nature portfolio

Peer Review File

Axial Nephron Fate Switching Demonstrates a Plastic System Tunable on Demand

Corresponding Author: Dr Nils Lindström

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

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Key Findings and Noteworthy Results
• The paper provides a compelling demonstration that a transient WNT^ON/BMP^OFF state during the pretubular aggregate (PTA) to renal vesicle (RV) transition strongly biases nephroid cells toward a distal nephron fate.
• The authors convincingly show that, in this “distal-enriched (DE)” state, cells produce increasing amounts ofFGF8, and that FGF signaling via FGFR1 is essential to lock in the distal fate in this WNT^OB/BMP^OFF setting. Crucially, inhibiting FGFR1 flips these nephroids back to more proximal-like trajectories, with podocytes and proximal tubules appearing once BMP/SMAD signaling is reactivated.
• The use of single-cell RNA-seq and spatial transcriptomics to map organoid lineages onto in vivo human nephron developmental stages stands out as an important methodological strength. These data support that the distalization is likely biologic and recapitulates important features of human distal cell types. The consistency between in vitro and in vivo data, especially regarding the spatiotemporal expression patterns of FGF8, strengthens the main conclusions.
• The data suggest a high degree of fate plasticity and open up the possibility of specifying distinct nephroid segments (proximal vs. distal) “on demand” using small-molecule-based manipulations of WNT, BMP, and FGF pathways.
Overall, these results advance our understanding of how nephroid segment boundaries and domain identities can be experimentally modulated—a major step forward for deriving specific renal lineages for disease modeling and potential therapeutic applications.

Significance to the Field and Relation to Existing Literature
• This study addresses a critical challenge in kidney organoid biology: the consistent generation of more distal tubular segments, which are often underrepresented in standard organoid protocols. Many previous reports have focused on proximal segments, glomeruli, or podocytes, leaving distal lineages comparatively elusive. The manuscript’s findings fill an important gap and expand the toolkit for directed nephron fate specification.
• The work builds on and refines well-established observations that (1) WNT activation can promote distalizing outcomes, (2) BMP signaling influences proximal nephron identities, and (3) FGF8 is involved in distal nephron formation in mice. However, the systematic way in which this study dissects these signals in human organoids—and the demonstration of robust flips between proximal and distal states—is novel.
• In light of other organoid protocols attempting to induce more mature tubular subtypes, this work is particularly timely. It provides a clear, concise mechanistic rationale for combinatorial small-molecule manipulations that readers can readily apply and compare to their own protocols.

Support of Conclusions and Potential Need for Additional Evidence
• The multi-omic approach (bulk RNA-seq, single-cell RNA-seq, immunofluorescence, spatial transcriptomics) and the consistency among these datasets make the conclusions well supported.
• One area that might benefit from additional discussion is the interplay between FGF8 and FGF10. Both are upregulated in the distal-enriched condition, but the study highlights FGF8 in detail, especially with in vivo spatial data. Clarifying whether FGF10 might also contribute to this distalizing signal—or whether it serves a separate or redundant function—would add depth to the FGF story.
• If feasible, more direct functional assays (e.g., ion transport assays to confirm thick ascending limb-like activity) would strengthen the notion that these distalized cells perform the specialized transport roles of loop of Henle segments. However, the paper already makes a strong case that they are, at minimum, on an appropriate developmental trajectory.

• As mentioned below, quantifying changes in expression patterns (especially for figure 4) would greatly improve the work.

Data Analysis, Interpretation, and Conclusions
• The data analysis—particularly the use of scRNA-seq with reference mapping to human fetal nephron datasets—is robust. The organoids' distal lineages cluster clearly with loop of Henle–like cells. The logic behind the scRNA-seq filtering and the DESeq2 thresholds ($p < 0.05$, log_2FC > 1.5) is sensible, and the volcano plots effectively highlight the major gene expression changes.
• The conclusion that BMP signaling is necessary for proximal identity, whereas FGF signaling is required for distal specification, is convincingly demonstrated using pharmacological inhibitors. These findings echo but extend earlier ex vivo rodent studies, offering new mechanistic clarity for human systems.
• The discussion section's model (where WNT/FGF-based cues and BMP/SMAD cues oppose one another to set up segment boundaries) is well reasoned. The proposed synergy between WNT and FGF is a point deserving continued investigation (e.g., the possibility that FGF8 might be a direct β-catenin target or that BMP modulates β-catenin output).

Methodological Rigor and Reproducibility
• The protocols for organoid generation are described in sufficient detail to be reproduced. Inclusion of alternative BMP inhibitors (LDN-193189 or Dorsomorphin) and PI3K inhibitors (LY294002 or GDC-0941), as well as the use of different iPSC lines, underscores the robustness of the approach.
• The single-cell datasets are clearly documented. Providing details about the gating strategy for live cells (DAPI^–/DRAQ5^+) and the sublibrary merges fosters confidence in the scRNA-seq pipeline.
• A small suggestion: including a table or short paragraph in Methods enumerating the sample size (e.g., how many replicate organoids per condition and day were used for each single-cell or bulk RNA-seq experiment) would further clarify reproducibility.
• GEO Datasets are supplied.

Further clarification and experiments
• The combination of live imaging or post-hoc lineage tracing (even with an HNF4A-YFP reporter) might be an interesting way to visualize cell fate reversibility in real time in future studies.
• Figure 3C: please make the IRX1 legend red color, if that is the intended color—it is difficult to disentangle it from DAPI—consider changing the pseudocolors?
• Please detail the code used in your bioinformatic analysis. Perhaps placing code in a github repository?
• It might be beneficial to explicitly acknowledge possible off-target effects from the pan–FGFR inhibitor SU-5402. Although the data strongly implicate FGFR1, a brief note on whether FGFR2/3 cross-inhibition is feasible and how that might (or might not) contribute to the reversion to proximal fate could address any residual concerns. Further, by inhibiting a receptor that is present in earlier cell states, it is difficult to contextualize whether this inhibitor is mediating its effect via on FGF8-> FGFR1. FGF18 is mentioned briefly as differentially expressed in DEs, but then never readdressed. Where is FGF18 localized?
• Labeling human fixed tissue as "in vivo" is probably confusing. It should probably be labeled "human"
• The authors should be careful in their language using WT1 as a definition of symmetry breaking in the text describing Fig1—WT1 may not be the earliest polarization marker.

1. Figure1. Clarify the operational definition used for "symmetric" vs. "asymmetric" WT1 labeling. For instance, do you score a nephron as asymmetric if one region is strongly WT1^+ and another region is clearly WT1^–? If these values represent two or more biological replicates, consider adding error bars or range. Otherwise, note if the results are from a single representative experiment with total nephron counts.
2. Figure 2. The figure legend mentions the final merged dataset, but it would be helpful to confirm if each condition includes multiple (biological) replicates that were pooled. If replicates are combined, a note on batch-correction or integration steps would reassure readers that the clustering isn't dominated by batch effects.
The UMAP cluster colors are visible but small. If you want to highlight specific cluster identities (e.g., podocyte-like, distal-like), adding an overlaid label or color key might help readers quickly see how you mapped them to known nephron subtypes.
3. It seems you've grouped markers according to known lineage (e.g., "podocyte precursors," "distal tubule precursors"), but the logic behind each set could be explained more. For example, do we truly know that each of these gene sets is exclusively marking that lineage at this stage? Many markers (e.g., PAX8, LHX1) can be active in multiple segments of early nephron development. A short explanation in the text or figure legend would help justify these "labels."
Some markers like JAG1 can mark both early proximal/medial compartments and even broader domains. Clarifying how you separated "podocyte precursors" from "proximal tubule precursors" strictly by JAG1 or WT1 expression might strengthen confidence in the classification.
• Fig2d. Interpreting the bar lengths can be tricky. For instance, if 60% of DE organoid cells express GATA3 vs. 10% in Control, this doesn't always reveal if the GATA3^+ cells in Control co-express proximal markers or if they're a separate subset. Some short discussion of "co-expression" vs. "exclusive expression" would help interpret these data in context of a single marker plot. Perhaps consider paired control and DE violin plots for each gene?
• The selection of genes here (TFAP2A, POU3F3, HNF1B, etc.) is appropriate for distal identity, but it might be valuable to add a couple of "ambiguous" genes that appear in both proximal and distal states. That could show the continuum or possible partial overlap.
4. Figure1 The figure references "week 14 and week 17" human kidney scRNA-seq. Briefly clarifying the sample size and cell count in that reference dataset would help readers appreciate coverage across nephron stages (PTA, RV, SSB, etc.). Mapping organoid cells to in vivo states can be complicated by differences in time-scale and environment. A short note in the figure or text about potential limitations (e.g., differences in gestational timing or incomplete representation of certain

lineages) would strengthen the interpretation. The figure does not explicitly state if or how you accounted for batch effects (e.g., SCTransform, Harmony, or Seurat's integration).
5. Figure 4D-G: Can you quantify positive cells to support your findings? I agree that subjectively the images appear to show this, but given variability between nephroids and subtle changes described, quantification would greatly improve this.
6. Figure 4G: this is a very interesting finding, but its interpretation is somewhat unclear. I
7. Directly comparing FigureS7F to control side-by-side would be helpful. Especially with quantification of cell populations based on IF.
1. Batch Effects and Integration
o The figure does not explicitly state if or how you accounted for batch effects (e.g., SCTransform, Harmony, or Seurat's integration). Readers should know whether the organoid data were first integrated across replicates before mapping.
2. Clarity of Legend
o The schematic arrow from "Nephroid (Control & DE)" to "Reference mapping" is clear, but adding a small legend explaining how "prediction scores" were computed or how "anchors" were identified (if using Seurat's reference mapping protocol) would be helpful.
8. Timing of FGF8 Expression
o The spatial transcriptomic data nicely show FGF8's spatiotemporal progression in the RV and SSB. It may be helpful to reiterate in the main text that FGF8 is transient during normal kidney development and highlight any parallels in organoid culture after Day 14 or Day 20 (i.e., do organoids downregulate FGF8 on Day 20 or Day 28 similarly to in vivo?).
9. Figure Clarifications
o In Figure 4h–i, consider adding the newly gleaned information about FGF10 or the expression of FGF9 in distal segments, to give readers a complete picture of the FGF ligand environment.
o In the immunofluorescence images, a scale bar or consistent color labeling across panels (for the same markers) would help unify the presentation.
10. Discussion of Notch Signaling
o Given the known importance of Notch in proximal tubule specification, the authors might briefly comment on whether Notch pathway manipulation could further sharpen the proximal-distal boundaries in these organoids. Although it is not the main focus, acknowledging its established role might interest readers seeking to refine tubule domains even further.
11. Functional Maturity of Distal Segments
o The authors note that the distal identities appear to represent early loop of Henle/macula densa lineages. Readers may wonder if these cells mature further (e.g., expression of crucial ion transporters such as ROMK, NKCC2, or others). A short note on typical organoid limitations to full functional maturity (versus early segment fate) would be valuable.

Overall Assessment
• The manuscript provides a compelling and well-supported set of experiments that establish a new, highly versatile method to "tune" nephron differentiation in human organoids.
• The demonstration of robust distal fates and the subsequent potential for reversing them to proximal fates by removing FGF signaling (or simultaneously inhibiting BMP) is a significant advance. It highlights the plasticity of the early nephron epithelium and provides a platform for studying segment-specific kidney physiology and diseases.
• I find the methodology to be sound, the analyses consistent, and the conclusions valid and well justified. The study will be a valuable resource for the kidney developmental and stem cell communities.
• The biggest limitation of the study is that it is unclear how the chemical perturbations performed in these organoids relate to the patterning in humans. While it seems likely that localized FGF8 driven by WNT signaling may be important for distal fates,

Reviewer #2

(Remarks to the Author)
This study established a tunable system to generate distalized kidney organoids (DE kidney organoids) from human pluripotent stem cells. Based on an established differentiation protocol, the authors identified a time window during which renal vesicle symmetry breaking is initiated and determined treatment conditions that effective promotes distalization of the kidney organoids. Through scRNA-seq and spatial transcriptomic, the authors compared compare human PSC-derived control and DE kidney organoid with developing human kidney, revealing the acquisition of distal identity in the DE organoids. The DE organoids can be further matured into the thick ascending limb of the loop of Henle. Notably, simultaneous suppression of FGF signaling reverts the organoids back to a proximal identity.
This is an interesting study that introduces a novel strategy for generating distalized kidney organoids. While a previous study developed a differentiation method to produce kidney organoids enriched with proximal tubules (PMID 37770563), the distal segments of human nephron play crucial roles in generating diluted urine as well as responding to changes in sodium levels delivered to the distal segment. This methodology may provide new opportunities to study renal pathophysiology specific to the distal segment. Below, please find several questions and comments to be addressed.
1. The tubular morphology in DE organoids at day 14 appears somewhat abnormal in certain images (e.g., Fig. 1i). Please provide whole-organoid images of the DE organoids at different time points (days 14, 20, and 28) to offer a comprehensive overview of their morphology. Ideally, H&E staining images should be included alongside nephron cell marker staining to confirm that these tubules are monolayer simple epithelial structures.
2. The authors utilized scRNAseq to compare day 14 control and DE organoids. Would it be possible to generate scRNAseq data for later-stage control and DE organoids (e.g. day 20 or day 28)? This could provide valuable insights into the final cell populations present in the DE organoids.
3. Is it possible to reverse the distal identity after day 14, once the distal identity is fully established?
4. Previous studies have demonstrated the successful derivation of REN+ cells (PMID: 32918942) and TAL cells (PMID: 38181751) without the need for distalizing kidney organoids. The authors should discuss whether maintaining a balanced

proximal-distal identity might be beneficial for generating a broader range of tubular epithelial cell types.
5. Is it possible to adjust the distalizing regimens to achieve a more balanced proximal-distal ratio? This could great enhance the applicability of this methodology.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors addressed all major reviewer critiques thoroughly, with new experiments, quantifications, and expanded discussion. Their responses were clear, scientifically rigorous, and improved the clarity and impact of the manuscript.

Reviewer #2

(Remarks to the Author)
In the revised manuscript, the authors have conducted additional experiments that satisfactorily address my previous concerns. In particular, they performed scRNA-seq on DD28 control and DE organoids, providing evidence that DE organoids are patterned toward distal nephron segments, including the thick ascending limb (TAL). These data strengthen the conclusion that the DE culture condition promotes bona fide distal segment differentiation, rather than generating epithelial tubules that merely acquire expression of some distal markers without adopting true distal segment identity.
I have no further comments.

Reviewer #1 (Remarks to the Author):

Key Findings and Noteworthy Results

The paper provides a compelling demonstration that a transient WNT^ON/BMP^OFF state during the pretubular aggregate (PTA) to renal vesicle (RV) transition strongly biases nephroid cells toward a distal nephron fate. The authors convincingly show that, in this “distal-enriched (DE)” state, cells produce increasing amounts of FGF8, and that FGF signaling via FGFR1 is essential to lock in the distal fate in this WNT^OB/BMP^OFF setting. Crucially, inhibiting FGFR1 flips these nephroids back to more proximal-like trajectories, with podocytes and proximal tubules appearing once BMP/SMAD signaling is reactivated.

The use of single-cell RNA-seq and spatial transcriptomics to map organoid lineages onto in vivo human nephron developmental stages stands out as an important methodological strength. These data support that the distalization is likely biologic and recapitulates important features of human distal cell types. The consistency between in vitro and in vivo data, especially regarding the spatiotemporal expression patterns of FGF8, strengthens the main conclusions. The data suggest a high degree of fate plasticity and open up the possibility of specifying distinct nephroid segments (proximal vs. distal) “on demand” using small-molecule-based manipulations of WNT, BMP, and FGF pathways.

Overall, these results advance our understanding of how nephroid segment boundaries and domain identities can be experimentally modulated—a major step forward for deriving specific renal lineages for disease modeling and potential therapeutic applications.

Significance to the Field and Relation to Existing Literature

This study addresses a critical challenge in kidney organoid biology: the consistent generation of more distal tubular segments, which are often underrepresented in standard organoid protocols. Many previous reports have focused on proximal segments, glomeruli, or podocytes, leaving distal lineages comparatively elusive. The manuscript’s findings fill an important gap and expand the toolkit for directed nephron fate specification.

The work builds on and refines well-established observations that (1) WNT activation can promote distalizing outcomes, (2) BMP signaling influences proximal nephron identities, and (3) FGF8 is involved in distal nephron formation in mice. However, the systematic way in which this study dissects these signals in human organoids—and the demonstration of robust flips between proximal and distal states—is novel.

In light of other organoid protocols attempting to induce more mature tubular subtypes, this work is particularly timely. It provides a clear, concise mechanistic rationale for combinatorial small-molecule manipulations that readers can readily apply and compare to their own protocols.

Thank you for your kind comments, insights, and suggestions. Please see our point-by-point responses below.

Support of Conclusions and Potential Need for Additional Evidence

The multi-omic approach (bulk RNA-seq, single-cell RNA-seq, immunofluorescence, spatial transcriptomics) and the consistency among these datasets make the conclusions well supported.

One area that might benefit from additional discussion is the interplay between FGF8 and FGF10. Both are upregulated in the distal-enriched condition, but the study highlights FGF8 in detail, especially with in vivo spatial data. Clarifying whether FGF10 might also contribute to this distalizing signal—or whether it serves a separate or redundant function—would add depth to the FGF story.

Thank you for your comments and suggestions. We now provide a more detailed characterization of FGF ligand/receptor expression patterns in developing and adult human kidneys, and in our kidney organoid models.

If feasible, more direct functional assays (e.g., ion transport assays to confirm thick ascending limb-like activity) would strengthen the notion that these distalized cells perform the specialized transport roles of loop

of Henle segments. However, the paper already makes a strong case that they are, at minimum, on an appropriate developmental trajectory.

As mentioned below, quantifying changes in expression patterns (especially for figure 4) would greatly improve the work.

Thank you for your comment, we have now quantified cell abundances as suggested (previous Figure 4 is now Figure 5) for a direct comparison of the changes observed (**Figure 5h**).

To tackle questions relating to function as raised by this and other reviewer, we profiled "end-point" differentiation nephroids from Day 28 organoids by single nucleus RNA sequencing. These data demonstrate robust expression of physiology imparting genes and as we map these nuclei to the Kidney Precision Medicine Project (KPMP) atlas the analyses show the cells generated correspond most closely to loop of Henle cell populations. We also include immunofluorescent stains showing strong detection of such proteins.

Data Analysis, Interpretation, and Conclusions

The data analysis—particularly the use of scRNA-seq with reference mapping to human fetal nephron datasets—is robust. The organoids' distal lineages cluster clearly with loop of Henle–like cells. The logic behind the scRNA-seq filtering and the DESeq2 thresholds ($p < 0.05$, log_2FC > 1.5) is sensible, and the volcano plots effectively highlight the major gene expression changes.

The conclusion that BMP signaling is necessary for proximal identity, whereas FGF signaling is required for distal specification, is convincingly demonstrated using pharmacological inhibitors. These findings echo but extend earlier ex vivo rodent studies, offering new mechanistic clarity for human systems.

The discussion section's model (where WNT/FGF-based cues and BMP/SMAD cues oppose one another to set up segment boundaries) is well reasoned. The proposed synergy between WNT and FGF is a point deserving continued investigation (e.g., the possibility that FGF8 might be a direct β-catenin target or that BMP modulates β-catenin output).

Thank you for your suggestion. In mice, *Fgf8* is indeed a direct target of β-catenin as demonstrated by ChIP (PMID: 22902740) and this is raised in the manuscript. Extending this to the human system will be an important future direction.

Methodological Rigor and Reproducibility

• The protocols for organoid generation are described in sufficient detail to be reproduced. Inclusion of alternative BMP inhibitors (LDN-193189 or Dorsomorphin) and PI3K inhibitors (LY294002 or GDC-0941), as well as the use of different iPSC lines, underscores the robustness of the approach.

• The single-cell datasets are clearly documented. Providing details about the gating strategy for live cells (DAPI^–/DRAQ5^+) and the sublibrary merges fosters confidence in the scRNA-seq pipeline.

• A small suggestion: including a table or short paragraph in Methods enumerating the sample size (e.g., how many replicate organoids per condition and day were used for each single-cell or bulk RNA-seq experiment) would further clarify reproducibility.

Thank you for your suggestion. Sample sizes of all quantitative and transcriptomic data in the manuscript have been described in the Methods section.

• GEO Datasets are supplied.

Further clarification and experiments

• The combination of live imaging or post-hoc lineage tracing (even with an HNF4A-YFP reporter) might be an interesting way to visualize cell fate reversibility in real time in future studies.

Thank you for your suggestion.

• Figure 3C: please make the IRX1 legend red color, if that is the intended color—it is difficult to disentangle it from DAPI—consider changing the pseudocolors?

Thank you for your comment. We have amended the color scheme of these panels to enhance contrast between the fluorescence signal and DAPI.

• Please detail the code used in your bioinformatic analysis. Perhaps placing code in a github repository?

Thank you for your suggestion. We have added the code used for bioinformatics to our lab's Github account (Github details provided in the manuscript).

• It might be beneficial to explicitly acknowledge possible off-target effects from the pan–FGFR inhibitor SU-5402. Although the data strongly implicate FGFR1, 5 brief note on whether FGFR2/3 cross-inhibition is feasible and how that might (or might not) contribute to the reversion to proximal fate could address any residual concerns. Further, by inhibiting a receptor that is present in earlier cell states, it is difficult to contextualize whether this inhibitor is mediating its effect via on FGF8-> FGFR1. FGF18 is mentioned briefly as differentially expressed in DEs, but then never readdressed. Where is FGF18 localized?

Thank you for your insight. We have expanded our analyses to describe expression of FGF ligands in vivo and in vitro. We now comment on a future need to resolve FGF and FGFR pairings during nephron patterning using genetic tools.

• Labeling human fixed tissue as "in vivo" is probably confusing. It should probably be labeled "human".

Thank you for your suggestion. We have amended our labeling of human samples.

• The authors should be careful in their language using WT1 as a definition of symmetry breaking in the text describing Fig1—WT1 may not be the earliest polarization marker.

Thank you for your comment. We now expand the use of antibody stains as well as our text to clarify the definition of symmetry breaking.

1. Figure 1. Clarify the operational definition used for "symmetric" vs "asymmetric" WT1 labeling. For instance, do you score a nephron as asymmetric if one region is strongly WT1+ and another region is clearly WT1-? If these values represent two or more biological replicates, consider adding error bars or range. Otherwise, note if the results are from a single representative experiments with total nephron counts.

Thank you for these comments and suggestions. We expanded on the definition of symmetry breaking to describe it within the context of protein markers (WT1, HNF1B, JAG1, POU3F3, and TFAP2A) that define the early PD axis. We now complement the WT1/JAG1/CDH1 panels with WT1/TFAP2A whole organoid overviews and magnified fields (**Figure S1f**) that clearly show that WT1 downregulation is a strong indicator of early asymmetry as it coincides with TFAP2A (distal marker). Error bars are now added to **Figure 1c**.
In general, this asymmetry matches to in vivo expectations.

2. Figure 2. The figure legend mentions the final merged dataset, but it would be helpful to confirm if each condition includes multiple (biological) replicates that were pooled. If replicates are combined, a not on batch-correction or integration steps would reassure readers that the clustering isn't dominated by batch effects.

Thank you for these comments. We have outlined the parameters of the RNA sequencing analyses and sample collection under the 'Methods' section. For each analysis, we have included a 'Sample Collection and Preparation' section that details samples sizes and 'Seurat Object Processing' section that outlines the script used to generate the UMAPs and related data. There was no batch variation between sublibraries or between conditions upon merging, therefore integration was not performed.

3. (Figure 2). The UMAP cluster colors are visible but small. If you want to highlight specific cluster identities (e.g., podocyte-like, distal-like), adding an overlaid label or color key might help readers quickly see how you mapped them to known nephron subtypes.

Thank you for your comments. We have added annotations to the UMAP cluster for easier identification of cluster identities (**Figure 2a, b**).

4. It seems you've grouped markers according to known lineage (e.g., "podocyte precursors", "distal tubule precursors"), but the logic behind each set could be explained more. For example, do we truly know that each of these gene sets is exclusively marking that lineage at this stage? Many markers (e.g., PAX8, LHX1) can be active in multiple segments of early nephron development. A short explanation in the text or figure legend would help justify these "labels". Some markers like JAG1 can mark both early proximal/medial compartments and even broader domains. Clarifying how you separated "podocyte precursors" from "proximal tubule precursors" strictly by JAG1 or WT1 expression might strengthen confidence in the classification.

Thank you for your comments. We have clarified the distribution of each marker and added references in support of their distribution.

5. Figure 2d. Interpreting the bar lengths can be tricky. For instance, if 60% of DE organoid cells express GATA3 vs. 10% in control, this doesn't always reveal if the GATA3+ cells in control co-express proximal markers or if they're a separate subset. Some short discussion of "co-expression" vs "exclusive expression" would help interpret these data in context of a single marker plot. Perhaps consider paired control and DE violin plots for each gene?

Thank you for these suggestions. We agree that the wording needs clarification. We have added clearer language to explain what the bars represent, i.e. that the tally is based on percent contribution rather than percent expression of each select gene, with an example. Regarding the point of co-expression, as single cell RNA-seq generates sparse data, we show this as co-expression at a cluster level **Figure 2b**.

6. The selection of genes here (TFAP2A, POU3F3, HNF1B, etc) is appropriate for distal identity, but it might be valuable to add a couple of "ambiguous" genes that appear in both proximal and distal states. That could show continuum or possible partial overlap.

Thank you for your suggestions. We have implemented them and included feature plots of general nephron markers, PAX2, PAX8 and LHX1 (**Figure S2e**) to demonstrate that both conditions express these genes with similar average expression.

7. Figure 3. The figure references "week 14 and week 17" human kidney scRNAseq. Briefly clarifying the sample size and cell count in that reference dataset would help readers appreciate coverage across nephron stages/ mapping organoid cells to *in vivo* states can be complicated by differences in timescale and environment. A short note in the figure or text about potential limitations (e.g., differences in gestational timing or incomplete representation of certain lineages) would strengthen the interpretation.

Thank you for your suggestion. We have added details of the cell counts of these data and added a comment acknowledging the limitations of reference mapping. Of note, these data contain all segments of the developing nephron, and we have previously described these in detail to ascertain the developmental ranges shown (PMID: 34428401, 31265809). We think they are as close a match as can be achieved for comparison with our *in vitro* model.

8. The figure does not explicitly state if or how you accounted for batch effects (e.g., SCTransform, Harmony or Seurat's integration).

Please see: Integration methods on Seurat in the Methods section under 'Reanalysis of Published Developing Human Single-cell RNA Sequencing Data' – we performed SCTransform integration on the human kidney dataset, which displayed batch variation between week 14 and week 17 data.

9. Figure 4D-G. Can you quantify positive cells to support your findings? I agree that subjectively the images appear to show this, but given variability between nephroids and subtle changes described, quantification would greatly improve this. Figure 4G: this is a very interesting finding, but its interpretation is somewhat unclear.

Thank you for your comments. We have included immunofluorescence quantifications to the figure. We have also added our interpretation of the "reversed (reversed)" phenotype observed in Figure 4G.

10. Directly comparing Figure S7F to control side-by-side would be helpful, especially with quantification of cell populations based on IF.

Thank you for your suggestions. We have included side-by-side panels of control organoids along with quantifications of immunofluorescence detection. Please note change in figure numbers.

11. Batch effects and integration: the figure doesn't explicitly state if or how you accounted for batch effects. Readers should know whether the organoid data were first integrated across replicates before mapping.

Thank you for your comments. We have dedicated sections under 'Methods' for each piece of data, detailing the methods of merging and integration, where applicable. Perhaps to our advantage, given that we were able to generate data from frozen organoids and were thus able to process them at the same time, we did not experience batch effects for either snRNAseq an scRNAseq data; as one could otherwise expect if processing and sequencing samples at different times and with different reagents.

12. Clarity of legend: the schematic arrow from "Nephroid (Control & DE)" to "Reference mapping" is clear but adding a small legend explaining how "prediction scores" were computed or how "anchors" were identified (if using Seurat's reference mapping protocol) would be helpful.

Thank you for your suggestions. We have included an explanation of the parameters used for reference mapping as well as how the prediction score is calculated under the 'Methods' section, which we refer to in the text.

13. Timing of FGF8 expression: the spatial transcriptomic data nicely show FGF8's spatiotemporal progression in the RV and SSB. It may be helpful to reiterate in the main text that FGF8 is transient during normal kidney development and highlight any parallels in organoid culture after Day 14 or Day 20 (i.e., do organoids downregulate FGF8 on Day 20 or Day 28 similarly to in vivo?)

Thank you for your insight. We have included illustrations and discussion of the transient expression of FGF8 in both human nephrons and our kidney organoids. Overall we have expanded on the expression of FGF ligands and receptors to provide more clarity to this point.

14. Figure clarifications: In Figure 4h-l, consider adding the newly gleaned information about FGF10 or the expression of FGF9 in distal segments, to give readers a complete picture of the FGF ligand environment.

Thank you for your comments. We have extended our coverage of FGF signaling in the nephron and included discussions of what their possible functions are.

15. In the immunofluorescence images, a scale bar or consistent color labeling across panels (for the same markers) would help unify the presentation.

Thank you for your comments. We have reviewed the images and included scale bars for each image.

16. Discussion of Notch signaling: Given the known importance of Notch in proximal tubule specification, the authors might briefly comment on whether Notch pathway manipulation could further sharpen the proximal-distal boundaries in these organoids. Although it is not the main focus, acknowledging its established role might interest readers seeking to refine tubule domains even further.

Thank you for your comments. We agree that Notch signaling is a crucial part of nephron cell type specification and we have included new data to address this point (**Figure S8g-j**). It is interesting to note that TFAP2A+ distal cells develop well in the presence of gamma-secretase inhibitor DAPT, while control organoids replicate expected loss of proximal phenotypes.

17. Functional maturity of distal segments: The authors note that the distal identities appear to represent early loop of Henle/macula densa lineages. Readers may wonder if these cells mature further (e.g., expression of crucial ion transporters such as ROMK, NKCC2, or others). A short note on typical organoid limitations to full functional maturity (versus early segment fate) would be valuable.

Thank you for your comments. We have included in-depth analysis of DD28 organoids and have included a panel of expression of ion transporters that are enriched in the thick ascending limb (**Figure 4i, S6e**).

Reviewer #2 (Remarks to the Author):

This study established a tunable system to generate distalized kidney organoids (DE kidney organoids) from human pluripotent stem cells. Based on an established differentiation protocol, the authors identified a time window during which renal vesicle symmetry breaking is initiated and determined treatment conditions that effective promotes distalization of the kidney organoids. Through scRNA-seq and spatial transcriptomic, the authors compared compare human PSC-derived control and DE kidney organoid with developing human kidney, revealing the acquisition of distal identity in the DE organoids. The DE organoids can be further matured into the thick ascending limb of the loop of Henle. Notably, simultaneous suppression of FGF signaling reverts the organoids back to a proximal identity.

This is an interesting study that introduces a novel strategy for generating distalized kidney organoids. While a previous study developed a differentiation method to produce kidney organoids enriched with proximal tubules (PMID 37770563), the distal segments of human nephron play crucial roles in generating diluted urine as well as responding to changes in sodium levels delivered to the distal segment. This methodology may provide new opportunities to study renal pathophysiology specific to the distal segment. Below, please find several questions and comments to be addressed.

Thank you for your comments, insights, and suggestions. Please see our point-by-point responses below.

1. The tubular morphology in DE organoids at day 14 appears somewhat abnormal in certain images (e.g., Fig 1i). Please provide whole-organoid images of the DE organoids at different time points (days 14, 20 and 28) to offer a comprehensive overview of their morphology. Ideally, H&E staining images should be included alongside nephron cell marker staining to confirm that these tubules are monolayer simple epithelial structures.

Thank you for your comments. We have replaced Figure 1i with a single-plane image rather than the maximal projection image previously included. This better represents the morphology of DE organoids. In addition, we now include overviews of organoids that show the morphologies at day 20 and 28 – (for instance **Figure**

3e – including ZO1 and SLC12A1 demonstrate a nice epithelial morphology with an apical surface enriched for a correctly localized solute carrier).

2. The authors utilize scRNAseq to compare day 14 control and DE organoids. Would it be possible to generate scRNAseq data for later-stage control and DE organoids (e.g., day 20 or day 28)? This could provide valuable insights into the final cell populations present in the DE organoids.

Thank you for your suggestions. We have now included snRNA-seq data on DD28 organoids that not only increases the yield of epithelial organoid cells but also allows for a more accurate comparison with adult human snRNAseq data.

3. Is it possible to reverse the distal identity after day 14 once the distal identity is fully established?

Our work indicates that nephron axial identities are plastic during the earliest stages of nephrogenesis and that after day 14 they progress along their set trajectory. Whether this is specified or determine would require additional manipulation after day 14, and our data suggests that withdrawal of factors (e.g., removing SU-5402 at day 14 from DE organoids) is insufficient to revert cells to a distal type as they persist to form podocytes (**Figure S8d**). Defining the capacity of programing mature cells would be of interest but is beyond the scope of this work.

4. Previous studies have demonstrated the successful derivation of REN+ cells (PMID: 32918942) and TAL cells (PMID: 38181751) without the need for distalizing kidney organoids. The authors should discuss whether maintaining a balanced proximal-distal identity might be beneficial for generating a broader range of tubular epithelial cell types.

Thank you for your comments. The goal of our study was to delineate axial patterning mechanisms and more specifically the regulatory program of the distal nephron, but we now comment on the general utility of organoids comprised of more homogenous cell-populations and the potential caveats for resolving cell-to-cell communications in these – given that the population complexity is reduced. We agree, both Shakar et al., and Liu et al., 2024 generate REN+ and TAL-like cells and we do not argue we are the first to describe these as they are expected to emerge in a kidney organoid. We now cite Liu in our discussion and discuss how in general, providing robust protocols for homogenous cell-populations can facilitate studies such as those conducted by Liu et al., where our protocol could facilitate decreasing organoid-to-organoid variability and enable large *in vitro* screening of PKD.

5. Is it possible to adjust the distalizing regimens to achieve a more balanced proximal-distal ratio? This could great enhance the applicability of this methodology.

Thank you for your suggestion. In the approach used in this study, which systemically changes signaling components, it is not feasible to tailor outcomes of both proximal and distal cell fates within the same organoid without providing spatially controlled inputs. Wew approaches such as our parallel BioRxiv work with spatial organizers (https://doi.org/10.1101/2024.11.30.626171) might pave the way for this.