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SOX4 Promotes ATOH1-independent Intestinal Secretory Differentiation Toward Tuft and Enteroendocrine Fates

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Abstract

Background & Aims: The intestinal epithelium is maintained by intestinal stem cells (ISCs), which produce post-mitotic absorptive and secretory epithelial cells. Initial fate specification toward enteroendocrine, goblet, and Paneth cell lineages requires the transcription factor ATOH1, which regulates differentiation of the secretory cell lineage. However, less is known about the origin of tuft cells, which participate in type 2 immune responses to parasite infections and appear to differentiate independently of ATOH1. We investigated the role of SOX4 in ISC differentiation

Methods: We performed experiments in mice with intestinal epithelial-specific disruption of *Sox4* (*Sox4*^{fl/fl};villCre;SOX4cKO) and mice without disruption of *Sox4* (control mice). Crypt- and single cell-derived organoids were used in assays to measure proliferation and ISC potency. Lineage allocation and gene expression changes were studied by immunofluorescence, real-time quantitative PCR, and RNA-seq analyses. Intestinal organoids were incubated with the type 2

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A.D.G. and S.T.M. designed the project and wrote the manuscript. A.D.G., L.A.S., M.J.F., D.C.T., and B.Z. carried out experiments. A.D.G., J.S., and J.R.R. carried out RNA-seq analysis and J.S. and S.T.M. generated t-SNE plots from scRNA-seq data. K.B. and R.L.R. consulted on and carried out helminth experiments. Y.L. and N.F.S. provided Atoh1^{GFP} tissues. S.T.M. supervised research.

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cytokine interleukin 13 (IL13) and gene expression was analyzed. Mice were infected with the helminth *Nippostrongylus brasiliensis* and intestinal tissues were collected 7 days later for analysis. Intestinal tissues collected from mice that express green fluorescent protein regulated by the *Atoh1* promoter (*Atoh1*GFP mice), and single-cell RNA-seq analysis, were used to identify cells that co-express *Sox4* and *Atoh1*. We generated SOX4-inducible intestinal organoids derived from *Atoh1*^{fl/fl};villCreER (ATOH1iKO) mice and assessed differentiation.

Results: SOX4cKO mice had impaired ISC function and secretory differentiation, resulting in decreased numbers of tuft and enteroendocrine cells. In control mice, numbers of SOX4⁺ cells increased significantly following helminth infection, coincident with tuft cell hyperplasia. *Sox4* was activated by IL13 in control organoids; SOX4cKO knockout mice had impaired tuft cell hyperplasia and parasite clearance following infection with helminths. In scRNA-seq analysis, *Sox4*⁺/*Atoh1*⁺ cells were enriched for ISCs, progenitors, and tuft cells; 12.5% of *Sox4*-expressing cells co-expressed *Atoh1* and were enriched for enteroendocrine cells. In organoids, overexpression of SOX4 was sufficient to induce differentiation of tuft and enteroendocrine cells—even in the absence of ATOH1.

Conclusions: We found SOX4 to promote tuft and enteroendocrine cell lineage allocation independently of ATOH1. These results challenge the long-standing model in which ATOH1 is the only regulator of secretory differentiation in the intestine, and are relevant for understanding epithelial responses to parasitic infection.

Abstract

Keywords

intestinal crypt; transcriptional regulation; stem cell; differentiation

Introduction

The intestinal epithelium is essential for both digestive and barrier function and contains a diverse complement of post-mitotic cells which carry out complex physiological functions critical for homeostasis. Functional cell types in the intestine can be broadly subdivided into: (a) absorptive enterocytes and (b) secretory lineages, which include antimicrobial-producing Paneth cells, mucus-producing goblet cells, and hormone-secreting enteroendocrine cells (EECs)¹. Tuft cells, which represent a less well-characterized lineage, initiate immune responses to parasitic infections in the intestine^{2–5}. With the exception of long-lived Paneth cells, these post-mitotic lineages are subject to the rapid (5–7 day) and continuous turnover of the intestinal epithelium. Therefore, their numbers must be maintained through constant proliferation and differentiation of the intestinal stem cells (ISCs), which reside in a specialized stem cell niche at the base of the intestinal crypts.

Extrinsic signals from the niche induce regulatory programs in ISCs and their immediate progeny, transit-amplifying progenitor cells (TAs), to drive commitment to different cellular

lineages. For example, loss of Notch signaling in ISCs and early progenitors is associated with the induction of secretory differentiation through the derepression of *Atoh1*, which is widely regarded as the master regulator of secretory fate^{6,7}. Certain pathological settings can shift lineage allocation in the intestine, as exemplified by goblet and tuft cell hyperplasia in the setting of type II immune responses. While tuft cells are also derived from ISCs, the transcriptional regulatory mechanisms governing their differentiation and hyperplastic response to infection remain less well characterized³. This is due in part to evidence that tuft cells differentiate independently of *Atoh1*, but still appear to require downregulation of Notch^{6,8,9}. Recent studies have demonstrated upregulation of tuft cell numbers following *Atoh1* deletion *in vivo*, and systems biology approaches have supported an *Atoh1*-independent differentiation program for this lineage¹⁰. Together, these data suggest an apparently paradoxical Notch-repressed, *Atoh1*-independent mechanism for tuft cell specification.

Sry-box containing (Sox) factors are transcription factors with broad regulatory roles in stem cell maintenance and differentiation in many adult tissues, including the intestinal epithelium^{11,12}. *Sox9* is known to contribute to Paneth cell differentiation, and previous work from our group has demonstrated that distinct expression levels of *Sox9* mark stem and progenitor cell populations from intestinal crypts, including label-retaining cells (LRCs)^{13–16}. The role of other Sox factors in ISC function and differentiation remain uncharacterized.

Sox4 has been associated with the ISC genomic signature and is correlated with increased invasion/metastasis and decreased survival in colon cancer, but its role in intestinal epithelial homeostasis is unknown^{17,18}. In the hematopoietic system, as well as the developing nervous system, pancreas, kidney, and heart, *Sox4* is required for both proper cell lineage allocation and maintenance of progenitor pools^{19–21}. The diverse regulatory potential of *Sox4*, coupled with its known role in a broad range of tissue-specific progenitor populations, led us to hypothesize that it may function as a regulator of ISC maintenance and differentiation. In this study we establish a role for *Sox4* in ISC homeostasis and regulation of tuft cell hyperplasia during parasitic helminth infection, which represents a novel pathway for tuft cell specification with important implications for human responses to parasitic disease.

Results

SOX4 is expressed at distinct levels in the ISC zone

To characterize *Sox4* expression, we first used *in situ* hybridization to detect *Sox4* mRNA. Consistent with previous reports, *Sox4* is restricted to the base of the intestinal crypts in the stem cell zone (Figure 1A)¹⁸. Since the cellular resolution of *in situ* hybridization is low, we leveraged a previously characterized *Sox9^{EGFP}* reporter mouse to examine the expression of *Sox4* in different crypt cell types. Distinct *Sox9^{EGFP}* levels differentially mark active ISCs (*Sox9^{low}*), secretory progenitors/reserve ISCs (*Sox9^{high}*), TA progenitors (*Sox9^{sublow}*), Paneth cells (*Sox9^{EGFP-neg}*;CD24⁺), and non-Paneth post-mitotic cells (*Sox9^{EGFP-neg}*;CD24^{neg})^{13,14,22,23}. *Sox9^{EGFP}* populations were isolated by fluorescence-activated cell sorting (FACS) and analyzed by RT-qPCR. *Sox4* was significantly enriched in

two populations: *Sox9^{low}*, which is consistent with active ISCs, and *Sox9^{high}*, which is consistent with secretory progenitors (Figure 1B) ^{23, 24}.

Next, we examined SOX4 protein expression by immunofluorescence. SOX4 protein was expressed in a more restricted pattern relative to mRNA. Like *Sox9*, we observed distinct expression levels of SOX4, which we characterized as “low” and “high” (Figure 1C). SOX4^{low} cells were associated with the crypt base columnar (CBC), active ISC population, in cell positions +1–3 counting from the base of the crypt (Figure 1C, D). Interestingly, SOX4^{high} cells were predominantly found in the supra-Paneth cell position, which is commonly associated with early secretory progenitors, at cell positions +4–7 (Figure 1C, D) ²⁵. Unlike SOX9, which is expressed in villus-based EECs/tuft cells, we did not detect any *Sox4* mRNA or protein outside of the crypts ¹³.

Since *Sox9^{high}* cells are known to represent EEC-like secretory progenitors, we asked if the SOX4^{high} population overlapped with *Sox9^{high}* cells. However, we found no co-localization between high levels of SOX4 protein and high levels of *Sox9* mRNA, as determined by *Sox9^{EGFP}*, which is an accurate surrogate of endogenous *Sox9* mRNA and protein expression. (*n* = 3 mice, 100 SOX4+ cells per mouse) (Figure 1E, E') ^{13, 26}. Of note, the *Sox9^{EGFP}* transgene is selectively silenced in Paneth cells, and SOX4 expression was never observed in the Paneth cell position. *Sox9^{high}*-expressing cells are consistent with a subset of label-retaining reserve ISCs (LRCs) located in the supra-Paneth cell position. However, not all LRCs are *Sox9^{high}* ²³. We reasoned that the supra-Paneth cell location of SOX4^{high} cells suggests that they may represent the non-*Sox9^{high}* subset of LRCs. To examine this, we counted the number of SOX4^{high} cells co-localizing with LRCs, as determined by 28 days of continuous labeling with 5-ethynyl-2-deoxyuridine (EdU), followed by 10 days of washout ^{23, 25}. Surprisingly, we found that no SOX4^{high} cells were LRCs (*n* = 3 mice, 100 SOX4+ cells per mouse) (Figure 1F). We observed rare co-localization between DCLK1, a tuft cell/ reserve ISC biomarker also associated with the +4 position, and SOX4 (5.0% ± 3.0%; Figure 1G). Finally, we addressed the possibility that SOX4^{high} cells may represent a quiescent population by co-staining with KI67, which marks all proliferating cells. 90.6 ± 3.2% of SOX4^{high} cells were found to be proliferative (Figure 1H). Together, these data suggest that SOX4^{high} cells represent ISCs or progenitors, and are distinct from LRC *Sox9^{high}* secretory precursors. Co-localization with DCLK1 suggests that a subset of SOX4^{high} cells may represent tuft cell or non-LRC secretory precursors.

Loss of Sox4 results in increased proliferation and decreased ISC function

Complete deletion of *Sox4* results in embryonic lethality ²⁷. To enable examination of *Sox4* function in adult intestinal epithelium, we crossed previously generated *Sox4^{fl/fl}* mice to mice expressing Cre recombinase driven by the *villin* promoter (*vilCre*), which is constitutively expressed in the intestinal epithelium beginning at E13.5 ^{28, 29}. To examine *Sox4* expression, we isolated CD44– and CD44+ populations from CD326 (EPCAM)+ control and *Sox4*cKO adult intestinal epithelium by FACS and confirmed CD44 enrichment by qPCR (Supplementary Figure 1A–C). CD44 marks crypt cells as well as EECs in the villi (Supplementary Figure 1A). As expected, control animals exhibited significant enrichment of *Sox4* in the CD44+ population, consistent with restricted expression of *Sox4* in the

intestinal crypts (Supplementary Figure 2A). Sox4cKO animals did not express detectable levels of *Sox4* mRNA or protein (Supplementary Figure 2A, B).

To assay the role of *Sox4* in ISC/TA proliferation, we examined KI67 and EdU incorporation, which was administered as a single, 2hr pulse. Sox4cKO crypts exhibited significantly higher percentages of total proliferating cells as well as cells in S-phase (Figure 2A, B). This increase in proliferation was reflected by increased crypt length in Sox4cKO intestines (Supplementary Figure 2C). In organoid cultures, Sox4cKO crypts grew at the same rate as control crypts, suggesting that hyperproliferation in the absence of *Sox4* may be masked by exogenous growth factors required for organoid culture (Figure 2C).

Due to its known role in maintenance of stem/progenitor pools in other tissues, we sought to determine if *Sox4* regulates the size or function of the ISC pool. OLFM4, which is an established marker of ISCs, was detected by immunofluorescence to assess ISC numbers, which were unchanged between control and Sox4cKO intestines (Figure 2D)³⁰. Next, we FACS-isolated CD44⁻ and CD44⁺ cells from control and Sox4cKO mice and conducted single cell organoid forming assays to test ISC function³¹. As expected, CD44⁻ cells failed to form organoids regardless of *Sox4* status (Figure 2E). CD44⁺ cells from Sox4cKO mice produced approximately 50% fewer organoids than controls, demonstrating a significant loss of stemness (Figure 2E).

To determine if this functional deficit was coincident with an increase in apoptosis in Sox4cKO samples, we examined cleaved-caspase-3 expression in the crypts of control and Sox4cKO intestines, as well as the number of apoptotic cells in CD44⁻ and CD44⁺ populations of cells prepared for FACS. In both cases, we found no significant difference in apoptosis between control and Sox4cKO samples (Supplementary Figure 3A, B). However, CD44⁺ cells from Sox4cKO mice expressed significantly lower levels of *Lgr5*, consistent with decreased stem cell function (Figure 2F). Consistent with immunofluorescence results, *Olfm4* mRNA levels were unchanged between control and Sox4cKO samples (Figure 2F). Together, these data indicate that *Sox4* does not regulate ISC numbers or apoptosis, but is required for normal ISC function and *Lgr5* expression in the intestinal crypt.

Sox4 regulates secretory lineage allocation

We next sought to determine if *Sox4* regulates differentiation and intestinal lineage allocation. Goblet cells, detected by expression of MUC2, were found at similar numbers between Sox4cKO and control intestines (Figure 3A). CHGA⁺ EECs were found in significantly reduced numbers in the villi of Sox4cKO mice (Figure 3B). Numbers of DCLK1⁺ tuft cells were significantly reduced in crypt and villus compartments of Sox4cKO intestines (Figure 3C). Paneth cells were increased in number in Sox4cKO intestines (Figure 3D), and located at higher positions in Sox4cKO crypts, but determined to be non-proliferative by co-staining with KI67 (Supplementary Figure 4A and 4B). Staining patterns for sucrose isomaltase (SIS), a brush-border enzyme associated with absorptive enterocytes, were unchanged between control and Sox4cKO samples (Supplementary Figure 5A). Additionally, villus height was unchanged, and no change was observed for *Hes1*, which specifies absorptive enterocyte fate (Supplementary Figure 2C and Supplementary Figure 5B).

In order to examine gene regulatory changes in Sox4cKO samples in an unbiased manner, we subjected FACS isolated CD44⁻ and CD44⁺ populations to RNA-seq. Consistent with histological observations, decreased expression of genes associated with EEC subpopulations, including pro-EEC transcription factors *Isl1*, *Nkx2.2*, and *Pax6*, was the predominant change observed in Sox4cKO CD44⁺ populations (Supplementary Figure 1D and Supplementary Table 1)^{32,33}. There were no significant gene expression changes in the CD44⁻ populations consistent with the restricted expression pattern of SOX4 in CD44⁺ intestinal crypts and CD44⁺ status of EECs (Supplementary Figure 1D). Gene set enrichment analysis (GSEA) revealed a decreased gene expression signature consistent with pancreatic beta cells, another well-characterized endocrine population (Supplementary Figure 1E and Supplementary Table 2). MAPK signaling, which was recently shown to inhibit EEC and tuft lineages, was enriched, as was IL-6 JAK/STAT signaling, which is associated with increased epithelial proliferation and may reflect increased Paneth cell numbers through Paneth cell-specific expression of IL-6R (Supplementary Figure 1E)³⁴⁻³⁶. Collectively, transcriptomic data support the phenotypic changes in lineage allocation observed in Sox4cKO mice, and indicate that these changes occur in CD44⁺ cells.

Since the EEC lineage is comprised of a diverse complement of subpopulations, we re-examined Sox4cKO intestines for the presence of hormones produced by specific EEC subtypes. Compared to the modest reduction in CHGA⁺ cell numbers, we found substantial and significant decreases in EEC subpopulations expressing GIP, CCK, SST, GCG, PYY, and GLP-2 (Figure 3E). Interestingly, numbers of 5-HT and SUBP-expressing populations remained unchanged (Figure 3E). Gene ontology (GO) analysis of significantly downregulated genes in Sox4cKO CD44⁺ cells was consistent with a loss of EEC populations, and yielded terms for biological processes involving hormone regulation, signaling, and nutrient homeostasis (Figure 3F).

Since Notch repression is required for EEC specification, we asked if *Sox4* is activated following downregulation of Notch. To do so, we treated wild type organoids with the gamma secretase inhibitor DAPT. Since *Sox4* has been reported to be regulated by Wnt in colon cancer cell lines and inhibited by Bmp in hair follicle progenitors, we also treated organoids with WNT3A and Noggin, a Bmp antagonist^{37,38}. While *Sox4* levels and cell numbers did not respond to Wnt activation or Bmp inhibition, we found that Notch inhibition drove a 2-fold increase in *Sox4* expression that coincided with decreased expression of *Ascl2*, and ISC marker, and increased expression of *Ngn3*, an early EEC transcription factor (Supplementary Figure 6). Together, these data demonstrate that *Sox4* is required for proper secretory lineage allocation. Furthermore, *Sox4* is upregulated following Notch inhibition, consistent with early secretory differentiation responses, and required for proper specification of EEC subpopulations.

Sox4 contributes to tuft cell differentiation and hyperplasia

Since a subset of SOX4^{high} cells were found to co-localize with DCLK1 and DCLK1⁺ tuft cells were decreased in Sox4cKO intestines, we sought to examine the regulatory role of *Sox4* in tuft cell differentiation more closely. IL-13 is known to induce tuft cell differentiation, and is produced by Group 2 innate lymphoid cells (ILC2) that are recruited

to the intestinal epithelium in response to tuft cell secreted IL-25. This mechanism produces an extrinsic feed-forward loop that signals through unknown intrinsic response elements in ISCs and progenitors to drive tuft cell hyperplasia following parasitic helminth infection^{2, 5}. We treated wild type intestinal organoids with IL-13 to determine its effect on *Sox4*. As previously reported, IL-13 induced expression of tuft cell-specific *Dclk1* (Supplementary Figure 7A). Compellingly, IL-13 also induced a dose-dependent upregulation of *Sox4*, driving an approximately 3-fold increase at the optimal dose (Supplementary Figure 7A). Levels of *Sox4* upregulation correlated closely with *Dclk1* induction. As expected, goblet cell *Muc2* was the only non-tuft lineage specific gene also upregulated in response to IL-13 (Supplementary Figure 7B)². To determine if *Sox4* is required for proper *Dclk1* upregulation in response to IL-13, we next treated Sox4cKO and control organoids with IL-13. While control organoids exhibited robust induction of *Dclk1* and the tuft cell transcription factor *Pou2f3*, Sox4cKO organoids failed to significantly upregulate these genes (Figure 4A).

Next, we asked if *Sox4*-driven tuft cell responses were physiologically necessary for clearance of helminth infection. Sox4cKO and control animals were infected with *N. brasiliensis*, a helminth known to induce tuft cell-mediated type II immune reactions^{2, 4, 5}. Intestines were examined 7 days post-infection (d.p.i.), at the peak of tuft cell hyperplasia, when most worms are typically cleared from wild type mice⁵. We first examined SOX4⁺ cells in control mice. Helminth infection drove a 4-fold increase in the number of SOX4⁺ cells per crypt (Figure 4B). As expected, DCLK1⁺ cell numbers were largely upregulated in control animals (Figure 4C). In contrast, Sox4cKO intestines exhibited an attenuated increase in tuft cell numbers (Figure 4C). To determine if impaired tuft cell hyperplasia in Sox4cKO animals affected worm burden at 7d.p.i., we quantified the number of adult worms present in the intestines of infected animals. In order to match worm clearance data with tuft cell hyperplasia in the same mouse, duodenal and ileal tissues were harvested to establish worm counts and jejunal tissues were used for histological studies, discussed above. Control animals exhibited a near-total clearance of adult worms, while Sox4cKO animals bore a significantly higher worm burden at 7d.p.i. (Figure 4D). These data implicate *Sox4* as a regulator of tuft cell differentiation at physiological baseline, with an important role in tuft cell hyperplasia during helminth infection.

Sox4- and Atoh1-expressing cells form overlapping and distinct populations

Early tuft cell specification remains a paradox in intestinal epithelial differentiation. *Atoh1* is accepted as the master regulator of early secretory fate in the intestine, and is de-repressed following loss of Notch signaling³⁹. While tuft cells fail to differentiate properly in the presence of constitutively active Notch, multiple lines of evidence demonstrate that a majority of tuft cells are not derived from *Atoh1*-expressing progenitors^{3, 6}. These data suggest the existence of Notch-repressed, *Atoh1*-independent tuft cell regulatory pathways that remain uncharacterized. Since our data demonstrate that *Sox4* is upregulated following Notch inhibition and is involved in tuft cell differentiation, we hypothesized that it might be expressed in an *Atoh1*-independent manner. We examined SOX4 expression in *Atoh1*^{GFP} intestines, and found that 55.3 ± 6.1% of SOX4^{high} cells were *Atoh1*-negative, with the remainder co-localizing with Atoh1-GFP (Figure 5A).

To examine *Sox4* and *Atoh1* populations more thoroughly, we reanalyzed previously published single cell RNA-seq (scRNA-seq) data from primary mouse intestinal epithelial cells⁴⁰. Cells were visualized by tSNE dimensionality reduction and classified into previously characterized phenotypic populations (Figure 5B)⁴⁰. Next, we queried the distribution of cell types within each of three populations of interest: (1) *Sox4*⁺*Atoh1*⁻ (*n* = 617 cells), (2) *Sox4*⁺*Atoh1*⁺ (*n* = 88 cells), and (3) *Sox4*⁻*Atoh1*⁺ (*n* = 162 cells). Within the *Sox4*⁺ population, 12.5% of cells were also *Atoh1*⁺. Next, we determined the cellular composition of each of our populations of interest. The *Sox4*⁺*Atoh1*⁻ population consisted primarily of ISCs and progenitor cells (88%), but also contained the highest proportion of tuft cells relative to other populations (Figure 5C). The *Sox4*⁺*Atoh1*⁺ population was a mix of ISCs and secretory cell types, and contained the highest proportion of EECs relative to other populations (Figure 5C). As expected, the *Sox4*⁻*Atoh1*⁺ population was comprised mostly of secretory cells, with the lowest proportion of ISCs and progenitor cells and the highest proportion of goblet and Paneth cells relative to other populations (Figure 5C). To ask the inverse question, we next binned scRNA-seq data by cell type and queried the percent distribution of *Sox4* and *Atoh1* within defined intestinal epithelial cell subpopulations. This analysis indicates that 46.1% of tuft cells are *Sox4*⁺*Atoh1*⁻ and 48.0% are negative for both *Sox4* and *Atoh1*, while *Sox4*⁻*Atoh1*⁺ and *Sox4*⁺*Atoh1*⁺ populations represent 2.9% of total tuft cell numbers, each (Supplementary Figure 8). These data demonstrate that *Sox4* and *Atoh1* form distinct and overlapping populations, and that *Sox4* is most strongly correlated with ISCs, progenitors, and tuft cells when expressed exclusively of *Atoh1* and more strongly correlated with EECs when co-expressed with *Atoh1*.

Sox4 drives tuft cell differentiation independently of Atoh1

We next sought to determine if *Sox4* is sufficient to drive secretory EEC and tuft cell differentiation. To this end, we generated stable transgenic organoids carrying a cumate-inducible *Sox4* overexpression vector (*Sox4*OE). Gain of function was validated by immunofluorescent detection of SOX4 in empty vector control and induced *Sox4*OE organoids (Supplementary Figure 9). To validate organoid models for assessment of *Sox4*-dependent differentiation phenotypes, we included organoids isolated from *Sox4*cKO mice in our analyses. As expected, *Sox4*cKO organoids exhibited very low numbers of DCLK1⁺ tuft cells and CHGA⁺ EECs (Figure 6A, B). In contrast, overexpression of *Sox4* resulted in a significant increase in numbers of both tuft cells and EECs, relative to empty vector controls (Fig 6A, B).

Since scRNA-seq demonstrated differential distribution of *Sox4* and *Atoh1* across progenitors and secretory cell types, we hypothesized that *Sox4* may function independently of *Atoh1* in secretory differentiation. To test this, we generated *Sox4*OE organoids derived from *Atoh1*^{fl/fl}:*vtCre*^{ER} mice. *Atoh1* recombination was induced by treating organoids with 1μM 4-OHT once a day for three days and collecting for analysis 24hr following final treatment with 4-OHT. Analysis of *Atoh1* expression confirmed the effectiveness of this strategy in generating *Atoh1*iKO organoids (Figure 6C). To assess whether residual ATOH1 protein was exerting continued effects in *Atoh1*iKO organoids, we next analyzed *Muc2* expression, which is associated with *Atoh1*-dependent goblet cell differentiation. We found

that *Muc2* levels were also significantly downregulated in *Atoh1* KO organoids, suggesting no residual ATOH1 function at the time of organoid analysis (Figure 6D).

Next, we examined the effect of *Atoh1* and *Sox4* on tuft and EEC differentiation. Surprisingly, *Dclk1* was significantly downregulated following loss of *Atoh1*, in direct conflict with reported *in vivo* data demonstrating an increase in tuft cells in *Atoh1*-null intestines (Figure 6E)¹⁰. However, treatment of *Atoh1* KO organoid cultures with IL13 restored *Dclk1* expression to control levels, in contrast to the loss of IL13 responsiveness in *Sox4* KO organoids (Supplementary Figure 10). Overexpression of *Sox4* was also sufficient to restore *Dclk1* expression levels in the absence of *Atoh1* (Figure 6E). Unexpectedly, *Chga* expression levels remained unchanged following loss of *Atoh1*, and were upregulated by *Sox4* in *Atoh1* KO organoids (Figure 6F). Notably, *Sox4* OE failed to rescue or upregulate *Muc2* expression levels, suggesting specificity to tuft and EEC lineages (Figure 6D). To refine these observations, we conducted whole mount staining on *Atoh1* KO and *Sox4* OE organoids and directly quantified tuft and EEC numbers in whole organoids. Quantification by immunofluorescence recapitulated gene expression results, demonstrating upregulation of DCLK1+ tuft cells and CHGA+ EECs in *Sox4* OE organoids regardless of *Atoh1* expression (Figure 6G and H). Together, these data demonstrate that *Sox4* is sufficient to drive an increase in tuft and EEC differentiation and can do so independently of *Atoh1*.

Dissecting regulatory roles for *Sox4* in intestinal stemness and lineage allocation

To further dissect the roles of *Sox4* and *Atoh1* in intestinal epithelial cell fate, we analyzed gene expression for known regulators of ISCs and differentiation in our *Sox4* OE: *Atoh1* KO organoids. First, we examined expression of ISC markers *Lgr5*, which is downregulated in *Sox4* KO intestines *in vivo*, and *Ascl2* and *Tcf4*, which activate a master ISC regulatory program together with beta catenin⁴¹. While loss of *Atoh1* results in significantly decreased *Lgr5* expression, *Sox4* is insufficient to upregulate *Lgr5* and cannot rescue *Lgr5* expression levels in the absence of *Atoh1* (Figure 7A). *Atoh1* KO organoids exhibit decreased *Ascl2* expression which is not rescued by *Sox4* OE and *Tcf4* is unaffected by loss of *Atoh1* or *Sox4* OE. Collectively, these data indicate that *Sox4* alone is insufficient to upregulate ISC core genes.

Next, we examined *Sox4* and *Atoh1* regulation of tuft cell-associated transcription factors *Gfi1b*, *Pou2f3*, and *Stat6*²⁻⁴. *Gfi1b* was not detected in any samples (data not shown). We found that *Pou2f3* is significantly downregulated in *Atoh1* KO organoids (Figure 7B). Interestingly, while *Sox4* is required for *Pou2f3* upregulation in response to IL13 treatment (Figure 5A), *Sox4* OE organoids did not exhibit increased *Pou2f3* expression, demonstrating that *Sox4* is necessary but not sufficient for *Pou2f3* induction (Figure 7B). *Stat6*, which is activated by tuft cell-associated cytokines IL-4 and IL-13, was weakly inhibited in the combined presence of normal levels of *Atoh1* and *Sox4* OE, but is otherwise unaffected by loss of *Atoh1* or *Sox4* OE (Figure 7B).

As EEC-related genes comprise the majority of transcriptomic changes in *Sox4* KO intestines, we sought to examine canonical secretory differentiation pathways in the setting of our *Sox4* OE: *Atoh1* KO organoid model. As expected, goblet and Paneth cell-associated transcription factors *Spdef* and *Gfi1* were found to be highly dependent on *Atoh1*

expression, exhibiting near total loss of expression in the setting of *Atoh1* KO (Figure 7C). Additionally, neither gene was responsive to Sox4OE, regardless of *Atoh1* expression. *Foxa1* and *Foxa2*, which are implicated in goblet and EEC differentiation, trended toward reduction following *Atoh1* KO and were unresponsive to Sox4OE (Figure 7C)⁴². *Hes1* was unaffected by either *Sox4* or *Atoh1* (Figure 7C). To assess EEC-specific transcriptional changes, we examined *Ngn3*, *Neurod1*, *Isl1*, *Pax6*, and *Nkx2-2*. All but *Nkx2-2* were expressed in Sox4OE:*Atoh1* KO organoids, as assessed by RT-qPCR. While *Ngn3* trended toward reduction following loss of *Atoh1*, this effect was not significant (Figure 7D). *Neurod1*, *Isl1*, and *Pax6* were also unaffected by loss of *Atoh1* (Figure 7D). Surprisingly, while Sox4OE had no effect on *Ngn3* or *Neurod1* expression, it significantly repressed *Isl1* and *Pax6* regardless of *Atoh1* expression and in contrast to observations made in Sox4cKO intestines (Figure 7D). To extend these observations, we next examined genes associated with terminally-differentiated EEC function. While none of the genes examined were significantly affected by loss of *Atoh1*, they could be categorized by differential responsiveness to Sox4OE. *Gip*, which was downregulated following loss of *Sox4* *in vivo*, was significantly upregulated by Sox4OE, regardless of *Atoh1* expression and consistent with the effect of *Sox4* on *Chga* (Figure 7E). Unexpectedly, *Cck*, *Gcg*, and *Nts*, which were all strongly downregulated in Sox4cKO intestines, were also significantly inhibited in the presence of Sox4OE (Figure 7F). *Tph1* and *Tac1*, which were not affected by loss of *Sox4* *in vivo*, were also unaffected by Sox4OE *in vitro* (Figure 7G). Together, these data suggest that transcriptional functions of *Sox4* in EEC regulation are largely independent of *Atoh1* and are exerted differentially at a gene-by-gene level, with portions of EEC genes being activated, repressed, or unaffected by *Sox4*. Collectively, the repression of late EEC transcription factors *Isl1* and *Pax6*, as well as a subset of EEC hormones, may suggest that *Sox4* exerts its effect on the EEC lineage by maintaining a progenitor state and preventing premature differentiation.

Discussion

Here we show that genetic ablation of *Sox4* in the intestinal epithelium results in increased numbers of proliferating cells and Paneth cells, as well as a reduction in EEC and tuft cell lineages. Our study supports *Sox4* mediated regulation of stem cells in other organ systems by demonstrating that *Sox4* regulates ISC function and differentiation toward EECs and tuft cells in the intestinal epithelium. Additionally, we demonstrate that *Sox4* has an essential role in pushing lineage allocation toward tuft cell differentiation in response to helminth infection, implying an important role for *Sox4* in host responses to pathogens.

Our group and others have reported *Sox4* expression in *Lgr5*⁺ CBCs, but a role for *Sox4* in ISCs has yet to be established^{18, 22}. We demonstrate that the numbers of ISCs quantified by OLFM4 immunostaining are unchanged between Sox4cKO and control samples. These data suggest that the associated increase in proliferating cells in Sox4cKO crypts is likely due to increased progenitor numbers and not more robust ISC activity. Rather, *in vitro* assay of ISC function demonstrated impaired organoid-forming efficiency of single ISCs isolated from Sox4cKO mice. As this dysfunction is not associated with a change in the number of apoptotic cells between control and Sox4cKO mice, it is likely that decreased organoid-forming efficiency is caused by loss of stemness and premature differentiation in *Sox4*

deficient ISCs. In this regard, Sox4cKO mice demonstrate reduced *Lgr5* mRNA. *Lgr5* is highly expressed in ISCs and serves as a receptor for R-Spondin ligands that potentiate Wnt-signaling, which is in turn required to maintain stemness⁴³. Interestingly, our gain-of-function experiments in organoids indicate that *Sox4* alone is insufficient to upregulate *Lgr5* and suggest that *Sox4* contributes to proper *Lgr5* expression through a co-regulatory manner involving other transcription factors or non-cell autonomous mechanisms.

In addition to loss of ISC function, Sox4cKO intestines also demonstrate decreased EEC and tuft cell numbers, implying that *Sox4* plays a key role in the specification of these secretory lineages. *In vivo*, RNA-seq analysis demonstrates that *Sox4* regulates the expression of the EEC transcription factors, including *Isl1*, *Pax6*, and *Nkx2-2*. *Isl1* deletion in the intestine is known to lead to loss of EECs subtypes expressing GIP, CCK, SST, GCG, PYY, and GLP-2³³. We demonstrate that loss of *Sox4* results in a marked reduction of *Isl1* expression with a concomitant reduction in the numbers of the same EEC subtypes. An unexpected result of our studies is the observation that *Sox4* overexpression is sufficient to drive an increase in CHGA+ cell numbers in the absence of *Atoh1*. This effect does not appear to be the result of residual ATOH1 activity or non-specific *Sox4* regulation of all secretory differentiation, as goblet cell-associated *Muc2* expression remains severely downregulated in *Atoh1*iKO organoids at the timepoint examined, regardless of *Sox4* expression status. These data raise interesting questions regarding the role of *Atoh1* as a master regulator of secretory differentiation. To our knowledge, no other studies to date have expressed transcriptional regulators of secretory differentiation that are normally expressed upstream of *Atoh1* in the absence of *Atoh1*. Further studies in this area would be necessary to determine if other transcription factors are capable of rescuing secretory differentiation in *Atoh1*iKO models.

Paradoxically, Sox4OE in organoid cultures results in the downregulation of many of the same genes that are downregulated in Sox4cKO *in vivo*, including *Isl1*, *Pax6*, *Cck*, and *Gcg*. Collectively, our *in vivo* and *in vitro* results indicate that *Sox4* is required for proper EEC specification through established EEC transcriptional regulators *Isl1*, *Pax6*, and *Nkx2-2* and suggest that *Sox4* must be downregulated in order for EEC differentiation to proceed along these pathways. As CHGA is expressed in both mature EECs and cycling EEC progenitors, the *Atoh1*-independent increase in CHGA+ cells in Sox4OE organoids may reflect an expansion of progenitors as opposed to mature EECs. Further, Sox4OE does not regulate *Ngn3* or *Neurod1*, suggesting that *Sox4* can promote an EEC progenitor state without upregulation of established EEC transcription factors, though the requirement of *Ngn3* and *Neurod1* in *Sox4*-mediated CHGA+ cell expansion remains unknown. In this way, *Sox4* may “prime” ISC and progenitor pools for differentiation while simultaneously inhibiting premature cell fate decisions. This role would be consistent with the crypt-restricted expression of *Sox4*, as well as *Sox4* knockout phenotypes in other tissues, including the developing heart, skeletal muscle, nervous, renal, and hematopoietic systems, which exhibit reduced progenitor and post-mitotic cell numbers due to precocious differentiation^{19, 20, 27, 44–46}.

Relative to EEC differentiation, the mechanisms that give rise to the tuft cell lineage are less well understood due to the relatively new characterization of tuft cells as a distinct lineage. The role of intestinal tuft cells was recently described by a handful of independent studies,

all of which demonstrated tuft cell hyperplasia following infection with parasitic helminths. Following infection, tuft cells initiate a type II immune response through ILC-2 cells^{2, 4, 5}. ILC-2s secrete IL-13, which induces tuft cell hyperplasia^{2, 4, 5}. *Pou2f3* and *Stat6* are known to be essential for tuft cell differentiation, but *Pou2f3* is also expressed by mature, villus-based tuft cells and *Stat6* is activated by type II responses and involved in tuft and goblet cell differentiation^{2, 4}. Other transcription factors involved in homeostatic tuft cell specification and hyperplasia remain unknown, and tuft cell progenitors have no distinct biomarkers².

Though SOX4^{high} cells are relatively rare in homeostasis, we observed a striking increase in SOX4^{high} cell numbers coincident with peak tuft cell hyperplasia following parasitic infection, suggesting that SOX4^{high} cells may represent a tuft cell progenitor population. We show that Sox4cKO epithelium demonstrates attenuated tuft cell hyperplasia, both in response to recombinant IL-13 *in vitro* and helminth infection *in vivo*, and that Sox4cKO mice are functionally impaired at clearing worms by 7d.p.i. Notably, IL13-mediated activation of *Pou2f3* is also impaired in Sox4cKO organoids, suggesting that *Sox4* acts upstream of this established tuft cell transcription factor. Since there is no reporter mouse model for *Sox4*, we leveraged existing scRNA-seq data to further interrogate the identity of *Sox4*⁺ cells. In agreement with histological data, we find that *Sox4* is enriched in ISC, progenitor, and secretory populations. Classifying *Sox4*⁺ cells by *Atoh1* expression reveals a strong correlation between *Sox4*⁺:*Atoh1*[−] cells and tuft cells and between *Sox4*⁺:*Atoh1*⁺ cells and EECs, supporting dual roles for *Sox4* in tuft and EEC differentiation. Functionally, this role is demonstrated by the ability of ectopic *Sox4* expression to induce increased tuft and EEC numbers in organoids.

Lineage specification of tuft cells from the ISC/progenitor pool remains a significant question in the field, as tuft cells are capable of being produced independently of *Atoh1*, but are inhibited by constitutive Notch signaling^{3, 6}. Further, it was recently shown that inducible knockout of *Atoh1* *in vivo* leads to increased tuft cell numbers, implying that *Atoh1* may suppress tuft cell differentiation¹⁰. Surprisingly, our *in vitro* analyses of *Atoh1*liKO organoids demonstrate the opposite effect. That is, loss of *Atoh1* *in vitro* leads to decreased tuft cell numbers. However, in contrast to Sox4cKO organoids, which fail to upregulate *Dclk1* in response to exogenous IL13, *Atoh1*liKO organoids retain IL13-responsiveness in terms of *Dclk1* expression. These data suggest that tuft cells may be specified by multiple signaling events and pathways, but that IL13-mediated tuft cell hyperplasia is *Sox4*-dependent and *Atoh1*-independent. In agreement, our gain-of-function studies reveal that, although *Atoh1*liKO organoids exhibit reduced tuft cell numbers relative to non-recombined controls, *Atoh1* is dispensable for *Sox4*-driven tuft cell differentiation.

The rarity of SOX4⁺ cells in homeostasis and diverse array of ISC, EEC, and tuft cell phenotypes in Sox4cKO mice presents a significant challenge for identifying molecular mechanisms for *Sox4* in the intestine. A common theme in our results is that *Sox4* appears to be required for IEC function and responses, such as proper *Lgr5* expression or *Pou2f3* upregulation in response to IL-13, without direct transcriptional regulation of these targets. One interpretation is that *Sox4* may act in concert with a number of co-regulatory factors to exert broad phenotypic effects, being required but insufficient to drive observed phenotypes, such as ISC function. Similar observations suggesting broad, co-factor dependent regulatory

roles for *Sox4* have been demonstrated in other tissues, with mechanistic work in pancreatic cancer showing that *Sox4* can drive either enhanced proliferation and tumorigenesis or apoptosis, dependent on co-expression of *Klf5*⁴⁷. Identification of direct transcriptional targets of SOX4 as well as potential cofactors is further hindered by a lack of antibodies appropriate for immunoprecipitation of native SOX4 protein. Future studies utilizing unique approaches will be required to dissect the seemingly complex *Sox4* regulatory network.

At the cellular level, SOX4^{low} cells overlap largely with ISCs, while SOX4^{high} cells appear to represent a cycling secretory progenitor population with EEC and tuft cell potential (see Graphical Abstract). The existence of *Atoh1*-independent lineage specification of tuft cells and EECs challenges the long-standing dogma of *Atoh1* as the master transcriptional regulator of secretory differentiation in the intestine. Our findings demonstrate that *Sox4* is upregulated following downregulation of Notch or in response to IL13, and drives tuft or EEC specification in a manner that can proceed independently of *Atoh1*. These findings implicate *Sox4* as an important regulator of host responses parasitic infection and shed further light on epithelial components of Type II immune responses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Sox4 is expressed in the ISC and early progenitor zone.

(A) *In situ* hybridization localizes *Sox4* mRNA to the base of small intestinal crypts. (B) FACS isolation of *Sox9*^{EGFP} populations demonstrates enrichment of *Sox4* in active ISCs (*Sox9*^{low}) and secretory progenitors/reserve ISCs (*Sox9*^{high}) (different letters indicate statistically significant differences, $p < 0.05$). (C) SOX4 protein is expressed in the ISC and supra-Paneth cell zones at variable levels, with (D) SOX4^{low} cells primarily localized to the CBC position (1–3) and SOX4^{high} cells occurring mainly in the supra-Paneth cell zone (4+). (E/E') SOX4^{high} and *Sox9*^{high} cells do not co-localize; arrows indicate SOX4^{hi} cells and arrowheads indicate *Sox9*^{high} cells ($n=3$ mice, 100 SOX4^{high} cells per mouse, scale bar

represents 20um). (F) SOX4^{high} cells are not LRCs by 28d continuous EdU labeling followed by 10d washout; white arrow indicates SOX4^{high} cell and yellow arrow indicates LRC (scale bar represents 20um; note: EdU detection kit reacts with Paneth cell granules). (G) A small portion of SOX4^{high} cells co-localize with the tuft cell marker DCLK1 and (H) a majority of SOX4^{high} cells are positive for proliferative marker KI-67.

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Figure 2. Loss of Sox4 results in increased proliferation and decrease in functional stemness, with no change in ISC numbers.

(A) Sox4cKO intestines exhibit increased total proliferation by KI67 staining, as well as (B) increased numbers of cells in S-phase, as indicated by EdU (scale bar represents 100 μm). (C) Whole crypts isolated from Sox4cKO intestines form organoids that undergo similar growth rates to control crypts (scale bar represents 100 μm). (D) Numbers of OLFM4+ ISCs remain unchanged in Sox4cKO crypts, while (E) *Lgr5* is downregulated in CD44+ populations from Sox4cKO intestines. Functional stemness, as assayed by organoid forming ability, is significantly decreased in CD44+ populations isolated from Sox4cKO intestines (scale bar represents 25 μm ; asterisks indicate significance; $p < 0.05$).

(A) MUC2+ goblet cells remain unchanged in Sox4cKO intestines, while (B) CHGA+ EECs are found at decreased numbers in Sox4cKO villi, and (C) DCLK1+ tuft cells are decreased in both crypt and villus compartments of Sox4cKO intestines (scale bar represents 100um). (D) Paneth cell numbers are increased in Sox4cKO crypts (scale bar represents 50um). (E) Assessment of EEC differentiation by immunofluorescence against markers associated with specific EEC subtypes reveals that *Sox4* is required for proper specification of a subset of EEC lineages. Conversely, numbers of cells positive for serotonin (5-HT) and Substance P (SUB-P) are unaffected in Sox4cKO intestines. (asterisks indicate significance; $p < 0.05$).

(F) The top 20 most significantly downregulated, GO-identified terms for biological processes in RNA-seq of Sox4cKO vs. control CD44+ populations are consistent with EEC function.

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Figure 4. Sox4 regulates tuft cell differentiation and is required for tuft cell hyperplasia following helminth infection.

(A) Tuft cell markers *Dclk1* and *Pou2f3* are upregulated in control, but not Sox4cKO organoids, in response to treatment with recombinant IL-13 (different letters indicate statistically significant differences, $p < 0.05$). (B) 7 days post-infection with *N. brasiliensis*, control intestinal crypts exhibit a significant expansion of SOX4^{high} cells (scale bar represents 20um). (C) Control mice demonstrate expected tuft cell hyperplasia at 7dpi, where Sox4cKO intestines have significantly fewer tuft cells as quantified by DCLK1, COX1, and COX2 (ND = no data; COX1+ cells are not found in intestinal crypts. Scale bar represents 100um). (D) Sox4cKO mice also fail to fully clear adult worms by 7dpi (asterisks indicate significance, $p < 0.05$, $n=3$ mice per group).

Figure 5. Atoh1+ and Sox4+ exhibit overlapping and exclusive expression in secretory cells and ISCs.

(A) Co-detection of SOX4 and *Atoh1*, using a transgenic *Atoh1^{GFP}* allele, reveals that $55.3 \pm 6.1\%$ of SOX4+ cells co-express *Atoh1* (yellow arrowhead indicates SOX4+/Atoh1+ cell; white arrowhead indicates SOX4+/Atoh1- cell. Scale bar represents 25um). (B) scRNA-seq ⁴⁰ identifies unique cell populations in the intestinal epithelium (C) Classifying cells based on *Sox4* and *Atoh1* expression status (*Sox4*+:*Atoh1*-, *Sox4*+:*Atoh1*+, and *Sox4*+:*Atoh1*+) reveals that *Sox4*+: populations contain the highest proportion of tuft cells, EECs, and ISCs, consistent with observed phenotypes *in vivo* and *in vitro*.

Figure 6. Sox4 drives tuft cell and EEC differentiation independently of Atoh1.

(A) Tuft cells, as identified by DCLK1, are rare in control organoids, absent in Sox4cKO organoids, and upregulated in Sox4OE organoids. (B) Similar results are observed for CHGA+ EECs, indicating that *Sox4* is sufficient to increase tuft and EEC differentiation (scale bars represent 50um; different letters indicate statistically significant differences, $p < 0.05$, $n=25$ organoids per group). (C) 4-OHT treatment downregulates *Atoh1* in control and Sox4OE organoids, resulting in (D) loss of *Muc2* expression, which demonstrates loss of *Atoh1*-driven transcription. (E) Loss of *Atoh1* in organoids also results in downregulation of *Dclk1*, which is rescued to normal levels in Sox4OE organoids, regardless of *Atoh1* expression. (F) *Chga* is not affected by *Atoh1* deletion in organoids, but is upregulated by Sox4OE in the absence of *Atoh1* (different letters indicate statistically significant differences, $p < 0.05$, $n=3$ different wells of organoids per group). Cell counts of (G) DCLK1+ tuft cells and (H) CHGA+ EECs demonstrate correlation between changes in gene

expression and lineage specification in *Atoh1*KO and Sox4OE organoids. Notably, CHGA+ cells numbers are increased in Sox4OE organoids both with and without *Atoh1* expression (scale bars represent 50um; different letters indicate statistically significant differences, $p < 0.05$, $n=25$ organoids per group).

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Figure 7. IEC gene regulation by Sox4 and Atoh1.

(A) ISC regulatory genes *Lgr5* and *Ascl2* are downregulated in *Atoh1*iKO and unaffected by *Sox4* overexpression, while *Tcf4* is unresponsive to *Atoh1*iKO or *Sox4*OE. (B) Tuft cell-associated transcription factors are differentially responsive to *Atoh1* and *Sox4*. *Pou2f3* requires *Atoh1* for normal expression levels and is not regulated by *Sox4*OE. *Stat6* is unresponsive to *Atoh1*iKO, but is mildly repressed by *Sox4*OE in an *Atoh1*-dependent manner. (C) Canonical IEC differentiation-associated transcription factors exhibit different requirements for *Atoh1* and *Sox4*. *Hes1*, which drives absorptive differentiation, is unaffected by *Atoh1* and *Sox4* expression. *Spdef* and *Gli1* are significantly downregulated in *Atoh1*iKO organoids and unaffected by *Sox4*OE. *Foxa1* and *Foxa2* trend toward downregulation, but are not significantly affected by loss of *Atoh1*, and are also unresponsive to *Sox4*OE. (D) Early EEC transcription factors *Ngn3* and *Neurod1* do not significantly change in *Atoh1*iKO or *Sox4*OE organoids, while late EEC transcription

factors *Isl1* and *Pax6* are significantly repressed by Sox4OE independently of *Atoh1* expression. EEC hormones exhibit differential responses, all independently of *Atoh1* expression, with Sox4OE resulting in (E) *Gip* upregulation and (F) *Cck*, *Gcg*, and *Nts* downregulation, and (G) having no significant effect on *Tph1* and *Tac1* (different letters indicate statistically significant differences, $p < 0.05$, $n=3$ different wells of organoids per group).

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