 2D human cell lines. Among these, 13 genes (13/20 = 65%) were classified as significant growth defect hits based on the analysis method used in this study **(see Reviewer Panel 1A below)**. Compared to the DepMap database (https://depmap.org/portal/), our library includes 26 targets classified as common essential genes, of which 14 (14/26 = 53.8%) were identified as growth defect hits in our screens in organoids **(see Reviewer Panel 1B below)**.



Reviewer Panel 1. A, The Venn diagram shows the 13 genes from the CRISPR KO screen identified as core fitness genes in a previous study (Hart et al., Cell 2014). **B,** The Venn diagram shows the 14 genes from the CRISPR KO screen identified as essential genes in DepMap.

It is worth noting that these “essential gene lists” can vary based on the criteria used for classification. For example, DepMap defines essential genes as follows:

"Common essential genes" are defined as: A gene which, in a large, pan-cancer screen, ranks in the top X most depleting genes in at least 90% of cell lines. X is chosen empirically using the minimum of the distribution of gene ranks in their 90th percentile least depleting lines."

Consequently, the CRISPR screen hits can vary based on the thresholds applied during the analysis. Furthermore, essential genes can differ across various cell types and may vary between 2D and 3D cultures (Han et al., Nature 2020, PMID: 32238925). Thus, in this study, we validated numerous hits individually to further evaluate the robustness of our designed CRISPR screens beyond solely relying on known gene functions **(Figure 1F)**. This underscored the significance of individual validation in CRISPR screening experiments.

It is important to note that we do not intend to test the congruency between CRISPR KO and CRISPRi screens in this study. However, we further leveraged the experience gained from the CRISPR KO screen to build upon this foundation by incorporating new iCRISPRi and iCRISPRa systems to study gene-drug interactions. According to the literature, many genes directly involved in DNA repair and cisplatin response encode DNA/RNA-binding proteins. Therefore, we selected an additional library for our iCRISPRi and iCRISPRa screens, which included DNA/RNA-binding proteins, epigenetic regulators, and chromatin remodeling genes. This comprehensive approach robustly validated the screen results, and our data further supported this conclusion.

2. The major issue with the authors' interpretation of the screens comes from Figure S2C. The authors implicitly claim that the screens can identify synergisms, but this is false. The screens cannot measure the effect of the drug alone (control gRNA frequency would be the same with and without the drug, even in the case of severe viability effects of the drug), thus, any effect of the gRNA in the presence of the drug might be additive or synergistic, and the screen cannot differentiate between the two. The chance this interaction is synergistic is higher if the gRNA effect alone is low, but in the case of severe gRNA effects, additive interaction of gRNA+drug cannot be excluded. This is nicely overcome in single-cell screening, where the effect of the drug alone can indeed be deduced, so proper synergism is correctly calculated (Figure S5A); no such possibility exists for bulk screening. I emphasize the point because it warrants caution in interpreting any hit that shows viability effects in the absence of the drug – TAF6L – most importantly. The only way to show synergism is to separate the effects of the drug and the gRNA in the validation experiments – this is normally done through colony formation assays or viability assays. However, the authors normalize viability assays in ways that prevent this. Figure 6B suggests that TAF6L KD does not affect viability, but I suspect this is the consequence of normalizing KD viability in the absence of the drug to 1, instead to control viability in the absence of the drug. The authors show that 84% of cells exit active cycling upon TAF6L KD (Figure 6E), which suggests quite a severe phenotype. In addition, TAF6L scores in 35% of all 2D cell lines (DepMap), having the strongest viability effects in lymphoma and gastric cancer cells. To what extent this contributes to the overall phenotype in the presence of cisplatin needs to be resolved.

RESPONSE: We want to thank Reviewer #1 for the comment, and we apologize for any lack of clarity. This type of synergism analysis has been extensively published before for both CRISPR and RNAi screens (PMID: 28985505, PMID: 25307932, and many others). During the analysis, we normalized the sgRNA growth effects. Specifically, we measured the relative abundance of each sgRNA in the treated population (with cisplatin) compared to the untreated population (without cisplatin). The untreated population reveals how each sgRNA affects growth in the absence of cisplatin. Conversely, the normalized difference in abundance between the treated and untreated populations for each sgRNA indicates the "sensitivity to cisplatin. " A sensitivity score greater than 0 suggests that the expression of the sgRNA provides protection against cisplatin, while a score less than 0 indicates sensitization.

As a toy example, we have:
Day 0
Day 10, no drug
Day 10, with drug

In a simplified explanation, if we compare Day 10 with drugs with Day 0, it is true that the effect of the drug alone cannot be deduced. However, if we compare Day 10 no drug with Day 10 with the drug, the effects of the growth of the sgRNA will be the same and normalized, leaving the "sensitivity to the drug."

We also thank Reviewer #1 for the suggestion regarding Figure 6B. Reviewer #1 is correct that *TAF6L* KD leads to decreased cell proliferation, resulting in a growth phenotype during long-term culture. However, we would like to clarify that because we used a doxycycline-inducible system, this significantly mitigates concerns regarding short-term culture experiments, such as individual validation experiments and functional assays. Following the reviewer's valuable suggestion, we have normalized all groups in Figure 6B to the control (no treatment) and provided **an updated Figure 6B**. As shown in the figure, *TAF6L* KD resulted in approximately a 15% decrease in cell viability in this experiment. This adjustment more accurately reflects the effect of *TAF6L* KD on cell growth and drug sensitivity. We sincerely appreciate Reviewer #1's insightful feedback.

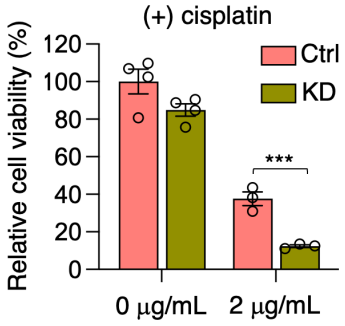


Figure 6B. Cell viability of Control (Ctrl) or *TAF6L* knockdown (KD) iCRISPRi TP53/APC DKO organoids after three days of cisplatin treatment. *TAF6L* KD shows significantly increased sensitivity to cisplatin. Relative cell viability is presented as means ± SEM of technical replicates from two independent experiments. Two-way ANOVA. ***, p<0.001.

3. While quality control for KO and CRISPRi screens can always be gauged through the discovery rate of essential genes, it is less clear how to assess the quality of CRISPRa screens. The authors show 10 sensitizing hits (Figure 3B and 3C), but the list (Table S6) shows that 12 control gRNAs scored as well, which is a substantial portion of all scoring gRNAs. What is more, the only persisting gRNAs in the screen are control gRNAs. Considering that the reproducibility of the CRISPRa screen was the lowest (Figure S2D), I am not convinced the quality of the screen is good. The authors also never chose to validate a single hit from the screen. Why?

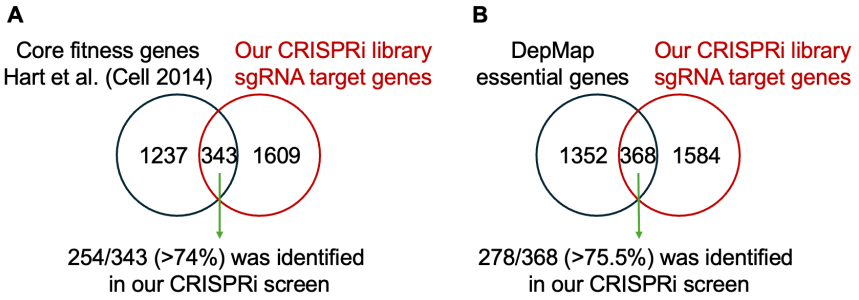
RESPONSE: We agree with Reviewer #1. CRISPRa screens are more challenging and require robust and consistent transcriptional activation, which can vary based on chromatin accessibility and basal gene expression levels. Moreover, there are significantly fewer published CRISPRa screens.

As noted in PMID: 30575746 (https://www.nature.com/articles/s41467-018-07901-8), false negatives in CRISPRa screens can occur when the target gene is not sufficiently overexpressed due to poor sgRNA design, incorrect TSS annotation, or an inaccessible chromatin environment. Unlike traditional ORF-based overexpression, CRISPRa employs endogenous promoters to modulate gene expression, making it a more physiologically relevant method; consequently, CRISPRa hits tend to be specific to cell types.

Despite these limitations, we successfully identified MYC as the top hit for CRISPRa, which aligns with the oncogene properties of *MYC* in promoting cancerous growth.

4. In the CRISPRi screen, it would be good to see how many essential genes have been identified in the absence of cisplatin. While validating the candidates, five genes could not be validated due to their essentiality (Figure S4B). However, only two of these five scored in the viability (no-drug) screen. Was the gRNA used for validation taken from the screen or a new one was designed? If the former, how do authors explain such a low level of reproducibility?

RESPONSE: We thank Reviewer #1 for the comment regarding the CRISPRi screen. Our CRISPRi sgRNA library, targeting 1,952 genes, includes 343 genes identified as core fitness genes by Hart et al. (*Cell*, 2014, PMID: 26627737). Among the results of our CRISPRi screen in the absence of cisplatin, 254 genes (hit rate = 254/343, >74%) were classified as causing significant growth defect phenotypes in this study **(see Reviewer Panel 2A below)**. When compared to the 1,720 common essential genes defined by DepMap, our library covers 368 genes, of which 278 were identified as significant hits in our study (hit rate = 278/368, >75.5%) **(see Reviewer Panel 2B below)**.



Reviewer Panel 2. A, The Venn diagram shows the 254 genes from the CRISPRi screen identified as core fitness genes in a previous study (Hart et al., Cell 2014). **B,** The Venn diagram shows the 278 genes from the CRISPRi screen identified as essential genes in DepMap.

For Figure S4B, only two of the five genes exhibiting growth phenotypes, including *POLR1D*, *CPSF2*, *BOD1L1*, *ZKSCAN2*, and *HMGN2*, were identified as hits in the absence of cisplatin. However, except for *HMGN2*, the remaining four genes had negative phenotype scores (see phenotype scores in Supplementary Table 5), indicating that knockdown of these genes tended to result in growth defect CRISPRi phenotypes. This discrepancy could be due to the cutoff threshold we set, which may have excluded some hits from being selected.

Our experiments, in fact, underscore the critical importance of individual validation in CRISPR screens. The primary purpose of the genetic screen is to identify top hits that can be functionally validated, while the validation process is designed to eliminate hits with low reliability. The sgRNAs used in our validation were selected from the primary screen. It is possible that results from a single sgRNA in pooled screens and individual validations may differ. For example, pooled screens involve multiple clones with different sgRNAs (usually hundreds or thousands of clones), which introduces factors such as competition between different clones that can influence the final results — a limitation inherent to genetic screening. In contrast, single sgRNA validation focuses on a homogeneous sgRNA population. Thus, these differences further emphasize the importance of performing single sgRNA validation to confirm and refine findings from the primary screen. This precisely aligns with the goal of our validation approach. Notably, in our validation, aside from excluding potential false-positive hits, we confirmed many previously reported hits, identified several novel hits, and conducted follow-up experiments to demonstrate their functions.

5. Single-cell CRISPR screen represents the most novel aspect of this work. The screen seems to be of a high quality and indeed, the synergistic effects could be properly uncovered. It would be good to provide a little more data on the cisplatin treatment and GMDS downregulation upon different hits perturbation. First, can GMDS downregulation be confirmed by a western blot? And second, the authors claim they discovered “a crosstalk between fucosylation and cisplatin sensitivity” (lines 381-382). In fact, such a crosstalk is not discovered, since there is no determined relationship between GMDS and sensitivity. If the authors knockdown GMDS and do drug curves for cisplatin, they would be closer to establishing such a link.

RESPONSE: We thank Reviewer #1 for their insightful suggestion. Consistent with our CROP-seq results, cisplatin treatment led to a decrease in *GMDS* mRNA expression. Additionally, at the protein level, we confirmed through Western blotting that cisplatin treatment reduced GMDS expression **(new Figure 5E)**.

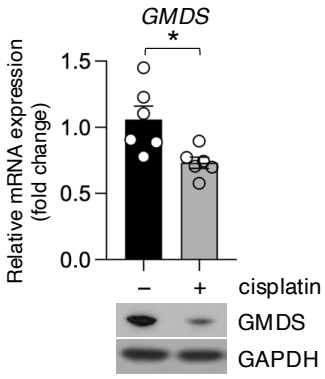


Figure 5E. Cisplatin treatment inhibited GMDS expression. Top panel: Quantification of *GMDS* mRNA expression by qPCR. The horizontal bar indicates the mean. The error bar represents SEM. Bottom panel: Western immunoblotting analysis demonstrated that cisplatin inhibited GMDS protein expression. Organoids were treated with 2 μg/mL cisplatin for 72 hrs.

We followed the reviewer's suggestion to functionally evaluate the crosstalk between GMDS, fucosylation, and cisplatin sensitivity. We generated a new lentiviral *GMDS* construct to establish organoid lines (GMDS OE) that stably express GMDS **(new Figure 5F)**. The expression of GMDS was confirmed by Western blotting, revealing that GMDS expression is sufficient to induce total cellular protein fucosylation **(new Figure 5F)** and to rescue the suppressed GMDS levels in cisplatin-treated organoids **(new Figure S6H)**. Most importantly, we observed that the gain-of-function *GMDS* OE organoids were more sensitive to cisplatin treatment than parental organoids **(new Figure 5G)**.

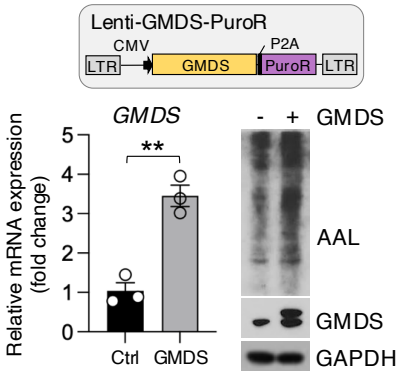


Figure 5F. Stable GMDS-overexpressing organoid lines were established by lentiviral transduction. Quantification of *GMDS* mRNA by qPCR indicated GMDS overexpression. The horizontal bar indicates the mean. The error bar represents SEM. T-test. **, p<0.005. Western immunoblotting analysis demonstrated that GMDS overexpression enhanced fucosylation. Total fucosylated proteins were detected by AAL Western blotting.

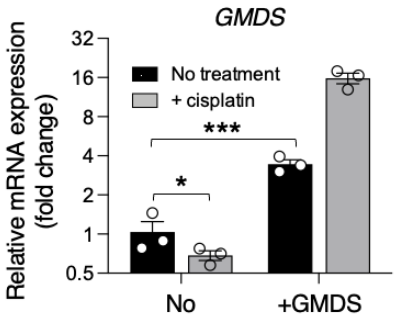


Figure S6H. GMDS overexpression rescued cisplatin-induced GMDS repression. Organoids were treated with 2 μg/mL cisplatin for 72 hrs. The horizontal bar indicates the mean. The error bar represents SEM.

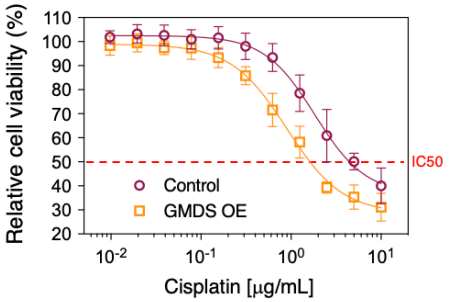


Figure 5G. Constitutive expression of GMDS sensitized the cisplatin sensitivity of organoids. A fully titrated cisplatin treatment was performed in three independent experiments (N=3) to determine cisplatin sensitivity.

Consistent with this result, nuclear pH2AX significantly increased in the combination of GMDS OE and cisplatin treatment, suggesting the accumulation of DNA damage **(new Figure 5H).** Notably, GMDS OE organoids did not compromise the baseline viability of parental organoids **(new Figure S6I)**, nor did they alter the expression of RAD51 and FANCD2 in the absence of cisplatin **(see Reviewer Panel 3 below)**, further ruling out nonspecific toxicity associated with elevated GMDS levels.

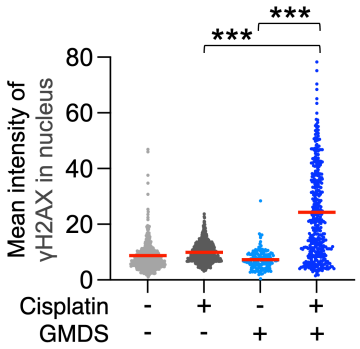


Figure 5H. Quantification of γH2AX immunofluorescence staining in nuclei. Each dot represents one nucleus. T-test. ***, p<0.001. The red bar represents the mean value of all data points. Organoids were treated with 2 μg/mL cisplatin for 48 hrs.

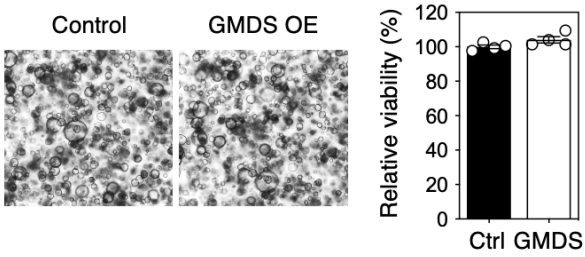
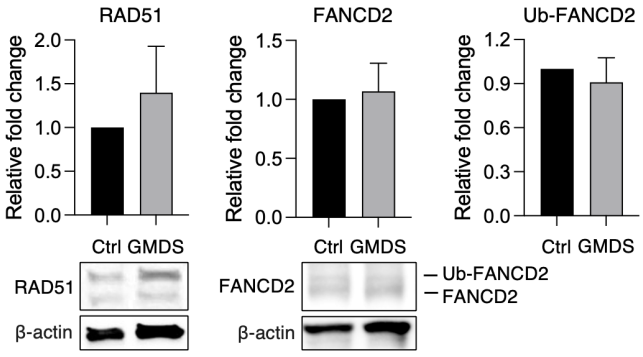


Figure S6I. GMDS overexpression did not inhibit the growth of organoids. Left panel: Brightfield images were taken for organoid growth on day 10 after the passage. Left panel: Quantification of metabolic activity was determined by Alamar blue assay on day 10 after passage.



Reviewer Panel 3. Quantification of RAD51, FANCD2, and Ub-FANCD2. At least two independent Western blotting experiments were performed.

To further investigate the effect of fucosylation on cisplatin sensitivity, we utilized 2F-PerAc-Fuc, an L-fucose analogue, to inhibit fucosylation by competitively saturating GDP-fucose biosynthetic enzymes **(new Figures S6E and S6J)**. We discovered that this treatment reversed the cytotoxicity of cisplatin **(new Figure S6K)**.

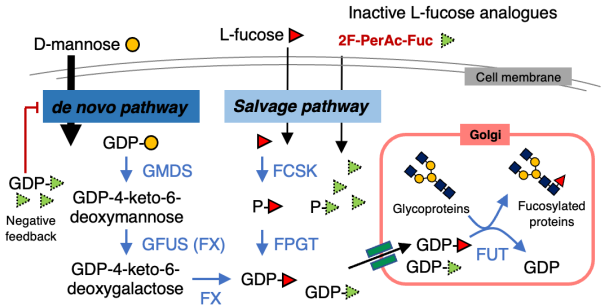


Figure S6E. Schematic of fucosylation biosynthetic pathways.

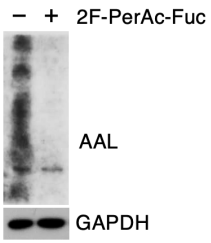


Figure S6J. Inactive L-fucose analogues, 2F-PerAc-Fuc inhibited fucosylation. Organoids were treated by 2F-PerAc-Fuc for 72 hrs. Total fucosylated proteins were determined by AAL Western blotting.

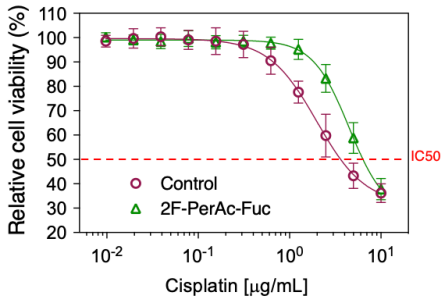


Figure S6K. Pharmacologic inhibition of fucose biosynthesis using rescued cisplatin sensitivity. The inactive L-fucose analogues, 2F-PerAc-Fuc, reduced cisplatin sensitivity in organoids. A fully titrated cisplatin treatment was performed in three independent experiments (N=3) to determine cisplatin sensitivity.

These new experiments reinforced the connection between GMDS, fucosylation, and cisplatin sensitivity. Based on the findings, we propose a potential model suggesting that the downregulation of GMDS-associated fucosylation likely acts as a DNA damage-induced pro-survival signal, which reduces cisplatin sensitivity **(new Figure S6L)**.

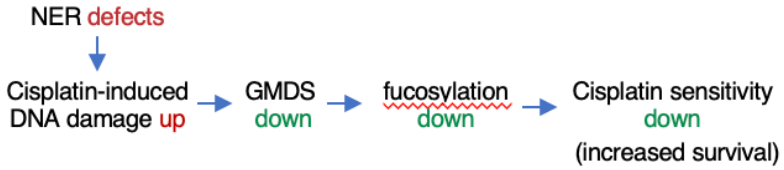


Figure S6L. Schematic of the working model. In response to either DNA damage induced by cisplatin alone or to the more severe stress of cisplatin and NER gene knockdown, *GMDS* repression acts as a pro-survival signal.

A new paragraph for these results has been added to the revised manuscript. See lines 387-413, reprinted below:

*"To gain deeper insight into the relationship between cisplatin sensitivity and GMDS expression, we first validated the effect of isolated cisplatin treatment on GMDS levels. Consistent with the Perturb-seq data **(Figure 5C)**, cisplatin treatment decreased GMDS expression **(Figure 5E)**. To determine whether GMDS expression functionally impacts cisplatin-induced cytotoxicity, we ectopically delivered a full-length GMDS into organoids (GMDS OE) using lentiviral transduction **(Figure 5F)**. As expected, GMDS expression was sufficient to induce total cellular protein fucosylation **(Figure 5F)**. Furthermore, GMDS expression effectively rescued the suppressed GMDS levels in cisplatin-treated organoids **(Figure S6H)** and resulted in GMDS OE organoids exhibiting an increased sensitivity to cisplatin treatment **(Figure 5G)**, accompanied by a significant increase in nuclear phospho-H2AX (**γ**H2AX) **(Figure 5H)**. Notably, GMDS OE organoids did not compromise the baseline viability of parental organoids in the absence of cisplatin **(Figure S6I)**, further ruling out nonspecific toxicity associated with elevated GMDS levels. This discrepancy thus dissociated GMDS downregulation from cisplatin-induced cytotoxicity and suggested that the synergistic suppression of GMDS observed with combined cisplatin treatment and NER gene knockdown was not inherently cytotoxic but rather reflected a positive feedback mechanism promoting cell survival following DNA damage.*

*Next, we tested the hypothesis that the downregulation of fucosylation could act as a pro-survival signal to protect cells from cisplatin-induced cytotoxicity. Inactive fucose analogues, such as 2F-PerAc-Fuc, are taken up by cells through the salvage pathway and inhibit fucosylation by competitively saturating GDP-fucose biosynthetic enzymes, potently depleting endogenous GDP-fucose **(Figure S6E)**. Accordingly, 2F-PerAc-Fuc inhibited total*

*protein fucosylation **(Figure S6J)** but reversed cisplatin cytotoxicity, evidenced by increased cisplatin IC_{50} **(Figure S6K)**. These results supported a potential working model in which the downregulation of GMDS-associated fucosylation functions as a DNA damage-induced pro-survival signal to reduce cisplatin sensitivity, independent of additional NER gene knockdown **(Figure S6L)**. Taken together, these findings reveal a previously uncharacterized crosstalk between GMDS expression and cisplatin sensitivity, highlighting a potential mechanism by which cancer cells modulate their response to cisplatin."*

6. Finally, some motivation for cisplatin as a drug of choice is warranted. The drug has been mostly replaced by oxaliplatin in gastric cancer treatment (PMID: 35130500).

RESPONSE: We thank Reviewer #1 for their suggestion. We agree with Reviewer #1's comment that cisplatin has largely been replaced by oxaliplatin in the treatment of gastric cancer. However, we would like to clarify that cisplatin and oxaliplatin share many similarities. For instance, both are platinum-based chemotherapy agents that induce cancer cell death by forming DNA crosslinks, primarily intrastrand crosslinks, which disrupt DNA replication and transcription. Furthermore, both drugs bind to guanine residues in DNA, activating the DNA damage response pathways. Our study utilized cisplatin to demonstrate the feasibility of using systematic CRISPR-based genetic screens in primary human 3D organoids to explore fundamental and translational biological questions related to gene-drug interactions. Similar approaches can be employed to study other drugs, including oxaliplatin. **We have added a sentence to the discussion in the revised manuscript to underscore this broader applicability. Please refer to lines 555-556 below:**

"Furthermore, the same research approach could also be applied to explore the effects of other anticancer drugs beyond cisplatin."

Minor comments:
1. Correlation coefficients should be provided in Figure S2D.

RESPONSE: We appreciate Reviewer #1's comments. We have incorporated the correlation coefficients in Figure S2D. The CRISPRa correlations are modest yet consistent, and they fall within the same range as previous CRISPRa screens (for example, PMID: 30575746 published by the Doench group).

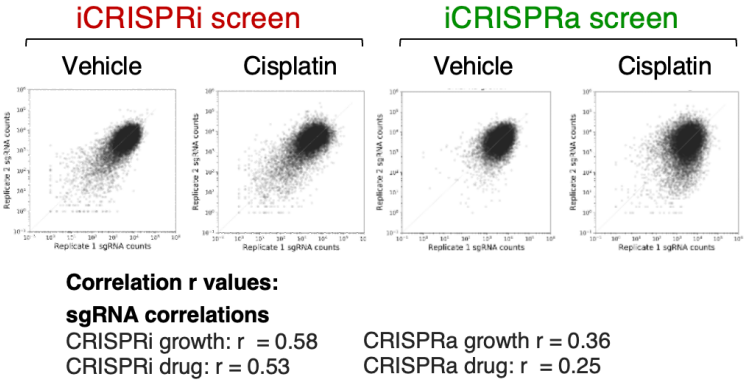


Figure S2D. Correlation of sgRNAs between two independent replicates for iCRISPRi and iCRISPRa screens.

2. Drug curves should be shown for Figure S4A, not the IC50 values only. In addition, the authors should clearly state in the methods section how the curves were calculated. Have they been normalized to no-drug condition for each gRNA or to no-drug condition of control gRNA? This is important, as I outline in major comment 2. The curves should be normalized to control gRNA no-drug condition so that any viability effect of the test gRNA can be deduced.

RESPONSE: We thank Reviewer #1 for the comments. In the revised manuscript, we have included the IC50 curves, featuring the updated Figure 3E (*ERCC4*, *ERCC6*, *GTF2H5*), the updated Figure 3F (*ELOF1*, *LEO1*, *ZNF677*), and the updated Figure S4A (*BRCA1*, *ZFP36L2*). As recommended by the reviewer, each data point in these new IC50 curves has been normalized to the no-drug condition for each gRNA. We have also provided additional details in the Materials and Methods section.

3. Some commenting on Figure 6E seems necessary. It is shown that cisplatin induces cell cycle arrest, decreasing a population of cycling cells by half. Furthermore, TAF6L KD has a similar, but stronger effect, decreasing the cycling population of cells by 84%. How do the authors explain the effects of the combination of cisplatin and TAF6L KD? It seems the KD rescues cisplatin's effects, or vice versa. Why would this be detrimental to cell viability?

RESPONSE: We thank Reviewer #1 for their detailed observations regarding Figure 6E and for this insightful comment. After carefully reviewing the issue, we have included quantitative data from three independent experiments in the revised manuscript to support our conclusions. These experiments were conducted independently, utilizing different passages, cisplatin treatments, and EdU assays. Consistent with our previous findings, the data demonstrate that *TAF6L* knockdown (KD) inhibits cell proliferation, while *TAF6L-GFP* re-expression significantly rescues the decrease in proliferation induced by cisplatin **(Figure 6E)**. Furthermore, the quantitative results from these triplicate experiments indicate that *TAF6L* KD combined with cisplatin leads to the lowest cell proliferation, which aligns with our iCRISPRi screen findings that identified *TAF6L* as a cisplatin sensitization target. We have provided new quantitative figures and employed appropriate statistical methods, including the absolute percentages of EdU+ cell populations for each group and the normalized fold-change relative to the control **(new Figure S7D)**.

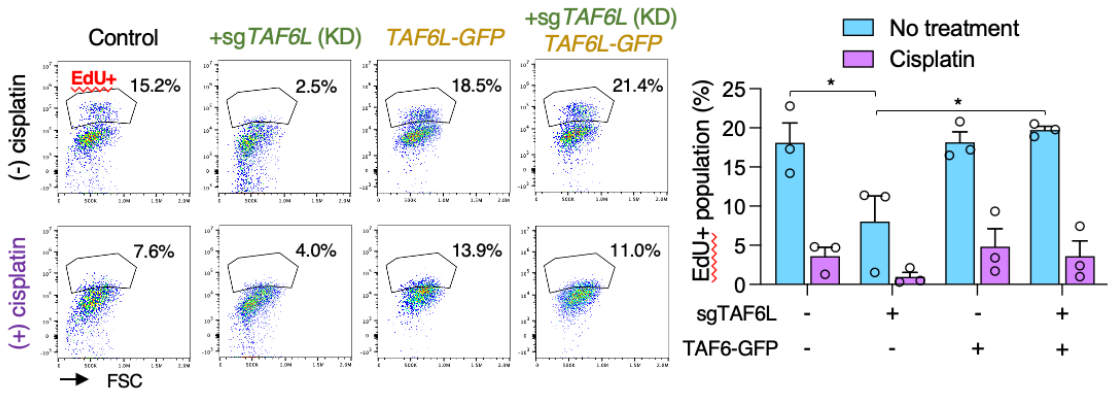


Figure 6E. EdU assay indicated the EdU+ cell population (percentage, %) in parental control, *TAF6L* KD, *TAF6L-GFP* OE, and TAF6L-GFP re-expression in *TAF6L* KD organoids, with or without cisplatin treatment. EdU+ cells were quantified using flow cytometry. Cells were treated with 2 μg/ml cisplatin for 24 hours. Quantification of three independent experiments was shown (N=3). T-test. *, p<0.01.

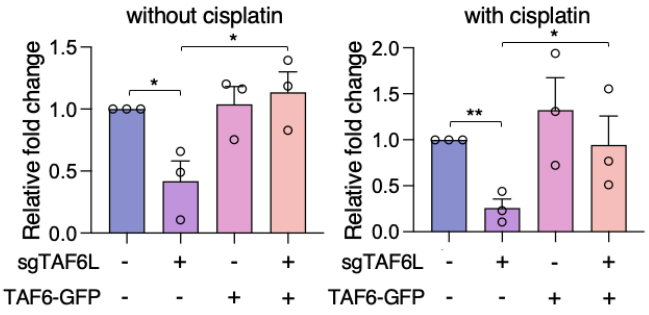


Figure S7D. Related to Figure 6E. EdU assay in parental control, *TAF6L* KD, *TAF6L-GFP* OE, and TAF6L-GFP re-expression in *TAF6L* KD organoids. The relative fold change of EdU+ cells was normalized to control with or without cisplatin treatment. Quantification based on three independent experiments was shown (N=3). T-test. *, p<0.01, **, p<0.05.

These findings have been incorporated into the revised manuscript. See lines 438-447, reprinted below:

*"After 48 hours of cisplatin treatment, the EdU+ cell population significantly decreased from an average of 18.1% to 3.6% in control organoid lines **(Figure 6E and S7D)**. However, TAF6L knockdown reduced the EdU+ cell population to 8.0% compared to control organoids (18.1% EdU+ on average), indicating that TAF6L depletion inhibited cell proliferation. Under TAF6L knockdown conditions, only about 1% of cells remained EdU+ following cisplatin treatment. In contrast, TAF6L-GFP expression enabled cells to maintain their ability to proliferate after cisplatin treatment, with an average of 4.8% EdU+ post-treatment. Notably, the re-expression of TAF6L-GFP was sufficient to rescue cell proliferation in TAF6L knockdown organoids."*

Reviewer #2 (Remarks to the Author): expert in Cisplatin response genes and CRISPR screens

The manuscript by Lo et al employs CRISPR technology to identify genes regulating cisplatin sensitivity. The manuscript present impressive technical innovations, as the authors employ CRISPR screens (involving cutting/knockout, interference/downregulation and activation/overexpression) in organoid cultures. This will be an important step towards expanding the use of CRISPR screens from 2D to 3D cultures. The authors employ rigorously-designed libraries and performed the screens in replicate. The data analyses are also solid. They find some known or expected hits, confirming that the screens in organoid cultures are feasible. They also identify new cisplatin regulators. While technological impressive, in my view the manuscript lacks in mechanistic findings.

Specific comments.

1. While the screens data is important for the field, the lack of mechanistic details is disappointing. The authors show that the GMDS gene involved in fucosylation and the TAF6L gene involved in recovery following cisplatin treatment are novel cisplatin sensitivity genes. However, how exactly they function is not investigated. At the very least, the authors should attempt DNA repair assays (quantification of DSBs and ICLs, impact on HR activation by RAD51 immunofluorescence, impact on FA pathway activation by FANCD2 ubiquitination, impact on replication fork stability) to test how these genes affect cisplatin-induced DNA repair.

RESPONSE: We thank Reviewer #2 for the comment. In the revised manuscript, we have provided a series of new mechanistic studies to support the conclusions of this research.

(1). GMDS: To functionally evaluate the crosstalk between GMDS, fucosylation and cisplatin sensitivity, we generated a new lentiviral *GMDS* construct to establish organoid lines (GMDS OE) that stably express GMDS **(new Figure 5F)**. The expression of GMDS was confirmed by Western blotting, and we found that GMDS expression is sufficient to induce total cellular protein fucosylation **(new Figure 5F)** and to rescue the suppressed GMDS levels in cisplatin-treated organoids **(new Figure S6H)**. Most importantly, we observed that the gain-of-function *GMDS* OE organoids were more sensitive to cisplatin treatment than the parental organoids **(new Figure 5G)**.

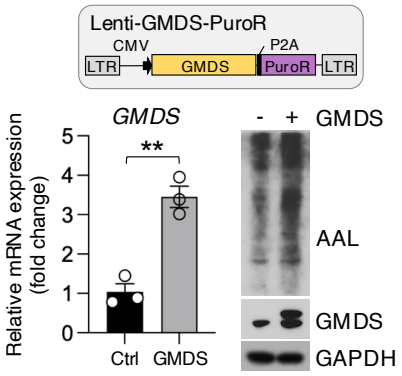


Figure 5F. Stable GMDS-overexpressing organoid lines were established by lentiviral transduction. Quantification of *GMDS* mRNA by qPCR indicated GMDS overexpression. The horizontal bar indicates the mean. The error bar represents SEM. T-test. **, p<0.005. Western immunoblotting analysis demonstrated that GMDS overexpression enhanced fucosylation. Total fucosylated proteins were detected by AAL Western blotting.

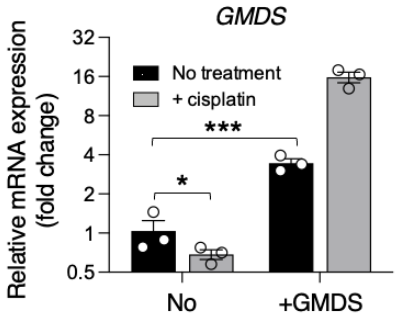


Figure S6H. GMDS overexpression rescued cisplatin-induced GMDS repression. Organoids were treated with 2 μg/mL cisplatin for 72 hrs. The horizontal bar indicates the mean. The error bar represents SEM.

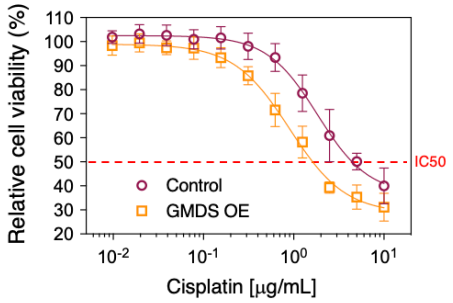


Figure 5G. Constitutive expression of GMDS sensitized the cisplatin sensitivity of organoids. A fully titrated cisplatin treatment was performed in three independent experiments (N=3) to determine cisplatin sensitivity.

Consistent with this result, nuclear γH2AX significantly increased in the combination of GMDS OE and cisplatin treatment, suggesting an accumulation of DNA damage **(new Figure 5H).** Notably, GMDS OE organoids did not compromise the baseline viability of parental organoids **(new Figure S6I)**, further ruling out nonspecific toxicity associated with elevated GMDS levels.

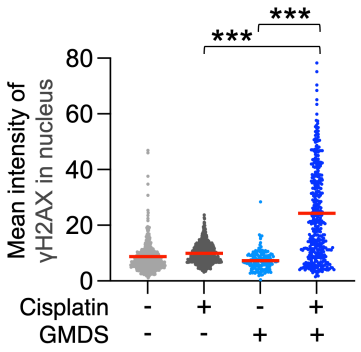


Figure 5H. Quantification of γH2AX immunofluorescence staining in nuclei. Each dot represents one nucleus. T-test. ***, p<0.001. The red bar represents the mean value of all data points. Organoids were treated with 2 μg/mL cisplatin for 48 hrs.

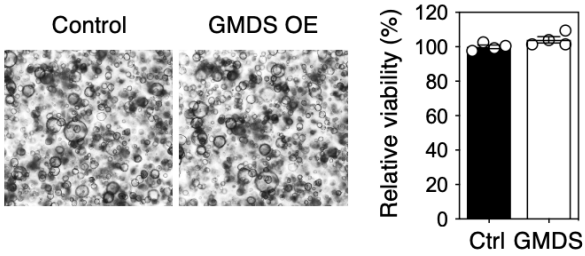
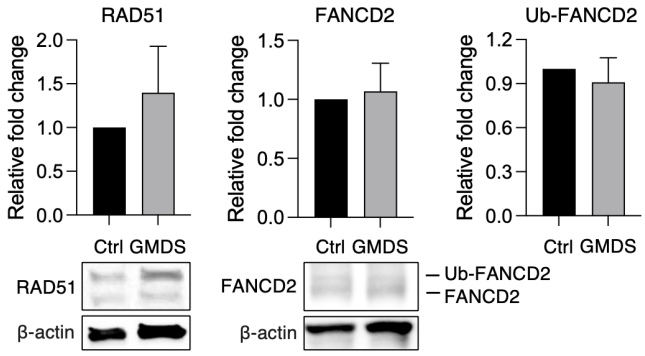


Figure S6I. GMDS overexpression did not inhibit the growth of organoids. Left panel: Brightfield images were taken for organoid growth on day 10 after the passage. Left panel: Quantification of metabolic activity was determined by Alamar blue assay on day 10 after passage.

We did not observe that GMDS OE significantly changes nuclear γH2AX **(new Figure 5H) or** the expression of RAD51, FANCD2, and Ub-FANCD2 **(see Reviewer Panel 3 below)**, suggesting that GMDS does not directly regulate DNA repair signaling activity.



Reviewer Panel 3. Quantification of RAD51, FANCD2, and Ub-FANCD2. At least two independent Western blotting experiments were performed.

To further investigate the effect of fucosylation on cisplatin sensitivity, we utilized 2F-PerAc-Fuc, an L-fucose analogue, to inhibit fucosylation by competitively saturating GDP-fucose biosynthetic enzymes **(new Figures S6E and S6J)**. We discovered that this treatment reversed the cytotoxicity of cisplatin **(new Figure S6K)**.

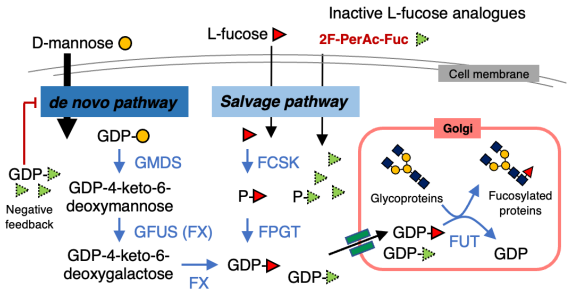


Figure S6E. Schematic of fucosylation biosynthetic pathways.

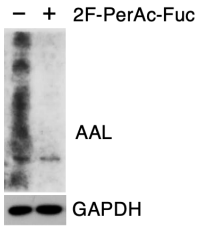


Figure S6J. Inactive L-fucose analogues, 2F-PerAc-Fuc inhibited fucosylation. Organoids were treated by 2F-PerAc-Fuc for 72 hrs. Total fucosylated proteins were determined by AAL Western blotting.

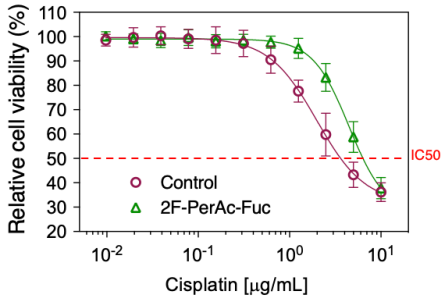


Figure S6K. Pharmacologic inhibition of fucose biosynthesis using rescued cisplatin sensitivity. The inactive L-fucose analogues, 2F-PerAc-Fuc, reduced cisplatin sensitivity in organoids. A fully titrated cisplatin treatment was performed in three independent experiments (N=3) to determine cisplatin sensitivity.

These new experiments reinforced the connection between GMDS, fucosylation, and cisplatin sensitivity. Based on the findings, we propose a potential model suggesting that the downregulation of GMDS-associated fucosylation likely acts as a DNA damage-induced pro-survival signal, which reduces cisplatin sensitivity **(new Figure S6L)**.

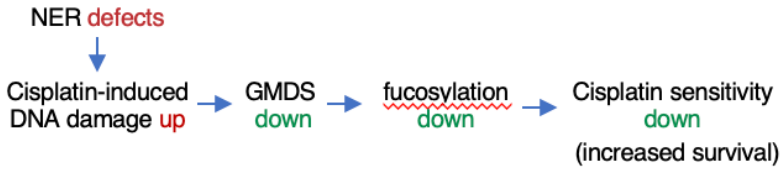


Figure S6L. Schematic of the working model. In response to either DNA damage induced by cisplatin alone or to the more severe stress of cisplatin and NER gene knockdown, *GMDS* repression acts as a pro-survival signal.

A new paragraph for these results has been added to the revised manuscript. See lines 387-413, reprinted below:

"To gain deeper insight into the relationship between cisplatin sensitivity and GMDS expression, we first validated the effect of isolated cisplatin treatment on GMDS levels. Consistent with the Perturb-seq data ***(Figure 5C)****, cisplatin treatment decreased GMDS expression* ***(Figure 5E)****. To determine whether GMDS expression functionally impacts cisplatin-induced cytotoxicity, we ectopically delivered a full-length GMDS into organoids (GMDS OE) using lentiviral transduction* ***(Figure 5F)****. As expected, GMDS expression was sufficient to induce total cellular protein fucosylation* ***(Figure 5F)****. Furthermore, GMDS expression effectively rescued the suppressed GMDS levels in cisplatin-treated organoids* ***(Figure S6H)*** *and resulted in GMDS OE organoids exhibiting an increased sensitivity to cisplatin treatment* ***(Figure 5G)****, accompanied by a significant increase in nuclear phospho-H2AX (γH2AX)* ***(Figure 5H)****. Notably, GMDS OE organoids did not compromise the baseline viability of parental organoids in the absence of cisplatin* ***(Figure S6I)****, further ruling out nonspecific toxicity associated with elevated GMDS levels. This discrepancy thus dissociated GMDS downregulation from cisplatin-induced cytotoxicity and suggested that the synergistic suppression of GMDS observed with combined cisplatin treatment and NER gene knockdown was not inherently cytotoxic but rather reflected a positive feedback mechanism promoting cell survival following DNA damage.*

Next, we tested the hypothesis that the downregulation of fucosylation could act as a pro-survival signal to protect cells from cisplatin-induced cytotoxicity. Inactive fucose analogues, such as 2F-PerAc-Fuc, are taken up by cells through the salvage pathway and inhibit fucosylation by competitively saturating GDP-fucose biosynthetic enzymes, potently depleting endogenous GDP-fucose ***(Figure S6E)****. Accordingly, 2F-PerAc-Fuc inhibited total protein fucosylation* ***(Figure S6J)*** *but reversed cisplatin cytotoxicity, evidenced by increased cisplatin* IC_{50} ***(Figure S6K)****. These results supported a potential working model in which the downregulation of GMDS-associated fucosylation functions as a DNA damage-induced pro-survival signal to reduce cisplatin sensitivity, independent of additional NER gene knockdown* ***(Figure S6L)****. Taken together, these findings reveal a previously uncharacterized crosstalk between GMDS expression and cisplatin sensitivity, highlighting a potential mechanism by which cancer cells modulate their response to cisplatin."*

(2). TAF6L: To further assess the role of *TAF6L* in DNA repair signaling, we evaluated the effect of TAF6L expression on RAD51, FANCD2, and Ub-FANCD2. However, we did not observe significant changes in *TAF6L* KD or *TAF6L-GFP* OE organoid lines **(new Figure S7B and S7C)**. This observation aligns with our initial conclusion that *TAF6L* does not directly regulate DNA repair gene expression (see the original manuscript, Lines 424-428). These new results further support our conclusion that *TAF6L* is essential for the proliferation necessary for cell recovery following cisplatin treatment (see the original manuscript, Lines 408-409).

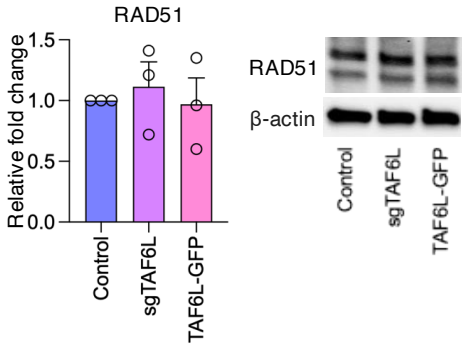


Figure S7B. Quantification of RAD51. At least three independent Western blotting experiments were performed.

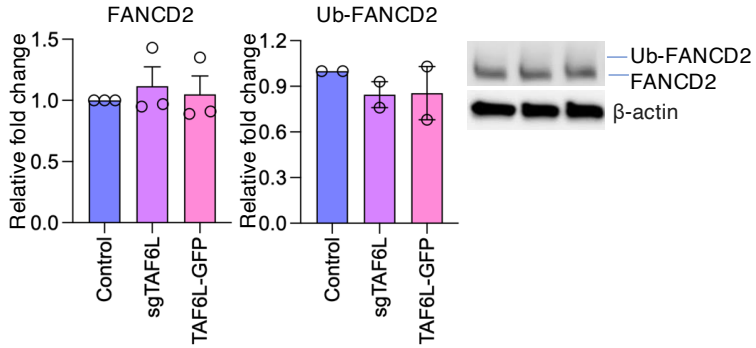


Figure S7C. Quantification of FANCD2. At least three independent Western blotting experiments were performed.

A new paragraph for these results has been added to the revised manuscript. See lines 431-447, reprinted below:

"To explore the molecular mechanism of TAF6L-associated cisplatin sensitivity, we first evaluated the role of TAF6L in DNA repair signaling. We examined whether TAF6L expression directly influences HRR and FA pathway activation. However, we did not observe significant changes in RAD51, FANCD2, or ubiquitinated FANCD2 (Ub-FANCD2) expression in TAF6L KD or TAG6L-GFP OE organoids lines ***(Figure S7B and S7C)****, suggesting that TAF6L does not directly regulate DNA repair signaling activity. Next, we tested the impact of TAF6L on cell proliferation using an EdU assay before and after cisplatin treatment. Viable EdU+ cells were quantified by flow cytometry* ***(Figure 6E and S7D)****. After 48 hours of cisplatin treatment, the EdU+ cell population significantly decreased from an average of 18.1% to 3.6% in control organoid lines* ***(Figure 6E and S7D)****. However, TAF6L knockdown reduced the EdU+ cell population to 8.0% compared to control organoids (18.1% EdU+ on average), indicating that TAF6L depletion inhibited cell proliferation. Under TAF6L knockdown conditions, only about 1% of cells remained EdU+ following cisplatin treatment. In contrast, TAF6L-GFP expression enabled cells to maintain their ability to proliferate after cisplatin treatment, with an average of 4.8% EdU+ post-treatment. Notably, the re-expression of TAF6L-GFP was sufficient to rescue cell proliferation in TAF6L knockdown organoids. These results suggested that TAF6L is critical for the proliferation required by cell recovery following cisplatin treatment."*

2. There is no justification for using libraries targeting transmembrane proteins in Fig1 and DNA and RNA binding proteins in Fig3. Particularly for the DNA and RNA binding proteins library, the authors should present more details in the main text detailing the biological functions present in this library (DNA repair, transcription etc..). Are the majority of DNA repair GO genes present in this library? What other GO biological processes are hyper-represented in the library?

RESPONSE: We thank Reviewer #2 for their comment. The Reviewer is correct that we should provide more details regarding the rationale behind selecting these sgRNA sub-libraries. In our CRISPR KO screen **(Figure 1)**, we selected a membrane protein sgRNA library for a pilot experiment to test the feasibility of performing a CRISPR screen in human organoids. This particular library was chosen because it had been extensively validated (a gift from Dr. Michael C. Bassik) and was readily available for use in the lab. We ensured that our pilot experiment provided sufficient sgRNA library information to assess the feasibility of conducting CRISPR screening in human organoids and proceeded with validating the hits identified. These included several essential genes **(see Reviewer Panel 1 above)** and the well-known tumor suppressor gene *LRIG1* **(Figure 1D)**. We further leveraged the experience gained from the CRISPR KO screen to build on this foundation by incorporating new iCRISPRi and iCRISPRa systems to study gene-drug interactions **(Figure 3)**. According to the literature, many genes directly involved in DNA repair and cisplatin response encode DNA/RNA-binding proteins. Therefore, we selected a new DNA/RNA-binding sgRNA library that contains GO terms such as RNA polymerase II complex, mediator complex, mRNA spliceosome, nucleotide excision repair, damaged DNA binding, histone modification, and chromatin remodeling complex. This comprehensive approach robustly validated the screen results, and our data further supported this conclusion.

We have added more details in the main text. See lines 135-138 and 190-197, reprinted below:

"In a pilot experiment to test the feasibility of CRISPR screens in organoids, we transduced a validated pooled lentiviral library of 12,461 sgRNAs targeting 1,093 membrane proteins, each with ~10 sgRNAs/gene, and 750 negative control non-targeting sgRNAs ***(Table S1)*** *into Cas9-expressing TP53/APC DKO organoids. "*

"Since many genes directly involved in DNA repair and cisplatin response encode DNA and RNA binding proteins, we designed two customized CRISPRi and CRISPRa sgRNA libraries specifically targeting an identical group of 1,952 genes encoding DNA and RNA binding proteins ***(Table S3)****. This targeted gene set includes key GO terms associated with cisplatin responses, such as RNA polymerase II complex, mediator complex, mRNA spliceosome, nucleotide excision repair, damaged DNA binding, histone modification, and chromatin remodeling complex."*

3. The choice of cisplatin exposure for the screens with this library is not justified (cisplatin treatment for 24h followed by 5 days of recovery, repeated for a total of 4 rounds). Why was this treatment scheme used? Is it clinically relevant? Were other cisplatin screens that the authors cited performed under similar conditions?

RESPONSE: We appreciate Reviewer #2 for highlighting this critical point. The treatment scheme was not designed to mirror clinical treatment regimens but was thoughtfully optimized for technical reasons. Our goal was to identify genes linked to cisplatin sensitization and persistence while balancing differential growth to secure a strong enough signal without causing excessive cell death. High-dose exposure could significantly reduce cell doubling, resulting in insufficient sequencing reads for each sgRNA at various time points, rendering statistical analysis unfeasible or introducing background noise that might affect the results.

To address this, we employed a repeated short-term treatment strategy using IC50 doses of cisplatin, allowing cells to recover between cycles to enhance experimental reliability. Specifically, cells were exposed to cisplatin for 24 hours, followed by a 5-day recovery period, repeated for 4 rounds. This regimen ensured that cisplatin-treated cells underwent approximately 6-7 fewer doublings than untreated cells, which was crucial for achieving high-quality screening results. This approach has been used in previous CRISPR screening studies, such as PMID: 28985505, in which rigosertib-treated cells were subjected to a similar pulsed treatment strategy to maintain sufficient differential growth. In that study, the authors stated:

"For rigosertib treatment, rigosertib was added to the cells at the concentrations indicated below and removed by centrifugation the following day. Additional pulses of rigosertib treatment were performed as needed until rigosertib-treated cells had undergone 5-7 fewer doublings than untreated cells. Cells were allowed to recover to > 80% cell viability, as measured on an Accuri bench-top flow cytometer (BD BioSciences), and harvested by centrifugation with a minimum 1000x library coverage."

Our results, which include the top hits identified and subsequent validations of each individual hit, demonstrated that this treatment approach is highly robust and effective for CRISPR screening.

4. A western blot needs to be shown for the TP53 and APC knockouts presented in Fig S1A.

RESPONSE: We thank Reviewer #2 for their suggestion. We apologize for not explaining this part clearly. We would like to clarify that the engineered *TP53/APC* DKO organoids used in this study were comprehensively characterized by WGS and scRNA-seq in our previous publication (Karlsson and Przybilla et al., *Nature* 2023, PMID: 37258665, Clone D3C2). These organoids underwent conditioned media selection, including Nutlin-3 treatment to enrich for *TP53* mutants and Wnt/R-Spondin withdrawal to select for *APC* mutants. Furthermore, in that study, we demonstrated that *TP53* loss is functional, showing that this mutation is sufficient to elicit progressive aneuploidy, including copy number alterations and structural variants commonly found in gastric cancers, with evident preferred orders. In this study, our Sanger sequencing re-confirmed CRISPR-induced indels **(Figure S1A)**. To further verify *TP53* and *APC* protein expression, we attempted to recover the *TP53* KO, *TP53/APC* DKO, and their parental normal organoid lines from DMSO stocks stored several years ago. Unfortunately, we were unable to recover the parental normal organoid line, and therefore we could not compare TP53 protein expression levels. However, we could compare APC expression between the *TP53* KO and *TP53/APC* DKO organoids. We have added the Western blot results showing that APC protein expression is lower in the *TP53/APC* DKO organoids compared to the *TP53* KO organoids in Figure S1A.

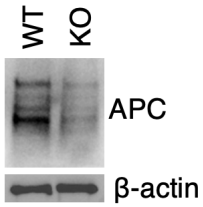


Figure S1A. The APC expression was detected by Western blotting in *APC* WT and *APC* KO organoids.

We have added a clarification and reference to the main text. See lines 123-125, reprinted below:

*"The TP53/APC double knockout (DKO) organoid line was established in our previous studies by sequentially disrupting TP53 and APC[34], two common oncogenic loci in gastric adenocarcinoma[35,36], from non-neoplastic human gastric organoids **(Figure S1A)**."*

5. The ranks in the individual replicate screens should be presented for all the hits chosen for validation in Figure S4A. The authors state that they randomly chose 14 hits for validation and they validated 9 of them. S4A only shows the results for these 9. The identity and the validation results for the other 5 genes picked for validation should be presented. Moreover, it is unclear why the authors selected these 14 genes randomly, as opposed to choosing the top hits based on rankings.

RESPONSE: We thank Reviewer #2 for the question. We selected genes randomly because we believed it would provide a more unbiased representation of the hits and validation of the screen. We have now included the ranks of all the hits in the Supplementary Tables. Our CRISPRi screen identified several top hits for cisplatin sensitization that are already known to play a role in DNA repair pathways, demonstrating the robustness of our CRISPRi screen in human organoids. To further explore the potential of our system, we opted to randomly select hits for validation to enhance the comprehensiveness of our validation rather than limiting it to well-studied top candidates. Additionally, we would like to clarify that out of the 14 selected hits, only 9 genes were used in fully titrated cisplatin IC50 experiments **(Figure S4A)**. The remaining 5 genes, including *POLR1D*, *CPSF2*, *BOD1L1*, *ZKSCAN2*, and *HMGN2,* were excluded from these experiments because knocking down these genes already showed significant growth defect phenotypes prior to cisplatin treatment. We want to emphasize that genetic screening is expected to identify some false positive hits that cannot be validated. The rationale for our selection process is discussed in the manuscript. **Please see lines 265-267, reprinted below:**

*"On the other hand, the remaining his, including POLR1D, CPSF2, BOD1L1, ZKSCAN2, and HMGN2, showed a rapid general growth defect phenotype after knockdown in the absence of cisplatin **(Figure S4B)** and were, therefore, not used for cisplatin sensitivity assay."*

6. Quantification of the growth defects presented as micrographs (Fig 1F, S4B) need to be presented, from multiple individual replicates with statistical analyses. S4B.

RESPONSE: We thank Reviewer #2 for their suggestion. In the revised manuscript, we quantified the number of organoids per well and their relative sizes. Consistent with the provided bright-field images, the knockdown or knockout of these genes caused organoid growth defects, resulting in a statistically significant reduction in both organoid numbers and sizes. We included this information in Figures S1D and S4B.

7. Fig 3A should also include details on cisplatin concentration and treatment timeline employed.

RESPONSE: We thank Reviewer #2 for their suggestion. In the manuscript, **Figure S2A** details the cisplatin concentration and treatment timeline used in **Figure 3A**, with specifics described in Lines 201-214 of the original text. In the revised manuscript, we have added the cisplatin concentration (IC50 treatment, 1.6 µg/mL) to enhance readers' understanding of the experimental setup. Please s**ee lines 203-216, reprinted below:**

*"We performed iCRISPRi and iCRISPRa screens in the presence of cisplatin in parallel with the cisplatin-naïve studies using an optimized protocol **(Figure S2A)**. After doxycycline induction, most organoids co-expressed dCas9 fusion proteins (mCherry+) and sgRNAs (GFP+) **(Figure S2B)**. In the treatment group, in each cycle, organoids were treated with cisplatin (IC50 treatment, 1.6 µg/mL) for 24 hours, followed by 5 days of recovery. A total of 4 rounds of cisplatin treatment cycles were repeatedly conducted to achieve approximately 6-7 doubling differences between the treated and untreated groups, which provided sufficient statistical power to measure gene-drug interactions – either buffering or sensitization effects **(Figure S2C)**. The genomic DNA was sequenced at day 2 (time point 0, T0) versus day 26 (time point 1, T1) after puromycin selection to enumerate the frequency of individual sgRNA lentiviral insertion. The relative abundance of each sgRNA at T1 versus T0 was quantitated to determine the degree of clonal expansion or contraction over the culture period, representing growth advantage or disadvantage, respectively."*

Reviewer #3 (Remarks to the Author): expert in single-cell CRISPR screens

In the presented manuscript entitled "Large-Scale CRISPR Screening in Primary Human 3D Gastric Organoids Enables Comprehensive Dissection of Gene-Drug Interactions", Lo and colleagues present a proof-of-study centered on the intersection of CRISPR technology and 3D organoid models to uncover gene-drug interactions, with a particular focus on cisplatin sensitivity in gastric cancer. The authors leverage diverse CRISPR platforms (CRISPR knockout, interference, activation, and single-cell CRISPR screens) to systematically identify genes contributing to cisplatin sensitivity. The study explores the use of expression readouts to identify synergistic combinations of genetic perturbation and drug treatment at the individual gene level, highlights TAF6L as a novel cisplatin sensitization gene, and provides mechanistic insights into its role in cell recovery after DNA damage. It also emphasizes the importance of single-cell approaches in unraveling high-dimensional gene-drug interactions and transcriptomic convergence within DNA repair pathways.

The manuscript has several strengths, but there are critical areas requiring further development. Despite these limitations, the paper addresses a compelling approach with high translational potential. Revisions to improve its clarity, generalizability, and overall impact would strengthen the manuscript.

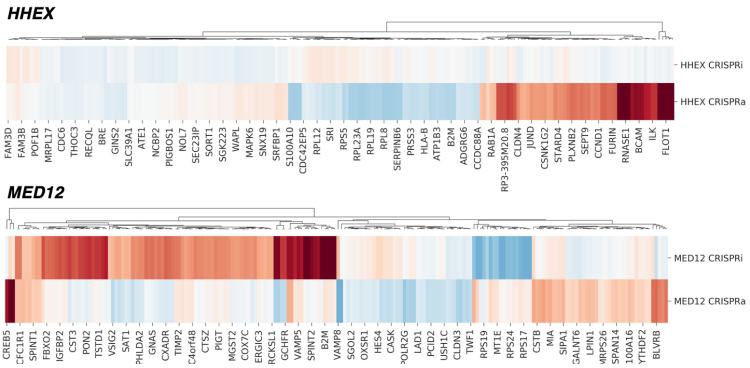
Strengths of the manuscript:
- Mechanistic Insights: The focus on TAF6L and its role in chromatin accessibility and cell proliferation following DNA damage provides a strong mechanistic foundation.

- Single-Cell Resolution: The use of single-cell CRISPR screening enhances the resolution of gene-drug interactions, with the potential to uncover synergistic effects. However, the approach to examining additive effects vs synergism is overly simplistic and is unlikely to generalize broadly (see major comments).

- Translational Potential: The study is a strong example highlighting how exhaustive genetic screening can identify potential therapeutic targets for enhancing drug sensitivity. In this case, cisplatin sensitivity in gastric cancer treatment.

Major Comments:
- The novelty of the manuscript lies in the combination of many CRISPR modalities, as there are multiple examples of genetic screens on patient-derived tumor models with drug perturbation. However, the authors do not present much in the way of integrated analyses across modalities in instances where the same set of targets were probed at the bulk or single-cell levels. As an example, in Figure 4C, some genotypes have been perturbed by both modalities, but the correlation analysis appears to be only within a modality (grey in the heatmap).

RESPONSE: We thank Reviewer #3 for their suggestion. The CRISPRi and CRISPRa Perturb-Seq libraries were designed separately and selected from their respective bulk screens. We did not originally intend to use integrated analysis to compare CRISPRi and CRISPRa. As a result, only *HHEX* and *MED12* overlapped between the CRISPRi and CRISPRa Perturb-Seq screens. Here, we demonstrate that we were able to identify the top differentially expressed genes and cluster them **(see Reviewer Panel 4 below)**. Similar approaches can be applied to appropriate studies in the future.



Reviewer Panel 4. Heatmaps of differentially expressed genes were identified from iCRISPRi and iCRISPRa Perturb-Seq screens for *HHEX* or *MED12*.

- It is unclear why the authors selected DNA/RNA-binding protein-targeting libraries or membrane protein-targeting libraries instead of a genome-wide screen.

RESPONSE: We thank Reviewer #3 for their comment. Reviewer #3 is correct in stating that we should provide more details regarding the rationale for selecting these sgRNA sub-libraries. In our CRISPR KO screen **(Figure 1)**, we selected a membrane proteins sgRNA library for a pilot experiment to evaluate the feasibility of conducting a CRISPR screen in human organoids. This particular library was chosen because it had been extensively validated (a gift from Dr. Michael C. Bassik) and was readily available for use in the lab. We ensured that our pilot experiment provided sufficient sgRNA library information to assess the feasibility of carrying out CRISPR screening in human organoids and proceeded with validating the hits identified. These included recognizing several essential genes, including the well-known tumor suppressor gene *LRIG1* **(Figure 1D)**. We further leveraged the experience gained from the CRISPR KO screen to build upon this foundation by incorporating new iCRISPRi and iCRISPRa systems to study gene-drug interactions **(Figure 3)**. According to the literature, many genes directly involved in DNA repair and the response to cisplatin encode DNA/RNA-binding proteins. Therefore, we selected a new DNA/RNA-binding sgRNA library that includes GO terms such as RNA polymerase II complex, mediator complex, mRNA spliceosome, nucleotide excision repair, damaged DNA binding, histone modification, and chromatin remodeling complex. This comprehensive approach robustly validated the screen results, and our data further supported this conclusion. **We have added more details in the revised manuscript. Please see lines 135-138 and 190-196, reprinted below:**

*"In a pilot experiment to test the feasibility of CRISPR screens in organoids, we transduced a validated pooled lentiviral library of 12,461 sgRNAs targeting 1,093 membrane proteins, each with ~10 sgRNAs/gene, and 750 negative control non-targeting sgRNAs **(Table S1)** into Cas9-expressing TP53/APC DKO organoids. "*

*"Since many genes directly involved in DNA repair and cisplatin response encode DNA and RNA binding proteins, we designed two customized CRISPRi and CRISPRa sgRNA libraries specifically targeting an identical group of 1,952 genes encoding DNA and RNA binding proteins **(Table S3)**. This targeted gene set includes key GO terms associated with cisplatin responses, such as RNA polymerase II complex, mediator complex, mRNA spliceosome, nucleotide excision repair, damaged DNA binding, histone modification, and chromatin remodeling complex."*

We did not consider using a genome-wide screen primarily because of budget constraints. Additionally, applying a targeted sgRNA sublibrary enables a higher resolution on genes of interest. We recommend utilizing such sublibraries for CRISPR screens in human organoids to achieve this goal. In the revised manuscript, we discuss the potential future application of genome-wide screens in organoids to investigate gene-drug interactions. **See lines 559-560, reprinted below:**

"Our study provides a valuable reference for future applications of large-scale chemical profiling or genome-wide CRISPR screens in primary human organoids."

- The study would be strengthened by broader validation. While the study demonstrates findings in TP53/APC DKO organoids, it is unclear if the response generalizes to other organoid models. Expanding validation to at least one additional model (patient-derived or more diverse organoid) will strengthen the work. In addition, the motivation to use the organoid model, which largely lacks the variability found in patient tumors, needs to be clarified.

RESPONSE: We thank Reviewer #3 for their comment. Our rationale for using engineered *TP53/APC* DKO organoids is grounded in their relatively homogeneous genetic background, which provides significant advantages for CRISPR-based genetic screening. In comparison to patient-derived organoids, the more controlled nature of the engineered organoids enhances the interpretability and reproducibility of our screening results, making them an effective system for systematically identifying oncogenic dependencies and treatment responses. This approach is aligned with our broader goal of establishing a robust CRISPR screening platform in organoids.

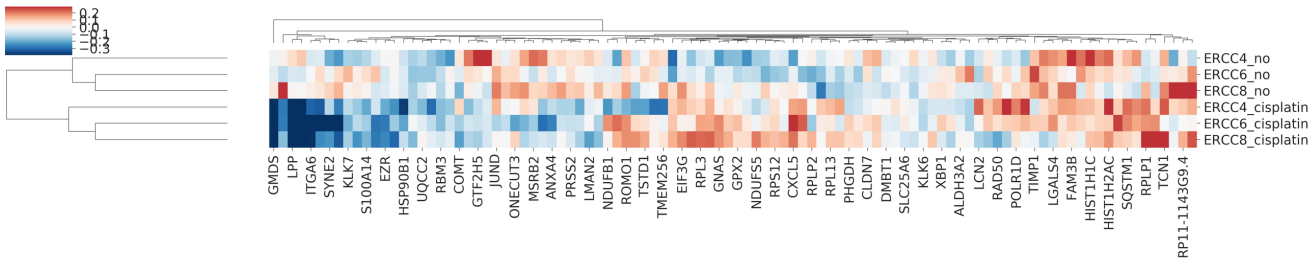
We completely agree that broader validation would enhance the study. However, due to the limited availability of patient-derived samples, we did not initially choose patient-derived organoids for this research. We have addressed these future directions in the manuscript, emphasizing our plans for further validation and translational applications. **See lines 126-128 and 551-563, reprinted below:**

"This engineered organoid model provides a relatively homogeneous genetic background, minimizing variability and enabling precise identification of gene-function relationships in CRISPR-based screens."

"Overall, our studies underscore the power of high-resolution approaches that link genotypes and phenotypes in human 3D organoids, enabling the mechanistic and unbiased dissection of gene-drug interactions. CRISPR screening platforms in CRISPR/Cas9-engineered human organoids could thus template the future exploration of multiplexed genetic interactions in primary 3D cultures. Although our current study employed oncogene-engineered tumor organoids, analogous studies could be performed in patient-derived organoids and induced pluripotent stem cell (iPSC)-induced organoids or be extended to compact genome-scale sgRNA libraries. Furthermore, the same research approach could also be applied to explore the effects of other anticancer drugs beyond cisplatin. Our study provides a valuable reference for future applications of large-scale chemical profiling or genome-wide CRISPR screens in primary human organoids. The application of organoid-based functional genomics to systematically evaluate mechanistic pathways should not only further the understanding of fundamental biological processes but also provide a robust avenue for drug-target interaction discovery."

- It is unclear what is driving the relationship between perturbation in 5B-C. The main grouping that appears upon treatment is the association of NER factors. Is this only due to viability phenotypes, or are there additional expression changes that characterize the grouping? Similarly, in 5B, are there pathways modulated in the absence of drug treatment by the genetic perturbation that make sense? Confidence that the associations are not random in 5B would strengthen the manuscript.

RESPONSE: We would like to thank Reviewer #3 for highlighting this point. We have now plotted the gene expressions for the main NER factors, both with and without cisplatin, demonstrating that specific changes in expression are likely to drive the grouping **(see Reviewer Panel 5 below)**.

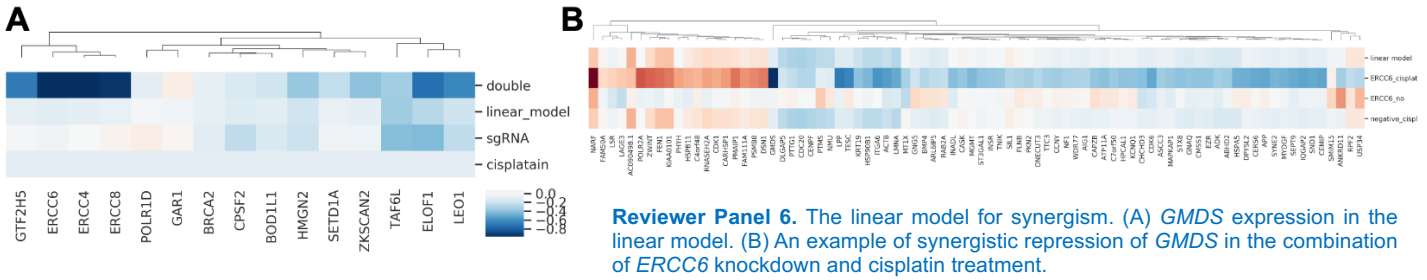


Reviewer Panel 5. Correlation of genes between two independent replicates for iCRISPRi and iCRISPRa screens.

- The approach to arrive at an additive model presented in Figure 5 is overly simplistic. How well can the additive model be generalizable by summing up two pseudobulk conditions given a high dropout rate scRNA seq and the number of cells captured per condition? For the extreme of GMDS, the assumption appears to work, but it is unclear if this would work for all genes. In addition, phenotypes like incomplete penetrance would lead to adding over heterogeneous groups of cells even if the actual effect is additive, and there are frameworks to explicitly test for synergism. The manuscript would be strengthened by demonstrating that the results in Figure 5D (3. Additive model) are not driven by noise.

RESPONSE: We thank Reviewer #3 for this comment. We have now implemented another sophisticated model for synergism and tested its results. Following the epistasis model developed by Norman et al., Science 2019, we can fit the model by linear robust regression **(see Reviewer Panel 6 below)**. Briefly, we sought to determine the extent to which each dual gene-drug phenotype (ab) could be explained by each independent perturbation of the gene and drug (a and b). We decomposed the transcriptional changes caused by dual perturbations into components explained by each of the single perturbations by fitting a robust Theil-Sen regressor (scikit learn TheilSenRegressor with parameters fit_intercept=False, max_subpopulation=1e5, max_iter=1000, random_state=1000): $\delta ab = c1\delta a + c2\delta b + e$. This model outputs a predicted dual-perturbation phenotype, which can then be compared to the observed phenotype. This allows the detection of neomorphic behavior (i.e., gene expression changes not explained by the phenotype of either single perturbation). However, regardless of the approach, single-cell methods have the limitation of high dropout rates. For lowly expressed genes, the prediction would inevitably be less accurate. We partially addressed this by considering only genes with a mean expression greater than 0.25 UMI per cell. Notably, regardless of whether it is an additive model or a linear model, GMDS is one of the most significantly repressed genes from our experiments. We agree with the reviewer that we

cannot entirely rule out whether these models would work for all genes. For our follow-ups, we only considered the genes with the largest effect size and provided functional validation related to GMDS to verify this finding.



Reviewer Panel 6. The linear model for synergism. (A) *GMDS* expression in the linear model. (B) An example of synergistic repression of *GMDS* in the combination of *ERCC6* knockdown and cisplatin treatment.

- A statement on data and code availability is lacking. There do not appear to be factors that would limit data sharing for the study. The authors should clarify if that is not the case.

RESPONSE: We thank Reviewer #3 for this important point. In the revised manuscript, we have included a section on data availability and deposited the raw sequencing data to GEO. All accession codes are now noted in the manuscript. **Please see lines 870-874, reprinted below:**

"Data Availability
The data sets generated in this study are available from the corresponding authors on reasonable request. Raw and processed sequencing data were deposited into the Gene Expression Omnibus under accession codes: RNA-seq (GSE280256), ATAC-seq (GSE280257), and scRNA-seq (GSE280506)."

Minor Suggestions:
- Overall, the choice of the drug is not well motivated. This is particularly important as the study does not highlight its application to the study of other drugs or large-scale chemical profiling.

RESPONSE: We thank Reviewer #3 for their suggestions. In our study, we specifically used cisplatin to demonstrate the feasibility of employing systematic CRISPR-based genetic screens in primary human 3D organoids to explore fundamental and translational biological questions related to gene-drug interactions. Similar approaches can be applied to investigate other drugs. We have added a sentence to the discussion to highlight this broader applicability. **See lines 557-560, reprinted below:**

"Furthermore, the same research approach could also be applied to explore the effects of other anticancer drugs beyond cisplatin. Our study provides a valuable reference for future applications of large-scale chemical profiling or genome-wide CRISPR screens in primary human organoids."

- Improving figure labeling and legends is important for accessibility. Figure 4C, in particular, seems low quality, which could be due to the import/export upon submission.

RESPONSE: We thank Reviewer #3 for their suggestions. Figure 4C looks fine on our computer, so this could be an image export issue. We will follow up with the journal to ensure the image quality is high enough for publication.

- Minimal preprocessing information is included for the scRNA data. Details of computational methods still need to be included.

RESPONSE: We thank the reviewer for this comment, and we apologize for the simplified description of our computational methods, which we followed as published methods. We have now significantly expanded the details of our computational methods. We have now included details of the code in the supplementary information, as well as links to GitHub pages with example, Python notebooks.

Reviewer #4 (Remarks to the Author): expert in ATAC-seq, CRISPR screens and single-cell OMICS

Large-Scale CRISPR Screening in Primary Human 3D Gastric Organoids Enables Comprehensive Dissection of Gene-Drug Interactions

Summary
Author present a large-scale CRISPR screen in gastric cancer organoids. They first show the validity and robustness of their screen, and inferring how each sgRNA affects cellular growth, with increasing or decreasing sgRNA abundance inferring growth advantages or disadvantages, respectively. They then design a custom panel of targeting genes which they suppress or activate in the subsequent CRISPR screens. They perform an activation or repression screen, both with and without cisplatin treatment. The analysis of the screen is well done with sufficient explanation of the reasoning behind each step. Drug sensitization is defined as a significant depletion in cell viability, i.e. lower number of gRNAs present in the dataset, compared to drug treatment in absence of the sensitization.

They end up with 41 novel cisplatin sensitization targets and focus on further validating one of them. However, this transition feels abrupt and requires a clearer connection. As TAF6L is not the top-ranked hit and showed substantial variability between replicates, the authors should provide a rationale for prioritizing this gene for further study. While it is reasonable to consider prior knowledge or biological relevance in selecting candidates, a brief explanation in the manuscript would help bridge the two parts of the study and ensure that the validation experiments are cohesively integrated into the overall narrative.

RESPONSE: We sincerely appreciate the positive comments from Reviewer #3. We selected TAF6L because all of our top 16 hits have previously been reported to have direct or indirect roles in DNA repair, ranging from ERCC6 to HELQ, as summarized in Figure 3D. Moreover, the ZKSCAN2 knockdown resulted in a significant growth defect (Figure S4B), which makes TAF6L the highest-ranking unreported hit on our screen. We have provided additional rationale in the revised manuscript to enhance the transition in this section. **Please see lines 416-419, reprinted below:**

*"To identify potential novel genes involved in cisplatin sensitivity, we focused on TAF6L, as it is the highest-ranking unreported cisplatin sensitization hits identified from our CRISPR screens **(Figure 3D)**, aside from ZKSCAN2, whose knockdown causes a growth defect phenotype **(Figure S4B)**."*

Major concerns
- Methods section requires more detail
The methods, particularly for bioinformatic analyses, are insufficiently described. Both the experimental and computational approaches need clearer documentation. Additionally, the authors should make their code publicly available (e.g., via GitHub) and provide detailed parameters within the manuscript. Specific issues include:

o ATAC-seq: The cut-off used to define differentially accessible peaks is not stated, nor are the parameters for peak calling. The reported number of differentially accessible peaks appears unusually high and should be clarified.

RESPONSE: We thank Reviewer #4 for raising this important point. We have added more details regarding ATAC-seq peak calling in the revised manuscript. **Please refer to lines 831-835, provided below:**

"Peak calling was performed using MACS2 callpeak (Galaxy Version 2.2.9.1+galaxy0) with the following parameters: --shift -100, --extsize 200 (to account for Tn5 transposase-specific 9 bp insertions), --gsize 2.7e9, --nomodel, and --qvalue 0.05. For identifying differentially accessible peaks, two BED files (treatment and control groups) were analyzed using MACS2 with the same cutoff (--qvalue 0.05)."

o scRNA-seq: The manuscript mentions that "a Random Forest classifier was trained" for differential gene expression analysis, but no further details are provided. As this is not a standard analysis for single-cell RNA-seq data, the authors should describe the process thoroughly and share the corresponding code.

RESPONSE: We thank Reviewer #4 for this comment, and we apologize for our simplified description of our computational methods, as we followed published methods. We have now significantly expanded the details of our computational methods. We have now included details of the code in the supplementary information, as well as links to GitHub pages with example Python notebooks.

- Biological and technical replicates
In both the CRISPR screens and several validation experiments, replicates are either absent, not shown, or not accounted for in the analysis. This pertains to experiments depicted in Figure 2D, Figure 2E, Figure 3E, Figure 3F and Figure 5D. Furthermore, for all western blot experiments, an uncut and unmodified image should be put in the supplement for all western blot experiments. Proper documentation and inclusion of replicates are essential to ensure the robustness of the findings.

RESPONSE: We thank Reviewer #4 for their comments and apologize for any lack of clarity. We would like to clarify that all our experiments include independent experiments or technical replicates, which are now explicitly noted in the manuscript or figure legends. For example, our CRISPR KO, CRISPRi, and CRISPRa screens were conducted with two completely independent screens (different transductions, and experiments were performed at different times), ensuring reproducibility. Furthermore, our CRISPRi and CRISPRa systems have been rigorously validated throughout this study, including using different sgRNAs to regulate various gene expressions (Figures 2D, 2E, 6A, etc.), demonstrating the robustness of our approach. In Figures 3E and 3F, we have incorporated error bars for each data point in the revised manuscript to help readers assess the variability of these experiments. We have carefully reviewed our work and included all relevant information in the revised manuscript to ensure clarity and transparency. We have made our best effort to provide unmodified Western blot images in Figure S8.

- Data availability
At the time of review, there is no mention of sequencing data (raw or processed) being deposited in a public repository. Additionally, the computational code has not been made accessible to reviewers or the wider scientific community. This omission undermines reproducibility and should be addressed prior to publication.

RESPONSE: We appreciate Reviewer #4 for highlighting this important point. In the revised manuscript, we have included a section on data availability and deposited the raw sequencing data to GEO. All accession codes are now provided in the manuscript. **Please refer to lines 870-874, reprinted below:**

"Data Availability
The data sets generated in this study are available from the corresponding authors on reasonable request. Raw and processed sequencing data were deposited into the Gene Expression Omnibus under accession codes: RNA-seq (GSE280256), ATAC-seq (GSE280257), and scRNA-seq (GSE280506)."

Minor concerns
- Data visualization improvements
o Figure 4C and Figure 5G: Ensure font sizes are consistent and legible across all figures.

Thank you. We have reviewed all the figures and will collaborate with the journal to ensure the image quality is suitable for publication.

o Figure 5A: Remove hits that were not tested with Perturb-seq to avoid confusion.

RESPONSE: We have labeled hits tested in Perturb-seq in yellow boxes (Figure 5A is now Figure S6C).

o Figure 4G and Figure 4H: These figures are difficult to interpret. Consider replotting the data as enrichment over the normal genomic distribution (e.g., a lollipop plot) to better visualize regions of significant enrichment and aid in data interpretation.

RESPONSE: Figures 4G and 4H are not in the manuscript. However, we have revisited all the figures to ensure that they will be of high quality upon publication.

o Figure 4F: The figure is unclear. Move the scale bar to the bottom for improved readability or try replotting for clarity.

RESPONSE: Thank you. Figure 4F is a bubble chart representing gene set enrichment analysis (GSEA). It highlights significant changes in the gene sets of individual perturbation subpopulations compared to control cells.

o Figure S1C: In addition to the overall agreement between replicates, a correlation plot for their top hits should be shown. This approach allows for a more detailed assessment of variability and consistency for their top hits. Visualizing data per gene would help identify specific genes with high or low agreement, which may be obscured when examining the overall distribution.

RESPONSE: We have now included correlation plots for both the sgRNA and gene levels **(Figure S2D)**. We recognize that the CRISPRa correlations are more modest but consistent and in the same range as previous CRISPRa screens (for example, PMID: 30575746 published by the Doench group). We have also included the rankings in the Supplementary Tables to assist readers in identifying genes with high or low agreement.

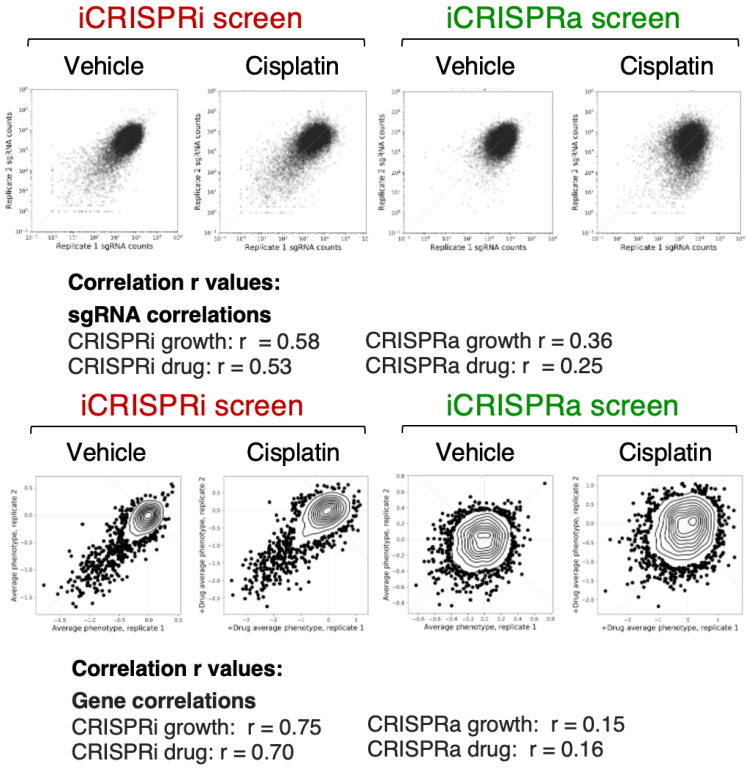


Figure S2D. Correlation of sgRNAs and genes between two independent replicates for iCRISPRi and iCRISPRa screens.

- Cell-type specific effects in tumor cells vs. healthy cells present in the tumor microenvironment Investigating how gene knockdown impacts different cell types (e.g., healthy vs. transformed cells) within the tumor microenvironment could strengthen the study. This would provide a deeper understanding of treatment vulnerabilities in gastric cancer. However, this is a suggestion rather than a requirement for publication.

RESPONSE: This is a good point. We thank Reviewer #4's suggestion.

Conclusion

Overall, this manuscript is well-written and presents significant findings that advance our understanding of gene-drug interactions in gastric cancer. While some improvements are necessary, particularly in the methods section, data availability, and figure clarity, the study is strong and deserving of publication.

RESPONSE: We are truly grateful to Reviewer #4 for these supportive comments.

Reviewer #5 (Remarks to the Author): ECR, co-reviewed with Reviewer #1

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 appreciate Reviewer #5's comments.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have submitted a revised manuscript on their work on cisplatin resistancy using CRISPR screening in gastric organoids. In my view the revised manuscript does not come with enough substantial changes. Although the data around cisplatin sensitivity and GMDS are indeed interesting and now read better, I do not see that previously raised issues regarding all other aspects of the work that were previously raised have been addressed. Details are below.

Major comments:
1. There is no good motivation for the KO screen (Figure 1). If it was done to show that drop-out screening is possible in organoids, such work has already been performed. The screen quality is suboptimal – around 50% of essential genes are accurately identified. The authors speculate this might have to do with 3D vs. 2D culture differences, yet the cited work to support this claim (PMID: 32238925) has not identified any differences in core essential genes, but rather in novel vulnerabilities in 3D culture.

RESPONSE: We thank Reviewer #1 for raising this point regarding the CRISPR KO screen. We would like to clarify that this concern was addressed in our original Response to Referees Letter (see Response to Reviewer #1, Question #1). Specifically, our screen identified **65%** (13/20) of essential genes based on the Hart et al. (2014) core fitness gene set, and **53.8%** (14/26) based on DepMap common essential genes, both higher than the "~50%" cited in the reviewer's comment.

We also acknowledge that direct comparisons across CRISPR screens can be influenced by multiple factors, including differences in analytical thresholds, gene sets, cell types, and culture conditions (e.g., 2D vs. 3D). **These were mentioned in our initial response not as definitive explanations, but as possible considerations for contextualizing screen performance.** To enhance clarity and transparency, **we have now included a brief discussion of these limitations in the revised manuscript (Lines 553-556).**

As emphasized previously, the KO screen was designed not to reproduce prior findings but to establish and optimize pooled CRISPR screening in 3D human organoids, a technically demanding platform that requires empirical fine-tuning. Despite reports of similar approaches in other contexts, adaptation to primary human organoids demands methodical validation. The identification of known essential genes, along with successful follow-up validation of selected hits, confirms the feasibility of our approach. This pilot experiment laid the groundwork for the development of our subsequent iCRISPRi and iCRISPRa platforms, which enabled the targeted dissection of gene-drug interactions in human organoids.

2. The authors have not included any comments about the CRISPRa screen, maintaining that the screen is of high quality. The two replicates have correlation of 0.25 for gRNAs and 0.16 for genes under the drug treatment. This means that each time a screen is performed largely a different set of hits is identified. I wonder why the quality of both KO and CRISPRa screens is subpar. The authors keep 1000x coverage after expansion but do not say what coverage is achieved on the day of infection. If the coverage at infection is 50x and cells are expanded to 1000x and maintained at 1000x, the real coverage is still 50x.

RESPONSE: We thank Reviewer #1 for their additional comments. We would like to clarify that this concern regarding the CRISPRa screen was already addressed in our original Response to Referees Letter (see Response to Reviewer #1, Question #3). As we stated, we fully agree that CRISPRa screens are more technically challenging and inherently less robust, likely due to variability in transcriptional activation that depends on chromatin accessibility and endogenous gene regulation. We also acknowledged in our response that the relatively low correlation between replicates is a known limitation in CRISPRa-based screening, as supported by prior studies (e.g., PMID: 30575746), and is not uncommon in the field. Nevertheless, our identification of *MYC* as the top hit validates the biological relevance of the screen and is consistent with known roles of *MYC* in cell proliferation. **In light of the reviewer's concern, we have further revised the manuscript to tone down our presentation of the CRISPRa screen and explicitly discuss its limitations in the Discussion section.** We hope this clarifies our position and demonstrates our careful consideration of the reviewer's feedback**. New sentences have been added or edited to the revised manuscript, reprinted below:**

Lines 185-187. *"Overall, the inducible genetic knockdown (iCRISPRi) and overexpression (iCRISPRa) systems enabled effective modulation of individual target gene expression in organoids, demonstrating their utility for precise gene regulation in a 3D context."*

Lines 216-219. ***"****Our data for each screen, which included two independent experiments as replicates, showed high consistency in the iCRISPRi screen, while the iCRISPRa screen exhibited lower replicate correlation, consistent with known technical challenges associated with CRISPRa-based perturbations[43]* ***(Figure S2D)****."*

Lines 258-259. ***"****These results suggested that the CRISPRi screens provide a strong capability to uncover genes associated with cisplatin sensitivity."*

Lines 504-510. "*In line with this, using iCRISPRi screens, we identified numerous hits already known to participate in DNA damage response to cisplatin treatment, such as genes involved in nucleotide excision repair (NER), homologous recombination repair (HRR), and Fanconi anemia (FA) pathways. Interestingly, most genes with strong effects on cisplatin sensitivity were identified exclusively in the iCRISPRi screens, but not in the iCRISPRa screen. This is consistent with the idea that genetic knockdown and overexpression of a given target may not produce reciprocal phenotypes due to saturation effects from homeostatic gene expression levels."*

Lines 558-561. *"In addition, CRISPRa-based screens are inherently less reproducible than CRISPRi or knockout screens[43]. These known technical limitations likely contributed to the lower correlation observed in our CRISPRa replicates. Nonetheless, the identification of biologically relevant hits such as* MYC *supports the potential utility of CRISPRa when cautiously interpreted."*

We also thank Reviewer #1 for this important question regarding the coverage of our CRISPR screens. Indeed, prior to lentiviral transduction, we plated a number of cells corresponding to >3000X library coverage. As noted in the Methods section, we used approximately 4×10^7 cells for lentiviral transduction, and the size of our sgRNA library was approximately 12,000 guides. Given that we used an initial lentiviral transduction rate of ~30% to deliver the sgRNA library, this ensured that the representation of the sgRNA library remained 1000X immediately after transduction. From that point onward, we consistently maintained ≥1000X coverage throughout the entire screen. This approach was applied uniformly across all experiments, including CRISPR knockout (CRISPR KO), CRISPR interference (CRISPRi), CRISPR activation (CRISPRa), and their respective biological replicates. **To further clarify this point, we have added the following description to the revised manuscript, reprinted below:**

Lines 138-142. *"Following lentiviral transduction, we ensured that the number of infected cells provided a cellular coverage of >1,000 cells per sgRNA from the outset. After harvesting a subpopulation 2 days post-puromycin selection (time point 0, T0), we continued to culture the remaining organoids at this same cellular coverage (>1,000 cells per sgRNA) throughout the screening until day 28 (time point 1, T1)."*

Lines 712-718. *"Approximately 4 x 10^7 suspension (d)Cas9-expressing cells were used for lentiviral transduction with the sgRNA library (~12,500 sgRNAs), corresponding to ~3000X coverage. Lentivirus was added at an infection rate of ~30%, resulting in an effective post-transduction coverage of ≥1000X. Lentiviral transduction of organoids was performed as previously described above. Following transduction, organoids were centrifuged at 600 x g for 5 minutes and resuspended in Matrigel at a density of 25,000-30,000 cells per 40μL Matrigel."*

3. The conclusions drawn from the TAF6L story are unsupported. First, the FACS plot values and bar plot values in Figure 6E do not match. Even the individual measurements in the bar plot do not correspond to FACS values, so it is difficult to know what values to look at. Judging by the bar plot that includes triplicates, TAF6L overexpression does not recover cells from cell cycle arrest under cisplatin, although it rescues the viability (compare Figure 6D and 6E bar plot). Thus, it seems the effects have little to do with the cell cycle, contrary to what the authors claim in lines 415, 446-447, and 511. Although RNA-seq and ATAC-seq experiments are subsequently performed on TAF6L-knockdown and overexpression organoids, no clues emerge from these results. Overall, it remains unclear how TAF6L interacts with cisplatin.

RESPONSE: We thank Reviewer #1 for pointing out the discrepancy between the FACS plots and bar plots in Figure 6E. We sincerely apologize for this oversight during the revision process. During the revision, we refined the gating strategy across all replicates to improve quantification accuracy and updated the bar plots accordingly. However, we inadvertently failed to replace the original FACS plots in Figure 6E to reflect the revised gates and the bar plots. This has now been corrected, and the updated FACS plots are included in the revised figure. We have also included the gating strategy in the revised supplementary figure **(new Figure S9)** for transparency.

Reviewer #1 raised concerns about the strength of the conclusions related to the TAF6L findings. We believe this critique may stem from a misinterpretation of our stated conclusions. **Importantly, we did not claim that TAF6L regulates "cell cycle arrest" or directly modulates the "cell cycle" in the manuscript, including in lines 415, 446-447, and 511 (please see the original text from those lines below for reference).** Our conclusions were carefully framed to reflect the observed effects on cell recovery and viability following cisplatin treatment. These interpretations were consistently described in conservative terms, fully supported by our data, and endorsed by Reviewers #2 through #5.

Line 415 (new Line 419). *"TAF6L is essential for cell recovery following cisplatin treatment."*

Lines 446-447. *"These results suggested that TAF6L is critical for the proliferation required by cell recovery following cisplatin treatment."*
We edited this sentence (new Lines 450-451). *"These results suggested that TAF6L is critical for the proliferation occurring during cell recovery following cisplatin treatment."*

Line 511 (new Line 515). "*... with its knockdown preventing cells from recovering after cisplatin-induced cell death, while re-expression rescued this effect."*

We respectfully note that Reviewer #1's own observation that "TAF6L overexpression does not recover cells from cell cycle arrest under cisplatin, although it rescues the viability" aligns with the key conclusion we intended to convey. If the phrase "cell recovery" introduced any ambiguity, we are more than willing to revise it to "cell viability" in the relevant sections to improve clarity for readers.

Regarding the RNA-seq and ATAC-seq datasets, these were included in the manuscript not to suggest that the mechanism is fully defined, but rather to provide data as a resource for interested readers and to support future hypothesis generation. We agree that the precise molecular mechanism by which TAF6L modulates cisplatin response remains to be elucidated. **To emphasize this point, we have added the following sentence to the Discussion.**

Lines 522-525. *"While our findings clearly demonstrate that TAF6L influences cellular viability and proliferation following cisplatin treatment, the precise molecular mechanism underlying this effect remains to be elucidated and warrants further investigation."*

4. I have asked the authors to provide direct evidence of GMDS protein downregulation under cisplatin and any of the NER genes gRNAs. This is their primary claim – that there is a synergism between NER disruption and cisplatin in downregulating GMDS. The authors responded by providing the western blot solely under cisplatin (Figure 5E), but not investigating the purported synergism. In addition, to validate the role of GMDS, the authors chose to overexpress it, which I find not an optimal solution. Namely, the GMDS dynamic is not preserved in OE organoids under cisplatin. The expression upon the drug treatment goes up, instead of down (Figure S6H), suggesting a substantially changed context in which experiments are conducted.

RESPONSE: We thank Reviewer #1 for their continued engagement and thoughtful comments. However, we would like to clarify several points that may have contributed to some misunderstandings.

GMDS overexpression (GMDS OE) was performed as a gain-of-function functional assay specifically designed to address the reviewer's original request to establish a link between fucosylation and cisplatin sensitivity. As is standard in functional genetics, overexpression studies are widely used to assess whether increasing the expression of a gene of interest can influence a specific phenotype. It was not intended to replicate the endogenous dynamics of GMDS under cisplatin treatment, but rather to test whether restoring GMDS expression

could modulate cisplatin response. This experiment served as one component of a broader set of mechanistic validations presented in the revised manuscript. Our data clearly demonstrate that GMDS OE increases total cellular fucosylation and enhances cisplatin-induced cytotoxicity (Figures 5F-H), without altering baseline viability or DNA repair gene expression (Figure S6I and Reviewer Panel 3).

While we agree that GMDS OE alters the dynamic regulation of the gene, given that the transgene is not driven by the endogenous promoter, we respectfully note this limitation applies broadly to any genetic perturbation, including overexpression, knockout, and knockdown approaches. Such interventions inherently modify the native regulatory context. **However, this does not diminish the value of the resulting GMDS OE phenotype in testing functional consequences.** The goal of our gain-of-function experiment was to determine whether restoring GMDS expression is sufficient to modulate the cisplatin response, and the observed phenotype following GMDS rescue provides direct evidence supporting this functional link.

The reviewer raised concerns that we did not directly test the synergy between NER gene knockdown and cisplatin on GMDS protein levels. While we did not perform Western blots specifically for GMDS protein levels under dual perturbation, we noted that this experiment is technically challenging due to substantial cell death under combined NER knockdown and cisplatin treatment. Nonetheless, we have demonstrated that GMDS expression is downregulated by cisplatin treatment alone, at both the mRNA and protein levels**.** We also provided direct experimental evidence in the original manuscript showing that this dual perturbation leads to a reduction in total fucosylated proteins as endpoint assays by measuring AAL and UEA-I lectin (Figure 5D and Figure S6F). These data provide a biologically meaningful and phenotypically relevant readout of GMDS pathway activity, supporting our proposed model of fucosylation suppression under dual perturbation conditions.

Taken together, we believe the combination of (1) GMDS downregulation at the mRNA and protein level following cisplatin treatment (Figures 5C-E), (2) Functional rescue with GMDS OE (Figures 5F-H), (3) Endpoint assays of synergistic reduction of fucosylation upon *ERCC4 knockdown + cisplatin* (Figure 5D and Figure S6F), and (4) Reversal of cisplatin cytotoxicity with the fucosylation inhibitor 2F-PerAc-Fuc (Figure S6K), collectively establish a functional link between fucosylation and cisplatin sensitivity. While we acknowledge that additional mechanistic details remain to be elucidated. In the revised Discussion, we acknowledged the need for further mechanistic investigation. As stated in the original manuscript, now **lines 542-543**: *"These unexpected results suggest a potential crosstalk between DNA repair and fucosylation, highlighting the need for further investigation into its mechanistic basis."* This reflects our recognition that while our data establish a strong functional link, the precise molecular details remain an important area for future study.

Reviewer #4 (Remarks to the Author):

The authors present a revised version of their manuscript entitled “Large-Scale CRISPR Screening in Primary Human 3D Gastric Organoids Enables Comprehensive Dissection of Gene-Drug Interactions”. While some improvements have been made since the initial submission, several important issues raised in the previous round of review remain unaddressed and continue to impact the overall assessment of the manuscript.

Major points:
• Data accessibility:
The authors have deposited the raw data to the GEO database, which is appreciated. However, the required access tokens for reviewers have not been provided. For the purposes of peer review, it is essential that reviewers can access and assess the deposited data. The authors should either include the access credentials in the next revision or make the dataset publicly available.

RESPONSE: We sincerely apologize for the oversight. The access tokens for the GEO datasets were included in our initial submission and were also provided to *Nature Communications* at the time of submission. However, we understand that the reviewer may not have had access to them. For convenience, we are re-listing the GEO accession numbers along with the reviewer access tokens below:

RNA-seq (GSE280256), token: **izuxweoqfvkjfkf**
ATAC-seq (GSE280257), token: **ebmleqqcrzovver**
scRNA-seq (GSE280506), token: **itudugwanpevvaz**

We thank the reviewer for bringing this to our attention and hope this resolves any access issues for the peer review process.

• Methods transparency and code availability:
Multiple reviewers, including myself, previously noted the lack of methodological detail and code availability. Although the authors have extended the Methods section, additional critical details are still lacking. In particular, the provided GitHub links point to example notebooks authored by others who performed similar analyses, rather than the actual code used in this study. For the sake of reproducibility and scientific integrity, it is imperative that the authors share the specific code used to generate the results reported in the manuscript. Without this, a thorough assessment of the analysis and its validity is not possible.

RESPONSE: We thank Reviewer #4 for emphasizing the importance of methodological transparency and reproducibility. In response, we have uploaded the code that can be used to reproduce the analyses reported in this study to a dedicated GitHub. The link (https://github.com/jinpjchen/organoid_GI) has been included in the revised manuscript under **the “Code Availability” section (Lines 899-901)**. We hope this update fully addresses the reviewer’s concern and facilitates independent validation of our results.

• Clarification on previous feedback:
I would like to apologize for a referencing error in my previous review. I referred to Figure 4, F-G, but intended to reference Figure 6, F-G. I kindly request that the authors consider re-plotting this data to show enrichment relative to a normal genomic distribution (e.g., via a lollipop plot) to enhance interpretability and highlight regions of significant enrichment. See attached figure for clarity.

RESPONSE: We thank Reviewer #4 for the clarification regarding the figure reference. We appreciate the suggestion to improve the interpretability of the ATAC-seq results shown in Figure 6F-G. In response, we have re-plotted the enrichment data to display regions of significant ATAC signal reduction relative to the background genomic distribution. A lollipop plot has been included in the revised figure to highlight genomic features associated with differential accessibility. We hope this new visualization enhances clarity and meets the reviewer’s expectations.

• Reproducibility of CRISPR screens:
The reproducibility of CRISPRi and particularly CRISPRa replicate screens remains unclear. The apparent variability raises concerns about the robustness of the results, and it is difficult to determine whether repeating

the experiment would yield consistent findings. This concern underscores the importance of a detailed analysis of replicate agreement (see next point).

RESPONSE: We appreciate Reviewer #4's concern regarding the reproducibility of our CRISPRi and CRISPRa screens. As clarified previously, **all screens were conducted as two completely independent experiments**, involving separate lentiviral transductions performed at different times. **These are not technical replicates of the same experiment, but rather fully independent biological replicates.** This distinction is important, as the observed variability reflects the real-world complexity and biological heterogeneity inherent to organoid-based screening platforms. This information is now explicitly noted in the Methods section and figure legends.

For the CRISPRi screen, although replicate correlation coefficients may not fully reflect signal strength in complex phenotypic assays, the results demonstrate strong biological validity. In particular, the top hits identified by the screen are highly enriched for genes in canonical DNA repair pathways, including NER, HRR, and FA. This pathway-level saturation of expected hits provides functional confirmation of screen performance and reinforces the robustness and specificity of the findings beyond statistical correlation metrics.

Regarding the CRISPRa screen, we fully agree that CRISPRa screens are more technically challenging and inherently less robust, likely due to variability in transcriptional activation that depends on chromatin accessibility and endogenous gene regulation. We also acknowledged in our response that the relatively low correlation between replicates is a known limitation in CRISPRa-based screening, as supported by prior studies (e.g., PMID: 30575746), and is not uncommon in the field. Nevertheless, our identification of *MYC* as the top hit validates the biological relevance of the screen and is consistent with known roles of *MYC* in cell proliferation. **In light of the reviewer's concern, we have further revised the manuscript to tone down our presentation of the CRISPRa screen and explicitly discuss its limitations in the Discussion section.** We hope this clarifies our position and demonstrates our careful consideration of the reviewer's feedback**. New sentences have been added or edited to the revised manuscript, reprinted below:**

Lines 185-187. *"Overall, the inducible genetic knockdown (iCRISPRi) and overexpression (iCRISPRa) systems enabled effective modulation of individual target gene expression in organoids, demonstrating their utility for precise gene regulation in a 3D context."*

Lines 216-219. ***"****Our data for each screen, which included two independent experiments as replicates, showed high consistency in the iCRISPRi screen, while the iCRISPRa screen exhibited lower replicate correlation, consistent with known technical challenges associated with CRISPRa-based perturbations[43]* ***(Figure S2D)****."*

Lines 258-259. ***"****These results suggested that the CRISPRi screens provide a strong capability to uncover genes associated with cisplatin sensitivity."*

Lines 504-510. "*In line with this, using iCRISPRi screens, we identified numerous hits already known to participate in DNA damage response to cisplatin treatment, such as genes involved in nucleotide excision repair (NER), homologous recombination repair (HRR), and Fanconi anemia (FA) pathways. Interestingly, most genes with strong effects on cisplatin sensitivity were identified exclusively in the iCRISPRi screens, but not in the iCRISPRa screen. This is consistent with the idea that genetic knockdown and overexpression of a given target may not produce reciprocal phenotypes due to saturation effects from homeostatic gene expression levels."*

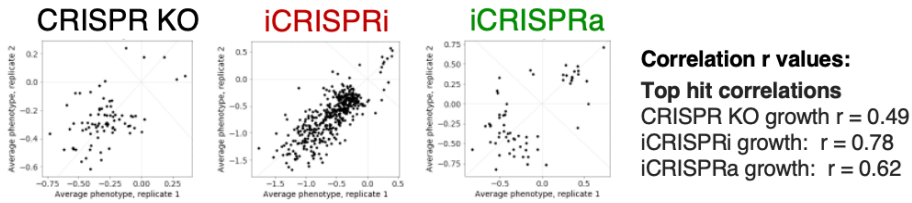
Lines 558-561. *"In addition, CRISPRa-based screens are inherently less reproducible than CRISPRi or knockout screens[43]. These known technical limitations likely contributed to the lower correlation observed in our CRISPRa replicates. Nonetheless, the identification of biologically relevant hits such as* MYC *supports the potential utility of CRISPRa when cautiously interpreted."*

• Analysis of replicate agreement:
A previously raised suggestion regarding Figure S1C has not been addressed. Specifically, it was recommended that the authors include a correlation analysis of the top hits between replicates. Such an analysis would allow for a more granular view of variability and would help identify genes with consistent versus inconsistent behavior across replicates. A focused plot showing correlation of the top 100 hits between replicates would significantly

improve the evaluation of the screen's performance, especially given potential sources of variability (e.g., transduction efficiency, gRNA efficacy, sequencing depth, and batch effects).

RESPONSE: We thank Reviewer #4 for the helpful suggestion. In response, we have included correlation analyses focusing on the top hits to improve the evaluation of replicate agreement. We used the average phenotype score to rank genes and generate the replicate correlation plots. Because the CRISPR KO and iCRISPRa screens yielded fewer than 100 significant hits, we plotted all significant hits for these datasets. For the iCRISPRi screen, which produced a larger number of significant hits. These data have been incorporated into the revised manuscript.

In addition, **we have provided complete ranking information for all hits in the supplementary tables**, including individual rankings from both independent replicates as well as the combined ranking. **This allows readers to evaluate any gene of interest within the screen results.** We hope this addresses the reviewer's concern and improves the clarity of the manuscript.



• Supplemental data (tables) description:
The supplemental tables require clearer annotation and integration with the main text. At present, it is difficult to discern what the reported values represent or how they were derived. For example, terms such as "average phenotype of strongest 3" are unclear without further methodological context. Again, this ties back to the need for code availability and improved transparency.

RESPONSE: We thank Reviewer #4 for the helpful suggestion. In response, we have reorganized and relabeled all supplemental tables to improve clarity and consistency. Regarding the term "average phenotype of strongest 3," we would like to clarify that this metric was defined in the original Methods section (now **lines 742–746**), where we stated:

"Phenotypes from sgRNAs targeting the same gene were collapsed into a single sensitivity phenotype for each gene using the average of the top three scoring sgRNAs (by absolute value, labeled as "ave phenotype of top 3 sgRNAs" in the supplementary tables) and assigned a p-value using the Mann-Whitney test of all sgRNAs targeting the same gene compared to the negative controls."

To further improve clarity, we have updated the column label in the supplemental tables to read "ave phenotype of top 3 sgRNAs." We hope these revisions address the reviewer's concerns regarding annotation and methodological transparency.

In conclusion, while the authors have taken some steps to improve the manuscript, several substantial issues remain unresolved. I encourage the authors to thoroughly address the points above, particularly regarding code availability, replicate reproducibility, and data presentation. These revisions are essential before the manuscript can be considered for publication.

We thank Reviewer #4 for the thoughtful and detailed feedback. We have carefully addressed all the points raised, including clarification of replicate reproducibility, improved data presentation, and clearer annotation of supplemental materials. We hope that these revisions have resolved the remaining concerns and clarified all outstanding issues.

Reviewer #2 (Remarks to the Author):

In the revised manuscript, the authors satisfactorily addressed the reviewers' concerns. They provide a large amount of new data and a thoughtful evaluation and interpretation of their results. The new data support the conclusions put forward by the authors.

We sincerely thank Reviewer #2 for the encouraging feedback and thoughtful evaluation, which we greatly appreciate

Reviewer #3 (Remarks to the Author):

The authors have addressed the concerns raised in my initial review. I appreciate the clarification of the rationale behind the library design choices and the implementation of a regression model, which strengthens the robustness of their approach. Additionally, the inclusion of preprocessing details and the statement on data and code availability enhances the transparency and reproducibility of the study. Overall, the revisions have improved the clarity and impact of the manuscript.

We are grateful to Reviewer #3 for the constructive comments and kind words regarding the improvements in clarity, transparency, and robustness.

Reviewer #5 (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 thank Reviewer #5 for their participation in the co-review process and for contributing to a thoughtful and constructive evaluation.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

Concerning my comments, nothing new has been added in terms of data, the authors have just changed the phrasing in a few places. All in all, the raised issues regarding different aspects of the work have therefore not been addressed.

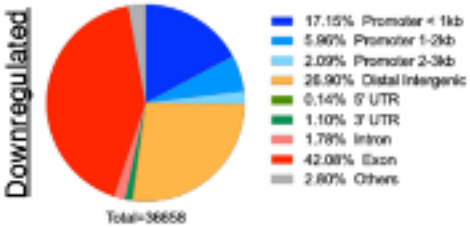
We thank Reviewer #1 for their feedback. As outlined in our previous detailed response letters, we have addressed all of the reviewer's concerns to the best of our ability, incorporating clarifications and revisions throughout the manuscript. We have no additional revisions at this time.

Reviewer #4 (Remarks to the Author):

I would like to thank the authors for carefully addressing my remaining concerns. They have taken all the necessary steps to ensure data sharing, code reproducibility, and have adequately responded to all outstanding questions. I fully support the publication of the manuscript.

We sincerely thank Reviewer #4 for their support.

TAF6L knockdown (KD)



G *TAF6L-GFP* (OE)