nature portfolio

Peer Review File

Single-nucleus multi-omics analysis of mouse small-intestinal Lgr5+ cell populations reveals Foxa3-induced Paneth cell-lineage differentiation

Corresponding Author: Professor Ning Jiang

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)
The manuscript by Deng and colleagues investigates the molecular heterogeneity of Lgr5-positive intestinal stem cells (ISCs) using single-cell RNA and ATAC sequencing. Their analysis identifies six distinct subpopulations within the Lgr5+ ISC compartment, including columnar base cells, label-retaining cells, Lgr5-low ISCs, transient amplifying cells, and enterocyte progenitors. Bioinformatic predictions suggest differentiation trajectories, with Lgr5-low ISCs giving rise to either absorptive or secretory lineages and label-retaining cells identified as secretory progenitors. Further transcription factor analysis highlights Foxa3 as a key regulator, with its knockdown impairing Paneth and goblet cell marker expression while increasing enterocyte gene expression in intestinal organoids.
This study presents valuable data on ISC heterogeneity; however, the lack of novel functional insights, the need for clearer interpretations of some results, and certain methodological details require further clarification. Addressing these concerns would strengthen the manuscript considerably.

Major concerns:
The study has little novelty. The subsets of ISCs were already previously described and functionally validated. Also, the differentiation paths were previously described. https://doi.org/10.1152/ajpgi.00188.2021 for the reference.
Limited Novelty: The study reiterates previously described ISC subtypes and differentiation trajectories without providing significant new functional insights. For example, the subpopulations and their roles have already been reported (e.g., DOI: 10.1152/ajpgi.00188.2021). The manuscript should clarify how this work expands upon prior knowledge.
Figure 2B: The finding that absorptive progenitors exhibit greater stemness potential than transient amplifying cells or CBCs is unexpected. How do the authors reconcile this with established models of ISC differentiation?
Supplementary Figure 8: The differences between CBCs, TA Hmgb2, and Lgr5-low ISCs appear subtle. Could the authors provide exact log2 fold changes and adjusted p-values for the expression differences between these clusters?
Supplementary Figure 9: The reported Z-score range of -1 to 1 suggests minimal variation. What are the corresponding log2 fold changes and FDR values for the differentially accessible TF binding sites?
Supplementary Figure 10b: How was the FDR for motif accessibility calculated? Please provide the FDR values for clarity.
Supplementary Figure 10c: The Pearson correlation appears weak and statistically insignificant for absorptive progenitors. How do the authors interpret this finding in the context of their conclusions?
Supplementary Figure 10d: The prediction of only 2 to 6 target genes per GO term suggests limited biological significance. Could the authors comment on this and provide additional validation?
Supplementary Figure 11: The weakest Pearson correlation between Foxa3 motifs and Regulon is observed in Lgr5+ label-retaining cells, despite the claim that these cells are Paneth cell precursors. If Foxa3 is crucial for Paneth cell differentiation, shouldn't the correlation be stronger?
Figure 5 It is unclear whether Foxa3 is required for ISC differentiation into the Paneth-Goblet lineage or if it primarily regulates Paneth cell maturation. Could the authors clarify this distinction?
Do the Foxa3 knockdown organoids form typical crypt-like structures, or do they resemble undifferentiated spheroids?
Does Foxa3 depletion affect other secretory cell types (e.g., goblet, enteroendocrine, or tuft cells), or is the effect specific to Paneth and goblet cells?
Supplementary Figure 12: The absence of Foxa3 expression in pre-Paneth cells is intriguing. How do the authors explain this discrepancy given its proposed role in secretory differentiation?

Minor Concerns:
In the sentence: “Four of these functional parenchymal cells were derived from Lgr5+ intestinal stem cells (ISCs),” please replace parenchymal with epithelial for accuracy.
Supplementary Figure 7: The methodology for defining gene expression modules needs further clarification. How were the modules established, and what is the log2 fold change threshold for differentially expressed genes?
The authors state: “Moreover, the primary function of the direct target genes of Hnf4a, as postulated in this experiment, was a regulatory role related to the binding of the structural domain (Supplementary Fig. 10d).” It is not clear what this sentence means.

Reviewer #2

(Remarks to the Author)
Cells that exit the crypt base initiate differentiation. Although Lgr5 remains expressed in these cells, various differentiation processes progressively evolve. During this transition, cell cycles are finely regulated, and chromatin accessibility is remodeled, enabling key transcription factors to access their downstream binding sites and activate differentiation programs, thereby committing cells to distinct fates. This study employs single-nucleus multi-omics (RNA + ATAC) to explore the heterogeneity of Lgr5+ small intestinal stem cells (ISCs), a powerful approach to elucidate this complex process. Regarding to the novelty
However, the authors make several assumptions based on the multi-omics profiling alone, and several critical questions remain unresolved, preventing definitive conclusions.

1. In line 175, the authors conclude that Lgr5-low stem cells exhibit much higher stem cell potential than Lgr5-high CBC cells, based on CytoTRACE analysis. However, the authors overlook the fact that both absorptive progenitor (AP) and transit-amplifying (TA) populations exhibit higher differentiation trajectories than CBC cells. Notably, the current dataset includes only sorted Lgr5+ cells, which lacks a comprehensive developmental hierarchy. Given that the dataset does not capture the entire spectrum of cell types across all differentiation stages, the reliability of CytoTRACE analysis in this context is uncertain. The authors should address whether CytoTRACE analysis is appropriate for the current dataset, which may not fully encompass the developmental complexities of ISCs.

2. The manuscript’s key finding—that Foxa3 is highly expressed in Lgr5+ LRC/secretory progenitor cells compared to the AP population, with Foxa3 motifs more enriched in the ATAC-seq dataset—suggests an important role for Foxa3 in regulating secretory cell differentiation. However, the authors propose that Foxa3 acts as a pioneer factor in this process without providing direct experimental evidence to support this hypothesis. To strengthen this claim, the authors should clarify the specific binding sites of Foxa3 in the genome. A ChIP-seq experiment may be needed. A comparison of the ChIP-seq peaks with the ATAC-seq data would be particularly informative, as it would demonstrate whether Foxa3 facilitates chromatin remodeling, thus promoting the accessibility of key regulatory regions during differentiation.

3. A recent study published in Nature (2023) identified FOXA3 as a master transcription factor for goblet cell differentiation in the human gut. Despite this, the authors of the current manuscript focus primarily on Foxa3 as a putative transcription factor for Paneth cell differentiation. This raises a critical question: why do the authors conclude that Foxa3 predominantly regulates Paneth cell differentiation rather than goblet cells, especially when the limited qPCR results presented in Figure 4b show similar downregulation of goblet cell markers? The manuscript should clarify why Foxa3 is exclusively associated with Paneth cell differentiation in this context, especially considering the broader literature suggesting its significant role in goblet cell differentiation. Addressing this discrepancy would strengthen the manuscript’s conclusions and provide a more comprehensive understanding of Foxa3’s role in secretory cell differentiation.

The manuscript would benefit from a more careful selection of terms and precise language. I recommend that the authors thoroughly review the entire manuscript for accuracy in terminology. Below are a few examples of suggested revisions:

1. Line 123:
The term "parenchymal cell" is not commonly used to describe differentiated cell types within the gut epithelium. I suggest the authors consider using a more appropriate term to accurately reflect the specific cell types being described.

2. Line 128:
The sentence "The ISCs were the most stemmed small intestinal cells" should not be used. I recommend rephrasing this sentence using more precise and scientifically appropriate language to convey the intended meaning.

3. Line 155:
It seems the authors aim to describe the heterogeneity within the Lgr5+ cell population, but referring to this as "cellular structure" is misleading. It would be more accurate to describe this variability as "cellular heterogeneity" among the Lgr5+ cells.

Version 1:

Reviewer comments:

Reviewer #2

(Remarks to the Author)
In the revised manuscript, the authors continue to assert that “Lgr5-low stem cells exhibit much higher stem cell potential than Lgr5-high CBC cells,” based solely on computational analysis (CytoTrace2). However, the score differences among Lgr5-low, Top2a+ TA, CBC, and Hmgb2+ TA populations are subtle and do not convincingly support such a strong conclusion. This statement appears overstated and insufficiently supported by the current data. I recommend that the authors substantially moderate or remove this claim unless robust experimental validation can be provided, particularly since this point is not the main focus of the manuscript.

The authors have strengthened their analysis of Foxa3-mediated regulation of Paneth cell differentiation by incorporating CUT&Tag-seq data. The new dataset identifies the Foxa3 binding motif and genome-wide binding sites, suggesting potential downstream interactions with PPAR transcription factors. While this addition provides some mechanistic depth, it largely reinforces existing knowledge. Furthermore, the authors’ own data indicate that Foxa3 is involved in both Goblet and Paneth cell differentiation, which weakens the specificity of their proposed model initially.
Overall, although the manuscript has improved technically, the novelty and strength of the conclusions remain limited. I would recommend acceptance after the authors appropriately temper their claims and clarify the distinct role of Foxa3 within secretory cell differentiation.

Reviewer #3

(Remarks to the Author)
In the manuscript “Single-nucleus multi-omics analysis of mouse small-intestinal Lgr5+ cell populations reveals Foxa3-induced Paneth cell-lineage differentiation” Deng and colleagues provide a deep analysis of small intestinal stem cells using multi-omics approaches. Notable additions to the revised manuscript include CUT&Tag analysis to strengthen the association between Foxa3 and PPAR to regulate Paneth cell differentiation.

Reviewer 1 comments:
1. Novelty: There were concerns regarding the novelty of the findings in the original manuscript. The authors have more clearly explained the advancement of knowledge of their manuscript in the introduction and discussion, which is largely the link between Foxa3 and secretory cell differentiation and that this is via PPAR transcriptional activity.
2. Figure 2B: results have changed in the revised manuscript, aligning with Reviewer 1’s query about increased stemness in absorptive progenitors.
3. Supplementary Figure 8: acceptable response
4. Supplementary Figure 9: acceptable response
5. Supplementary Figure 10: acceptable responses
6. Supplementary Figure 11: The authors provide a valid response to the question regarding the correlation between Foxa3 expression and motif accessibility. However, in the revised manuscript, the authors need to more accurately describe the results. The authors should state that the correlation of 0.99 is achieved when pooling cells within a cell type, but no correlation is seen within individual cell types.
7. Figure 5: acceptable response
8. Supplementary Figure 12: acceptable response
9. Minor changes: acceptable responses

Version 2:

Reviewer comments:

Reviewer #2

(Remarks to the Author)
The authors have appropriately toned down their original claim that “Lgr5-low stem cells exhibit much higher stem cell potential than Lgr5-high CBC cells”, and now frame their results as indicating a lack of a strict correlation between Lgr5 expression levels and inferred developmental potential.
I find this revision satisfactory, and I think it is OK with proceeding with acceptance of the manuscript.

Dear Editors and Reviewers,

Thank you very much for the valuable comments from the two reviewers and the opportunity to revise our manuscript entitled "Single-nucleus multi-omics analysis of mouse small-intestinal Lgr5+ cell populations reveals Foxa3-induced Paneth cell-lineage differentiation" (Manuscript ID: COMMSBIO-25-0669). We sincerely appreciate the time and effort of the reviewers and editors who have dedicated to evaluating our work, which have substantially improved the clarity, rigor and novelty of our work.

We have carefully addressed all the reviewers' comments and have made substantial revisions to improve the manuscript accordingly. Below, we provide a point-by-point response to each comment. In particular, for the comments on the novelty and addition of Chip-seq experiment raised by the reviewers, we supplemented experiments of CUT&Tag-seq (to map Foxa3 binding sites) and bulk RNA-seq (to assess gene expression changes), and further revealed that Foxa3 regulates Paneth cell differentiation by targeting the peroxisome proliferator-activated receptor (PPAR) gene family. Extant studies have established that Ppard promotes Paneth cell differentiation, and miR-802 knockout mice (with elevated Ppard expression) have increased Paneth cell numbers. Our findings link Foxa3 to this PPAR-mediated pathway, refining our understanding of secretory lineage regulation.

All authors have approved the response letter and the revised version of the manuscript. We hope that the revised version of the manuscript will now be suitable for publication in *Communications Biology*.

Thank you for your time and consideration.

Sincerely,

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Associate Professor

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School of Life Sciences

Fudan University, Shanghai, China

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Reviewer #1 (Remarks to the Author):

The manuscript by Deng and colleagues investigates the molecular heterogeneity of Lgr5-positive intestinal stem cells (ISCs) using single-cell RNA and ATAC sequencing. Their analysis identifies six distinct subpopulations within the Lgr5+ ISC compartment, including columnar base cells, label-retaining cells, Lgr5-low ISCs, transient amplifying cells, and enterocyte progenitors. Bioinformatic predictions suggest differentiation trajectories, with Lgr5-low ISCs giving rise to either absorptive or secretory lineages and label-retaining cells identified as secretory progenitors. Further transcription factor analysis highlights Foxa3 as a key regulator, with its knockdown impairing Paneth and goblet cell marker expression while increasing enterocyte gene expression in intestinal organoids.

This study presents valuable data on ISC heterogeneity; however, the lack of novel functional insights, the need for clearer interpretations of some results, and certain methodological details require further clarification. Addressing these concerns would strengthen the manuscript considerably.

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Limited Novelty: The study reiterates previously described ISC subtypes and differentiation trajectories without providing significant new functional insights. For example, the subpopulations and their roles have already been reported (e.g., DOI: 10.1152/ajpgi.00188.2021). The manuscript should clarify how this work expands upon prior knowledge.

Response: Thanks for the valuable comment. As noted, prior studies have reported that Lgr5+ ISCs can differentiate into transient amplifying (TA) cells

(which further mature into absorptive precursors) or Lgr5+ label-retaining cells (LRCs, which mature into secretory lineages). Our revised manuscript advances this knowledge through three key novel contributions, enabled by the scRNA-seq/ATAC-seq datasets and also additionally supplemented experiments of CUT&Tag-seq (to map Foxa3 binding sites) and bulk RNA-seq (to assess gene expression changes):

First, this is the first study to analyze enriched mouse intestinal Lgr5+ ISCs using integrated scRNA-seq and scATAC-seq based on 10× Genomics Multiome technology. Unlike conventional single-omics approaches, 10× Genomics Multiome technology generates a unified map of a cell's transcriptome and epigenome. This dual-modality integration enables deeper insights into cellular physiological states, uncovers intracellular regulatory mechanisms, and provides a comprehensive view of Lgr5+ ISCs, ultimately allowing more precise identification of drivers of gene expression differences during differentiation.

Second, using this bimodal dataset, we not only validated the two canonical differentiation pathways of crypt base columnar (CBC) cells at both transcriptomic and epigenomic levels (a step beyond prior transcriptome-only studies) but also identified a novel Lgr5-low ISC subpopulation in mice. This novel subpopulation is consistent with recent findings in human intestinal stem cell research [1,2]. The Lgr5-low subpopulation exhibits comparable differentiation potential to Lgr5-high ISCs, with the capacity to differentiate into both absorptive progenitors and Lgr5+ ISC precursors, expanding our understanding of ISC developmental pathways beyond the classic CBC-centric model.

Third, we uncovered a novel regulatory mechanism of Foxa3 in Paneth cell differentiation. By joint analysis of gene expression dynamics and chromatin accessibility motif enrichment along differentiation trajectories, we identified Foxa3 as a key transcription factor for secretory lineage commitment. Additionally, based on the valuable suggestions from the reviewers, we

supplemented experiments of CUT&Tag-seq (to map Foxa3 binding sites) and bulk RNA-seq (to assess gene expression changes) to explore the specific regulatory mechanisms of Foxa3 during Paneth cell differentiation:

1. Foxa3 knockdown organoid samples exhibited significant downregulation of genes' expression within the Peroxisome Proliferator-Activated Receptor (PPAR) signaling pathway, including Peroxisome Proliferator-Activated Receptors (Ppara, Ppard, and Pparg).
2. Concurrently, the 3 kb upstream regions of all downregulated genes exhibited significant enrichment for binding motifs corresponding to the PPARs gene family (Ppara, Ppard, and Pparg) transcription factors.
3. At the epigenomic level, CUT&Tag-seq data targeting Foxa3 revealed distinct peaks in the 3 kb region upstream of the TSSs of the PPARs gene family, indicating Foxa3 protein binding in these regions.
4. The 3 kb upstream regions of these PPARs family genes (candidate promoters) were predicted to contain Foxa3 transcription factor binding sites based on JASPAR DATABASE.
5. Motif enrichment analysis of the 3 kb upstream region of PPAR pathway genes also confirmed the presence of Foxa3 binding sites upstream of the transcription start sites (TSS) of these genes.

These findings demonstrated that Foxa3 could regulate Paneth cell differentiation by targeting the peroxisome proliferator-activated receptor (PPAR) gene family. Extant studies have established that Ppard promotes Paneth cell differentiation [3,4], and miR-802 knockout mice (with elevated Ppard expression) have increased Paneth cell numbers [5]. Our findings link Foxa3 to this PPAR-mediated pathway, refining our understanding of secretory lineage regulation.

In summary, our work complements prior studies by providing a multi-omics validation of ISC differentiation pathways, identifying a novel Lgr5-low ISC subpopulation, and uncovering a Foxa3-PPAR regulatory axis in Paneth cell differentiation.

Reference 1. Malagola, E. et al. Isthmus progenitor cells contribute to homeostatic cellular turnover and support regeneration following intestinal injury. Cell 187, 3056-3071 e3017 (2024).
Reference 2. Capdevila, C. et al. Time-resolved fate mapping identifies the intestinal upper crypt zone as an origin of Lgr5+ crypt base columnar cells. Cell 187, 3039-3055 e3014, doi:10.1016/j.cell.2024.05.001 (2024).
Reference 3. Varnat F, Heggeler BB, Grisel P, Boucard N, Corthésy-Theulaz I, Wahli W, Desvergne B. PPARbeta/delta regulates paneth cell differentiation via controlling the hedgehog signaling pathway. Gastroenterology. 2006 Aug;131(2):538-53. doi: 10.1053/j.gastro.2006.05.004. PMID: 16890607.
Reference 4. Peters, J. M., Hollingshead, H. E., & Gonzalez, F. J. (2008). Role of peroxisome-proliferator-activated receptor β/δ (PPARβ/δ) in gastrointestinal tract function and disease. Clinical Science, 115(4), 107–127. https://doi.org/10.1042/cs20080022
Reference 5. Goga A, Yagabasan B, Herrmanns K, Godbersen S, Silva PN, Denzler R, Zünd M, Furter M, Schwank G, Sunagawa S, Hardt WD, Stoffel M. miR-802 regulates Paneth cell function and enterocyte differentiation in the mouse small intestine. Nat Commun. 2021 Jun 7;12(1):3339. doi: 10.1038/s41467-021-23298-3. PMID: 34099655; PMCID: PMC8184787.

Figure 2B: The finding that absorptive progenitors exhibit greater stemness potential than transient amplifying cells or CBCs is unexpected. How do the authors reconcile this with established models of ISC differentiation?

Response: We appreciate the reviewer's comments and have improved the methodology for assessing stem cell scores within small intestinal stem cell subpopulations. A detailed explanation of these modifications is provided in the revised manuscript (lines 181–186 and Figure2), and is summarized as follows: We employed CytoTrace2 to analyze the complete lineage of small intestinal cell types across all differentiation stages, encompassing various stem cell subpopulations and six intestinal cell lineages. Unlike CytoTrace, which evaluates stemness based on a single input dataset, CytoTrace2 is an interpretable deep learning framework that integrates 31 scRNA-seq datasets from human and mouse sources, covering 28 tissue types. This comprehensive dataset enables CytoTrace2 to generalize experimentally validated potency levels and differentiation states across the entire cellular developmental spectrum [6].

In our analysis of stemness among small intestinal stem cell subpopulations, we first included fully differentiated functional cells of the small intestine in the overall assessment before comparing differences between the subpopulations. While this analytical approach may result in more pronounced differences in

stemness between small intestinal stem cells and absorptive or secretory lineage cells, and relatively smaller differences between the stem cell subpopulations (Figure r1), we believe that this method provides a more accurate reflection of the true stemness patterns among these cells.

The updated findings indicate that Lgr5-low stem cells exhibit the highest degree of stemness among small intestinal stem cell populations, followed by Lgr5-high CBC cells and transit-amplifying (TA) cells. Among the six identified stem cell clusters, secretory progenitor cells-Lgr5+ label-retaining cells (LRCs) and absorptive progenitor cells (APs) display relatively lower levels of stemness (Figure r2).

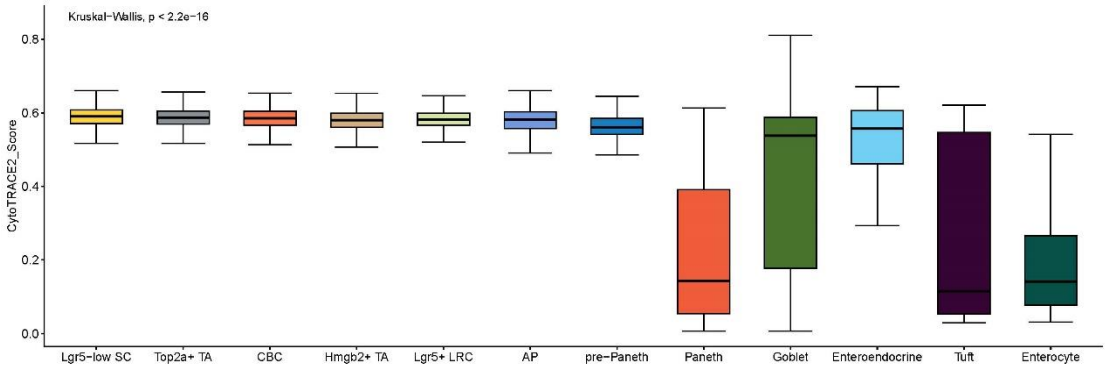

Figure r1: CytoTrace2 prediction results for the differentiation potential of small intestinal epithelial cells.

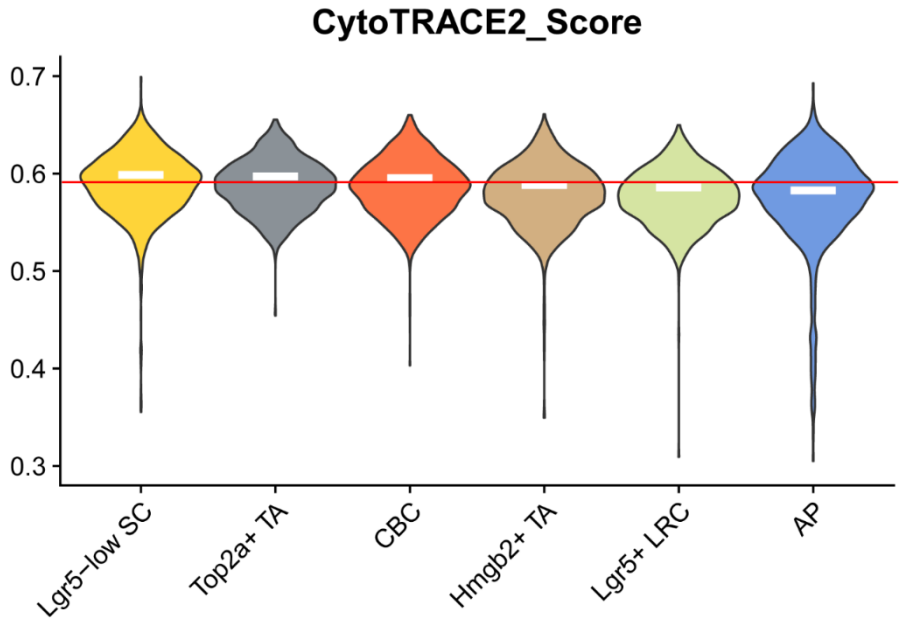

Figure r2: CytoTrace2 prediction results for the differentiation potential of small intestinal stem cells.

Reference 6. Kang M, Armenteros JJA, Gulati GS, Gleyzer R, Avagyan S, Brown EL, Zhang W, Usmani A, Earland N, Wu Z, Zou J, Fields RC, Chen DY, Chaudhuri AA, Newman AM. Mapping single-cell developmental potential in health and disease with interpretable deep learning. bioRxiv [Preprint]. 2024 Mar 21:2024.03.19.585637. doi: 10.1101/2024.03.19.585637. PMID: 38562882; PMCID: PMC10983880.

Supplementary Figure 8: The differences between CBCs, TA Hmgb2, and Lgr5-low ISCs appear subtle. Could the authors provide exact log2 fold changes and adjusted p-values for the expression differences between these clusters?

Response: Thanks for the valuable comment. As suggested, the log2 fold change values and adjusted p-values (corrected by Benjamini-Hochberg method) for the differential expression of specific transcription factors among the three stem cell subtypes (CBCs, TA Hmgb2, and Lgr5-low ISCs) presented in Supplementary Figure 8 were supplemented (Supplementary Data 1). Furthermore, the log2 fold change values and adjusted p-values (corrected by Benjamini-Hochberg method) for the differences in regulon activity scores (AUC values) of transcription factors and their target genes across the three subpopulations were calculated based on pySCENIC results and are summarized in Supplementary Data 2.

Supplementary Figure 9: The reported Z-score range of -1 to 1 suggests minimal variation. What are the corresponding log2 fold changes and FDR values for the differentially accessible TF binding sites?

Response: Thanks for the valuable comment. As suggested, we supplemented the log2 fold change values and adjusted p-values (corrected by Benjamini-Hochberg method) for differences in chromatin accessibility scores for the top 10 accessible TF binding sites in each small intestine stem cell subpopulation in Supplementary Data 3.

Supplementary Figure 10b: How was the FDR for motif accessibility calculated? Please provide the FDR values for clarity.

Supplementary Figure 10c: The Pearson correlation appears weak and

statistically insignificant for absorptive progenitors. How do the authors interpret this finding in the context of their conclusions?

Supplementary Figure 10d: The prediction of only 2 to 6 target genes per GO term suggests limited biological significance. Could the authors comment on this and provide additional validation?

Response: Thank you for the question. A series of Wilcoxon rank-sum tests were conducted between each of the six subgroups. The Benjamini-Hochberg method was employed to correct for the error rate resulting from multiple comparisons. The specific p-values and false discovery rate (FDR) for the differential analysis among the Lgr5-low SC, Lgr5+ LRC, and AP groups are detailed in the table below. Furthermore, the p-values in Figure 4c, d and Supplementary Figure 11b have been recalculated to reflect p.adjust.

CellType1	CellType2	p	p.adjust
Lgr5-low SC	Lgr5+ LRC	1.64e-75	2.7e-75
Lgr5-low SC	AP	2.89e-97	5.4e-97
Lgr5+ LRC	AP	1.37e-13	1.7e-13

In the present study, the correlation between gene expression levels and corresponding motif openness in individual cells for various transcription factors was investigated. The primary focus was on the correlations between different stem cell subpopulations. Specifically, based on the average expression levels and openness of TFs in each subpopulation, we compared their differences across various types of small intestine stem cells to simulate the dynamic changes in cellular development and differentiation processes. At the single-cell resolution, our results also indicate a significant correlation between the gene expression levels and motif open states of Hnf4α across all intestinal stem cells. However, this correlation is weaker within the AP cell cluster. This phenomenon is primarily attributed to the high variability in gene expression levels that is typically observed in single-cell sequencing data. Cells within the same subpopulation exhibit a high degree of similarity, however, single-cell

resolution has been observed to exaggerate the differences between cells within the same subpopulation. This subtle cellular heterogeneity has the potential to influence the stability of data when calculating correlations. This is particularly evident in the AP cluster, which exhibits the smallest total cell count of 708 cells. Technical errors in small sample sizes have the potential to mask true biological signals, resulting in weaker correlations and non-significant statistical tests.

Notably, the six subpopulations were divided into high-expression (AP, Top2a+ TA, Lgr5+ LRC) and low-expression groups based on Hnf4a expression. Correlation was non-significant across the three high-expression Hnf4a subpopulations, whereas correlations between expression and chromatin accessibility were significant within the three low-expression subpopulations. This may be due to the fact that, in the three groups with low accessibility, the accessibility of the Hnf4α motif plays a relatively important role in determining gene expression. Conversely, in the three groups exhibiting high accessibility, Hnf4α expression levels may have already surpassed the threshold necessary for transcription factors to effectively bind DNA and initiate transcription. A subsequent opening of the Hnf4α motif no longer induces specific transcription factor binding or a linear increase in target gene expression, resulting in a reduced correlation. At this juncture, disparities in Hnf4α expression may be attributable to alternative factors, such as post-transcriptional regulation.

In consideration of the inherent limitations of single-cell sequencing data and the primary focus of this study on the regulation of gene expression by chromatin accessibility as an epigenetic feature, it is hypothesized that treating the various subpopulations of ISCs as a whole to compare differences in gene expression and motif accessibility better reflects the dynamic regulatory processes of ISCs during early developmental differentiation in vivo.

With regard to the results of the GO analysis of the genes contained within the Hnf4α regulatory network, the limited number of enriched genes can be attributed to the fact that the regulatory network obtained through pySCENIC

software analysis exclusively includes genes that are directly targeted by Hnf4α. The entire regulatory network comprises a total of 13 genes. Consequently, the enrichment results indicate that each term comprises fewer genes. It is acknowledged that preceding studies have comprehensively examined the function of Hnf4α in the context of enterocyte differentiation. Consequently, the present study merely serves to corroborate these findings, devoid of any additional profound scrutiny.

Supplementary Figure 11: The weakest Pearson correlation between Foxa3 motifs and Regulon is observed in Lgr5+ label-retaining cells, despite the claim that these cells are Paneth cell precursors. If Foxa3 is crucial for Paneth cell differentiation, shouldn't the correlation be stronger?

Response: Thank you for the question. It is our belief that treating the various ISC subpopulations as a whole to compare differences in gene expression and motif accessibility between them better reflects the dynamic regulatory processes of ISC during early developmental differentiation in vivo. The correlations within each subpopulation are generally low, approximately 0.1, due to technical noise and anomalous amplified cellular heterogeneity among similar cells. Within each subpopulation, the highest correlation for Foxa3 is 0.14, and in the Lgr5+ LRC subpopulation, it is 0.085, with the actual values not differing significantly. Fluctuations in correlation R values may not accurately reflect true biological differences. Similarly, Foxa3 demonstrates analogous phenomena with Hnf4a from the preceding question. It is conceivable that the highest Foxa3 expression observed in the Lgr5+ LRC subpopulation may already exceed the threshold required for effective DNA binding and transcriptional initiation. Such disparities in gene expression levels may be attributable to post-transcriptional regulation or other associated factors. In consideration of the inherent limitations of single-cell sequencing data and the primary focus of this study on the regulation of gene expression via chromatin accessibility as an epigenetic feature, it is hypothesized that the synergistic

increase in average Foxa3 expression and motif openness in the Lgr5+ LRC subpopulation compared to the Lgr5-low SC subpopulation better reflects the upregulation phenotype of Foxa3 in Paneth cell differentiation and its important role. The study has also been supplemented with relevant CUT&Tag-seq (to map Foxa3 binding sites) and bulk RNA-seq sequencing experiments to further validate conclusions (Detailed information is described in Figures 5 and 6 of the revised manuscript.).

Figure 5 It is unclear whether Foxa3 is required for ISC differentiation into the Paneth-Goblet lineage or if it primarily regulates Paneth cell maturation. Could the authors clarify this distinction?

Do the Foxa3 knockdown organoids form typical crypt-like structures, or do they resemble undifferentiated spheroids?

Does Foxa3 depletion affect other secretory cell types (e.g., goblet, enteroendocrine, or tuft cells), or is the effect specific to Paneth and goblet cells?

Response: We thank the reviewer for this important suggestion. In order to further clarify the function of Foxa3 in small intestinal stem cell development, our revised manuscript was supplemented with target-specific cleavage and labelling (CUT&Tag-seq) with RNA sequencing (RNA-seq) analysis. In consideration of the extant results and antecedent studies, it is hypothesized that Foxa3 exerts a pivotal function in the differentiation of Paneth cells and goblet cells. More detailed descriptions were added in revised manuscript (lines 437-496 and Figure6) and also as below:

Initially, RNA-seq was performed on two normal small intestinal organoid samples and two Foxa3 knockdown organoid samples. The Foxa3 knockdown organoids were derived from small intestinal crypts enriched with small intestinal stem cells, which were established using the Wnt signal agonist CHIR-99021. A differential gene expression analysis was performed between the knockdown group and the control group, which revealed that Foxa3 knockdown significantly downregulated the expression of Paneth cell markers

(*Mmp7*, *Ang4*) (Figure r3).

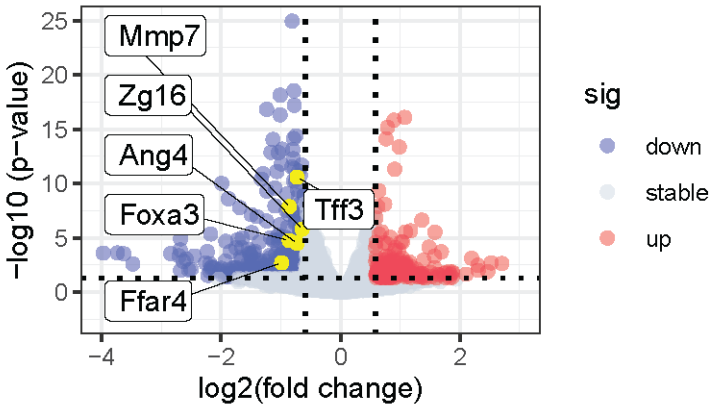

Figure r3: Volcano plot of differentially expressed genes in Foxa3 knockdown small intestinal organoids in comparison with normal small intestinal organoids.

Further KEGG analysis revealed that Foxa3 knockdown resulted in the downregulation of core genes in the PPAR signaling pathway, including Ppara, Ppard, and Pparg (Figure r4). Furthermore, the motif enrichment analysis of the 3 kb upstream regions of all downregulated genes in the knockdown group also enriched Ppara, Ppard and Pparg transcription factor binding motifs (Figure r5). The present findings indicate that Foxa3 exerts significant transcriptional regulation over the PPAR signaling pathway, particularly targeting PPARs ligands.

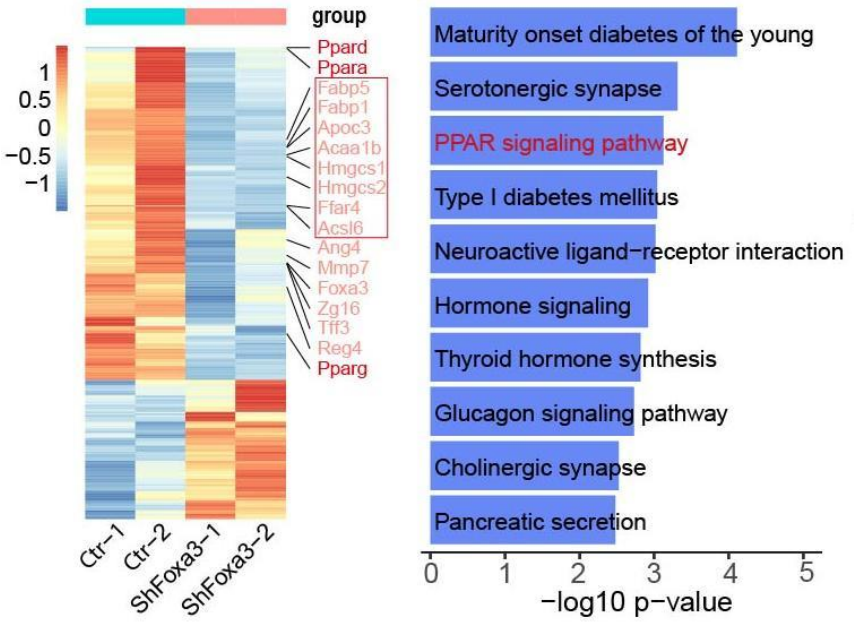

Figure r4: Heatmap of differentially expressed genes in Foxa3 knockdown small

intestinal organoids in comparison with normal small intestinal organoids. (left) KEGG functional enrichment analysis results for downregulated genes in the Foxa3 knockdown group. (right)

TF	Motif	P-Value
Foxa3		1e-6
Ppara/d		1e-55
Pparg		1e-49

Figure r5: Homer motif enrichment analysis results for downregulated genes in the Foxa3 knockdown group.

In order to provide further elucidation on potential Foxa3-PPAR interactions at the epigenomic level, a screening of CUT&Tag-seq data was conducted for Foxa3 protein binding within the 3 kb upstream region of PPARs gene TSSs. As hypothesized, the results were consistent with those obtained from bulk RNA-seq. The upstream regions of the transcription start sites (TSS) for multiple genes in the PPAR signaling pathways, including Ppara, Ppard, Hmgcs1, and Fabp5, exhibit Foxa3 peaks. This indicates that the Foxa3 protein binds to DNA within these regions (Figure r6). Subsequently, the 3 kb upstream region of these gene transcription start sites (candidate promoter regions) was extracted in order to predict Foxa3 transcription factor binding sites. The results obtained from JASPAR database confirmed the presence of the Foxa3-specific binding motif "GTAAACA" within the 3 kb upstream regions of these genes (Figure r7). Motif enrichment analysis of the 3 kb upstream regions of PPAR pathway genes also revealed significant enrichment of FOXA family transcription factors, including Foxa3 (Figure r8). The collective conclusion drawn from this analysis is that a regulatory relationship exists between Foxa3 and PPARs during the development of small intestinal stem cells.

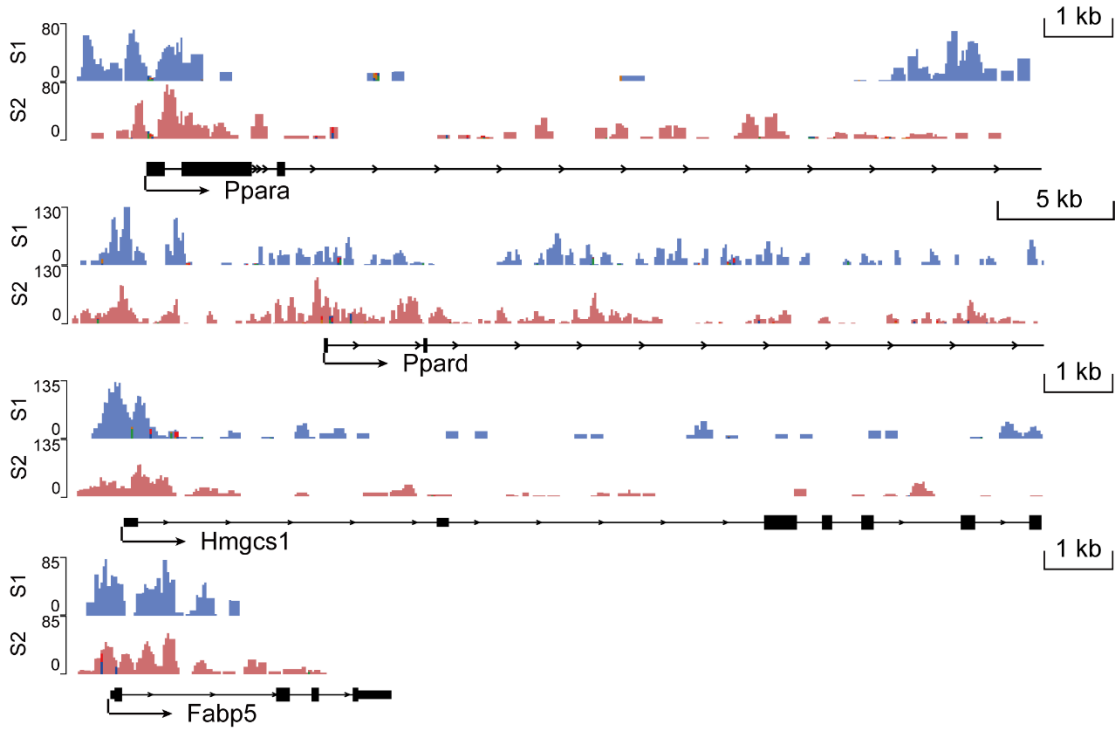

Figure r6: Gene tracks showing CUT&Tag-seq data for genes in the PPAR signaling pathway.

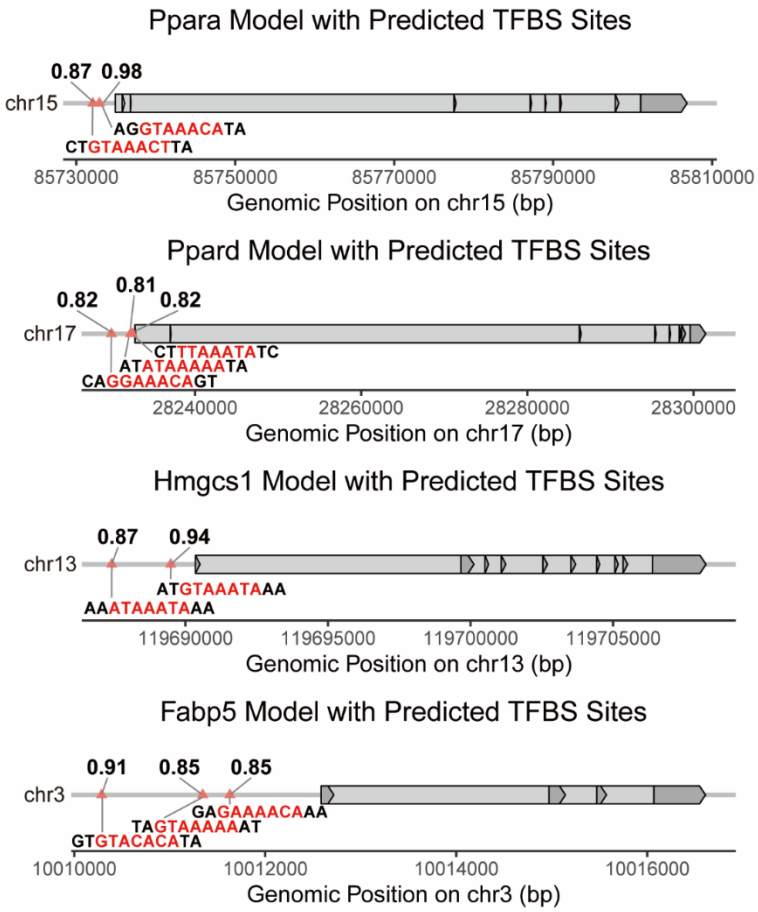

Figure r7: Model of Predicted Transcription Factor Binding Sites in the Upstream Regions of Genes in the PPAR Signaling Pathway

TF	Motif	P-Value
Foxa3		1e-2
Foxa2		1e-8
Foxa1		1e-9

Figure r8: Homer motif enrichment analysis results for genes in the PPAR pathway.

It has been demonstrated by preceding studies that Peroxisome Proliferator-Activated Receptors (PPARs, Ppara, Ppard, Pparg), are intimately linked to the process of Paneth cell differentiation[3,4,5]. Consequently, we reveal that Foxa3 predominantly promotes Paneth cell differentiation in the small intestine by regulating the expression of core genes within the PPAR signaling pathway, particularly members of the PPARs gene family.

We cultured both Foxa3 knockdown and control group organoids, and both were capable of budding. Furthermore, using bulk RNA-seq data, we analyzed the gene expression profile following Foxa3 knockdown and found that the expression of proliferation-related genes (i.e., Mki67, Mcm9, and Cdkn2d) showed moderate downregulation (Supplementary Data 4). These results indicate that Foxa3 knockdown suppresses stem cell proliferation, consistent with the observed morphology of the organoids.

The effects of Foxa3 on other small intestinal secretory cells are discussed below. The qPCR results obtained in this study suggest that the knockdown of Foxa3 results in a reduction in the expression levels of goblet cell markers, including *Tff3* and *Reg4*. Moreover, the supplementary bulk RNA-seq data demonstrate that genes that are significantly downregulated in the Foxa3 knockdown group compared to the control group also encompass goblet cell markers such as *Zg16*, *Tff3*, *Ffar4* and *Reg4* (Figures r3 and r4). At the epigenome level, supplementary CUT&Tag-seq data also revealed Foxa3 transcription factor binding peaks within the gene regulatory region upstream

of the *Tff3* gene TSS by 3 kb (candidate promoter). Foxa3 TFBS were indeed detected within the 3 kb upstream region of the *Tff3* gene (Figure r9). In consideration of these findings and a recent study published in Nature (2023) which identified FOXA3 as the master transcription factor for human intestinal goblet cell differentiation[7], it is proposed that Foxa3 also plays a significant role in the differentiation of goblet cells.

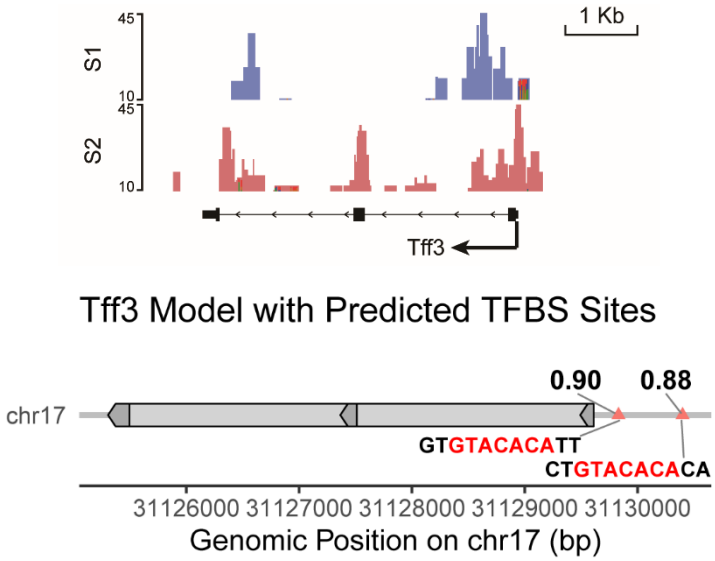

Figure r9: Gene tracks showing CUT&Tag-seq data for marker genes of goblet cells (top).
Model of predicted Transcription Factor Binding Sites in the upstream regions of Foxa3 (bottom).

In conclusion, our study reveals that Foxa3 influences Paneth cell differentiation by regulating the expression of PPARs ligands. It also provides further evidence to confirm that Foxa3 has an impact on goblet cell differentiation.

Reference 7. Hickey JW, Becker WR, Nevins SA, Horning A, Perez AE, Zhu C, Zhu B, Wei B, Chiu R, Chen DC, Cotter DL, Esplin ED, Weimer AK, Caraccio C, Venkataraaman V, Schürch CM, Black S, Brbić M, Cao K, Chen S, Zhang W, Monte E, Zhang NR, Ma Z, Leskovec J, Zhang Z, Lin S, Longacre T, Plevritis SK, Lin Y, Nolan GP, Greenleaf WJ, Snyder M. Organization of the human intestine at single-cell resolution. Nature. 2023 Jul;619(7970):572-584. doi: 10.1038/s41586-023-05915-x. Epub 2023 Jul 19. PMID: 37468586; PMCID: PMC10356619.

Supplementary Figure 12: The absence of Foxa3 expression in pre-Paneth cells is intriguing. How do the authors explain this discrepancy given its proposed role in secretory differentiation?

Response: We appreciate the reviewer's constructive suggestions. More details on the expression of Foxa3 in pre-Paneth cells can be found below.

Firstly, the overall expression of Foxa3 in intestinal epithelial cells was examined. The results of the feature plot indicate that Foxa3 is expressed in all intestinal epithelial cell types with the exception of AP and Enterocyte (Figure r10). Specifically for pre-Paneth cells, the data confirmed that only approximately 25% of pre-Paneth cells exhibited Foxa3 expression levels greater than zero. In addition, within the Lgr5+ LRC subpopulation, approximately 50% of cells exhibited Foxa3 expression levels that exceeded zero. Consequently, we hypothesize that this paucity in gene expression may be attributable to intrinsic characteristics of single-cell transcriptomics: the saturation of sequencing data is insufficient in single-cell RNA-seq. Furthermore, the pre-Paneth subpopulation displays increased cellular heterogeneity in comparison to other subpopulations. This phenomenon may be attributed to substantial disparities between ISCs and cells within the small intestinal secretory lineage, resulting in the pre-Paneth cluster becoming dispersed between ISCs and Paneth cells during unsupervised clustering.

In light of the observed low saturation of gene expression in single-cell data, it is believed appropriate to employ FeaturePlot and Dotplot. The expression of Foxa3 in the pre-Paneth cell cluster within this dataset appears as zero in the violin plot, which could be misleading. Consequently, a FeaturePlot illustrating Foxa3 expression across different subpopulations has been incorporated into the revised manuscript.

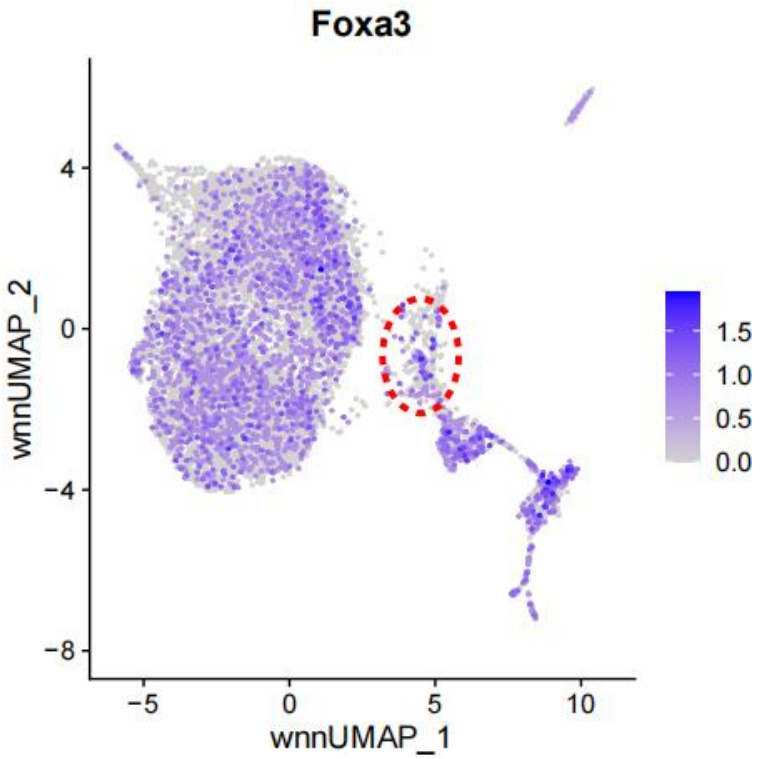

Figure r10: FeaturePlot of Foxa3 expression in small intestine epithelial cells

Minor Concerns:

In the sentence: “Four of these functional parenchymal cells were derived from Lgr5+ intestinal stem cells (ISCs),” please replace parenchymal with epithelial for accuracy.

Response: Thank you for the question. In accordance with your suggestion, this sentence on line 123 of the primary text has been revised.

Supplementary Figure 7: The methodology for defining gene expression modules needs further clarification. How were the modules established, and what is the log2 fold change threshold for differentially expressed genes?

Response: Thank you for the question. We used the graph_test function in the monocle3 package and set the “neighbor_graph” parameter to “principal_graph” to calculate whether each gene has related expression in cells at similar positions along the pseudo-time developmental trajectory. The results are saved as Moran's I index, with values ranging from -1 to 1. A value of 0 indicates

that this gene has no spatial co-expression effect, while a value of 1 indicates that this gene has highly similar expression values in cells with similar spatial distances. Ultimately, genes with a Moran's I index greater than 0.1 and a adjusted p-value (corrected by Benjamini-Hochberg method) of 0 are selected as highly differentially expressed genes along the pseudo-time trajectory for the formation of gene expression modules. These genes have a log2 fold change greater than 0.3.

Then, the aggregate_gene_expression function was used to cluster these genes into small modules based on their expression patterns via community analysis using the Louvain algorithm. The small modules were merged through an appropriate number of iterations to form gene modules specifically expressed by each cell subpopulation during development.

The authors state: “Moreover, the primary function of the direct target genes of Hnf4a, as postulated in this experiment, was a regulatory role related to the binding of the structural domain (Supplementary Fig. 10d).” It is not clear what this sentence means.

Response: Thank you for the question. In this section, an explanation is provided as to why the Hnf4α regulatory element is not directly enriched in term related to small intestinal epithelial cell differentiation. Rather, it is enriched in functions that affect transcription, such as the regulation of chromatin structure. This is due to the fact that the pySCENIC software analysis results exclusively retain target genes upstream that contain direct binding sites for the Hnf4α motif. Consequently, effector genes that result from subsequent changes in chromatin structure are not included.

Moreover, the present findings are in accordance with those of preceding studies, which demonstrate that HNF4 has the capacity to influence chromatin accessibility and regulate gene expression by modulating the dynamic occupancy of RNA polymerase II, thereby driving enterocytes differentiation [8,9]. The present results merely provide preliminary validation of genes directly

activated by Hnf4α for transcription, and no further investigation has been made into their complex regulatory processes involving enterocyte markers. Further details can be found in lines 280 to 290 of the revised manuscript.

Reference 8. Chen, L., Cao, W., Aita, R., Aldea, D., Flores, J., Gao, N., Bonder, E. M., Ellison, C. E., & Verzi, M. P. (2021). Three-dimensional interactions between enhancers and promoters during intestinal differentiation depend upon HNF4. Cell Reports, 34(4), 108679. https://doi.org/10.1016/j.celrep.2020.108679

Reference 9. Vemuri, K., Kumar, S., Chen, L., & Verzi, M. P. (2024). Dynamic RNA polymerase II occupancy drives differentiation of the intestine under the direction of HNF4. Cell Reports, 43(6), 114242. https://doi.org/10.1016/j.celrep.2024.114242

Reviewer #2 (Remarks to the Author):

Cells that exit the crypt base initiate differentiation. Although Lgr5 remains expressed in these cells, various differentiation processes progressively evolve. During this transition, cell cycles are finely regulated, and chromatin accessibility is remodeled, enabling key transcription factors to access their downstream binding sites and activate differentiation programs, thereby committing cells to distinct fates. This study employs single-nucleus multi-omics (RNA + ATAC) to explore the heterogeneity of Lgr5+ small intestinal stem cells (ISCs), a powerful approach to elucidate this complex process. Regarding to the novelty However, the authors make several assumptions based on the multi-omics profiling alone, and several critical questions remain unresolved, preventing definitive conclusions.

1. In line 175, the authors conclude that Lgr5-low stem cells exhibit much higher stem cell potential than Lgr5-high CBC cells, based on CytoTRACE analysis. However, the authors overlook the fact that both absorptive progenitor (AP) and transit-amplifying (TA) populations exhibit higher differentiation trajectories than CBC cells. Notably, the current dataset includes only sorted Lgr5+ cells, which lacks a comprehensive developmental hierarchy. Given that the dataset does not capture the entire spectrum of cell types across all differentiation stages, the reliability of CytoTRACE analysis in this context is uncertain. The authors

should address whether CytoTRACE analysis is appropriate for the current dataset, which may not fully encompass the developmental complexities of ISCs.

Response: We appreciate and totally agree with the reviewer's insightful comment. As suggested, we refined the analytical methodology for assessing stemness scores within small intestinal stem cell subpopulations, resulting in a more precise and biologically relevant assessment of cellular stemness. A detailed description of these modifications can be found in the revised manuscript (lines 181–186 and Figure2), as summarized below:

Following your suggestion, we used CytoTrace2 to analyze the complete lineage of small intestinal cell types across all differentiation stages, including various small intestinal stem cell subpopulations and functional cells.

As you know, CytoTrace predicts single-cell orderings based on the number of detectable expressed genes in each cell within a single dataset, with its results primarily reflecting the relative stemness ranking of cells in that dataset. In contrast, CytoTrace2 is an interpretable deep learning framework designed to characterize cell potency and differentiation states on an absolute scale using scRNA-seq data. What's notable is that the software itself includes 31 scRNA-seq datasets from humans and mice, covering 28 tissue types. These datasets allow it to recapitulate experimentally determined potency levels and differentiation states, spanning the entire spectrum of cellular ontogeny. This, in turn, enables more accurate assessment of stemness for the cells in our input data [10].

Additionally, when analyzing the stemness of small intestinal stem cell subpopulations, we included fully differentiated small intestinal functional cells in the overall analysis (Figure r11). This, we believe, further enhances the integrity of the input data when simulating the developmental level of the small intestine.

The updated results demonstrate that Lgr5-low stem cells retain the highest stemness among small intestinal stem cells, followed by Lgr5-high CBC cells

and transit-amplifying (TA) cells. Among the six stem cell clusters, the secretory lineage and absorptive lineage progenitor cells, Lgr5+ label-retaining cells (LRCs) and absorptive progenitors (APs), exhibit relatively the lowest stemness (Figure r12). Additionally, the stemness of each small intestinal stem cell subpopulation is higher than that of Paneth cell precursor cells and other parenchymal functional cells.

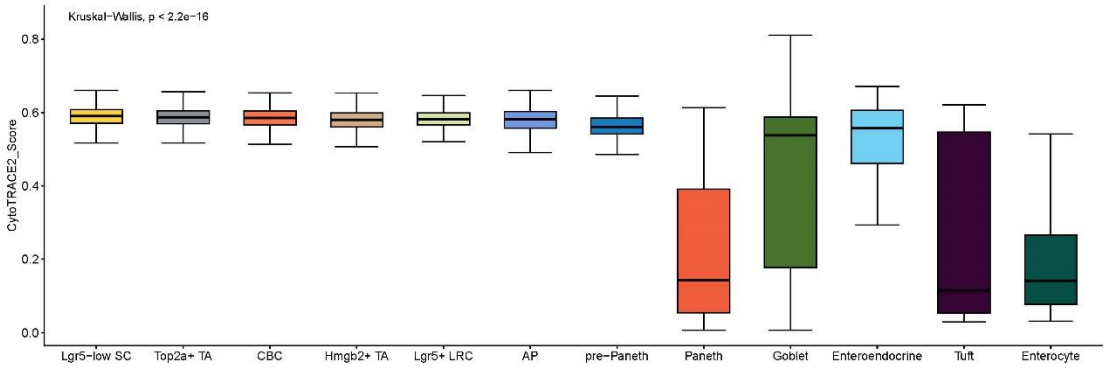

Figure r11: CytoTrace2 prediction results for the differentiation potential of small intestinal epithelial cells.

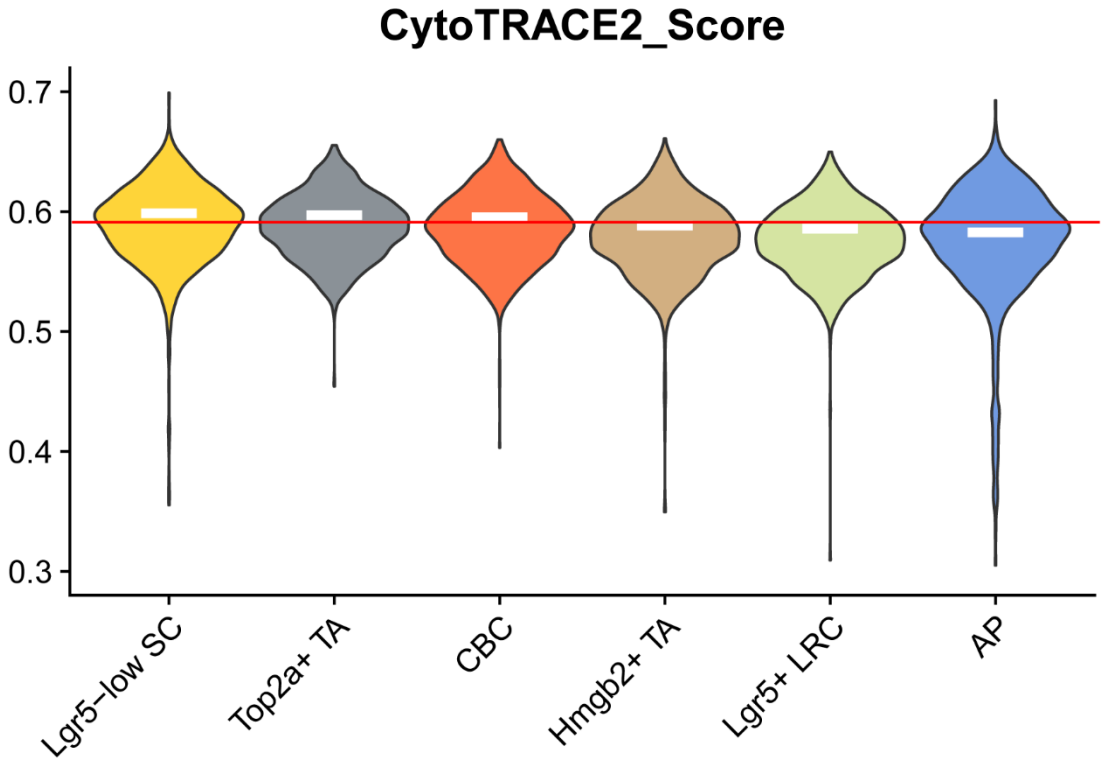

Figure r12: CytoTrace2 prediction results for the differentiation potential of small intestinal stem cells.

Reference 10. Kang M, Armenteros JJA, Gulati GS, Gleyzer R, Avagyan S, Brown EL, Zhang W, Usmani A, Earland N, Wu Z, Zou J, Fields RC, Chen DY, Chaudhuri AA, Newman AM. Mapping single-cell developmental potential

in health and disease with interpretable deep learning. bioRxiv [Preprint]. 2024 Mar 21:2024.03.19.585637. doi: 10.1101/2024.03.19.585637. PMID: 38562882; PMCID: PMC10983880.

2. The manuscript's key finding—that Foxa3 is highly expressed in Lgr5+ LRC/secretory progenitor cells compared to the AP population, with Foxa3 motifs more enriched in the ATAC-seq dataset—suggests an important role for Foxa3 in regulating secretory cell differentiation. However, the authors propose that Foxa3 acts as a pioneer factor in this process without providing direct experimental evidence to support this hypothesis. To strengthen this claim, the authors should clarify the specific binding sites of Foxa3 in the genome. A ChIP-seq experiment may be needed. A comparison of the ChIP-seq peaks with the ATAC-seq data would be particularly informative, as it would demonstrate whether Foxa3 facilitates chromatin remodeling, thus promoting the accessibility of key regulatory regions during differentiation.

Response: We thank the reviewer for this important suggestion. To further clarify the specific binding sites of Foxa3 and its downstream regulatory mechanisms, we combined target-specific cleavage and labeling (CUT&Tag-seq) with RNA sequencing (RNA-seq) analysis for screening and exploration. More detailed descriptions for the results from these two complementary experiments were added in revised manuscript as below: (lines 437-496 and Figure6)

As you suggested, the CUT&Tag sequencing technique was employed to investigate Foxa3 activity in two organoid samples derived from small intestinal crypts. The experimental results indicate that analysis of all peaks in the CUT&Tag-seq data using the Homer pattern enrichment tool revealed significant enrichment of Foxa3 and other FOXA family transcription factors (Figure r13). It was evident that both samples exhibited peaks of protein-binding in the promoter region of the Foxa3 transcription factor (Figure r14). Furthermore, the analysis of the 3 kb region upstream of the Foxa3 gene revealed the presence of the Foxa3 binding motif "GTAAACA" in the upstream

region (Figure r15). These findings not only confirmed the efficacy of the CUT&Tag targeting approach but also indicated that Foxa3 transcription and regulation are actively involved in the differentiation and development of small intestinal stem cells.

TF	Motif	P-Value
Foxa3		1e-10
Foxa2 (GSE47459*)		1e-13
Foxa2 (GSE25694*)		1e-5
Foxa1		1e-3

Figure r13: Homer motif enrichment analysis results for peaks in the CUT&Tag-seq data. * from NCBI Chip-seq experiments.

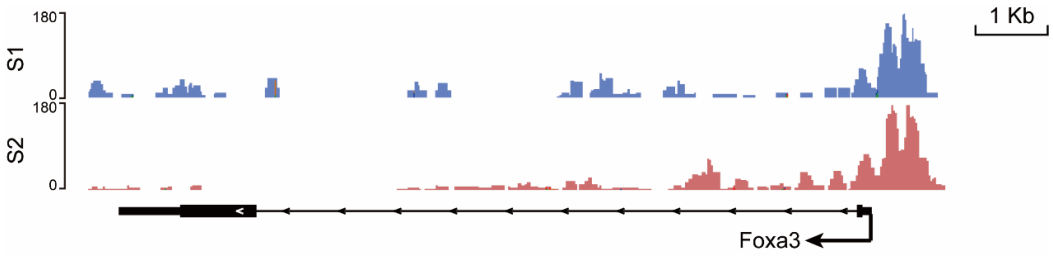

Figure r14: Gene tracks showing CUT&Tag-seq data for Foxa3.

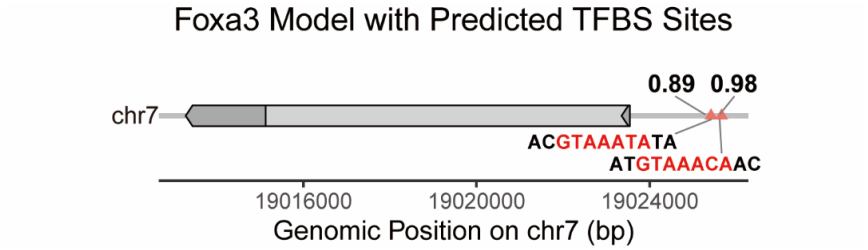

Figure r15: Model of predicted Transcription Factor Binding Sites in the upstream regions of Foxa3.

A further comparison was made between the consistency of CUT&Tag-seq peaks and scATAC-seq peaks. The initial stage of the study involved the enumeration of the number of genes exhibiting peaks within the gene body of each sample, as well as in the 10 kb regions upstream and downstream from it. The results indicate that all genes corresponding to CUT&Tag-seq peaks had matching peaks in the scATAC-seq data (Figure r16). Furthermore, by calculating the read density within the same peak regions after MAnorm normalization of both CUT&Tag-seq and scATAC-seq data, a comparison was

made of signal intensity across identical peak regions in both datasets. The findings reveal no substantial discrepancy in peak signal intensity between the two datasets, with the overall peak distributions exhibiting a high degree of consistency (Figure r17).

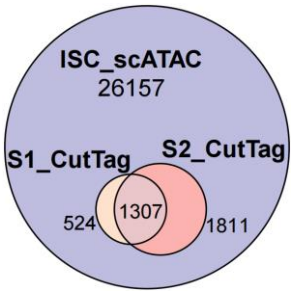

Figure r16: Statistical Venn diagram with peaks falling within genes and their regulatory regions

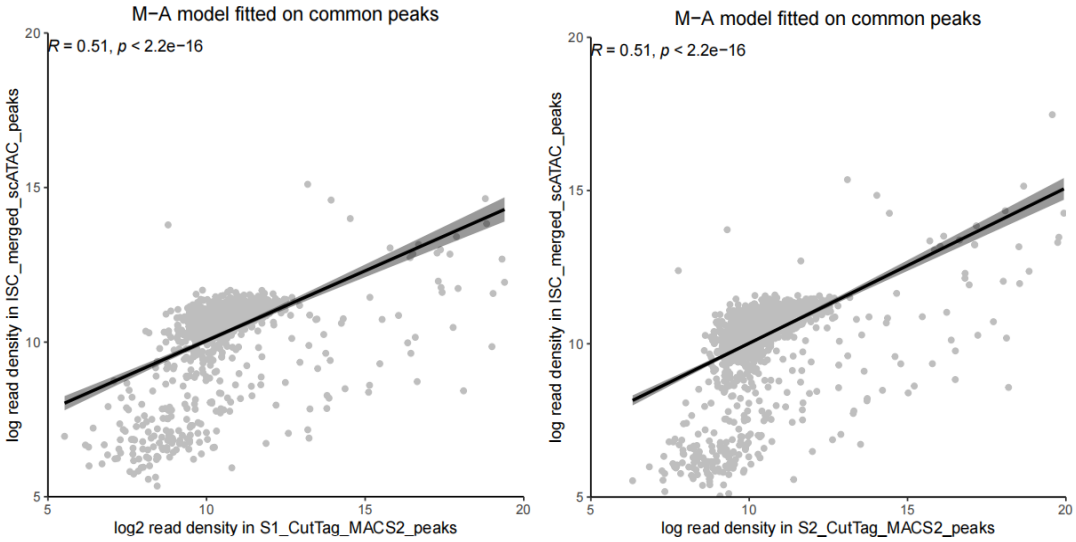

Figure r17: Correlation between peak signal intensities in CUT&Tag-seq data and scATAC seq data

In accordance with the preceding analysis, a screening process was initiated for markers of the secretory cell lineage. The results of this process indicate the presence of peaks suggestive of Foxa3 transcription factor binding within the candidate promoter region, specifically the 3 kb upstream of the transcription start site (TSS) for certain Paneth cell markers such as Mmp7. However, it is important to note that the overall binding strength in these regions is relatively weak (Figure r18). It is hypothesized that Foxa3 exerts only a limited influence

on Paneth cell differentiation by directly regulating the expression of genes such as Mmp7.

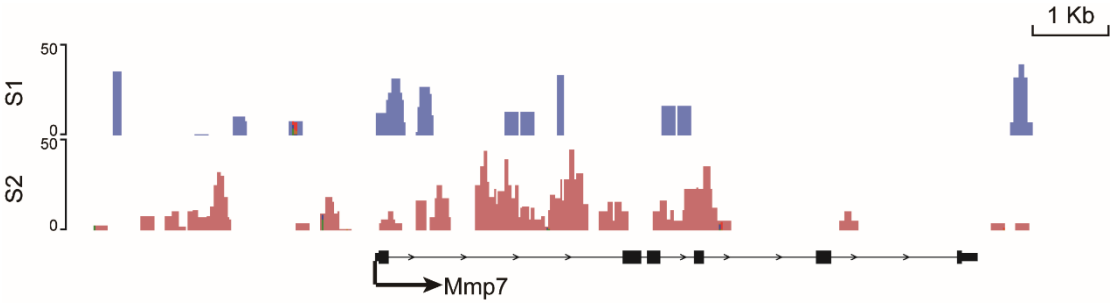

Figure r18: Gene tracks showing CUT&Tag-seq data for Mmp7.

In order to further elucidate the regulatory mechanism of Foxa3, RNA sequencing was performed on two normal intestinal organoid samples and two Foxa3 knockdown intestinal organoid samples. Differential gene expression analysis across both groups revealed a significant reduction in the expression of Paneth cell markers (*Mmp7*, *Ang4*) in Foxa3-knockdown organoids (Figure r19).

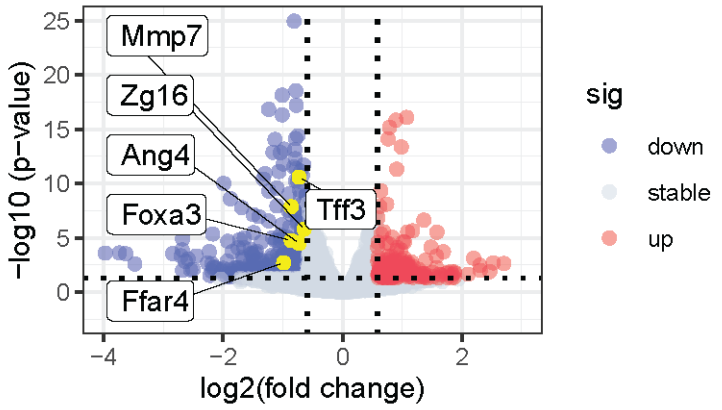

Figure r19: Volcano plot of differentially expressed genes in Foxa3 knockdown small intestinal organoids in comparison with normal small intestinal organoids

Further KEGG analysis revealed that Foxa3 knockdown resulted in the downregulation of core genes in the PPAR signaling pathway, including Ppara, Ppard, and Pparg (Figure r20). Furthermore, the motif enrichment analysis of the 3 kb upstream regions of all downregulated genes in the knockdown group also enriched Ppara, Ppard and Pparg transcription factor binding motifs

(Figure r21). The present findings indicate that Foxa3 exerts significant transcriptional regulation over the PPAR signaling pathway, particularly targeting PPARs ligands.

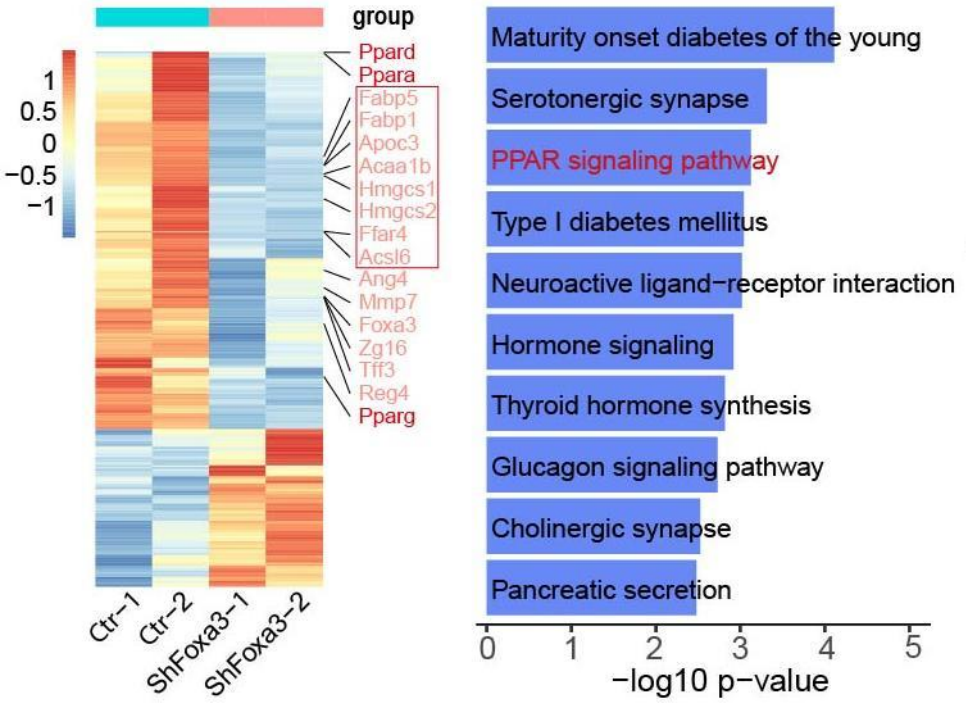

Figure r20: Heatmap of differentially expressed genes in Foxa3 knockdown small intestinal organoids in comparison with normal small intestinal organoids. (left)
KEGG functional enrichment analysis results for downregulated genes in the Foxa3 knockdown group. (right)

TF	Motif	P-Value
Foxa3		1e-6
Ppara/d		1e-55
Pparg		1e-49

Figure r21: Homer motif enrichment analysis results for downregulated genes in the Foxa3 knockdown group.

In order to provide further elucidation on potential Foxa3-PPAR interactions at the epigenomic level, a screening of CUT&Tag-seq data was conducted for Foxa3 protein binding within the 3 kb upstream region of PPARs gene TSSs.

As hypothesized, the results were consistent with those obtained from bulk RNA-seq. The upstream regions of the transcription start sites (TSS) for multiple genes in the PPAR signaling pathways, including Ppara, Ppard, Hmgcs1, and Fabp5, exhibit peaks. This indicates that the Foxa3 protein binds to DNA within these regions. (Figure r22). Subsequently, the 3 kb upstream region of these gene transcription start sites (candidate promoter regions) was extracted in order to predict Foxa3 transcription factor binding sites. The results confirmed the presence of the Foxa3-specific binding motif "GTAAACA" within the 3 kb upstream regions of these genes (Figure r23). Motif enrichment analysis of the 3 kb upstream regions of PPAR pathway genes similarly revealed significant enrichment of FOXA family transcription factors, including Foxa3 (Figure r24). The collective conclusion drawn from this analysis is that a regulatory relationship exists between Foxa3 and PPARs during the development of small intestinal stem cells.

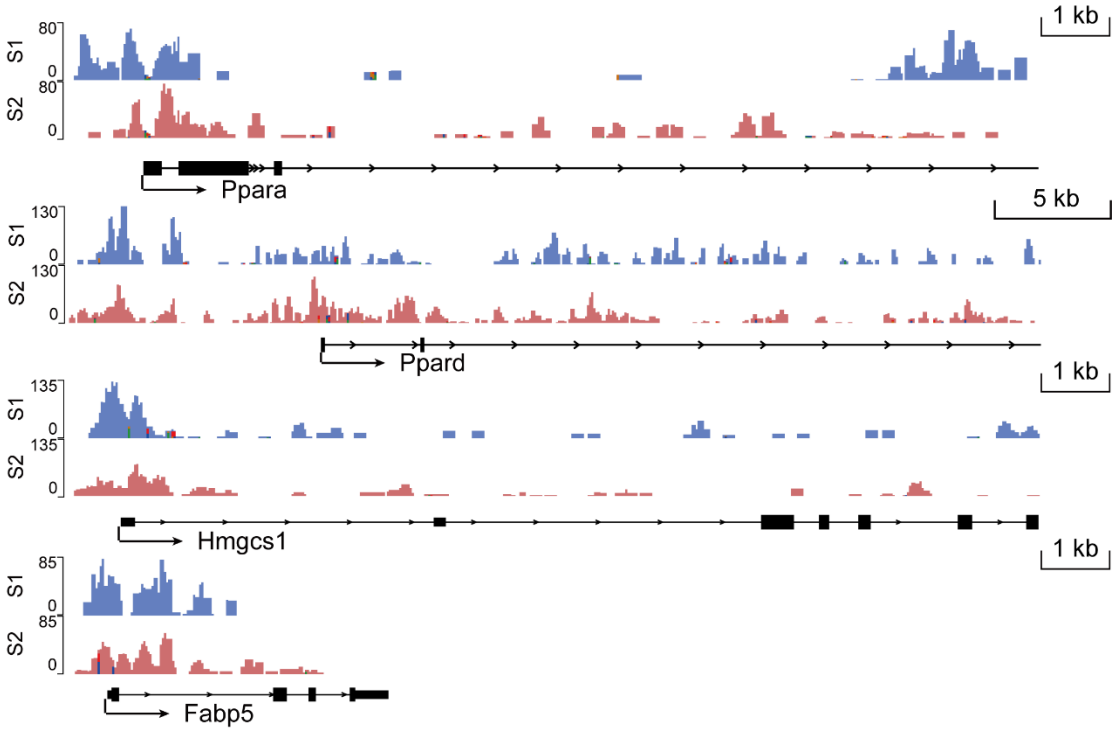

Figure r22: Gene tracks showing CUT&Tag-seq data for genes in the PPAR signaling pathway.

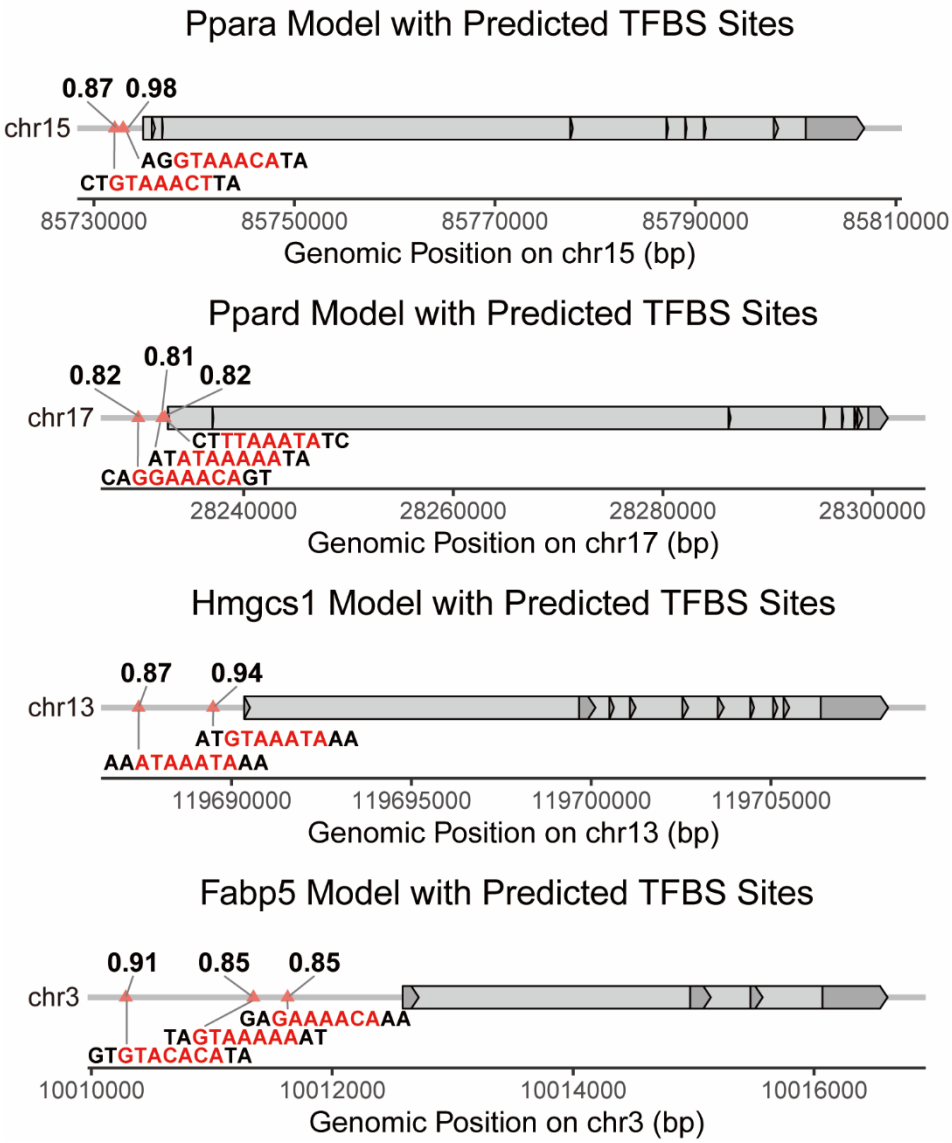

Figure r23: Model of predicted Transcription Factor Binding Sites in the upstream regions of genes in the PPAR signaling pathway.

TF	Motif	P-Value
Foxa3		1e-2
Foxa2		1e-8
Foxa1		1e-9

Figure r24: Homer motif enrichment analysis results for genes in the PPAR pathway.

Previous studies have demonstrated that Ppard promotes Paneth cell

differentiation [11, 12]. Consistently, miR-802-deficient mice exhibit increased Paneth cell numbers and elevated expression of Ppard, a known positive regulator of Paneth cell differentiation and maturation [13]. These findings collectively suggest a strong association between the PPARs and Paneth cell differentiation.

In summary, we hypothesize that Foxa3 primarily promotes Paneth cell differentiation in the small intestine by regulating the expression of core genes within the PPAR signaling pathway, specifically members of the PPARs family.

Reference 11. Varnat F, Heggeler BB, Grisel P, Boucard N, Corthésy-Theulaz I, Wahli W, Desvergne B. PPARbeta/delta regulates paneth cell differentiation via controlling the hedgehog signaling pathway. Gastroenterology. 2006 Aug;131(2):538-53. doi: 10.1053/j.gastro.2006.05.004. PMID: 16890607.

Reference 12. Peters, J. M., Hollingshead, H. E., & Gonzalez, F. J. (2008). Role of peroxisome-proliferator-activated receptor β/δ (PPARβ/δ) in gastrointestinal tract function and disease. Clinical Science, 115(4), 107–127. https://doi.org/10.1042/cs20080022

Reference 13. Goga A, Yagabasan B, Herrmanns K, Godbersen S, Silva PN, Denzler R, Zünd M, Furter M, Schwank G, Sunagawa S, Hardt WD, Stoffel M. miR-802 regulates Paneth cell function and enterocyte differentiation in the mouse small intestine. Nat Commun. 2021 Jun 7;12(1):3339. doi: 10.1038/s41467-021-23298-3. PMID: 34099655; PMCID: PMC8184787.

3. A recent study published in Nature (2023) identified FOXA3 as a master transcription factor for goblet cell differentiation in the human gut. Despite this, the authors of the current manuscript focus primarily on Foxa3 as a putative transcription factor for Paneth cell differentiation. This raises a critical question: why do the authors conclude that Foxa3 predominantly regulates Paneth cell differentiation rather than goblet cells, especially when the limited qPCR results presented in Figure 4b show similar downregulation of goblet cell markers? The manuscript should clarify why Foxa3 is exclusively associated with Paneth cell differentiation in this context, especially considering the broader literature suggesting its significant role in goblet cell differentiation. Addressing this discrepancy would strengthen the manuscript's conclusions and provide a more comprehensive understanding of Foxa3's role in secretory cell differentiation.

Response: We appreciate the reviewer's constructive suggestions. More

detailed descriptions for Foxa3's role in secretory cell differentiation were added in revised manuscript as below: (lines 398-413,469-475)

As you mentioned, our qPCR results indicate that Foxa3 knockdown leads to reduced expression levels of goblet cell markers, including *Tff3* and *Reg4*. Furthermore, the supplementary bulk RNA-seq data demonstrate that genes significantly downregulated in the Foxa3 knockdown group compared to the control group also encompass goblet cell markers such as *Zg16*, *Tff3*, *Ffar4* and *Reg4* (Figure r19, r20). At the epigenome level, supplementary CUT&Tag-seq data also revealed Foxa3 transcription factor binding peaks within the gene regulatory region upstream of the *Tff3* gene TSS by 3 kb (candidate promoter). Foxa3 TFBS were indeed detected within the 3 kb upstream region of the *Tff3* gene. (Figure r25). Based on these findings and previous studies [14], we propose that Foxa3 also plays a significant role in the differentiation of goblet cells.

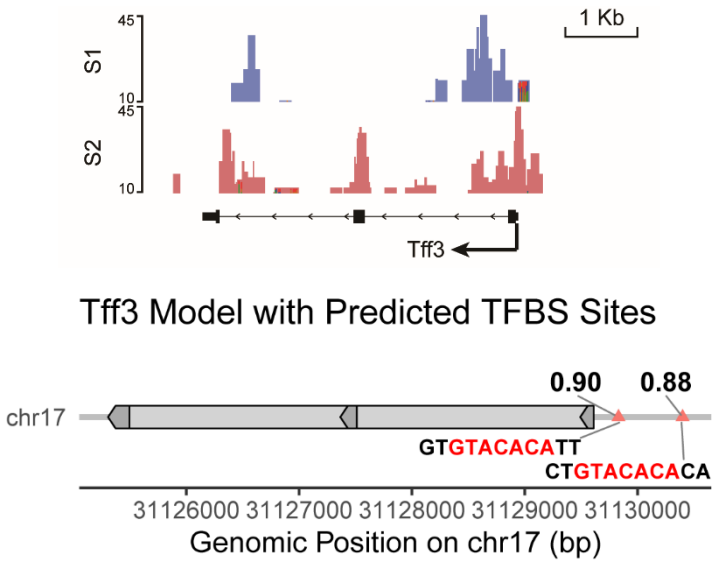

Figure r25: Gene tracks showing CUT&Tag-seq data for marker genes of goblet cells (top).
Model of predicted Transcription Factor Binding Sites in the upstream regions of Foxa3 (bottom).

It is important to note that the present study provides further evidence for Foxa3's role in regulating goblet cell differentiation. In addition, it identifies and validates during analysis that Foxa3 can influence Paneth cell differentiation by modulating the expression of related ligands, such as PPARs. This contributes

to the expansion of our understanding of Foxa3's role in the differentiation process of cells within the small intestinal secretory lineage.

Reference 14. Hickey JW, Becker WR, Nevins SA, Horning A, Perez AE, Zhu C, Zhu B, Wei B, Chiu R, Chen DC, Cotter DL, Esplin ED, Weimer AK, Caraccio C, Venkataraaman V, Schürch CM, Black S, Brbić M, Cao K, Chen S, Zhang W, Monte E, Zhang NR, Ma Z, Leskovec J, Zhang Z, Lin S, Longacre T, Plevritis SK, Lin Y, Nolan GP, Greenleaf WJ, Snyder M. Organization of the human intestine at single-cell resolution. Nature. 2023 Jul;619(7970):572-584. doi: 10.1038/s41586-023-05915-x. Epub 2023 Jul 19. PMID: 37468586; PMCID: PMC10356619.

The manuscript would benefit from a more careful selection of terms and precise language. I recommend that the authors thoroughly review the entire manuscript for accuracy in terminology. Below are a few examples of suggested revisions:

1. Line 123:

The term "parenchymal cell" is not commonly used to describe differentiated cell types within the gut epithelium. I suggest the authors consider using a more appropriate term to accurately reflect the specific cell types being described.

Response: Thank you for the question. We have revised the sentence in line 123 of the main text to read: “Four of these functional epithelial cells were derived from Lgr5+ intestinal stem cells (ISCs)”.

2. Line 128:

The sentence "The ISCs were the most stemmed small intestinal cells" should not be used. I recommend rephrasing this sentence using more precise and scientifically appropriate language to convey the intended meaning.

Response: Thank you for the question. We have revised the sentence in line 128 of the main text to read: “The ISCs located at the base of the crypt reside within a permissive niche that fosters their identity. As these cells move upward and exit the niche, there is a gradual decrease in their stemness, ultimately leading to their differentiation into absorptive or secretory cell lineage cells.”

3. Line 155:

It seems the authors aim to describe the heterogeneity within the Lgr5+ cell population, but referring to this as "cellular structure" is misleading. It would be more accurate to describe this variability as "cellular heterogeneity" among the

Lgr5+ cells.

Response: Thank you for the question. We have revised the sentence in line 155 of the main text to read: “These findings collectively indicate cellular heterogeneity among small intestinal stem cells.”

Dear Editors and Reviewers,

Thank you very much for the valuable comments from the two reviewers and the opportunity to revise our manuscript entitled "Single-nucleus multi-omics analysis of mouse small-intestinal Lgr5+ cell populations reveals Foxa3-induced Paneth cell-lineage differentiation" (Manuscript ID:COMMSBIO-25-0669A). We are sincerely grateful to the reviewers for their thoughtful critiques and constructive suggestions, which have substantially improved the clarity, rigor and quality of our work. We have carefully addressed the 3 concerns and have improved the manuscript accordingly. Below, we provide a point-by-point response to each comment.

Reviewer #2 (Remarks to the Author):

In the revised manuscript, the authors continue to assert that “Lgr5-low stem cells exhibit much higher stem cell potential than Lgr5-high CBC cells,” based solely on computational analysis (CytoTrace2). However, the score differences among Lgr5-low, Top2a+ TA, CBC, and Hmgb2+ TA populations are subtle and do not convincingly support such a strong conclusion. This statement appears overstated and insufficiently supported by the current data. I recommend that the authors substantially moderate or remove this claim unless robust experimental validation can be provided, particularly since this point is not the main focus of the manuscript.

Response: We appreciate the reviewer’s constructive suggestions. We have restructured and substantially moderated the claim of the stemness diversity in the six identified Lgr5+ ISC subgroups in Result section (Page 7, Line 175-181) and Discussion section (Page 25, Line 518-525):

“CytoTRACE2 was used to infer cell stemness among the six identified stem cell clusters, revealing that Lgr5-low-expressing cells may possess relatively higher developmental potential. Besides, secretory progenitor cells (Lgr5+ LRCs) and APs exhibited relatively lower stemness (Fig. 2b). Notably, Lgr5 was differentially

expressed across distinct ISC subpopulations. These findings further support the lack of a strict correlation between Lgr5 expression levels and inferred developmental potential (Supplementary Fig. 2b, d). Meanwhile, this is consistent with recent studies that have detected the highest levels of cellular potency in some Lgr5-low cells[30,32].”

“The present study employs single-cell multi-omics analysis of enriched Lgr5+ ISCs to define ISC subtypes with heterogeneous differentiation potential and progenitor cells with unidirectional differentiation potential (AP and Lgr5+ LRC), inferring differentiation trajectories among various ISC subtypes. Notably, Lgr5 expression levels do not show a strict correlation with developmental stemness among these ISC subtypes. This is consistent with previous studies that ISC subtype with Lgr5-low expression have relatively highest level of cellular potency[30,32].”

The authors have strengthened their analysis of Foxa3-mediated regulation of Paneth cell differentiation by incorporating CUT&Tag-seq data. The new dataset identifies the Foxa3 binding motif and genome-wide binding sites, suggesting potential downstream interactions with PPAR transcription factors. While this addition provides some mechanistic depth, it largely reinforces existing knowledge. Furthermore, the authors' own data indicate that Foxa3 is involved in both Goblet and Paneth cell differentiation, which weakens the specificity of their proposed model initially.

Overall, although the manuscript has improved technically, the novelty and strength of the conclusions remain limited. I would recommend acceptance after the authors appropriately temper their claims and clarify the distinct role of Foxa3 within secretory cell differentiation.

Response: We appreciate and totally agree with the reviewer's insightful comment. To clarify the distinct role of Foxa3 within secretory cell differentiation, we have provided detailed supplementary information in Discussion section (Page 26, Line 543-576):

“Existing studies have established that the PPAR gene family plays critical roles in the differentiation and physiological functions of Paneth cells in the small intestine. Specifically, Ppard has been shown to promote Paneth cell differentiation by regulating

the Hedgehog signaling pathway[50]. In aged Paneth cells, reduced Ppara activity leads to increased Notum expression, thereby impairing the regenerative capacity of senescent intestinal epithelial cells[54]. Mice deficient in Pparg exhibit decreased expression of antimicrobial peptide-encoding genes and increased bacterial colonization between villi[55]. However, the precise mechanisms by which PPARs and their associated regulatory factors form complex regulatory networks to govern Paneth cell differentiation remain unclear. The present study, focusing on the level of transcription factors, has identified Foxa3 as a potential upstream regulator of PPARs in Paneth cell differentiation, advancing our molecular understanding of this process.

Concurrently, the present study further confirms the role of Foxa3 in goblet cell differentiation, which is consistent with previous findings[29]. Although both goblet cells and Paneth cells are classified as members of the small intestinal secretory lineage, notable differences exist between them in terms of physiological localization, specific functions, and differentiation regulatory mechanisms. Existing studies have established that these two cell types are regulated by shared transcription factors, which in turn activate distinct downstream transcription factors to modulate their respective differentiation and functions. For instance, ATOH1 is a pivotal initiator that specifies all secretory cell lineages in the small intestine: it drives goblet cell differentiation and function by activating SPDEF, while regulating Paneth cell differentiation through the activation of SOX9[19]. However, the specific molecular regulatory mechanisms governing the differentiation of small intestinal stem cells into these two secretory cell types remain poorly understood. Our study has identified Foxa3 as a transcription factor that simultaneously regulates both goblet and Paneth cell differentiation. Moreover, we found that Foxa3 modulates Paneth cell differentiation by regulating the expression of PPARs. The existing body of research suggests that PPARs appear to be dispensable for goblet cell differentiation and functional execution. Consequently, further elucidation is required to determine the precise role of Foxa3 in goblet cell differentiation. In summary, our integrated analysis identifies Foxa3 as a transcription factor enriched in secretory progenitor cells, capable of modulating marker gene expression during Paneth cell and goblet cell differentiation."

Reviewer #3 (Remarks to the Author):

6. Supplementary Figure 11: The authors provide a valid response to the question regarding the correlation between Foxa3 expression and motif accessibility. However, in the revised manuscript, the authors need to more accurately describe the results. The authors should state that the correlation of 0.99 is achieved when pooling cells within a cell type, but no correlation is seen within individual cell types.

Response: We thank the reviewer for this important suggestion. We have added more detailed and accurate descriptions in Result section (Page 14, Lines 298-304):
"When pooling all ISC cells within a single cell type, the overall expression of the Foxa3 gene regulatory module and the degree of chromatin accessibility at the Foxa3 motif exhibit a high correlation (R=0.99). In contrast, no such significant correlation was observed within every individual ISC cell-subtypes, suggesting that dynamic variations in Foxa3 gene expression and chromatin accessibility between ISC subtypes are primarily driven by inter-lineage differences rather than intra-lineage differences. (Fig. 4a, Supplementary Fig. 12)."

Thank you for your time and consideration.

Sincerely,

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