Cell Systems

Transparent Peer Review Record

Systematically quantifying morphological features reveals constraints on organoid phenotypes

Lauren E. Beck, Jasmine Lee, Christopher Coté, Margaret C. Dunagin1, Ilya Lukonin, Nikkita Salla, Marcello K. Chang, Alex J. Hughes, Joseph D. Mornin, Zev J. Gartner, Prisca Liberali, Arjun Raj

Summary

Initial Submission: Received Jan 13, 2021
Preprint: doi.org/10.1101/2021.01.08.425947
Scientific editor: Bernadett Gaal, DPhil

First round of review: Number of reviewers: 3
3 confidential, 0 signed
Revision invited March 16, 2021
Major changes anticipated
Revision received Feb 15, 2022

Second round of review: Number of reviewers: 1
1 original, 0 new
1 confidential, 0 signed
Accepted May 26, 2022

This Transparent Peer Review Record is not systematically proofread, type-set, or edited. Special characters, formatting, and equations may fail to render properly. Standard procedural text within the editor's letters has been deleted for the sake of brevity, but all official correspondence specific to the manuscript has been preserved.

Editorial decision letter with reviewers' comments, first round of review

Dear Arjun,

I'm enclosing the comments that reviewers made on your paper, which I hope you will find useful and constructive. As you'll see, they express interest in the study, but they also have a number of criticisms and suggestions. Based on these comments, it seems premature to proceed with the paper

in its current form; however, if it's possible to address the concerns raised with additional experiments and analysis, we'd be interested in considering a revised version of the manuscript.

As a matter of principle, I usually only invite a revision when I'm reasonably certain that the authors' work will align with the reviewers' concerns and produce a publishable manuscript. You will see in this case that while all three reviewers see value in the paper, they recommend quite a large range of extensions. I have outlined below what we see as a reasonable path forward.

Reviewers 1 & 2 recommend extending the work to other cell lines and a multi-cell-type organoid. We appreciate that the relative morphological simplicity of the MDCK cyst system is helpful for the proof of principle demonstration that is the purpose of this study, and we wouldn't necessarily expect you to include other experimental systems.

That said, what we think would be valuable is a more through analysis of the space of perturbations and morphologies of the MDCK cyst system, including exploration of where and how the relationships you've identified break down. This is along the lines of Reviewer 1's advice to study the effects of genetic perturbations and compounds that are likely to affect morphogenetic processes, as well as to look at the effect of variation in ECM and in culture conditions.

Also, Reviewer 3 raises some points that we think would be important and appropriate to address here, precisely because they are more clearly addressable in a simpler system, and because addressing them would strengthen the core of this proof of concept work. The first is the question of whether the PCs are independently regulated by a range of genetic perturbations. The second is to use a non-linear approach in addition to PCA - if the assumptions of PCA are problematic in this context and non-linear approaches could generate more insight, then this would be important to work out in the proof of concept study before application to more complex organoids.

In addition to the concerns I've touch on above, the reviewers raise some technical concerns and questions about inconsistencies within the results, all of which we hope you will able to address.

As you address these concerns, it's important that you and I stay on the same page. I'm always happy to talk, either over email or Zoom, if you'd like feedback about whether your efforts are moving the manuscript in a productive direction. Do note that we generally consider papers through only one major round of revision, so the revised manuscript would be either accepted or rejected based on the next round of comments we receive from the reviewers. If you have any questions or concerns, please let me know. More technical information and advice about resubmission can be found below my signature. Please read it carefully, as it can save substantial time and effort later.

I look forward to seeing your revised manuscript.

All the best,

Bernadett

Bernadett Gaal, DPhil
Scientific Editor, Cell Systems

Reviewers' comments:

Reviewer #1:

I fully agreee with the authors that the question of dimensionality in organoids may not have been covered to full satisfaction previously; and thus represents a restriction of both our knowledge and possibilities for investigating their "functional space". For example, this would be critical to impleement organoids formed by non-transformed cell lines like MDCK cells as a valuable assays format to investigate the effects of small molecule inhibitors on fundamental pocesses like morphogenesis. Assuming, that form follows function and morphologies reflect intrinsic differentiation programs.

One of the obvious restrictions of this articles relates to the likely restricted functional differentiation potential of MDCK cells. These are likely to be very specific for this cell line (Right? We do not know this for sure...). The observed, striking homogeneity of organoids/acini formed possibly reflects the lack of heterogeneity in this specific cell line has contributed to the rather uniform features formed in 3D cultures. This, however, may be a special feature of MDCK cells, and may ot necessarily apply for other cell lines. Thus, many of the conclusions may be of limited value, as cellular heterogeneity and composition is actually a common feature observed in many widely used cell lines...which also form circular hollow organoids. This is not even considering the complex morphologies formed by gastrointestinal cells; but even primary breast or prostate epithelial cells show more heterogeneity in the organoids they form.

The morphology of these equally rounded organoids may thus reflect the intrinsic biological differences between cell populations and their variable differentiation potential; typically a spectrum of features is observed. The same applies for many epithelial tumour lines - showing more restricted organoid formation processes than primary cells e.g. from the gut and which may yet be more complex than MDCK cells. As a consequence, the morphologies formed in other epithelial-lineage cells or cell lines may very well show very different dimensionality. So I am not entirely convinced that "our results demonstrate a general strategy for determining the ways in which organoid morphologies are either constrained or free to vary".

In addition, by expanding the scope to similar but still different, additional cell lines, one does not risk to develop a somewhat restricted practical value for other researchers. Expanding the scope to at least one or a few additional cell lines would generate a) more convincing data whatever these conclusions would be , b) raise the general interest for readers in this field of science, and c) increase the depths of biological insights that can be drawn from these observations.

The authors come closest to these imporrtant biological questions in the compound screens, and selected drug/growth factor treatments; although even these show that in most cases, MDCK organoids show rrather uniform morphologies and restricted morphometric dimensionality. Again, maybe this is simply a characteristic of MDCK cells that may or may not apply for other cell types and organoids.
The findings shown in Fig. 3 are interesting, nevertheless. However, my guess would be that most of the effects observed more or less dirently relate to altered cell proliferation, which naturally affects organoi size, and also morphologies. There seem to be few compounds that affect morphogeneisis independent of cell proliferation? (Can the authors cdiscuss this issue?).

Thus, in my opinion the biological or even biochemical relevance of the findings could be improved by

using either genetic or biochemical perturbations that are known to affect and disturb or even block acinar differentiation, for example ROCK inhibitors, Rho/Rac inhibitors, or inhibitors affecting the cortical actin cytoskeleton formation. Most of the hits are "usual suspects" affecting proliferation-promoting and differentiation-blocking pathways such as PI3Kinase, AKT and mTOR signalling; almost none of them affect morphogenetic processes or actin cytokeleton which have direct effects on morphogenesis - often without affecting proliferation and growth. Thus, I believe the value of these biochemical perturbations could be improved by increasing not the size of the screen/library, but by hand-picking compounds that affect these morphometric processes mentioned above.

This is partly addressed in Fig. 4 using known biologically relevant disturbants such as HGF, but in my opinion still lacks a focus on morphometric processes. Again, HGF may be one of the most interesting observtions here BECAUSE it seems to have a direct effect on epithelial differentiation, formation of the actin cytoskeleton...but this may be secondary as the cells may undergo a prominent trans-differentiation, away from the epithelial lineage. The results are therefore again somewhat difficult to interpret and relate to the formation of acini/organoids. Therefore, it may also not be surprising that HGF-treatment is one of the few deviations of the general low dimensionality observed in MDCK organoids. For the same reasons, I dont think that the drug combinations tested here, tgether with HGF treatment, have a very strong biological relevance. They are combining proliferation-blocking drugs with activation of a pathway that fundamentally changes epithelial differentiation potential. I think this is somehow creating a "mixed bag" situation that confuses interpretation.

A few other aspects could be implemented, or at least discussed that reflect additional factors for organoid development and differentiation. For example, it is rather well known that the microenvironment affects the way how the lumen forms (apoptosis? anoikis?), how large the lumen becomes, and the polarization of the outer cell layer. All of this may be very strongly dependent on the nature of the extracellular matrix (ECM), its density and rigidity, or even strictly dependent simply on the presence and concentration of laminins (e.g. in laminin-rich ECM preparations like Matrigel) over collagens or synthetic fiber materials. Depending on all of these external parameters, or environmental factors, the dimensionality may fundamentally change even for MDCK cells. Instead, the authors use multiple parallel approaches to show that the dimensionality in MDCK cells is INDEED restricted... which I have no doubt. But what does that tell us about the biology going on in these cultures? This is a strictly technically inclined manuscript (nothing wrong with that); it would strongly increase the significance of the paper if SOME variablilty of ECM/microenvironment were implemented. Or the other aspects outlined above.

By the way, this aspects are also partly dependent on the imaging parameters. In confocal imaging, it may pay off try reducing the typical "meniscus problem" by placing the orrganoids in a layered formation, with standard distance to the bottom of the well...for more uniform distribution and easier segmentation.

The authors mention several time the possibility to "engineer organoids", but they dont explain how this should work, or whatt would be the purpose? Isnt any mrphometric transformation already some kind of "engineering" that reflects fundamental biological processes.

Reviewer #2:
In the manuscript from Beck et al. the authors describe a method to quantify morphological features of 3D MDCK cysts and extract few morphological constrains. The manuscript is interesting but has

major points that at this point would prevent us to accept it. Major revision would be required.

1. The authors speak most of the time about organoids but they only analysis MDCK cysts. Therefore, the authors should include real organoids data or completely restate the aim of the manuscript. Moreover, MDCK cysts have been extensively analysed so the novelty of the manuscript would need to be evaluated in the results extracted from the morphospace.
2. The authors use a semi-automated method for segmentation; however, it is not clear what is the actual improvement to other 3D method. The authors should compare it to other available methods and dataset to test the applicability to other systems.
This point is not compared to other segmentation in 3D using neuronal networks. In the discussion the authors say: "but we found that most methods applied to our data would produce very good results 80% of the time, and poor results 20% of the time, which was an insufficient level of accuracy for the conclusions we wanted to draw." This is very speculative. Given the authors performed what they describe as a high throughput drug screen, they should be more thorough on evaluating and interpreting results of their screen
2. The authors use half of the MDCK cysts to then extrapolate the results to a full cysts. Whereas it is correct to extrapolate the cyst and lumen size and volume from the half of the cyst, it seems problematic to do that for the number of micro-lumens in poly-lumen phenotypes. The authors should provide the comparison for the extrapolated and real data in terms of precision for the cysts that have been acquired entirely?
3. The author then ask if: "the number of cells scale with the size of the cyst? Or, did larger cysts have the same number of cells as smaller cysts, but with larger component cells?". This biological question is a well-studied problem, and the authors should provide some literature references that make them doubt that cell size would be invariant and that the cyst growth is driven by cell division.
4. The authors claim that peripheral cell height and width are constant. This seems to not be supported by the data presented in Fig 1J, as PC1 seems to be positively correlated with cyst volume and anti-correlated with nuclear size. Could authors please elaborate on this discrepancy?
5. The author define a constant cell density constrain. This part of the study would benefit from a quantitative model that uses these constraints to model the cyst growth and show that with the found constraints they have identified a predictive model of this biological process.
6. Could authors elaborate what the mechanisms are that would dictate the minimum lumen volume? At which step in the cyst development do lumens appear, does this minimum volume require a certain number of cells?
Given that authors claim the constraints they measured govern the MDCK cyst morphogenesis, they should be able to use a model to predict when lumens would appear and how many cells are required to build a lumen.
7. In the manuscript the authors use for the features describing lumens, the mean across all lumens in the cyst, e.g. mean lumen volume. This seems problematic given the presence of cysts with either a single or multiple lumens. Authors should show whether the results would be different if they used cumulative lumen volume instead.
8. The authors show that when lumen get larger nuclei get smaller. Doesnt this contradict the cell size constraint? This might be confusing. Given that authors use number of nuclei in relation to cyst size to claim that cyst growth is mediated by increase in cell numbers rather than increased size of individual cells, this seems to be hard to consolidate.
9. The authors conclude that MDCK cysts can be represented by a limited number of dimensions. While this could be partially true, this is a little bit a trivial observation: this principle underlies virtually any dimensionality reduction method. What is misleading in this sentence is the logical jump between variance being explained by a set of principle components and morphologies being represented by a

limited number of dimensions, as all of the measured features were used to calculate the principle components mentioned and these describe covariance of multiple measurements as seen in Figure 1J.
10. The full paragraph on age dependencies is hard to interpret and follow. Moreover, there is no biological interpretation of any kind.
11. The authors say they use 1ul of the drugs They should state the concentrations.
12. The fixation timepoint for the screen was 7 days (that is not 3 or 9 as in all the previous part of the manuscript. Why this time point? in the previous section comparison was to day 9 cysts, could authors please explain why particular time points were used? And can it be then comparable to the previous analysis
13. How was the functional annotation enrichment performed on the hits? Was it controlled by the library composition?
14. Authors show that almost no compound perturbed the constrains. However, above the authors have seen a change in the constraints depending on the cyst age. Assuming the cysts grow in size over time and that age correlates with growth, drugs inhibiting cell proliferation should also affect the cell-denisy-constraint. Could authors please elaborate on this?
15. All the drug perturbation analysis is very dry and consist of a list of hits with no depth in description or analysis of the underlying biological question. Why such compound affect the constrain. What these few compounds do to change these constrains?
16. In the HGF stimulated cysts the author claim that cells organize in multilayers. How often is this observed, is this feature restricted to HGF-treated cysts? could authors please provide a figure reference? Moreover, they say that the cells are taller? Is the the full epithelium thicker with more layered cells or the cells are taller? How would HGF change these behaviors?
17. They then say that HGF qualitatively change some feature but this seems in contrast to the full quantitative workflow and they should show how this is
18. What is a "spindly cyst", Authors do not introduce this term and then start using it without much explanation.
19. The authors say: "Taken together, the morphological changes induced by HGF and another perturbation suggest that the effects of individual perturbations do not necessarily combine additively when administered simultaneously." However, it is not clear why authors chose to combine HGF stimulation with these particular inhibitors. To be able to make the authors claim at this point, they should provide evidence that crosstalk between HGF and ErbB2 /EGFR can be excluded or choose another drug to combine with that acts on an HFG-unrelated pathway.
20. The author show that some drugs are able to "cancel-out" the effect of others. Using terms like rescue might be more appropriate. Moreover, the authors should absolutely discuss the molecular events triggered by HFG stimulation and how the drugs they combine with the treatment can influence these.
21. The author conclude: "While many potential mechanisms may be compatible with our experimental data, perturbations will be required to exclude certain classes of models and establish causality". The current study restricts itself to a rather phenomenological observation. However the performed experiments include a screen, would it be possible for authors to use the data they collected to propose mechanisms that contribute to the constraints that they define?
22. The authors also say "However, it is also possible that the complexity of the underlying molecular pathways is too great and multi-faceted to ever fully relate to these constraints in an easily understood manner". We disagree with this view. While our understanding of biological systems, and multicellular systems in particular, is not complete, it is, in our opinion, still feasible (and pivotal) to relate the observations with potential underlying mechanisms. This can be achieved, for example, using parallel perturbations, an assay that was performed by the authors of this study.
23. The authors say: "It may also be possible, with sufficient perturbation, to destroy a constraint, for

example to completely decouple cyst volume from the number of cells." This is very speculative. Given the authors performed a high throughput drug screen, they should be more thorough on evaluating and interpreting results of their screen.
24. In the conclusion authors say: "Future work that quantifies what degree of segmentation accuracy is needed for a given question may guide efforts to develop segmentation algorithms". Given that this study restricts itself to a more technical angle, authors could actually answer the question that they raise in this point of discussion and elaborate more on the performance of their segmentation algorithms. Considering the authors are publishing the code they used, investing more effort into characterising their image processing package would be crucial and would benefit the community.
25. The final sentence is:" It will be interesting in the future to apply this framework to such multi-cell-type organoids to see what constraints are obeyed by the much richer feature sets associated with multi-cell-type interactions." The authors should show whether the constraints they identify and describe would be valid in at least one organoid model system that features cell type heterogeneity. Otherwise the claim the authors make in the abstract ("This quantitative framework for identifying constraints on organoid morphologies may inform future efforts to engineer organoids") remains purely speculative and unsubstantiated.

Reviewer #3: Fig 1I How did you randomize data? Is it marginal resampling or something else?

Fig 1: I am not suggesting further experiments but perhaps just a comment on whether you believe the PCs are indepdnetly regulated themselves? Meaning, do you have a sense of whether mutations would preferentially impact individually PCs or do you expect a mutation to impact all of the features in a complex fashion? Said another way, PCA, by construction, must decompose the data into orthogonal collective features but this does not mean that the system abides by this orthogonality. What evidence, or thoughts, do you have regarding this? In the absence of mutant data to address this question I think the reader would be interested to hear the authors speak to this issue. Too often the non-mathematically oriented reader sees the orthogonal axes of PCA and maps that onto an orthogonal set of regulatory mechanisms. This confusion should be preempted and addressed in my view. Furthermore, its interesting.

Fig 1: Given the paucity of data it might seem reasonable to regularize PCA. This might indeed help the authors in more categorically identifying the loading vectors. As the authors are no doubt aware, unregularized algorithms will assign non-zero entries to every coeffiecient to soak up as much variance as it can. Since data is limited I believe a regularization based analysis would bolster the statistical claims the authors make.

Fig1: A suggestion, perhaps beyond the context of this paper, is to consider a manifold learning approach. This would be a nonlinear attempt at explaining the variance. This might indeed reveal more decomposable features. I could recommend either an isomer approach or an auto encoder. Again, if the authors decide that this is beyond the scope of this paper then perhaps this suggestion should be taken as something for a future study. My rationale for this again being the standard linearity assumption inherent to PCA, and their orthogonality, can raise many issues. Perhaps before going fully nonlinear the authors, for the sake of this paper, could attempt Non-negative matrix factorization (NMF) where you know to look for 3 features. This approach would at least respect the posivitivyt of the data and NMF gives features that aren't orthogonal. This might help interpretation.

Figure 2: Why not do a sliding window for days rather than blocks? This way you can leverage the

data to its max

Fig2: I think the authors should make a stronger case for themselves. At all ages the constraints are present but their quantitative features evolve. This might be lost on the non technical reader that might interpret the language as saying "sometimes there are constraints, sometimes there aren't". The result really is that there are always constraints, at all ages, but its quantitative features evolve. This should not be lost on the non-mathematically oriented reader. Perhaps the authors at this point can suggest would might be the cause of this quantitative variation in constraint traits?

Fig2: If one did PCA on all the 3-17 day data do you recover similar PCs with one of them now correlating with time? Said another way, when you do PCA on the temporal ensemble vs a fixed day ensemble do you see quantitatively similar features or rather different? Said yet another way, does the intraday variance line up with the interday variance? There is good reason to think this since cysts live in their own world, and need not abide by our counting of days. Hence the variance at a given day should account for some of the temporal variance you see.

Fig4: The expectation of additivity is something that one can only anticipate to hold in the perturbative limit. More technically, superposition is a consequence of a linear theory. Said more plainly, one should only expect approximate additivity if the drug dose is small. Meaning small amount of one drug + small amount of other drug should even be anticipated to give an additive phenotypic effect. Large doses probe nonlinear response and all bets are off. So I believe the straw man that is constructed here is made of too much straw.

Authors' response to the reviewers' first round comments

Attached.

Editorial decision letter with reviewers' comments, second round of review

Dear Arjun,

I'm very pleased to let you know that we now have the feedback we need on your revised manuscript, the peer-review process is complete, and only a few minor, editorially-guided changes are needed to move forward towards publication. Reviewers 2 and 3 were unable to review the revised version of the manuscript, but Reviewer 1 kindly agreed to look through your responses to Reviewer 2's concerns and to give broad feedback on the revised manuscript. We are also satisfied with your revisions in response to Reviewer 3.

I've made some suggestions about your manuscript within the "Editorial Notes" section, below. Please consider my editorial suggestions carefully, ask any questions of me that you need, make all warranted changes, and then upload your final files into Editorial Manager. ***We hope to receive your***

files within 5 business days, but we recognize that the COVID-19 pandemic may challenge and limit what you can do. Please email me directly if this timing is a problem or you're facing extenuating circumstances.

As you look forward to acceptance, please do consider submitting one of the protocols you've developed in this paper to STAR Protocols, or extending this offer to one of your trainees. STAR Protocols is geared towards trainees and its key purpose is to provide complete and consistent instructions for how to conduct reproducible experiments. If you have any questions, please email starprotocols@cell.com.

I'm looking forward to going through these last steps with you. Although we ask that our editorially-guided changes be your primary focus for the moment, you may wish to consult our FAQ (final formatting checks tab) to make the final steps to publication go more smoothly. More technical information can be found below my signature, and please let me know if you have any questions.

All the best,

Bernadett

Bernadett Gaal, DPhil
Editor-in-Chief, Cell Systems

Editorial Notes

Transparent Peer Review: Thank you for electing to make your manuscript's peer review process transparent. As part of our approach to Transparent Peer Review, we ask that you add the following sentence to the end of your abstract: "A record of this paper's Transparent Peer Review process is included in the Supplemental Information." Note that this ***doesn't*** count towards your 150 word total!

Also, if you've deposited your work on a preprint server, that's great! Please drop me a quick email with your preprint's DOI and I'll make sure it's properly credited within your Transparent Peer Review record.

Figures and Legends:

Please look over your figures keeping the following in mind:

- Please ensure that every time you have used a graph, you have defined "n's" specifically and listed statistical tests within your figure legend.
- Please ensure that if you include representative images within your figures, a "representative of XXX individual cells"-type statement is made in the legend.

STAR Methods:

Please note that the STAR Methods section should be structured and the order of sections should follow the guidance outlined in the guidelines here.

Note that Cell Press has recently changed the way it approaches "availability" statements for the sake of ease and clarity. Please revise the first section of your STAR Methods as follows, noting that the particular examples used might not pertain to your study. Please consult the STAR Methods guidelines for additional information. I think you may find the guidelines here particularly helpful.

RESOURCE AVAILABILITY

Lead Contact: Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Jane Doe (janedoe@qwerty.com).

Materials Availability: This study did not generate new materials. *-OR-* Plasmids generated in this study have been deposited at [Addgene, name and catalog number]. *-OR-* etc.

Data and Code Availability:

- **Source data statement** (described below)
- **Code statement** (described below)
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

Data and Code Availability statements **have three parts and each part must be present. Each part should be listed as a bullet point, as indicated above.**

Instructions for section 1: Data. The statements below may be used in any number or combination, but at least one must be present. They can be edited to suit your circumstance. Please ensure that all datatypes reported in your paper are represented in section 1. For more information, please consult this list of standardized datatypes and repositories recommended by Cell Press.

• [Standardized datatype] data have been deposited at [datatype-specific repository] and are publicly available as of the date of publication. Accession numbers are listed in the key resources table.

• [Adjective] data have been deposited at [general-purpose repository] and are publicly available as of the date of publication. DOIs are listed in the key resources table.

• This paper analyzes existing, publicly available data. These accession numbers for the datasets are listed in the key resources table.

• [Adjective or all] data reported in this paper will be shared by the lead contact upon request.

Instructions for section 2: Code. The statements below may be used in any number or combination, but at least one must be present. They can be edited to suit your circumstance. ***If you are using GitHub, please follow the instructions here to archive a "version of record" of your***

GitHub repo at Zenodo, then report the resulting DOI. Additionally, please note that the Cell Systems strongly recommends that you also include an explicit reference to any scripts you may have used throughout your analysis or to generate your figures within section 2.

• All original code has been deposited at [repository] and is publicly available as of the date of publication. DOIs are listed in the key resources table.

• All original code is available in this paper's supplemental information.

• This paper does not report original code.

Instructions for section 3. Section 3 consists of the following statement: Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

In addition,

STAR Methods follows a standardized structure. Please reorganize your experimental procedures to include these specific headings in the following order: LEAD CONTACT AND MATERIALS AVAILABILITY (including the three statements detailed above); EXPERIMENTAL MODEL AND SUBJECT DETAILS (when appropriate)*;* METHOD DETAILS (required); QUANTIFICATION AND STATISTICAL ANALYSIS (when appropriate); ADDITIONAL RESOURCES (when appropriate). We're happy to be flexible about how each section is organized and encourage useful subheadings, but the required sections need to be there, with their headings. They should also be in the order listed. Please see the STAR Methods guide for more information or contact me for help.

Please ensure that the standardized datasets generated in this paper has been archived in at least one datatype-specific repository recommended by Cell Press (e.g. GEO, PRIDE, etc.). If your data are not standardized, we recommend that you deposit them in a general purpose repository recommended by Cell Press. Please provide your datasets' accession numbers/DOIs in Deposited Data section of the Key Resources Table. Thank you!

Please ensure that original code has been archived in a general purpose repository recommended by Cell Press and that its DOI is provided in the Software and Algorithms section of the Key Resources Table. If you've chosen to use GitHub, please follow the instructions here to archive a "version of record" of your GitHub repo at Zenodo, complete with a DOI. Thank you!

Currently, you don't have a **Key Resources Table** (KRT). Note that the key resources table is required for manuscripts with an experimental component, and if a purely computational manuscript links to any external datasets (previously published or new), code-containing websites (e.g. a GitHub repo, noting that DOIs are strongly preferred), or uses non-standard software, it needs to include a key resources table that details these aspects of the paper. Purely computational or theoretical papers that don't contain any external links and use standard software don't require a key resources table, although you're welcome to include one if you like. For details, please refer to the Table Template or feel free to ask me for help.

Thank you!

Reviewer comments:

Reviewer #1: The addition of intestinal organoids (or enteroids) as a 2nd, independent data set significantly expands the scope of the manuscript, and also the interest of potential readers in the manuscript. At the same time, it does not unnecessarily expand the length of the manuscript and is therefore a very constructive addition (and addresses the reviewers comments/suggestions directlymore complex). IN essence, it does not add any novel morphologies, or phenotypes, to the full picture, and researchers from the field of cancer research - often facing issues such as invasive phenotypes displayed by organoids - may find the software less appealing or useful for this reason alone. But I take it that the focus of the manuscript is not on this issue, and thats acceptable. The authors also address this issue in the text: more complex organoid cultures/systems will show a higher degree of dimensionality and non-linearity and will require more complex solutions.

This probably also aplies to applications in high content drug screening. The scope of the manuscript concerning different types of drugs, and mechanisms of actions with different outcome (beyond proliferation of organoids increasing their size) is limited, but it is acceptable as it will be impossible to address everything under the sun, when it comes to different morphologies that can be observed with organoids. In this regard, it is very positive for the scope of the paper that drug treatments were added that essentially change the actin cytoskeleton functions, with direct consequences for cell- and therefore organoid shapes. I am pleased the authors have picked up this specific suggestion of the reviewer as this has been beneficial also for a number of other, older studies related to the phenotypic analysis of organoids. It is therfore to be considered (in my opinion) as a standard set of compounts that adds a certain level of different mechanisms impacting on organoid integrity, shape, size and textures... potentially all at once. The authors have then also brought this in context with the HGF signalling discussed in the 1st bversion of the manuscript, which again expands the necessary biological and physiological scope of the fetures addressed here. And again, I believe this will not only make readers more confident into the presented analytical methods, it will also increase how often this manuscript will be refered/cited by others. Which should be in the interest of both the authors and the journal.

Reviewer 1

I fully agree with the authors that the question of dimensionality in organoids may not have been covered to full satisfaction previously; and thus represents a restriction of both our knowledge and possibilities for investigating their "functional space". For example, this would be critical to implement organoids formed by non-transformed cell lines like MDCK cells as a valuable assays format to investigate the effects of small molecule inhibitors on fundamental processes like morphogenesis. Assuming, that form follows function and morphologies reflect intrinsic differentiation programs.

We thank the reviewer for their enthusiasm for our work.

One of the obvious restrictions of this articles relates to the likely restricted functional differentiation potential of MDCK cells. These are likely to be very specific for this cell line (Right? We do not know this for sure...). The observed, striking homogeneity of organoids/acini formed possibly reflects the lack of heterogeneity in this specific cell line has contributed to the rather uniform features formed in 3D cultures. This, however, may be a special feature of MDCK cells, and may not necessarily apply for other cell lines. Thus, many of the conclusions may be of limited value, as cellular heterogeneity and composition is actually a common feature observed in many widely used cell lines...which also form circular hollow organoids. This is not even considering the complex morphologies formed by gastrointestinal cells; but even primary breast or prostate epithelial cells show more heterogeneity in the organoids they form.

The morphology of these equally rounded organoids may thus reflect the intrinsic biological differences between cell populations and their variable differentiation potential; typically a spectrum of features is observed. The same applies for many epithelial tumour lines - showing more restricted organoid formation processes than primary cells e.g. from the gut and which may yet be more complex than MDCK cells. As a consequence, the morphologies formed in other epithelial-lineage cells or cell lines may very well show very different dimensionality. So I am not entirely convinced that "our results demonstrate a general strategy for determining the ways in which organoid morphologies are either constrained or free to vary".

The reviewer has brought up a great point about the potential limitations of the MDCK model and consequently the potential lack of generality of our conclusions. To address this concern, we obtained a very large dataset on small intestine organoids (enteroids) from Prisca Liberali's recent work (Lukonin et al, Nature, 2020) and subjected those data to a similar analysis as we had done for our own MDCK data. Enteroids grow from cells with variable differentiation potential and exhibit considerable morphological variability in both their shape and cell types, which makes them an ideal system for further testing our framework. We found that the number of cells increases linearly with enteroid area, which we also found to be true for MDCK cysts. We also found that specific cell types also increase linearly with enteroid area, and that various small molecule perturbations could shift the exact nature (slope or intercept) of this relationship. Overall, we think the addition of these data has greatly expanded the scope of our work and its range of applicability, and we thank the reviewer for the suggestion. In addition, we have expanded on the possible limitations of our approach in the Discussion (see below).

We focused primarily on MDCK cysts for our proof of concept because of their simplicity, both morphologically and in terms of the number of cell types involved (in this case, just one cell type). We also applied our methodology to the more complex enteroid system that has several cell types that interact in various ways; however, the feature set available for each enteroid was relatively less rich due to the complexities of quantifying those features in a complex three dimensional set of cells. ***We have***

demonstrated, in principle, that our approach can determine the ways in which organoid morphologies are either constrained or free to vary. However, it is important to note that more complex organoid systems may have higher degrees of dimensionality and non-linearities that may require more sophisticated approaches and analyses. It will be interesting in the future to apply this framework to such organoids to see what constraints are obeyed, as well as to map the relationship between an organoid's morphological constraints to an organoid's functional characteristics.

In addition, by expanding the scope to similar but still different, additional cell lines, one does not risk to develop a somewhat restricted practical value for other researchers. Expanding the scope to at least one or a few additional cell lines would generate a) more convincing data whatever these conclusions would be, b) raise the general interest for readers in this field of science, and c) increase the depths of biological insights that can be drawn from these observations.

The reviewer has made a great point that the use of additional systems could help expand the generality of our findings. As highlighted above, we have now included an entirely different organoid system in our paper (enteroids), which we think will make our work more practically relevant for a broader set of researchers. In terms of the use of additional cell lines specifically, there are no particular other cell lines with an origin and behavior similar to MDCK cells (indeed, there are other MDCK cell lines, but they do not form cysts). Nevertheless, we hope the use of enteroids in our manuscript alleviates the reviewer's concerns about generality.

The authors come closest to these important biological questions in the compound screens, and selected drug/growth factor treatments; although even these show that in most cases, MDCK organoids show rather uniform morphologies and restricted morphometric dimensionality. Again, maybe this is simply a characteristic of MDCK cells that may or may not apply for other cell types and organoids.

We appreciate the reviewer's point about the relative simplicity of MDCK cysts. As mentioned, we have now included data from an entirely new and considerably more complex organoid system, enteroids (small intestine organoids). While enteroids certainly have more ways in which their morphology may vary, we nonetheless found that they obeyed similar constraints to MDCK cysts. For example, in both systems, the number of cells scaled strongly with the size of the cyst/organoid. Using the Liberali lab's screen data, we were also able to show that these constraints are often conserved, even upon perturbation. We believe these additional data provide further support for the generality of our approach.

The findings shown in Fig. 3 are interesting, nevertheless. However, my guess would be that most of the effects observed more or less directly related to altered cell proliferation, which naturally affects organoid size, and also morphologies. There seem to be few compounds that affect morphogenesis independent of cell proliferation? (Can the authors discuss this issue?).

We thank the reviewer for their interest in our findings. They also raise an excellent point about whether the cell proliferation is the underlying intermediate between the perturbations and organoid size, which we now discuss in the paper. Of the 9 perturbations we chose, 6 had some relationship to proliferation, whereas the other three targeted HDAC, a serotonin receptor class, and nuclear export. While some of these perturbations undoubtedly affect proliferation, and that may be the mechanism by which organoid morphology was affected, it is also the case that many compounds that probably affected proliferation in our screen elicited no effect, so proliferation alone cannot be the sole determinant of changes in

organoid size and morphology. We have now added some discussion of this point to the relevant section of the results (see below). We also saw similar obeying of constraints upon perturbation in the intestinal organoid data that we have now analyzed (Fig. 5), suggesting that such behavior is universal. Also, we would like to highlight that our study is primarily focused on identification of the constraints as a “grammar” for organoid morphologies. The mechanisms that may underlie those constraints are undoubtedly of major interest, but at this point, we can only speculate on them.

We further manually grouped hits for smaller and larger cysts according to their targets (Supp. Table 3-4). We selected four drugs from our list of hits from the screen that increased cyst size from groups targeting mammalian target of rapamycin, aurora kinase, phosphodiesterase, and serotonin. Similarly, we selected three drugs that made cysts smaller from groups targeting epidermal growth factor receptor, histone deacetylases, and exportin-1. Given the relatively small number of factors we were able to rigorously test, we did not perform enrichment analysis on the categories of factors that came up as targets of our drug screen. We additionally used the following perturbations we thought likely to change MDCK cyst morphology based on the literature: idelalisib, oratinib, Y-27632, NSC23766, and blebbistatin. We plated MDCK cells to form cysts, immediately added these drugs at a range of concentrations, and then grew the cysts for 9 days (Table 2). Additionally, we tested two non-drug perturbations, cell seeding density (by culturing MDCK cysts with a higher initial cell density) and dilute Matrigel. We then fixed, stained, and imaged the perturbed cysts as described above, after which we measured the same set of morphological features (Fig. 3B-C). We found that the screen hits that we expected to make cysts smaller did indeed lead to smaller cysts, but none of the ones predicted to make them larger did so. We found that increased seeding density nor dilute Matrigel had no effect on the size of the cysts. ***Note that many of the hits from the screen targeted proliferation, suggesting that perhaps effects on size were a necessary consequence of changes to proliferative capacity. However, many other drugs in the screen also targeted proliferation but had no effect on cyst size, arguing against this possibility.***

Thus, in my opinion the biological or even biochemical relevance of the findings could be improved by using either genetic or biochemical perturbations that are known to affect and disturb or even block acinar differentiation, for example ROCK inhibitors, Rho/Rac inhibitors, or inhibitors affecting the cortical actin cytoskeleton formation. Most of the hits are "usual suspects" affecting proliferation-promoting and differentiation-blocking pathways such as PI3Kinase, AKT and mTOR signalling; almost none of them affect morphogenetic processes or actin cytoskeleton which have direct effects on morphogenesis - often without affecting proliferation and growth. Thus, I believe the value of these biochemical perturbations could be improved by increasing not the size of the screen/library, but by hand-picking compounds that affect these morphometric processes mentioned above.

We thank the reviewer for the great suggestion of looking at these other pathways to perturb that are independent of proliferation. Following their suggestion, we perturbed MDCK cysts with drugs that inhibit ROCK (Y-27632), Rac (NSC 23766), and myosin II (blebbistatin). We found that Y-27632 changed all three constraints on the number of cells, cell size, and number of lumens, while, blebbistatin changed only the constraints on number and cell size, and NSC 23766 changed only the constraint on cell size. These results suggest that these qualitatively different perturbations can have the same effects on constraints as the perturbations we used previously, and thus strengthens our conclusions.

This is partly addressed in Fig. 4 using known biologically relevant disturbants such as HGF, but in my opinion still lacks a focus on morphometric processes. Again, HGF may be one of the most interesting observations

here BECAUSE it seems to have a direct effect on epithelial differentiation, formation of the actin cytoskeleton...but this may be secondary as the cells may undergo a prominent trans-differentiation, away from the epithelial lineage. The results are therefore again somewhat difficult to interpret and relate to the formation of acini/organoids. Therefore, it may also not be surprising that HGF-treatment is one of the few deviations of the general low dimensionality observed in MDCK organoids. For the same reasons, I don't think that the drug combinations tested here, together with HGF treatment, have a very strong biological relevance. They are combining proliferation-blocking drugs with activation of a pathway that fundamentally changes epithelial differentiation potential. I think this is somehow creating a "mixed bag" situation that confuses interpretation.

The reviewer has made a great point about the relevance of combining proliferation-blocking drugs with HGF. In addition to the perturbation combinations with HGF, we have now tested a few other drugs that do not affect proliferation in combination with each other. We tested sumatriptan succinate (a serotonin receptor inhibitor) in combination with Y-27632 (a ROCK inhibitor) and NSC 23766 (a Rac inhibitor); none of these drugs would be expected to directly affect proliferation *per se*. We chose to combine these drugs with sumatriptan succinate because sumatriptan succinate altered the constraints of MDCK cysts when used alone. Much like the perturbation combinations we tested with HGF, we found that the effects of these perturbations on constraints do not add or average out when combined. For example, while both sumatriptan succinate and Y-27632 alone did not change the constraint on cell volume, when used in combination the constraint on cell volume increased relative to unperturbed controls. Overall, we believe these experiments provide further evidence to support our general claim that combinations can have unpredictable effects. We have also included a discussion of this point, saying:

We then wondered how the constraints of cysts perturbed with one drug changed when the cysts were exposed to a second drug. One possibility is that doubly-perturbed cysts obeyed a set of constraints that averaged the constraints obeyed by singly-perturbed cysts (assumption of linearity). Another possibility is that doubly-perturbed cysts obeyed the same set of constraints as only one of the perturbations, suggesting that some drugs may be able to override the effects of others, or some other non-linear interaction. One might expect that linearity would hold for small doses of drug, but that nonlinear aspects of the regulatory processes may appear for larger doses. We found that sometimes one perturbation overrode the effects of the other and that sometimes doubly-perturbed cysts did not obey the same constraints that the singly-perturbed cysts did (Fig. 4J-L). Perturbations overrode the effects of one another in many combinations and for both constraints. We observed that while HGF alone obeyed a different constant-cell-density constraint, when used in combination with either lapatinib or oratinib the cysts obeyed the same constraint as unperturbed cysts. We likewise found that the effects of Y-27632 on the lumen-number-cap constraint and the effects of NSC23766 on the constant cell density constraint were both canceled out by sumatriptan succinate. We additionally found many examples where singly-perturbed MDCK cysts obeyed the same constraints, but when those perturbations were used in combination the cysts did not obey the same constraints. We found that while neither HGF, lapatinib, nor oratinib alone changed the lumen-number-cap constraint, cysts perturbed with both HGF and lapatinib or oratinib had higher lumens per cyst volume (Fig. 4J-L). We also found this to be true for sumatriptan succinate and Y-27632 for the constant-cell-density constraint. In totality, the many differences between the constraints obeyed by double-perturbed cysts and single-perturbed cysts suggests that the effects of any given perturbation do not appear to simply add together, but rather can combine in unanticipated ways. ***It is important to note that many of the perturbations affected particular classes of biological processes, such as proliferation (e.g. lapatinib, oratinib, etc) and those affecting morphological processes (e.g. HGF, Y-27632, etc). It is possible that the use of different combinations with drugs perturbing the same processes could have more predictable effects.***

A few other aspects could be implemented, or at least discussed that reflect additional factors for organoid development and differentiation. For example, it is rather well known that the microenvironment affects the way how the lumen forms (apoptosis? anoikis?), how large the lumen becomes, and the polarization of the outer cell layer. All of this may be very strongly dependent on the nature of the extracellular matrix (ECM), its density and rigidity, or even strictly dependent simply on the presence and concentration of laminins (e.g. in laminin-rich ECM preparations like Matrigel) over collagens or synthetic fiber materials. Depending on all of these external parameters, or environmental factors, the dimensionality may fundamentally change even for MDCK cells. Instead, the authors use multiple parallel approaches to show that the dimensionality in MDCK cells is INDEED restricted... which I have no doubt. But what does that tell us about the biology going on in these cultures? This is a strictly technically inclined manuscript (nothing wrong with that); it would strongly increase the significance of the paper if SOME variability of ECM/microenvironment were implemented. Or the other aspects outlined above.

The reviewer has brought up a great point about the potential that ECM could have to influence the constraints we identified. Indeed, we were very interested in the potential for ECM to affect these identified constraints. Thus, in addition to standard Matrigel conditions, we also tried to culture MDCKs cysts in dilute Matrigel, collagen, and dilute collagen. While we were unable to get MDCK cysts to grow in collagen gels, we did observe that MDCK cysts grow in dilute Matrigel obeyed the same set of constraints. These results suggest that the constraints governing MDCK cyst formation may require a certain baseline ECM composition but could be invariant to some aspects of the ECM, although certainly more work would be required to explore these concepts further. We think these points are very important and thank the reviewer for the suggestion.

By the way, these aspects are also partly dependent on the imaging parameters. In confocal imaging, it may pay off try reducing the typical "meniscus problem" by placing the organoids in a layered formation, with standard distance to the bottom of the well...for more uniform distribution and easier segmentation.

The reviewer has given many great suggestions for how to improve the imaging. Given that we have already collected and analyzed a large amount of our data, we decided not to recollect the data in this new manner, but we agree that future researchers looking to build on this work could benefit from this idea. To that end, we have added these potential improvements to quality and throughput to the discussion (see below). We will certainly explore these possibilities as we follow up on the work.

One principal technical challenge in the scaling of approaches such as the one we took here is the extraction of annotations of MDCK cyst structures from microscopy images. Our assumption was that we would need highly accurate annotations to reveal subtle constraints on MDCK cyst morphologies, and those annotations proved difficult to fully automate. For this reason, we chose to build an interface that enabled us to manually correct annotations from any algorithm. Our hope is that this approach and the software is of use to others looking to annotate images, structures, or tissues for which automated solutions have yet to be developed. Deep learning has produced great advances in automatic image segmentation (Moen et al. 2019), and it is possible that the application of these methods, once fully automated and of very high quality, would allow us to obtain much larger numbers of annotations compared to our combination of automated algorithms and manual annotation review. It is also worth considering what level of segmentation accuracy is needed for the question at hand. Future work that quantifies what degree of segmentation accuracy is needed for a given question may guide efforts to develop segmentation algorithms. ***In addition, it is possible that alternative strategies for culturing or imaging the cysts might make the images easier to segment. One such example would be to***

plate all MDCK cysts at the same distance from the bottom of the well. Future work could also use the large and highly accurate segmentations produced in this work to train deep learning models specific to this task.

The authors mention several times the possibility to "engineer organoids", but they dont explain how this should work, or what would be the purpose? Isn't any morphometric transformation already some kind of "engineering" that reflects fundamental biological processes.

The reviewer rightly pointed out that we were very vague with the term “engineer organoids”. Our goal was to develop a set of phenomenological rules that organoids could obey, with the idea being that once those rules are known, one could use those rules to manipulate organoids—for instance, if you want a bigger organoid, what rules are followed, and based on those rules, how might you be able to perturb organoids in order to achieve the intended effect? We have now clarified this point in our revised discussion (see below).

We also found that while some perturbations altered cyst parameters within constraints, others changed the nature of the constraint. ***Knowledge of which types of perturbations lead to which type of effect might aid in the development of an instruction manual for building designer organoids, potentially existing in very different parts of parameter space than normal organoids. Our framework may reveal the parameters one may be able to manipulate organoids using the rules learned by these systematic perturbations. Such organoids may have properties that make them more useful for particular applications in regenerative medicine or as disease models.*** *It may also be possible, in principle if not in practice, to destroy a constraint with sufficient perturbation. For example, the right perturbation might completely decouple cyst volume from the number of cells. Future work could search for perturbations with such effects by combining high throughput drug screens with our detailed quantification of organoid morphologies. With the ability to decouple morphological constraints, we might be able to engineer organoids to adopt entirely novel configurations.*

Reviewer 2

In the manuscript from Beck et al. the authors describe a method to quantify morphological features of 3D MDCK cysts and extract few morphological constraints. The manuscript is interesting but has major points that at this point would prevent us to accept it. Major revision would be required.

1. The authors speak most of the time about organoids but they only analyze MDCK cysts. Therefore, the authors should include real organoids data or completely restate the aim of the manuscript. Moreover, MDCK cysts have been extensively analysed so the novelty of the manuscript would need to be evaluated in the results extracted from the morphospace.

The reviewer has brought up a great point about the limitations of using only MDCK cysts as a model for our approach. To address this concern, we obtain a large dataset of enteroids, both normal and perturbed with ~2,500 drugs, from Prisca Liberali’s recent work (Lukonin et al, Nature, 2020). We applied our approach to this dataset and found that, similar to MDCK cysts, the number of cells increases linearly with enteroid area. We also found this to be true for specific cell types (enterocytes and Paneth cells). We think extending our approach to an additional system has greatly expanded the scope of our work and its range of applicability, and we thank the reviewer for the suggestion.

2. The authors use a semi-automated method for segmentation; however, it is not clear what is the actual improvement to other 3D method. The authors should compare it to other available methods and dataset to test the applicability to other systems.
This point is not compared to other segmentation in 3D using neuronal networks. In the discussion the authors say: "but we found that most methods applied to our data would produce very good results 80% of the time, and poor results 20% of the time, which was an insufficient level of accuracy for the conclusions we wanted to draw." This is very speculative. Given the authors performed what they describe as a high throughput drug screen, they should be more thorough on evaluating and interpreting results of their screen.

The reviewer has brought up a great point as to how our approach to segmentation compares to other methods. For the field of image segmentation manually reviewed segmentations are the gold standard by which segmentation algorithms can be compared. For this reason we thought it unfair to compare the segmentation results from other algorithms to our segmentation results, as our results have all been manually reviewed. Further, while fully automated segmentation algorithms do exist for segmenting nuclei and the whole organoid, we also segmented lumens, and there are no algorithms which segment lumen structures.

Nonetheless, we agree with the reviewer that our claims about the performance of other algorithms on our data was unfairly speculative. Our sole intention was to convey that the combination of automated algorithms and manual review worked best for our data, not that our approach is any better or worse than other approaches. We have revised this section of the discussion accordingly (see below).

One principal technical challenge in the scaling of approaches such as the one we took here is the extraction of annotations of MDCK cyst structures from microscopy images. Our assumption was that we would need highly accurate annotations to reveal subtle constraints on MDCK cyst morphologies, and those annotations proved difficult to fully automate. For this reason, we chose to build an interface that enabled us to manually correct annotations from any algorithm. Our hope is that this approach and the software is of use to others looking to annotate images, structures, or tissues for which automated solutions have yet to be developed. ***Deep learning has produced great advances in automatic image segmentation (Moen et al. 2019), and it is possible that the application of these methods, once fully automated and of very high quality, would allow us to obtain much larger numbers of annotations compared to our combination of automated algorithms and manual annotation review. It is also worth considering what level of segmentation accuracy is needed for the question at hand.*** *Future work that quantifies what degree of segmentation accuracy is needed for a given question may guide efforts to develop segmentation algorithms. In addition, it is possible that alternative strategies for culturing or imaging the cysts might make the images easier to segment. One such example would be to plate all MDCK cysts at the same distance from the bottom of the well. Future work could also use the large and highly accurate segmentations produced in this work to train deep learning models specific to this task.*

2. The authors use half of the MDCK cysts to then extrapolate the results to a full cysts. Whereas it is correct to extrapolate the cyst and lumen size and volume from the half of the cyst, it seems problematic to do that for the number of micro-lumens in poly-lumen phenotypes. The authors should provide the comparison for the extrapolated and real data in terms of precision for the cysts that have been acquired entirely?

We thank the reviewer for pointing out a potential problem with extrapolating morphological features of a full cyst from annotations of only half of a cyst. We agree completely, and should have more accurately described our approach. We only annotated half of each cyst and measured the

morphological features of the structures contained in that half. For example, if half of the cyst contained 3 lumens then we considered the cyst to have 3 lumens, not 6. It is of course possible that when considering the full cyst there are more (or less) than 3 lumens. As such, our approach may be adding noise to our data. However, we were unable to image the cysts in their entirety and thus it is difficult to speculate as to how much noise we are adding.

3. The author then ask if: "the number of cells scale with the size of the cyst? Or, did larger cysts have the same number of cells as smaller cysts, but with larger component cells?". This biological question is a well-studied problem, and the authors should provide some literature references that make them doubt that cell size would be invariant and that the cyst growth is driven by cell division.

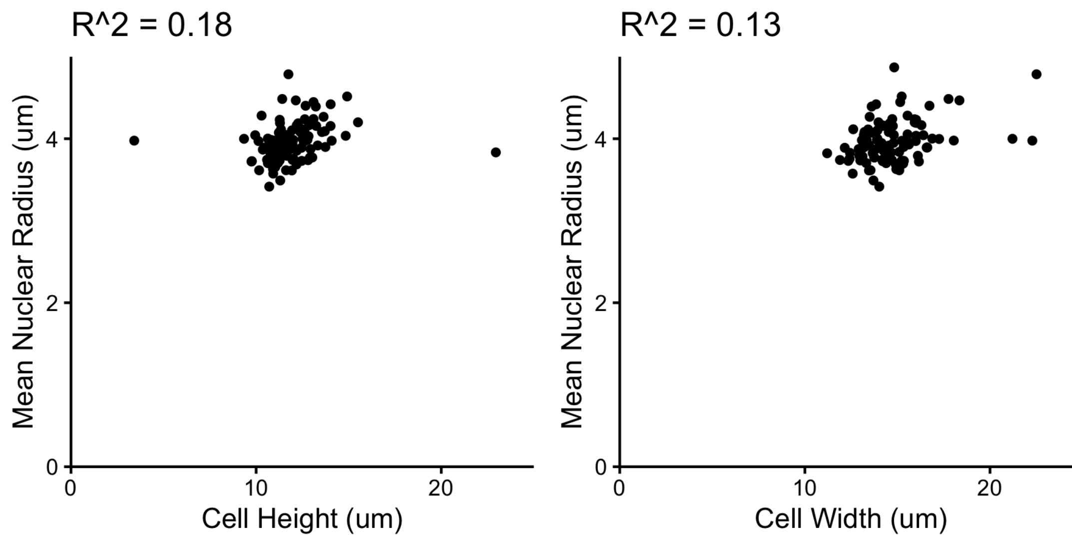
The reviewer is absolutely right that there is an extensive body of literature on these problems, and we regret the omissions. We have added a couple references in the text. We agree that the expectation would be that the number of cells would scale with the size of the cyst, and have modified our language to deemphasize the alternative hypothesis (see below).

We then wanted to find relationships between features that could potentially reflect biological constraints. For example, did the number of cells scale with the size of the cyst, as is typically the case in mammalian systems (Hafen and Stocker 2003; Savage et al. 2007)? Or, did larger cysts have the same number of cells as smaller cysts, but with larger component cells? *We used the number of nuclei as a proxy for the number of cells and found that larger cysts had proportionally more nuclei (Fig. 1B). Because cells peripheral to the lumen(s) had different morphology than those internal to the lumens, we wondered whether their number scaled differently with cyst volume. We found that the number of peripheral nuclei scaled sublinearly with cyst volume (Supp. Fig. G). Surprisingly, the number of internal cells scaled superlinearly with cyst volume, thus ensuring that the total number of cells scaled linearly with cyst volume. Given that the number of cells scaled with the cyst volume, we predicted that cell size should be independent of cyst size. We found that despite increases in cyst volume the peripheral cell height and width are fairly constant at ~9-13 μm and ~12-18 μm, respectively (Fig. 1C-D). Together, we called this set of constraints the constant-cell-density constraint.*

4. The authors claim that peripheral cell height and width are constant. This seems to not be supported by the data presented in Fig 1J, as PC1 seems to be positively correlated with cyst volume and anti-correlated with nuclear size. Could authors please elaborate on this discrepancy?

We thank the reviewer for bringing this potential confusion to our attention. We did indeed interpret cell height and cell width to be constant over a range of cyst sizes as seen in Figure 1C-D. The reviewer is right that there is a small amount of variation in cell height (~9-13 μm) and cell width (12-18 μm), which we have now included in the main text (see below).

It is definitely true that PC1 shows a strong anti-correlation with nuclear size. Nuclear size is, in principle, a different feature than cell size, and so need not be correlated to cell height or width. Indeed, we find nuclear size and cell height and width to not be correlated (see below). There is indeed some positive correlation of PC1 with cyst volume, but the magnitude of the correlation is relatively weak as compared to many of the other features.



We then wanted to find relationships between features that could potentially reflect biological constraints. For example, did the number of cells scale with the size of the cyst, as is typically the case in mammalian systems (Hafen and Stocker 2003; Savage et al. 2007)? Or, did larger cysts have the same number of cells as smaller cysts, but with larger component cells? We used the number of nuclei as a proxy for the number of cells and found that larger cysts had proportionally more nuclei (Fig. 1B). Because cells peripheral to the lumen(s) had different morphology than those internal to the lumens, we wondered whether their number scaled differently with cyst volume. We found that the number of peripheral nuclei scaled sublinearly with cyst volume (Supp. Fig. G). Surprisingly, the number of internal cells scaled superlinearly with cyst volume, thus ensuring that the total number of cells scaled linearly with cyst volume. Given that the number of cells scaled with the cyst volume, we predicted that cell size should be independent of cyst size. We found that despite increases in cyst volume the peripheral cell height and width are ***fairly constant at ~9-13 μm and ~12-18 μm****, respectively (Fig. 1C-D). Together, we called this set of constraints the constant-cell-density constraint.*

5. The authors define a constant cell density constraint. This part of the study would benefit from a quantitative model that uses these constraints to model the cyst growth and show that with the found constraints they have identified a predictive model of this biological process.

We thank the reviewer for this suggestion. We agree that a model would be helpful, but we believe creating and validating a quantitative model in a satisfying way would merit its own independent paper, given the complexities of such an undertaking. We do think this is a very exciting avenue for future research, though, and have added a line about that in the discussion.

While many potential mechanisms may be compatible with our experimental data, perturbations will be required to exclude certain classes of models and establish causality. Such molecular mechanisms, if identified, are enormously powerful and are a critical ultimate goal for molecular biology. Given the relatively small number of perturbations, it was difficult to connect pathways with phenotypes. It is possible that more extensive sets of perturbations may ultimately reveal the underlying molecular mechanisms responsible for particular constraints. However, it is also possible that the complexity of the underlying molecular pathways is too great and multi-faceted to ever fully relate to these constraints in an easily understood manner (Mellis and Raj 2015). Nevertheless, these constraints and others like them may constitute an effective “grammar” of organoid morphology that one may be able to build upon irrespective of the molecular details. ***Knowledge of the building blocks of organoid morphologies***

may also inform generative quantitative models of morphogenesis that could be evaluated for their ability to recapitulate such constraints.

6. Could authors elaborate what the mechanisms are that would dictate the minimum lumen volume? At which step in the cyst development do lumens appear, does this minimum volume require a certain number of cells? Given that authors claim the constraints they measured govern the MDCK cyst morphogenesis, they should be able to use a model to predict when lumens would appear and how many cells are required to build a lumen.

The reviewer has raised a very interesting point about being able to estimate the minimum number of cells required for a lumen to form. Our dataset actually contained several small cysts that had no lumen, so we graphed those vs. both cyst size and number of cells (Supp. Fig U). While there were some outliers, we found that there was a clear shift from 0 to 1 lumens at around 10,000 μm^3 and at 7 cells. Thus, our data suggest that a minimum of 7 cells is required to form a lumen. We have updated the main text as well:

We wondered whether other constraints were similarly affected by age. We looked at the age dependence of the lumen-number-cap constraint (Fig. 2F, Supp. Fig. N). We again found that cysts of all ages obeyed a constraint on the number of lumens per cyst volume. However, this constraint changed with age: we found that younger cysts cultured for 3 days had a higher maximum number of lumens per cyst volume, and cysts cultured for 13-17 days had a lower maximum number of lumens per cyst volume. The decrease in the number of lumens per cyst as cysts age beyond 9 days suggests that multiple lumens in a cyst are either merging or disappearing as cysts grow older. It is difficult to speculate what mechanisms might govern this age-dependent quantitative change in constraints without perturbations. However, given that very young cysts seem to obey a different set of constraints than older cysts, it may be that some of these differences are due to the establishment of apico-basal polarity around the lumen considering the work of Vasquez et al (Vasquez et al. 2021). ***Also, we noticed in young cysts that the threshold of size and number of nuclei in order to form a lumen was 10,000 μm^3 and 7 nuclei, respectively (Supp. Fig. U).*** *Together, our results point to constraints as being dynamic entities that can change as cysts grow and develop.*

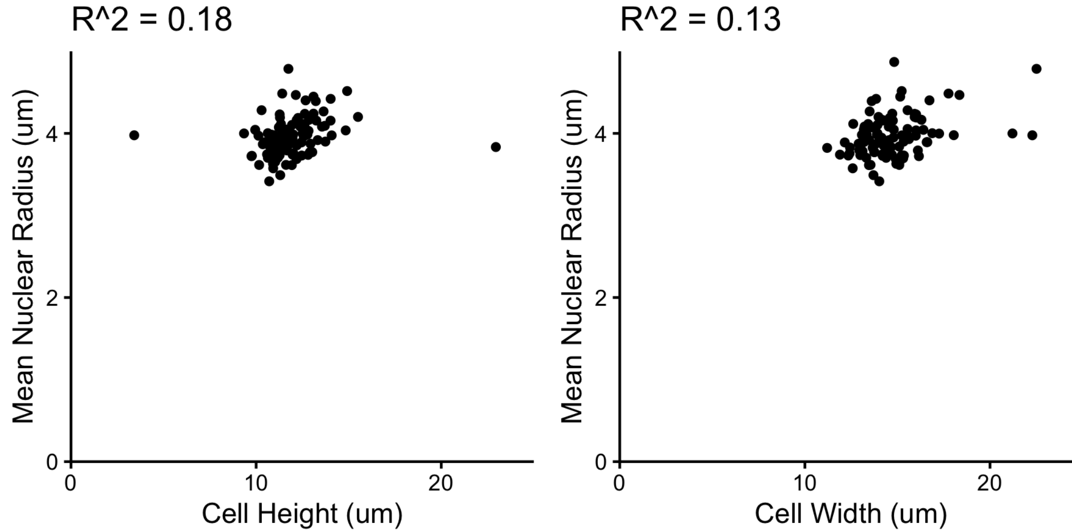
7. In the manuscript the authors use for the features describing lumens, the mean across all lumens in the cyst, e.g. mean lumen volume. This seems problematic given the presence of cysts with either a single or multiple lumens. Authors should show whether the results would be different if they used cumulative lumen volume instead.

We thank the reviewer for this helpful suggestion. We agree that using mean lumen volume may not entirely reflect all of the morphological variation in lumens, especially for organoids with one versus many lumens. For this reason, we included cumulative lumen volume (though we referred to it as total lumen volume) as one of the features we measured. As an example of what cumulative lumen volume correlates with, we observed an increase in cumulative lumen volume with cyst volume (Figure 1E).

8. The authors show that when lumens get larger nuclei get smaller. Doesn't this contradict the cell size constraint? This might be confusing. Given that authors use number of nuclei in relation to cyst size to claim that cyst growth is mediated by increase in cell numbers rather than increased size of individual cells, this seems to be hard to consolidate.

The reviewer brings up an excellent point that the trend between nuclear and lumen size and the cell size constraint seem contradictory. The cell size constraint suggests that the number, but not size of

cells, increases with increasing cyst size. However, the size of the cell and the size of the nuclei are two different properties and it need not be that larger cells have larger nuclei. We find these properties to not be correlated (see below). We have added this clarification to the text (see below).



Given that MDCK cysts obey a number of constraints, we then wondered whether these constraints are coupled. In other words, might there be a single dimension (or a few dimensions), each of which may comprise several correlated features, along which all MDCK cyst morphologies fall (Fig. 1H)? To identify dimensions in the space of MDCK cyst morphologies we performed principal component analysis (PCA) on the set of 77 cysts and their 66 morphological features. In order to apply PCA to our data, we needed to supply a single value for each feature for each cyst. For all features describing nuclei we used both the mean and standard deviation across all nuclei within the cyst, e.g. mean nuclear volume and standard deviation of nuclear volume. For features describing lumens we used the mean across all lumens in the cyst, e.g. mean lumen volume. We didn't include other higher order statistics like standard deviation because it was impossible to do so for the many cysts that had only one lumen. We found that the first three principal components respectively explain 28%, 19%, and 10% of the variation in MDCK cyst morphologies (Fig. 1I). We then wondered whether the principal components reflected any of the constraints we had previously identified. We found that the first principal component represented lumen size and inversely nuclear size, reflecting the fact that as lumens get larger, nuclei get smaller (Fig. 1J-L, Supp. Fig. H). ***Note that this trend is consistent with our earlier finding of relatively constant cell size because cell size and nuclear size are different properties.*** *The second principal component represents cyst size and number of nuclei, reflecting that increased cyst size was associated with increased number of nuclei, a relationship we previously identified as the constant-cell-density constraint. The third principal component represented the trade-off between lumen size and the number of lumens, reflecting that, for a given cyst size, in order to have more lumens, the individual lumens must be smaller (rather than there is a maximum lumen size which is independent of the number of lumens). The third principal component also represented a trade-off between nuclear size and the density of nuclei, reflecting that, for a given cyst size, in order to have more nuclei the nuclei must be smaller (Supp. Fig. I). (Consistent with PC1, we also found that nuclear size anti-correlated with lumen size.) Beyond those three principal components, the remaining components accounted for less variation than components calculated from randomized data, suggesting that those PCs likely do not reflect substantial variation in the data. In addition to performing conventional PCA on our data, we also used a sparse PCA method (Benjamin Erichson et al. 2018), which can aid with interpretation because it tries to reduce small contributions to principal components*

to zero when possible. We found that the principal components from such an analysis (after discarding the first principal component, which is most likely technical and batch variability) recapitulated similar contributions to the axes of variation revealed by conventional PCA. This analysis also had a more straightforward interpretation owing to the principal components having just a few primary contributing features (Supp. Fig. T). Thus, despite quantifying a large number of features, MDCK cyst morphologies can thus be represented by a limited number of dimensions.

9. The authors conclude that MDCK cysts can be represented by a limited number of dimensions. While this could be partially true, this is a little bit a trivial observation: this principle underlies virtually any dimensionality reduction method. What is misleading in this sentence is the logical jump between variance being explained by a set of principle components and morphologies being represented by a limited number of dimensions, as all of the measured features were used to calculate the principle components mentioned and these describe covariance of multiple measurements as seen in Figure 1J.

The reviewer has raised some interesting issues regarding our interpretation of PCA in terms of morphologies. While PCA is often used for dimensional reduction, it is by no means assured to reduce dimension; if the variance were equally spread out between a number of independent variables, it would not be possible to reduce dimension via PCA. In our case, the fact that there are a few dominant components suggests that variance can be captured by a few limited dimensions, and that suggests that one can capture all the morphologies with just a couple variables. It is certainly possible that we are not capturing all the variance with our set of features. Perhaps we are misunderstanding the reviewer's comment. Regardless, here is where we make this point in our main text:

Given that MDCK cysts obey a number of constraints, we then wondered whether these constraints are coupled. In other words, might there be a single dimension (or a few dimensions), each of which may comprise several correlated features, along which all MDCK cyst morphologies fall (Fig. 1H)? To identify dimensions in the space of MDCK cyst morphologies we performed principal component analysis (PCA) on the set of 77 cysts and their 66 morphological features. In order to apply PCA to our data, we needed to supply a single value for each feature for each cyst. For all features describing nuclei we used both the mean and standard deviation across all nuclei within the cyst, e.g. mean nuclear volume and standard deviation of nuclear volume. For features describing lumens we used the mean across all lumens in the cyst, e.g. mean lumen volume. We didn't include other higher order statistics like standard deviation because it was impossible to do so for the many cysts that had only one lumen. We found that the first three principal components respectively explain 30%, 19%, and 10% of the variation in MDCK cyst morphologies (Fig. 1I). We then wondered whether the principal components reflected any of the constraints we had previously identified. We found that the first principal component represented lumen size and inversely nuclear size, reflecting the fact that as lumens get larger, nuclei get smaller (Fig. 1J-L, Supp. Fig. H). The second principal component represents cyst size and number of nuclei, reflecting that increased cyst size was associated with increased number of nuclei, a relationship we previously identified as the constant-cell-density constraint. The third principal component represented the trade-off between lumen size and the number of lumens, reflecting that, for a given cyst size, in order to have more lumens, the individual lumens must be smaller (rather than there is a maximum lumen size which is independent of the number of lumens). The third principal component also represented a trade-off between nuclear size and the density of nuclei, reflecting that, for a given cyst size, in order to have more nuclei the nuclei must be smaller (Supp. Fig. I). (Consistent with PC1, we also found that nuclear size anti-correlated with lumen size.) Beyond those three principal components, the remaining components accounted for less variation than components calculated from randomized data, suggesting that those PCs likely do not

reflect substantial variation in the data. It is possible that if we were to measure additional morphological features we might find additional axes of variability. Nonetheless, despite quantifying a large number of features, MDCK cyst morphologies can thus be represented by a limited number of dimensions.

10. The full paragraph on age dependencies is hard to interpret and follow. Moreover, there is no biological interpretation of any kind.

We thank the reviewer for pointing out that the section on age dependencies is hard to follow. We have made substantial changes to that section to improve readability. Further, we have added some potential biological interpretations to the end of the section. We think that these changes make the section much easier to follow and thank the reviewer for the suggestion.

*We wondered whether other constraints were similarly affected by age. We looked at the age dependence of the lumen-number-cap constraint (Fig. 2F, Supp. Fig. N). We again found that cysts of all ages obeyed a constraint on the number of lumens per cyst volume. However, this constraint changed with age: we found that younger cysts cultured for 3 days had a higher maximum number of lumens per cyst volume, and cysts cultured for 13-17 days had a lower maximum number of lumens per cyst volume. The decrease in the number of lumens per cyst as cysts age beyond 9 days suggests that multiple lumens in a cyst are either merging or disappearing as cysts grow older. I**t is difficult to speculate what mechanisms might govern this age-dependent quantitative change in constraints without perturbations. However, given that very young cysts seem to obey an entirely different set of constraints than older cysts, it may be that some of these differences are due to the establishment of apico-basal polarity around the lumen considering the work of Vasquez et al (Vasquez et al. 2021).** Together, our results point to constraints as being dynamic entities that can change as cysts grow and develop.*

11. The authors say they use 1ul of the drugs They should state the concentrations.

We thank the reviewer for pointing out this omission; we have now added all drug concentrations to the Methods section (see Methods - MDCK Cyst Perturbation Experiments and Supp. Table 2).

12. The fixation timepoint for the screen was 7 days (that is not 3 or 9 as in all the previous part of the manuscript. Why this time point? in the previous section comparison was to day 9 cysts, could authors please explain why particular time points were used? And can it be then comparable to the previous analysis

The reviewer has spotted an important technical point. At the outset of the project we decided we were interested in quantifying the effects of cyst age and drug perturbations on MDCK cyst constraints. We chose 9 days as a reference point because that would give us plenty of timepoints for younger (3, 5, and 7 days) and older (11, 13, 15, and 17 days) cysts. Unfortunately, for the screen, we were limited to 7 days because the screening facility was concerned that evaporation in the plate format could ruin the experiment. We decided that for followup experiments for drugs from the screen it would be more convenient to compare the effects of age and drug perturbations if they had the same reference time point (9 days). In addition, we hypothesized that any drugs which affected MDCK cyst morphology for 7 days would continue to do so for another 2 days. We have now highlighted this difference in the Methods section (see below).

MDCK Cyst Perturbation Experiments

MDCK cysts were cultured using the above technique with the following exceptions. For drug perturbations, cysts were cultured in media containing drug throughout their entire growth (Supp. Table 2). Media was replaced every other day. For the high cell density perturbation, the cysts were plated from a cell-Matrigel suspension containing 100,000 cells/mL. Cysts were fixed and imaged on the 9th day using the protocols described above. ***Note that in the drug screen the cysts were fixed on the 7th day (because the screening facility was concerned that longer timepoints would risk media evaporation). However, to more easily compare the effects of drug perturbations with age perturbations we decided to use the same reference age (9 days).***

13. How was the functional annotation enrichment performed on the hits? Was it controlled by the library composition?

The reviewer has posed a good question about analysis of the hits from our drug compound screen. While functional enrichment would in principle be very exciting, we elected not to perform such an analysis because the screen itself consisted of assays that were relatively primitive, so it would be hard to ascribe function in a rigorous way to the constraints we identified. Moreover, it would have been very hard to validate the large number of hits required to make a strong statement in this regard. In the end, we manually grouped hits together based on common targets and then picked a candidate or two from each group for further analysis, reasoning that these would be the most promising for downstream validation. We have now made this point more clear in the Results section:

We further ***manually*** *grouped hits for smaller and larger cysts according to their targets (Supp. Table 3-4). We selected four drugs from our list of hits from the screen that increased cyst size from groups targeting mammalian target of rapamycin, aurora kinase, phosphodiesterase, and serotonin. Similarly, we selected three drugs that made cysts smaller from groups targeting epidermal growth factor receptor, histone deacetylases, and exportin-1.* ***Given the relatively small number of factors we were able to rigorously test, we did not perform enrichment analysis on the categories of factors that came up as targets of our drug screen.*** *We additionally used the following perturbations we thought likely to change MDCK cyst morphology based on the literature: idelalisib, oratinib, Y-27632, NSC23766, and blebbistatin. We plated MDCK cells to form cysts, immediately added these drugs at a range of concentrations, and then grew the cysts for 9 days (Table 2). Additionally, we tested two non-drug perturbations, cell seeding density (by culturing MDCK cysts with a higher initial cell density) and dilute Matrigel. We then fixed, stained, and imaged the perturbed cysts as described above, after which we measured the same set of morphological features (Fig. 3B-C). We found that the screen hits that we expected to make cysts smaller did indeed lead to smaller cysts, but none of the ones predicted to make them larger did so. We found that increased seeding density nor dilute Matrigel had no effect on the size of the cysts. Note that many of the hits from the screen targeted proliferation, suggesting that perhaps effects on size were a necessary consequence of changes to proliferative capacity. However, many other drugs in the screen also targeted proliferation but had no effect on cyst size, arguing against this possibility.*

14. Authors show that almost no compound perturbed the constraints. However, above the authors have seen a change in the constraints depending on the cyst age. Assuming the cysts grow in size over time and that age correlates with growth, drugs inhibiting cell proliferation should also affect the cell-density-constraint. Could authors please elaborate on this?

We thank the reviewer for bringing up this excellent point. We agree with the reviewer that cysts grow in size over time and that age correlates with growth. However, it need not be that a drug that made the cysts smaller (by inhibiting cell proliferation) would change the constraints. It's quite possible, and indeed what we found for many perturbations, that a drug can make the cysts smaller while still obeying the same constraints as the unperturbed cysts of the same age. This is why, in Figure 3, we compared the constraints of perturbed cysts to the constraints of cysts of different ages. We concluded that a perturbation changes the constraints of the cyst only if that constraint was different from the constraints of cysts of *any* age.

15. All the drug perturbation analysis is very dry and consist of a list of hits with no depth in description or analysis of the underlying biological question. Why such compound affect the constraint. What these few compounds do to change these constrains?

We thank the reviewer for raising this point. On a philosophical level, one aspect of our work was to perform perturbations in a relatively mechanism-agnostic way. Indeed, we performed a drug screen to find perturbations that would affect morphology regardless of the molecular pathways involved. Our goal was to uncover the general principles of how morphologies could change in response to perturbations of any kind. That said, we agree that the mechanisms are of great potential interest. As we point out in our response to point 21 below, we are unfortunately undersampled to make conclusive claims, but we have now included language to make clear that mechanistic underpinnings is a very promising avenue for future research.

16. In the HGF stimulated cysts the author claim that cells organize in multilayers. How often is this observed, is this feature restricted to HGF-treated cysts? could authors please provide a figure reference? Moreover, they say that the cells are taller? Is the the full epithelium thicker with more layered cells or the cells are taller? How would HGF change these behaviors?

We thank the reviewer for these suggestions. To clarify, we have added a supplemental figure (Supp. Fig. Q) that gives examples of cells in HGF-treated cysts organizing in multilayers. To be specific, we have used the term multilayers to describe the presence of more than one cell layer between the lumen and the boundary of the cyst. (We are not considering a cyst to have multilayers when there are cells in the space between lumens, which is often the case in unperturbed cysts.) When we compared 15 HGF-treated cysts and 15 randomly-chosen unperturbed cysts, we observed multilayers in 8 of the 15 HGF-treated cysts and not at all in unperturbed cysts. These results suggest that multilayers are unique to HGF-treated cysts. The multilayers themselves are quite apparent to the eye, which we have now explicitly referenced by adding a reference to Supp. Fig. Q in the results.

The reviewer has raised an important point about measuring the morphological properties of cells in the HGF conditions. It is difficult to make hard conclusions in this case because the morphologies were so radically different and harder to analyze. What we could say definitively is that the total cell volume (i.e., the non-lumen volume occupied by the organoid) was larger, both in absolute terms and per cell, in cysts treated with HGF. Thus, we made the claim that cell volume was larger. We then speculated as to potential causes for this increased volume. It could be that each cell is taller, or that the organization of the cells is different within the multi-layer structure. Given our analysis challenges, we were unable to determine which of these possibilities was the case, so we left the point speculative.

17. They then say that HGF qualitatively changes some features but this seems in contrast to the full quantitative workflow and they should show how this is.

We thank the reviewer for catching this typo. We indeed did intend to say that HGF quantitatively changes some features of MDCK cysts and have updated the text accordingly.

18. What is a "spindly cyst", authors do not introduce this term and then start using it without much explanation.

We thank the reviewer for this suggestion. We used the term “spindles” to refer to the cellular protrusions that emanate from the spherical portion of MDCK cysts exposed to HGF (see arrows in Figure 4A). There does not appear to be consistent terminology in the literature for these tubule precursors (“cytoplasmic processes” in Montesano et al, Cell, 1991 and “extension” in O’Brien et al, Nat Rev Mol Cell Biol, 2002). Nonetheless, we have changed our terminology from “spindles” to “extensions” to be more consistent with the recent literature, and have defined it more clearly in the main text.

19. The authors say: "Taken together, the morphological changes induced by HGF and another perturbation suggest that the effects of individual perturbations do not necessarily combine additively when administered simultaneously." However, it is not clear why authors chose to combine HGF stimulation with these particular inhibitors. To be able to make the authors claim at this point, they should provide evidence that crosstalk between HGF and ErbB2 /EGFR can be excluded or choose another drug to combine with that acts on an HFG-unrelated pathway.

The reviewer has raised an excellent point about the possibility of crosstalk between HGF and lapatinib (an EGFR inhibitor). We have now discussed this possibility (see below), and we have tested two additional drug combinations, Y-27632 (a ROCK inhibitor) with sumatriptan succinate (a serotonin receptor inhibitor) and NSC 23766 (a Rac inhibitor) with sumatriptan succinate. We found that the effects of these drug combinations did not add or average out when combined, much like the effects of drug combinations we tested involving HGF. For example, while sumatriptan succinate and Y-27632 alone do not change the constraint on cell volume, when used in combination the constraint on cell volume increases relative to unperturbed controls. We thank the reviewer for this suggestion as we believe it has provided additional evidence to support our claim that combinations can have unpredictable effects.

Given that HGF qualitatively changed some features of MDCK cysts, we wondered what the morphological effects would be upon combining HGF with the previously used perturbations that engendered more quantitative changes. For example, would a perturbation that produces spindly cysts (HGF) and a perturbation that produces smaller cysts yield small, spindly cysts? We perturbed MDCK cysts for nine days with either HGF alone or HGF in combination with lapatinib or orantinib (Fig. 4G). ***To ensure that any differences we noticed could not be explained by possible cross-talk between HGF and EGFR (Jo et al. 2000), we additionally perturbed MDCK cysts with sumatriptan succinate alone or in combination with Y-27632 or NSC23766.*** *We found that cysts exposed to HGF and lapatinib or oratinib had lower solidity than cysts exposed to only one of these perturbations (Fig. 4H). We found that cysts exposed to HGF, alone or in combination, were also larger, on average, than control cysts (Fig. 4I). Taken together, the morphological changes induced by HGF and another perturbation suggest that the effects of individual perturbations do not necessarily combine additively when administered simultaneously.*

20. The author show that some drugs are able to "cancel-out" the effect of others. Using terms like rescue might be more appropriate. Moreover, the authors should absolutely discuss the molecular events triggered by HFG stimulation and how the drugs they combine with the treatment can influence these.

We thank the reviewer for suggesting different terminology. We have retained the “cancel-out” terminology because the term rescue implies rescue from a deficiency of some kind (as with a deleterious phenotype of a genetic mutation). We have no notion of what “deficiency” or “normality” is for MDCK cysts, so we prefer the more neutral language; we would be happy to revisit this point if the reviewer has strong feelings on it.

21. The author conclude: "While many potential mechanisms may be compatible with our experimental data, perturbations will be required to exclude certain classes of models and establish causality". The current study restricts itself to a rather phenomenological observation. However the performed experiments include a screen, would it be possible for authors to use the data they collected to propose mechanisms that contribute to the constraints that they define?

We thank the reviewer for this suggestion. Although our data were relatively in scope so as to make comprehensive statements difficult (our screen only yielded a few strong candidates), we did try to speculate on how the mechanisms might affect the constraints. For instance, HDAC, PI3K and Aurora A kinase appeared to affect the lumen-number-cap constraint. We have added a discussion of these mechanisms to the Results and Discussion sections (see below).

Results

We then wondered how a perturbation which does change a constraint influences other constraints—if cysts perturbed with drug X do not obey the constant-cell-density constraint, must they also not obey the lumen-number-cap constraint? We found that cysts perturbed with givinostat (a histone deacetylase inhibitor), idelalisib (a phosphoinositide 3-kinase delta isoform inhibitor), sumatriptan succinate, Aurora A Inhibitor I, Y-27632, and blebbistatin had more lumens in a given cyst volume than unperturbed cysts of any age (Fig. 3F). Thus, cysts perturbed with these drugs do not obey the same lumen-number-cap constraint of unperturbed cysts, instead they obey a constraint with a larger slope. We found that some perturbations (selinexor, givinostat, idelalisib, Aurora A Inhibitor I, and NSC 23766) changed only one constraint, but others (sumatriptan succinate, Y-27632, and blebbistatin) changed both. ***Given the targets of these perturbations, we can hypothesize as to which mechanisms may regulate these constraints. We saw that exportin 1 was important for the constant-cell-density constraint, HDAC, PI3K, and Aurora A kinase for the lumen-number-cap constraint, and serotonin receptors were important to both constraints.*** *We similarly found some mechanisms perturbed MDCK cysts along a single axis in principal component space, while others perturb cysts along two axes (Supp. Fig. S), suggesting that the axes of MDCK cyst variation may not be governed by independent mechanisms.* ***These factors all work through quite different pathways; however, given the relatively small number of perturbations we analyzed, it was difficult to uncover any general rules on which pathways affected which constraints.*** *In combination, this suggests that the set of morphologies available to MDCK cysts is richer than unperturbed cysts would suggest.*

Discussion

While many potential mechanisms may be compatible with our experimental data, perturbations will be required to exclude certain classes of models and establish causality. Such molecular mechanisms, if

identified, are enormously powerful and are a critical ultimate goal for molecular biology. ***Given the relatively small number of perturbations, it was difficult to connect pathways with phenotypes. It is possible that more extensive sets of perturbations may ultimately reveal the underlying molecular mechanisms responsible for particular constraints.*** *However, it is also possible that the complexity of the underlying molecular pathways is too great and multi-faceted to ever fully relate to these constraints in an easily understood manner (Mellis and Raj 2015). Nevertheless, these constraints and others like them may constitute an effective "grammar" of organoid morphology that one may be able to build upon irrespective of the molecular details. Knowledge of the building blocks of organoid morphologies may also inform generative quantitative models of morphogenesis that could be evaluated for their ability to recapitulate such constraints.*

22. The authors also say "However, it is also possible that the complexity of the underlying molecular pathways is too great and multi-faceted to ever fully relate to these constraints in an easily understood manner". We disagree with this view. While our understanding of biological systems, and multicellular systems in particular, is not complete, it is, in our opinion, still feasible (and pivotal) to relate the observations with potential underlying mechanisms. This can be achieved, for example, using parallel perturbations, an assay that was performed by the authors of this study.

We agree with the reviewer that it is possible, as well as important, to relate observations to the underlying molecular mechanisms, and have added a sentence to our discussion accordingly. That said, we also believe that the formal possibility remains that the potential for multiple mechanisms acting simultaneously can lead to emergent phenomena that are difficult to describe to molecular details (as an example, while one can completely solve the hydrogen atom from first principles of quantum mechanics, it is not currently possible to solve larger atoms in that same way). Either way, we think it is largely a matter of opinion at this point.

While many potential mechanisms may be compatible with our experimental data, perturbations will be required to exclude certain classes of models and establish causality. ***Such molecular mechanisms, if identified, are enormously powerful and are a critical ultimate goal for molecular biology.*** *Given the relatively small number of perturbations, it was difficult to connect pathways with phenotypes. It is possible that more extensive sets of perturbations may ultimately reveal the underlying molecular mechanisms responsible for particular constraints. However, it is also possible that the complexity of the underlying molecular pathways is too great and multi-faceted to ever fully relate to these constraints in an easily understood manner (Mellis and Raj 2015). Nevertheless, these constraints and others like them may constitute an effective "grammar" of organoid morphology that one may be able to build upon irrespective of the molecular details. Knowledge of the building blocks of organoid morphologies may also inform generative quantitative models of morphogenesis that could be evaluated for their ability to recapitulate such constraints.*

23. The authors say: "It may also be possible, with sufficient perturbation, to destroy a constraint, for example to completely decouple cyst volume from the number of cells." This is very speculative. Given the authors performed a high throughput drug screen, they should be more thorough on evaluating and interpreting results of their screen.

We thank the reviewer for making this point. We have now highlighted that this point we are making is completely speculative with some text modification (see below). We agree that a high throughput drug screen could reveal perturbations that break constraints (for example by decoupling the number of cells and cyst size). Unfortunately, in our high throughput drug screen we were limited to using low

magnification. At this magnification we were only able to quantitatively assess cyst size and not the number of nuclei. However, we now discuss how future high throughput screens done at higher resolution would enable such an analysis.

We also found that while some perturbations altered cyst parameters within constraints, others changed the nature of the constraint. Knowledge of which types of perturbations lead to which type of effect might aid in the development of an instruction manual for building designer organoids, potentially existing in very different parts of parameter space than normal organoids. Our framework may reveal the parameters one may be able to manipulate organoids using the rules learned by these systematic perturbations. Such organoids may have properties that make them more useful for particular applications in regenerative medicine or as disease models. ***It may also be possible, in principle if not in practice, to destroy a constraint with sufficient perturbation. For example, the right perturbation might completely decouple cyst volume from the number of cells. Future work could search for perturbations with such effects by combining high throughput drug screens with our detailed quantification of organoid morphologies. With the ability to decouple morphological constraints, we might be able to engineer organoids to adopt entirely novel configurations.***

24. In the conclusion authors say: "Future work that quantifies what degree of segmentation accuracy is needed for a given question may guide efforts to develop segmentation algorithms". Given that this study restricts itself to a more technical angle, authors could actually answer the question that they raise in this point of discussion and elaborate more on the performance of their segmentation algorithms. Considering the authors are publishing the code they used, investing more effort into characterising their image processing package would be crucial and would benefit the community.

We thank the reviewer for this suggestion. Our approach to segmentation involved manually reviewing the candidate segmentations from various algorithms, and correcting the segmentations as needed. As such, we think our results are perhaps less about the algorithm we chose (because the data are ultimately manually curated for the analysis anyway), but rather may be of use as a gold-standard for future studies. In our revision, we now emphasize (see below) that our software has an interface for manual correction of segmentations, which we think may be the most useful part of our pipeline for others to use.

One principal technical challenge in the scaling of approaches such as the one we took here is the extraction of annotations of MDCK cyst structures from microscopy images. Our assumption was that we would need highly accurate annotations to reveal subtle constraints on MDCK cyst morphologies, and those annotations proved difficult to fully automate. ***For this reason, we chose to build an interface that enabled us to manually correct annotations from any algorithm. Our hope is that this approach and the software is of use to others looking to annotate images, structures, or tissues for which automated solutions have yet to be developed.*** *Deep learning has produced great advances in automatic image segmentation (Moen et al. 2019), and it is possible that the application of these methods, once fully automated and of very high quality, would allow us to obtain much larger numbers of annotations compared to our combination of automated algorithms and manual annotation review. It is also worth considering what level of segmentation accuracy is needed for the question at hand. Future work that quantifies what degree of segmentation accuracy is needed for a given question may guide efforts to develop segmentation algorithms. In addition, it is possible that alternative strategies for culturing or imaging the cysts might make the images easier to segment. One such example would be to plate all MDCK cysts at the same distance from the bottom of the well.*

Future work could also use the large and highly accurate segmentations produced in this work to train deep learning models specific to this task.

25. The final sentence is:" It will be interesting in the future to apply this framework to such multi-cell-type organoids to see what constraints are obeyed by the much richer feature sets associated with multi-cell-type interactions." The authors should show whether the constraints they identify and describe would be valid in at least one organoid model system that features cell type heterogeneity. Otherwise the claim the authors make in the abstract ("This quantitative framework for identifying constraints on organoid morphologies may inform future efforts to engineer organoids") remains purely speculative and unsubstantiated.

The reviewer has brought up a great point about the limitations of applying our approach only to MDCK cysts. To address this concern we obtained a large dataset on small intestine organoids (enteroids) from Prisca Liberali's group (Lukonin et al, Nature, 2020). We subjected the enteroids to similar analysis as we had for MDCK cysts. We identified a set of constraints that enteroids obey, including a linear relationship between the number of cells and enteroid area, as well as small molecule drug perturbations that change the nature of these constraints. Notably, the enteroids have multiple cell types and we found that at least two of those types (enterocytes and Paneth cells) also scale with enteroid area. We think extending our analytical framework to an additional organoid system has greatly improved the scope of our paper and we thank the reviewer for the suggestion.

Reviewer 3

Figure 1I: How did you randomize data? Is it marginal resampling or something else?

We thank the reviewer for pointing out this omission. Our data was stored in a table where each row represented one MDCK cyst and each column represented one morphological feature. For our randomization analyses, we shuffled each column of the table. We have now clarified this in the Methods section (see below).

PCA and Linear Models

In order to run PCA we first standardized the units of our features. We took the cube root of all volume features, the square root of all surface area features, and the inverse of the number of lumens. We then z-score normalized each feature. We ran PCA using the prcomp function from the R's stats package (https://www.rdocumentation.org/packages/stats/versions/3.6.2). To estimate how much variance we could expect to be explained due to chance, we also ran PCA on randomized data. ***To randomize the data, we shuffled each column of a table where each row represents one cyst/enteroid and each column represents one morphological feature.***

Figure 1: I am not suggesting further experiments but perhaps just a comment on whether you believe the PCs are independently regulated themselves? Meaning, do you have a sense of whether mutations would preferentially impact individually PCs or do you expect a mutation to impact all of the features in a complex fashion? Said another way, PCA, by construction, must decompose the data into orthogonal collective features but this does not mean that the system abides by this orthogonality. What evidence, or thoughts, do you have regarding this? In the absence of mutant data to address this question I think the reader would be interested to hear the authors speak to this issue. Too often the non-mathematically oriented reader sees the orthogonal axes of PCA and maps that onto an orthogonal set of regulatory mechanisms. This confusion should be preempted and addressed in my view. Furthermore, its interesting.

The reviewer has raised an excellent point about the mapping between principal components and mechanism. We completely agree with the reviewer that just because PCA decomposes that data into an orthogonal set of features, it need not be the case that those features are regulated orthogonally or correspond to distinct mechanisms. To get at this possibility, we looked at how well the effects of drugs corresponded to the principal components themselves (Supp. Fig. S). We found that some drugs affected just one principal component or the other, while others affected both. The results are consistent with at least the possibility that the effects of drugs correspond to principal components, although we hesitate to draw a firm conclusion based on the limited data. In any case, we agree that it is critical to make this clear to readers who may misinterpret these results in that way, and we have updated the main text accordingly:

Results:

We then wondered how a perturbation which does change a constraint influences other constraints—if cysts perturbed with drug X do not obey the constant-cell-density constraint, must they also not obey the lumen-number-cap constraint? We found that cysts perturbed with givinostat (a histone deacetylase inhibitor), idelalisib (a phosphoinositide 3-kinase delta isoform inhibitor), sumatriptan succinate, Aurora A Inhibitor I, Y-27632, and blebbistatin had more lumens in a given cyst volume than unperturbed cysts of any age (Fig. 3F). Thus, cysts perturbed with these drugs do not obey the same lumen-number-cap constraint of unperturbed cysts, instead they obey a constraint with a larger slope. We found that some perturbations (selinexor, givinostat, idelalisib, Aurora A Inhibitor I, and NSC 23766) changed only one constraint, but others (sumatriptan succinate, Y-27632, and blebbistatin) changed both. Given the targets of these perturbations, we can hypothesize as to which mechanisms may regulate these constraints. We saw that exportin 1 was important for the constant-cell-density constraint, HDAC, PI3K, and Aurora A kinase for the lumen-number-cap constraint, and serotonin receptors were important to both constraints. ***We similarly found some mechanisms perturbed MDCK cysts along a single axis in principal component space, while others perturb cysts along two axes (Supp. Fig. S), suggesting that the axes of MDCK cyst variation may not be governed by independent mechanisms.*** *These factors all work through quite different pathways; however, given the relatively small number of perturbations we analyzed, it was difficult to uncover any general rules on which pathways affected which constraints. In combination, this suggests that the set of morphologies available to MDCK cysts is richer than unperturbed cysts would suggest.*

Discussion:

It remains unclear what underpins the constraints on cyst (or, more generally, organoid) morphologies. One could imagine any number of potential mechanisms, any one of which might be critical to a constraint by itself or in combination with many others. Such mechanisms may be based upon conventional biochemical signaling (such as signaling between cells to control proliferation), or may involve mechanical sensing of variables such as membrane curvature. A related question is whether these mechanisms map in a one-to-one manner onto each of the principal components of variation we detected. I.e., is there one mechanism that governs cell size and related variables, and another mechanism that governs lumen number? In principle, there is no need for any one mechanism to map in such a direct way onto a particular axis of variation. ***Our data with various drugs suggests that it is entirely possible for a mechanism to affect just one or multiple principal components, so there is no reason to predict that individual mechanisms would be restricted to affecting just a single axis.***

Figure 1: Given the paucity of data it might seem reasonable to regularize PCA. This might indeed help the authors in more categorically identifying the loading vectors. As the authors are no doubt aware, unregularized algorithms will assign non-zero entries to every coeffiecient to soak up as much variance as it can. Since data is limited I believe a regularization based analysis would bolster the statistical claims the authors make.

We thank the reviewer for this excellent suggestion. We ran a sparse PCA on our dataset to see whether it would strengthen our claims. We found that it indeed recapitulated the core results we found, and indeed made those results even more clear (Supp. Fig. T). We have now included a paragraph on this result as included below. It is important to note that the first principle component is dense, which often captures technical and batch variability, as has been observed (Sun et al, Ann. Appl. Stat., 2012 and Leek et al, PNAS, 2008). We thank the reviewer for suggesting this analysis.

Given that MDCK cysts obey a number of constraints, we then wondered whether these constraints are coupled. In other words, might there be a single dimension (or a few dimensions), each of which may comprise several correlated features, along which all MDCK cyst morphologies fall (Fig. 1H)? To identify dimensions in the space of MDCK cyst morphologies we performed principal component analysis (PCA) on the set of 77 cysts and their 66 morphological features. In order to apply PCA to our data, we needed to supply a single value for each feature for each cyst. For all features describing nuclei we used both the mean and standard deviation across all nuclei within the cyst, e.g. mean nuclear volume and standard deviation of nuclear volume. For features describing lumens we used the mean across all lumens in the cyst, e.g. mean lumen volume. We didn't include other higher order statistics like standard deviation because it was impossible to do so for the many cysts that had only one lumen. We found that the first three principal components respectively explain 28%, 19%, and 10% of the variation in MDCK cyst morphologies (Fig. 1I). We then wondered whether the principal components reflected any of the constraints we had previously identified. We found that the first principal component represented lumen size and inversely nuclear size, reflecting the fact that as lumens get larger, nuclei get smaller (Fig. 1J-L, Supp. Fig. H). Note that this trend is consistent with our earlier finding of relatively constant cell size because cell size and nuclear size are different properties. The second principal component represents cyst size and number of nuclei, reflecting that increased cyst size was associated with increased number of nuclei, a relationship we previously identified as the constant-cell-density constraint. The third principal component represented the trade-off between lumen size and the number of lumens, reflecting that, for a given cyst size, in order to have more lumens, the individual lumens must be smaller (rather than there is a maximum lumen size which is independent of the number of lumens). The third principal component also represented a trade-off between nuclear size and the density of nuclei, reflecting that, for a given cyst size, in order to have more nuclei the nuclei must be smaller (Supp. Fig. I). (Consistent with PC1, we also found that nuclear size anti-correlated with lumen size.) Beyond those three principal components, the remaining components accounted for less variation than components calculated from randomized data, suggesting that those PCs likely do not reflect substantial variation in the data. ***In addition to performing conventional PCA on our data, we also used a sparse PCA method (Benjamin Erichson et al. 2018), which can aid with interpretation because it tries to reduce small contributions to principal components to zero when possible. We found that the principal components from such an analysis (after discarding the first principal component, which is most likely technical and batch variability) recapitulated similar contributions to the axes of variation revealed by conventional PCA. This analysis also had a more straightforward interpretation owing to the principal components having just a few primary contributing features (Supp. Fig. T).*** *Thus, despite quantifying a large*

number of features, MDCK cyst morphologies can thus be represented by a limited number of dimensions.

Figure 1: A suggestion, perhaps beyond the context of this paper, is to consider a manifold learning approach. This would be a nonlinear attempt at explaining the variance. This might indeed reveal more decomposable features. I could recommend either an isomer approach or an auto encoder. Again, if the authors decide that this is beyond the scope of this paper then perhaps this suggestion should be taken as something for a future study. My rationale for this again being the standard linearity assumption inherent to PCA, and their orthogonality, can raise many issues. Perhaps before going fully nonlinear the authors, for the sake of this paper, could attempt Non-negative matrix factorization (NMF) where you know to look for 3 features. This approach would at least respect the positivity of the data and NMF gives features that aren't orthogonal. This might help interpretation.

We thank the reviewer for this suggestion. We agree that nonlinear approaches might reveal more decomposable features. To account for at least some of the obvious non-linear relationships that likely exist between features, we standardized the units of our features before applying PCA. For example, we took the cube root of all features with units μm^3 and the square root of all features with units μm^2. Of course, it is possible there are non-obvious non-linear relationships that our approach will miss. We also tried NMF (see below), and found that it gave us results that were similar to the results we obtained with standard PCA, but were somewhat harder to interpret, so we elected not to include those results. We also feel as though we don't have enough data to properly run autoencoders. We hope to pursue these possibilities in future work once we have larger datasets in hand.

Weights of each feature for three factors from NMF:

Figure 2: Why not do a sliding window for days rather than blocks? This way you can leverage the data to its max

We thank the reviewer for this suggestion. We tested sliding windows for Figure 2 (see below). Ultimately, we decided to keep our original use of blocks because we felt it was easier to see the change in constraints over time because the sliding windows “blurred” some of the effects that were visible when we binned by individual days.

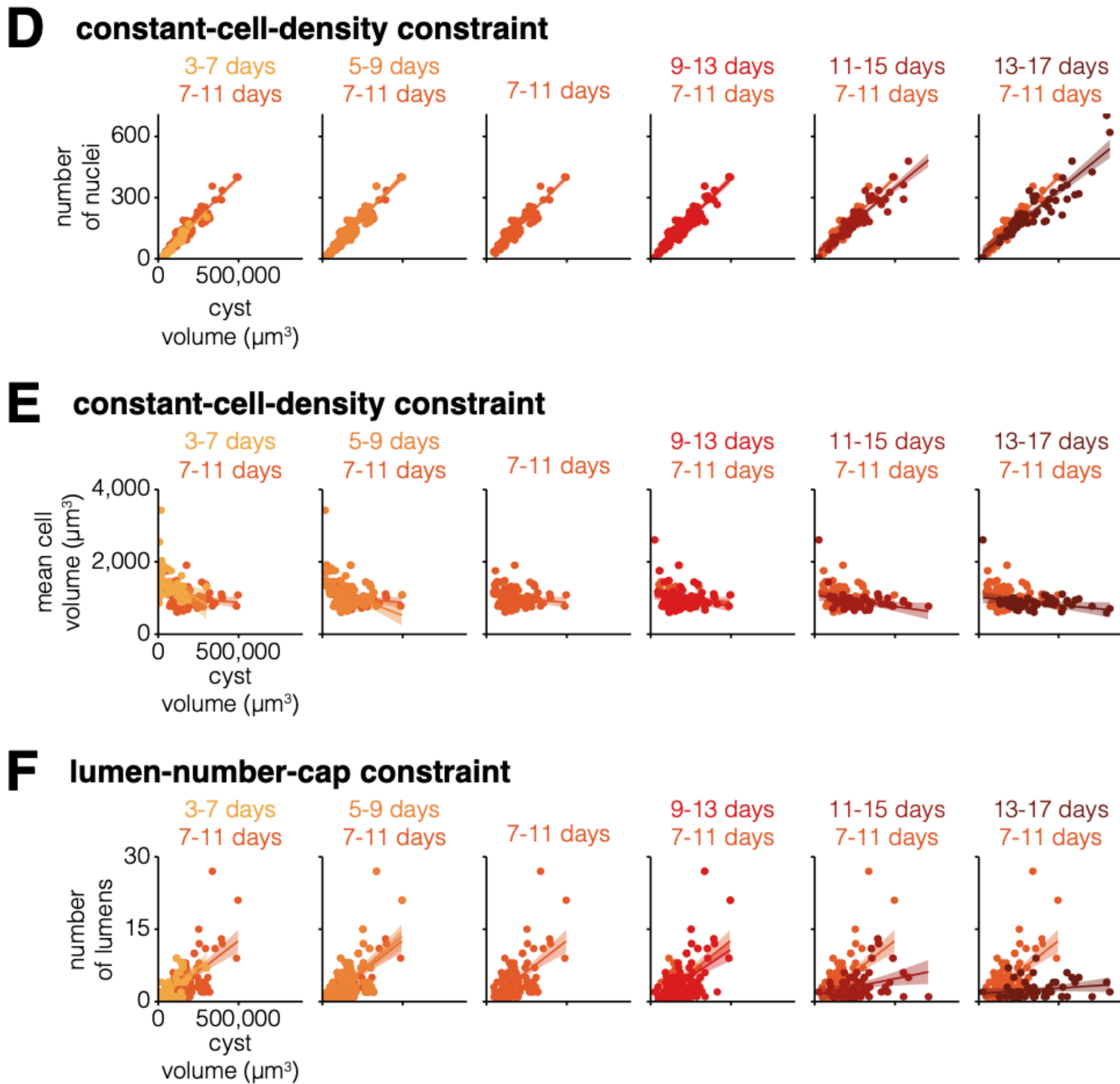


Figure 2: I think the authors should make a stronger case for themselves. At all ages the constraints are present but their quantitative features evolve. This might be lost on the non technical reader that might interpret the language as saying "sometimes there are constraints, sometimes there aren't". The result really is that there are always constraints, at all ages, but its quantitative features evolve. This should not be lost on the non-mathematically oriented reader. Perhaps the authors at this point can suggest would might be the cause of this quantitative variation in constraint traits?

The reviewer makes an excellent point, echoed by reviewer 2. We have re-written this section to clarify that MDCK cysts of all ages do obey a set of constraints, but that the exact nature of those constraints (their intercept and/or slope) varies with MDCK cyst age. We have additionally included a discussion of what mechanisms may underlie this quantitative variation (see below).

We wondered whether other constraints were similarly affected by age. We looked at the age dependence of the lumen-number-cap constraint (Fig. 2F, Supp. Fig. N). We again found that cysts of all ages obeyed a constraint on the number of lumens per cyst volume. However, this constraint changed with age: we found that younger cysts cultured for 3 days had a higher maximum number of lumens per cyst volume, and cysts cultured for 13-17 days had a lower maximum number of lumens per cyst volume. The decrease in the number of lumens per cyst as cysts age beyond 9 days suggests

that multiple lumens in a cyst are either merging or disappearing as cysts grow older. ***It is difficult to speculate what mechanisms might govern this age-dependent quantitative change in constraints without perturbations. However, given that very young cysts seem to obey an entirely different set of constraints than older cysts, it may be that some of these differences are due to the establishment of apico-basal polarity around the lumen considering the work of Vasquez et al (Vasquez et al. 2021).*** *Also, we noticed in young cysts that the threshold of size and number of nuclei in order to form a lumen was 10,000 μm3 and 7 nuclei, respectively (Supp. Fig. U). Together, our results point to constraints as being dynamic entities that can change as cysts grow and develop.*

Figure 2: If one did PCA on all the 3-17 day data do you recover similar PCs with one of them now correlating with time? Said another way, when you do PCA on the temporal ensemble vs a fixed day ensemble do you see quantitatively similar features or rather different? Said yet another way, does the intraday variance line up with the interday variance? There is good reason to think this since cysts live in their own world, and need not abide by our counting of days. Hence the variance at a given day should account for some of the temporal variance you see.

The reviewer has posed an excellent question about what "time" corresponds to for cysts. We ran the PCA they suggested for all the time points put together, and we found that the PCs were virtually the same, but their order was switched in that the PC for size now accounted for more of the total variance (Supp. Fig. P). This result is what would be expected if the intraday variance lined up with the interday variance. That is, there is a large variability in size from young to aged cysts, but that variability in size is the same in kind as that between large and small cysts in just one age category. That said, it is entirely possible that there were more subtle differences between ages that was difficult to pick up in an *en masse* PCA. To look for such effects, we plotted each constraint itself (i.e. number of nuclei vs. cyst size) for each time point. While the general trend was the same, there were subtle but observable differences in the trend over time (Fig. 2). Given that this more directed analysis revealed age differences, we have kept that analysis in the paper. We hope our updated text in the section describing these results makes the connection to the reviewer's excellent question more clear.

We first wondered if the constant-cell-density constraint varied for cysts of different ages (Fig. 2D, Supp. Fig. L). We found that cysts of all ages obeyed the constant-cell-density constraint on the number of nuclei and cyst volume. ***This relationship was confirmed by PCA run on the complete dataset including all time points, in which the principal components remained the same, but the principal component for cyst size accounted for more total variance due to size differences with age (Supp. Fig. P).*** *However, the exact nature of this constraint, specifically the slope and intercept of the linear relationship, varied with the age of the cyst. Cysts cultured for 3-5 or 13-17 days obeyed a constant-cell-density constraint with a smaller slope; i.e., they had fewer nuclei per cyst volume. As cysts age, they get larger, so in principle it could be that older cysts could have a lower cell density because all cells are larger, or it could be that there is a threshold volume beyond which cell density decreases. We found that amongst older cysts with smaller sizes (that matched those of middle-aged cysts), the densities were similar to those of middle-aged cysts, suggesting the latter threshold scenario (Fig. 2E, Supp. Fig. M). Younger cysts, however, had a uniformly lower cell density than middle-aged cysts.*

Figure 4: The expectation of additivity is something that one can only anticipate to hold in the perturbative limit. More technically, superposition is a consequence of a linear theory. Said more plainly, one should only expect approximate additivity if the drug dose is small. Meaning small amount of one drug + small amount of other

drug should even be anticipated to give an additive phenotypic effect. Large doses probe nonlinear response and all bets are off. So I believe the straw man that is constructed here is made of too much straw.

The reviewer makes an excellent point that one may not expect that the effects of our drug perturbations would combine additively except in the limit of “small” perturbations, and that nonlinearity may very well be the more natural expectation. We have now modified the text to reduce the amount of straw:

We then wondered how the constraints of cysts perturbed with one drug changed when the cysts were exposed to a second drug. ***One possibility is that doubly-perturbed cysts obeyed a set of constraints that averaged the constraints obeyed by singly-perturbed cysts (assumption of linearity). Another possibility is that doubly-perturbed cysts obeyed the same set of constraints as only one of the perturbations, suggesting that some drugs may be able to override the effects of others, or some other non-linear interaction. One might expect that linearity would hold for small doses of drug, but that nonlinear aspects of the regulatory processes may appear for larger doses.*** *We found that sometimes one perturbation overrode the effects of the other and that sometimes doubly-perturbed cysts did not obey the same constraints that the singly-perturbed cysts did (Fig. 4J-L). Perturbations overrode the effects of one another in many combinations and for both constraints. We observed that while HGF alone obeyed a different constant-cell-density constraint, when used in combination with either lapatinib or oratinib the cysts obeyed the same constraint as unperturbed cysts. We likewise found that the effects of Y-27632 on the lumen-number-cap constraint and the effects of NSC23766 on the constant cell density constraint were both canceled out by sumatriptan succinate. We additionally found many examples where singly-perturbed MDCK cysts obeyed the same constraints, but when those perturbations were used in combination the cysts did not obey the same constraints. We found that while neither HGF, lapatinib, nor oratinib alone changed the lumen-number-cap constraint, cysts perturbed with both HGF and lapatinib or oratinib had higher lumens per cyst volume (Fig. 4J-L). We also found this to be true for sumatriptan succinate and Y-27632 for the constant-cell-density constraint. In totality, the many differences between the constraints obeyed by double-perturbed cysts and single-perturbed cysts suggests that the effects of any given perturbation do not appear to simply add together, but rather can combine in unanticipated ways. It is important to note that many of the perturbations affected particular classes of biological processes, such as proliferation (e.g. lapatinib, oratinib, etc) and those affecting morphological processes (e.g. HGF, Y-27632, etc). It is possible that the use of different combinations with drugs perturbing the same processes could have more predictable effects.*