

Intestinal tuft cell subtypes represent successive stages of maturation driven by crypt-villus signaling gradients

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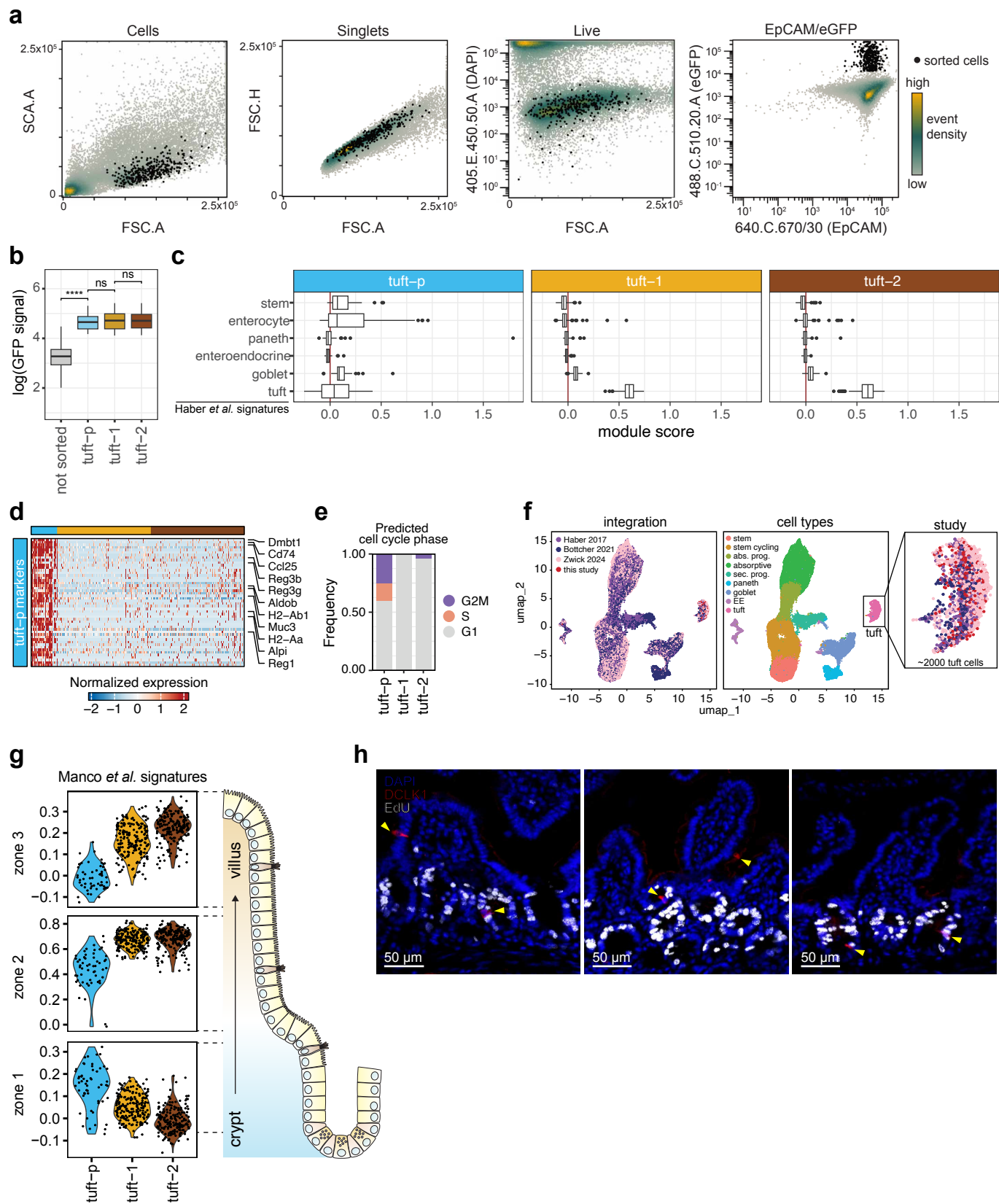
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Supplementary Information

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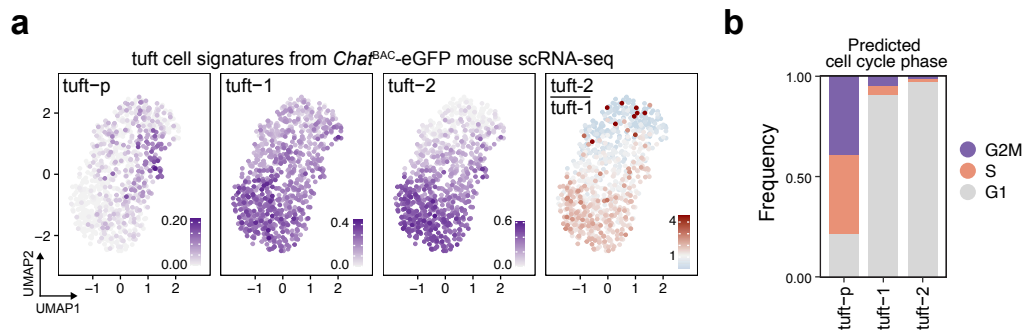
Supplementary Figures 1-12

Supplementary Table 1



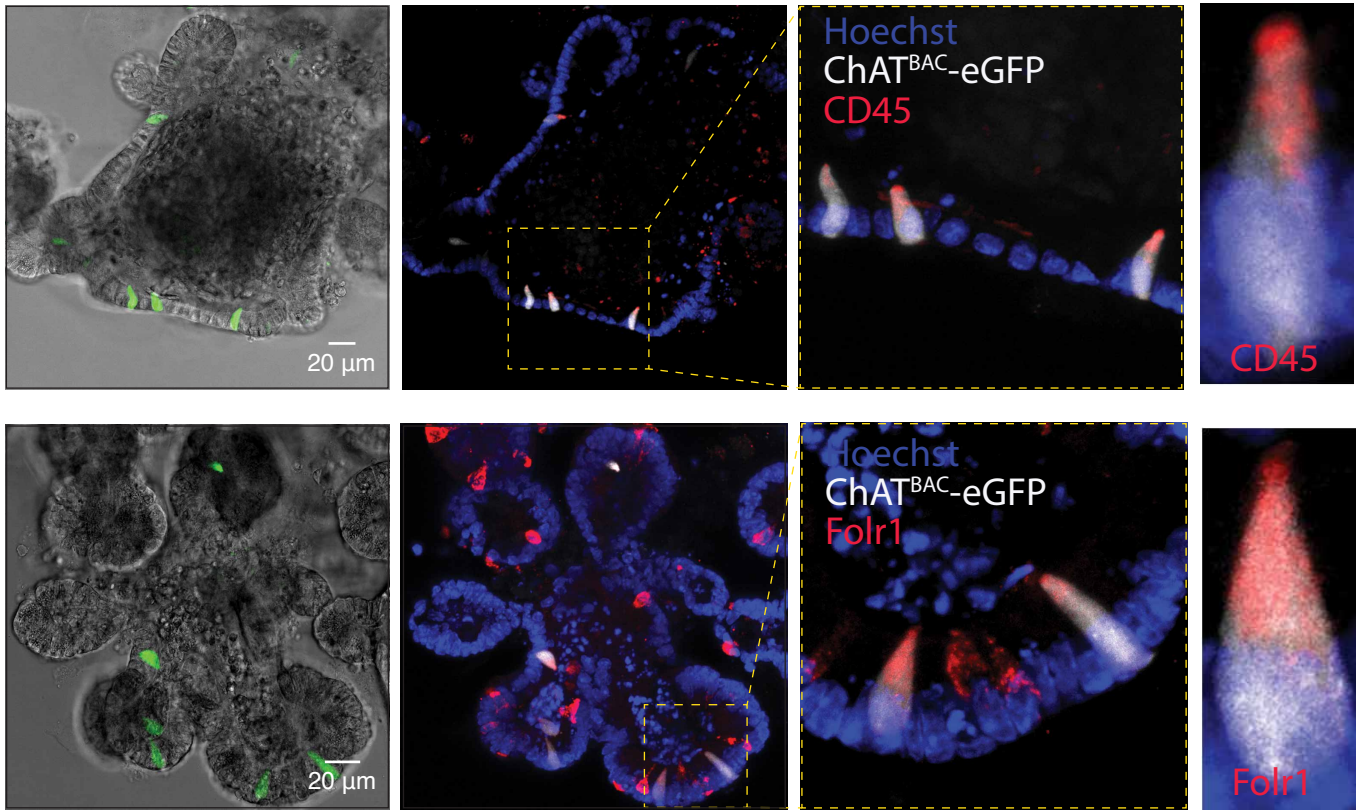
Supplementary Figure 1 | scRNA-seq of mouse small intestinal *Chat*^{BAC}-eGFP+ tuft cells Related to Figure 1.

a Gating strategy used during FACS prior to plate-based scRNA-sequencing of EpCAM+*Chat*^{BAC}-eGFP+ tuft cells isolated from mouse small intestinal tissue. Event density is indicated by color gradient. Sorted events are denoted in black ($n = 3$ mice, pooled). **b** Mean fluorescence signal (eGFP) of cells from indicated UMAP clusters measured by FACS prior to plate-based scRNA-sequencing (ANOVA < 0.001 , ns not significant, **** $p < 0.001$, Tukey HSD test). **c** Box plot indicating the scores by which various small intestinal cell type signatures¹ can be detected in single cells of the indicated tuft clusters as identified in the *Chat*^{BAC}-eGFP dataset. **d** Gene expression profile of tuft-p cells. The top 50 differentially expressed genes with the highest fold-change are plotted and selected genes are highlighted (adjusted p-value < 0.01 , Wilcoxon rank-sum test with Bonferroni correction). **e** Distribution of computationally predicted cell-cycle phase per cluster. **f** UMAP of integrated single-cell transcriptomic datasets of mouse small intestinal epithelium. Cells are colored by study origin (left) or cell type (middle). Right: zoom-in of tuft cell cluster (~2000 cells) colored by study origin. EE: enteroendocrine cells. Abs. prog.: absorptive progenitors, Sec. prog.: secretory progenitors. **g** Violin plots indicating the scores by which the zonated expression signatures along the crypt-villus axes² are detected in single cells of the three tuft clusters found in the *Chat*^{BAC}-eGFP dataset. **h** Representative fluorescence microscopy images of EdU staining on mouse small intestinal tissue after 4-hour EdU pulse. Yellow arrows indicate DCLK1+ cells ($n = 3$ mice).



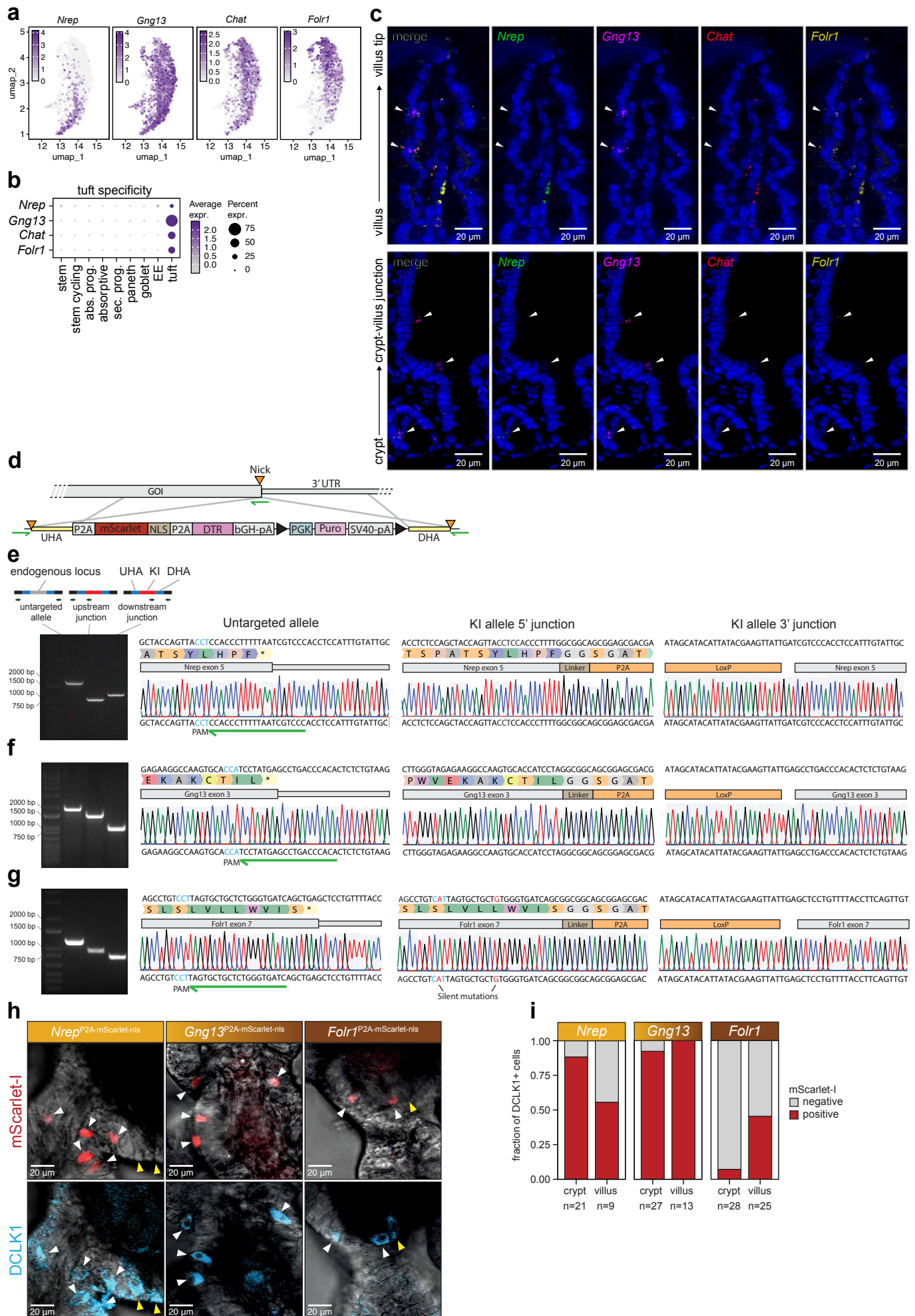
Supplementary Figure 2 | Analysis of transcriptomic heterogeneity within the human small intestinal tuft cell pool Related to Figure 2.

a UMAP of re-clustered human tuft cells overlaid with tuft-p, tuft-1 and tuft-2 gene signatures extracted from the *Chat*^{BAC}-eGFP mouse scRNA-seq dataset from Fig. 1. The right most panel shows the ratio of tuft-2 over tuft-1 signature expression. **b** Distribution of computationally predicted cell-cycle phase per cluster.



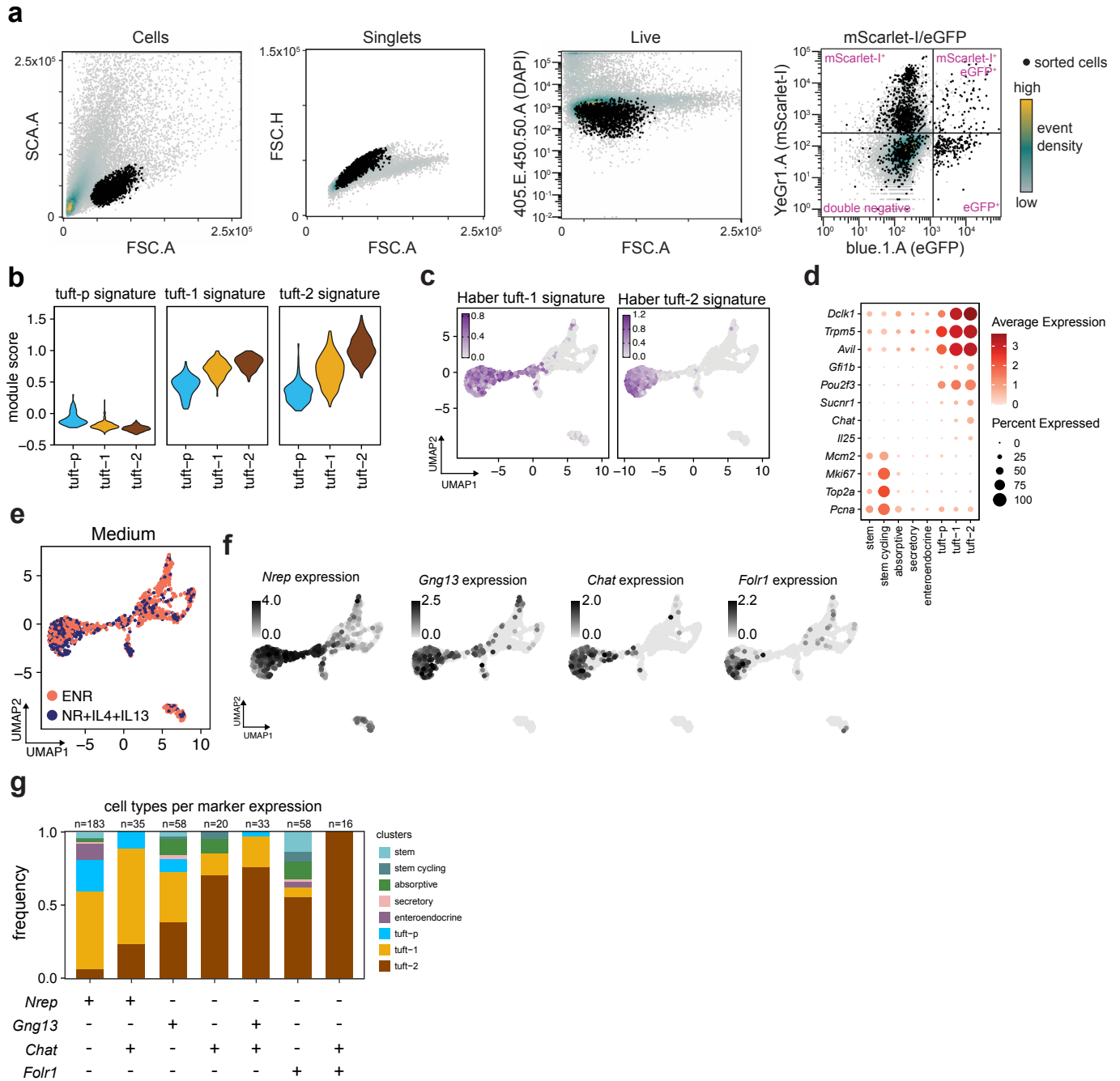
Supplementary Figure 3 | Mature tuft cell markers are expressed in mouse small intestinal organoids
Related to Figure 3.

Microscopy pictures of mouse small intestinal organoids from *Chat*^{BAC}-eGFP mice showing brightfield image (grey) overlaid with fluorescent GFP signal (green). From each organoid the corresponding fluorescent images are shown in three magnifications (GFP signal in white) with immunofluorescence staining against indicated tuft cell markers CD45 or FOLR1 (red). For counterstaining, nuclei (blue) are labelled with Hoechst 3342.

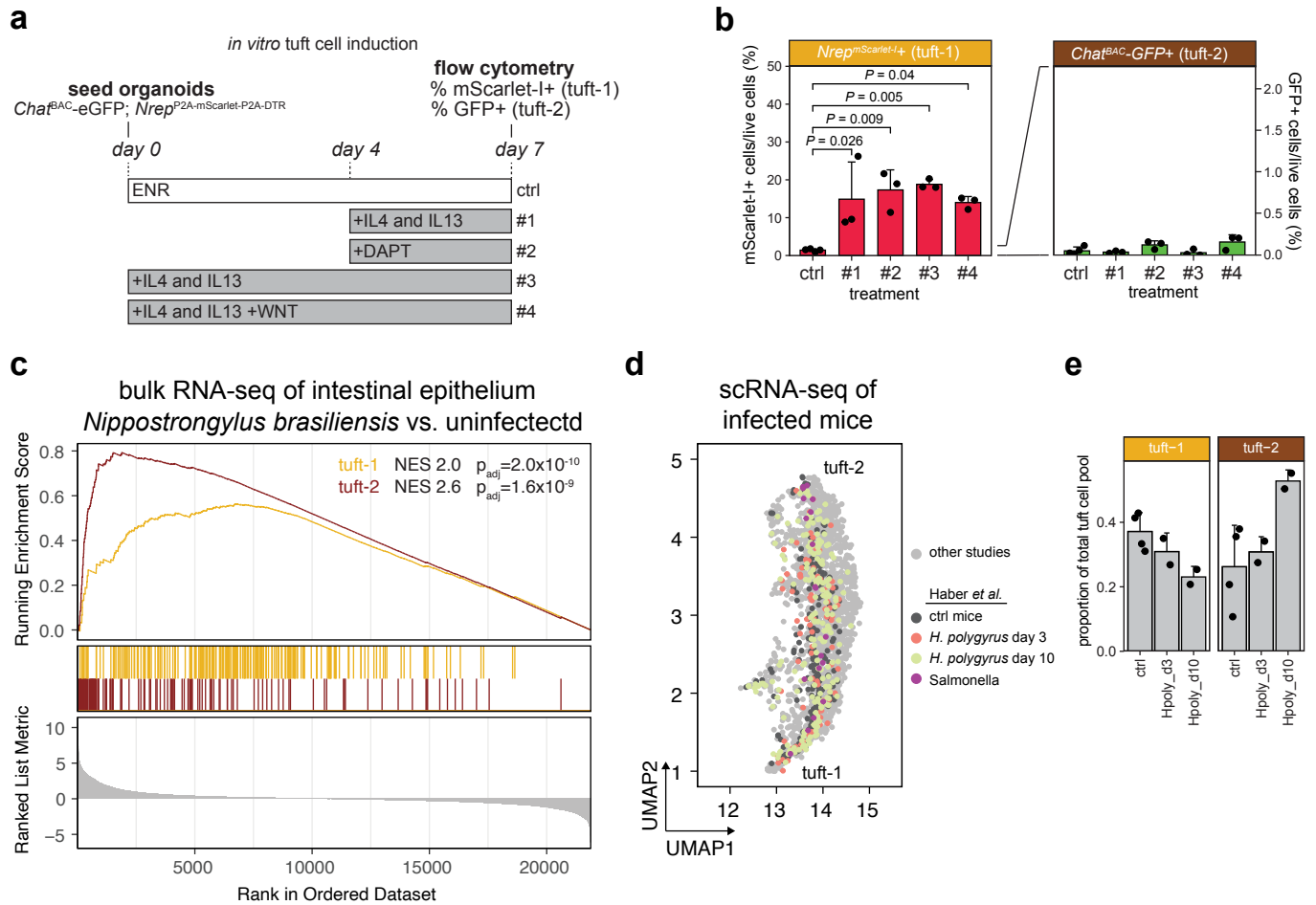


Supplementary Figure 4 | Generation of tuft subtype-specific reporter knock-ins in mouse small intestinal organoids Related to Figure 3.

a UMAP of integrated *in vivo* tuft cell cluster (Fig. 1d), with color coding showing relative expression of selected reporter genes for prospective tuft type-specific knock-ins. **b** Relative expression level of selected tuft subtype-specific reporter genes in various intestinal cell types featured in the integrated *in vivo* mouse small intestine single-cell transcriptome dataset (Fig. 1d). Average expression is represented by dot color. Percentage of expressing cells is denoted by dot size. EE: enteroendocrine, abs. prog.: absorptive progenitor, sec.prog.: secretory progenitor. **c** Representative fluorescence microscopy images of RNAscope targeting indicated prospective tuft subtype markers. White arrows indicate cells positive for at least one of these markers. **d** Schematic representation of genetic knock-in strategy using in-trans paired nicking, where Cas9D10A nickase generates single strand breaks ('nick', orange triangles) in genomic target site, as well as at the extremities of the homology arms in the knock-in donor template. Guide RNA (green arrows) targets vicinity of C-terminal STOP-codon. Black triangles indicate LoxP sites. UTR: untranslated region, UHA: upstream homology arm, DHA: downstream homology arm, NLS: nuclear localization signal, DTR: diphtheria toxin receptor, bGH-pA: polyadenylation signal of bGH, PGK: PGK promotor, Puro: puromycin resistance cassette and SV40-pA: polyadenylation signal of SV40. **e** Validation of correct genomic integration of heterozygous knock-in in *Nrep* by PCR (left) and Sanger sequencing of the corresponding PCR amplicons (primers can be found in Supplementary Table 1). Untargeted allele remains unaffected with in-trans paired nicking. UHA: upstream homology arm, DHA: downstream homology arm, KI: integrated knock-in, PAM: protospacer adjacent motif. Guide RNAs are indicated with green arrows. **f** As in (e), but for *Gng13*. **g** As in (e), but for *Folrl*. **h** Representative brightfield microscopy images overlayed with fluorescence of indicated tuft cell subtype reporter knock-in lines (mScarlet-I, red) stained with anti-DCLK1 (blue). White arrows indicate co-expression, yellow arrows indicate DCLK1+ cells without co-expression of tuft subtype marker. **i** Quantification of (h).

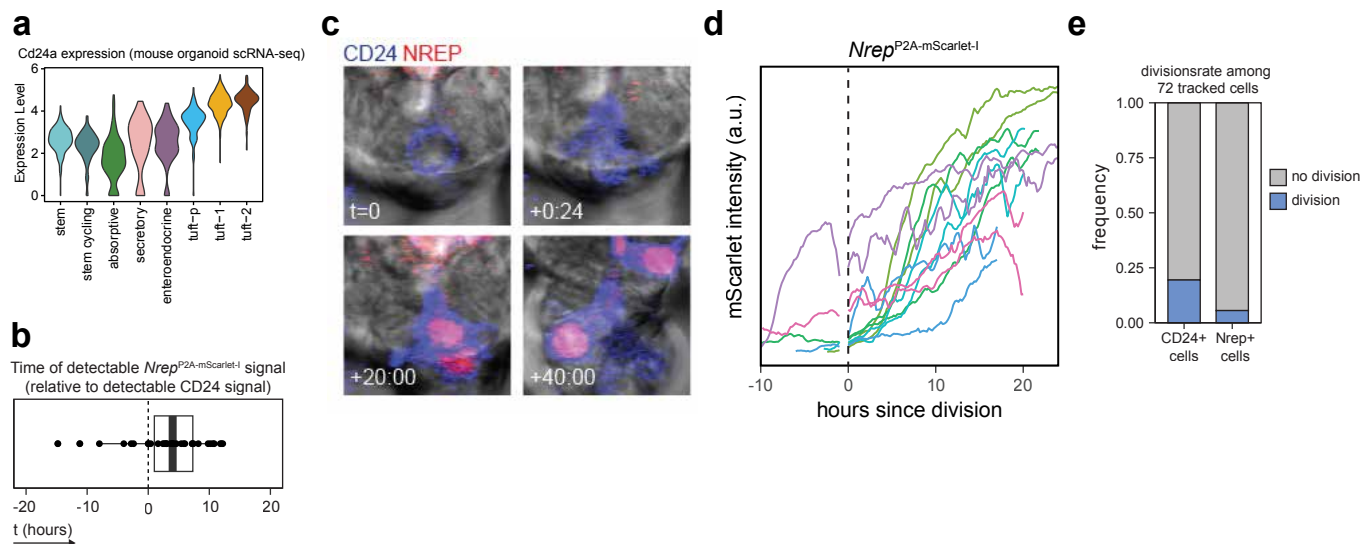


Supplementary Figure 5 | scRNA-seq of tuft subtype-specific reporter knock-in organoids Related to Figure 3. **a** Gating strategy used during FACS prior to plate-based scRNA-sequencing of fluorescent cell populations from tuft subtype-specific reporter organoids. Event density is indicated by color gradient. Sorted events are denoted in black. Plot includes subsampling of events from all three organoid reporter lines and both medium conditions. **b** Violin plot depicting tuft-p, tuft-1 and tuft-2 signature expression in *in vitro* organoid scRNA-seq tuft cell clusters. **c** UMAP of organoid single-cell transcriptomic dataset overlaid with relative tuft-1 and tuft-2 signature scores¹. **d** Single cell dot plot showing expression of tuft cell and proliferation markers per tuft cell cluster in *in vitro* organoid scRNA-seq dataset. Average expression is represented by dot color while the percentage of expressing cells is denoted by the dot size. **e** UMAP of organoid single-cell transcriptomic dataset, colored by medium condition. **f** UMAP of organoid single-cell transcriptomic dataset, overlaid with normalized RNA expression of indicated tuft cell subtype markers. **g** Barplot showing cell types from organoid single-cell transcriptomic dataset positive (robust expression detected) for indicated tuft subtype markers.



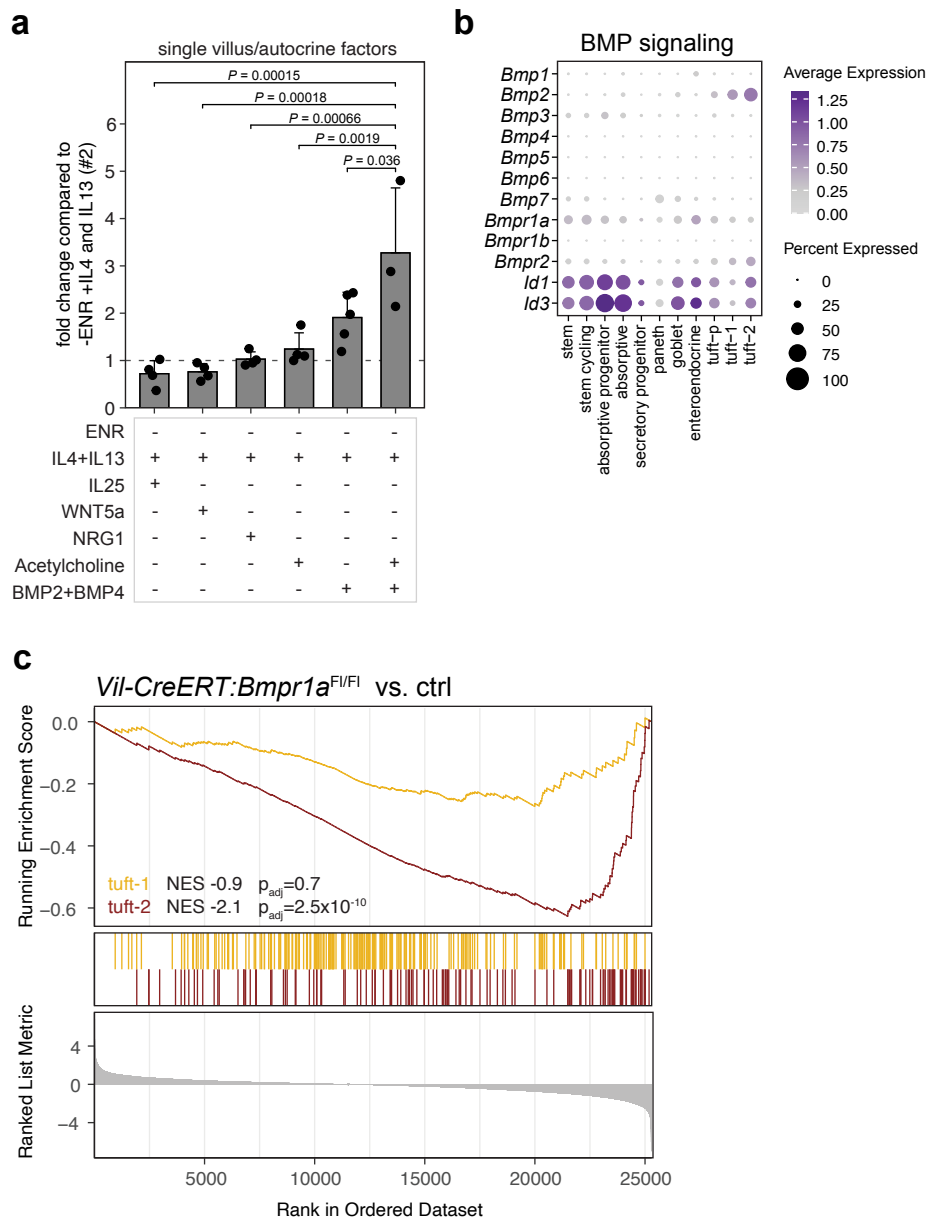
Supplementary Figure 6 | Type 2 cytokine treatment hardly yields tuft-2 cells *in vitro* Related to Figure 4.

a Schematic representation of experimental setup for *in vitro* tuft cell induction. *Chat*^{BAC-eGFP}; *Nrep*^{P2A-mScarlet-I} organoids were treated by various regimens (#1- #4) and evaluated by flow cytometry on the 7th day after seeding. **b** Percentage of mScarlet-I+ and GFP+ cells per indicated treatment shown in (a). Data are represented as mean $\pm$ SD (ANOVA mScarlet+ cells $p < 0.01$, ANOVA GFP+ cells not significant, Tukey HSD test). **c** Gene set enrichment analysis of tuft-1 and tuft-2 signatures¹ in mice infected with *Nippostrongylus Brasiliensis*. Bulk RNA-sequencing data of infected and control mice was obtained from E-MTAB-9183³. **d** Single-cell RNA sequencing profiles of tuft cells isolated from mice infected with *Heligmosomoides polygyrus* (obtained from GSE92332¹) highlighted in UMAP of tuft cell cluster from integrated mouse *in vivo* single-cell transcriptome dataset (Fig. 1d). **e** Proportion of post-mitotic tuft-1 and tuft-2 subtypes during infection with *Heligmosomoides polygyrus* (obtained from GSE92332¹). Data are represented as mean $\pm$ SD. Data points represent individual mice.



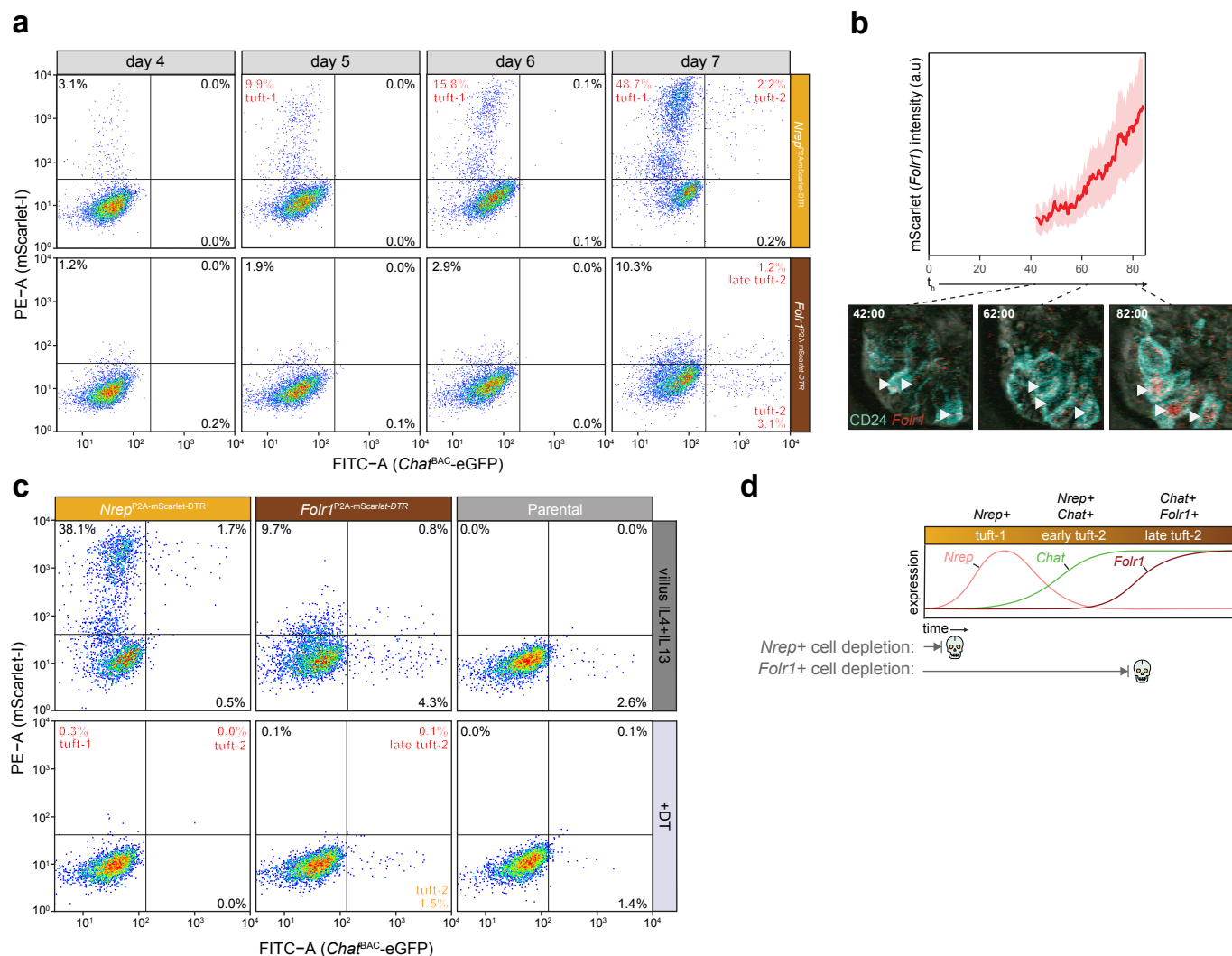
Supplementary Figure 7 | Live imaging of *Chat*^{BAC}-eGFP;*Nrep*^{P2A-mScarlet-I} organoids with anti-CD24 Related to Figure 4.

a Violin plot showing Cd24a (CD24) expression in organoid scRNA-seq dataset. **b** Boxplot depicting timing of first detectable *Nrep*^{P2A-mScarlet} fluorescence signal relative to first detectable anti-CD24 signal. **c** Representative timelapse sequence showing brightfield images overlaid with anti-CD24 (blue) and *Nrep*^{P2A-mScarlet} (red) fluorescence signals. Here, anti-CD24 signal was detectable before division, while *Nrep*^{P2A-mScarlet} was first detected after division. **d** *Nrep*^{P2A-mScarlet-I} fluorescence signal intensity over time in rare dividing tuft progenitor cells ($n = 6$ cells). Tracks are synchronized such that $t = 0$ marks division timepoint (dashed line). **e** Frequency of cells dividing while positive for CD24 or *Nrep* during live microscopy experiments ($n = 72$ cells).



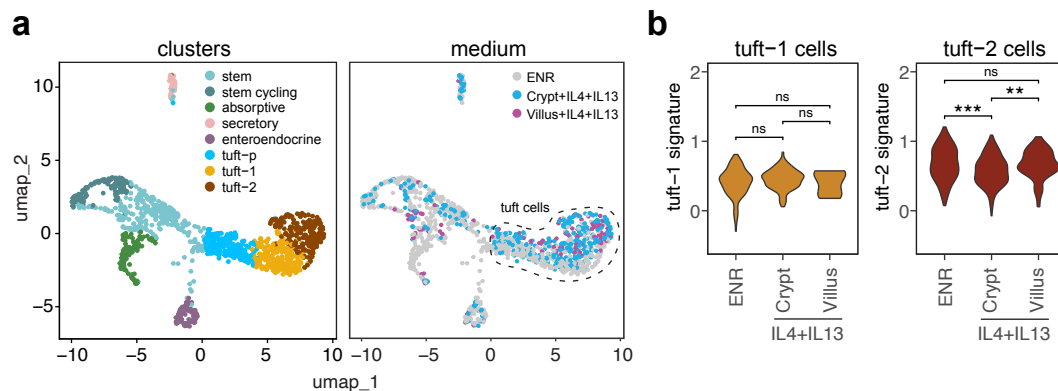
Supplementary Figure 8 | Tuft cell maturation to tuft-2 phenotypes depends on BMP signaling Related to Figure 5.

a Fold-change induction of *Chat*^{BAC}-eGFP+ cells after addition of single villus/autocrine-inspired factors compared to cytokine-pretreated organoids without ENR (condition #2 in Fig. 5a-b). Data are represented as mean $\pm$ SD (ANOVA $p < 0.0001$, Tukey HSD test). **b** Expression level of BMP ligands, receptors and BMP responsive genes in indicated cell types extracted from the integrated *in vivo* mouse small intestine single-cell transcriptome dataset (Fig. 1d). Average expression is represented by dot color. Percentage of expressing cells is denoted by dot size. **c** Gene set enrichment analysis of tuft-1 and tuft-2 signatures¹ in bulk RNAseq dataset of intestinal epithelium depleted for *Bmpr1a* (*Vil-CreERT:Bmpr1a^{FL/FL}*) vs. control mice⁴. For this analysis, RNA data from multiple diets were included in the comparison between *Bmpr1a* knockout and control groups. Upon perturbed BMP signaling, tuft-2 signature genes are the most downregulated.



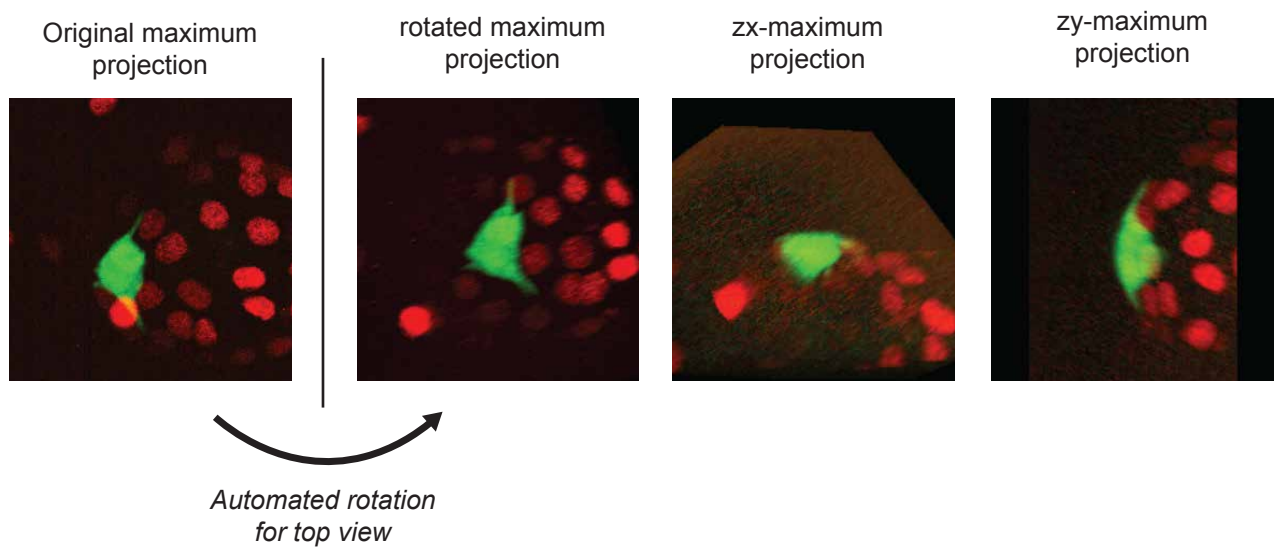
Supplementary Figure 9 | Temporal dynamics of tuft cell subtype-specific markers in villus medium and depletion with DT Related to Figure 5.

a Representative flow cytometry time course analysis of fluorescent cell populations in $Chat^{BAC}$ -eGFP; $Nrep^{P2A}$ -mScarlet-I and $Chat^{BAC}$ -eGFP; $Folr1^{P2A}$ -mScarlet-I organoids treated with villus-inspired maturation regimen (Fig. 5a-b #3). Combined data of 3 biological replicates are shown, 5000 cells are shown per plot. **b** Top: mScarlet($Folr1$) signal over time as determined by live imaging in organoids treated with villus-inspired regimen indicated in Fig. 5a (#3). Bottom: microscopy stills from indicated timepoints (red: mScarlet($Folr1$); cyan: anti-CD24). Imaging was started after addition of cytokines on the 3rd day after seeding ($n = 10$ cells from a representative experiment). White arrows indicate cells that are mScarlet($Folr1$)+ at the end of timelapse microscopy. **c** Representative flow cytometry analysis of fluorescent cell populations in $Chat^{BAC}$ -eGFP; $Nrep^{P2A}$ -mScarlet-I-DTR, $Chat^{BAC}$ -eGFP; $Folr1^{P2A}$ -mScarlet-I-DTR and parental no-knockin control organoids ($Chat^{BAC}$ -eGFP) treated with villus-inspired maturation regimen (Fig5ab #3) in the presence or absence of Diphtheria toxin (DT). Combined data of 3 biological replicates are shown, 3000 cells are shown per plot. **d** Schematic with model of sequential expression of $Nrep$, $Chat$, and $Folr1$ within a single cell during tuft cell differentiation. Below, the expected time of death in case of tuft cell differentiation in the presence of DT.



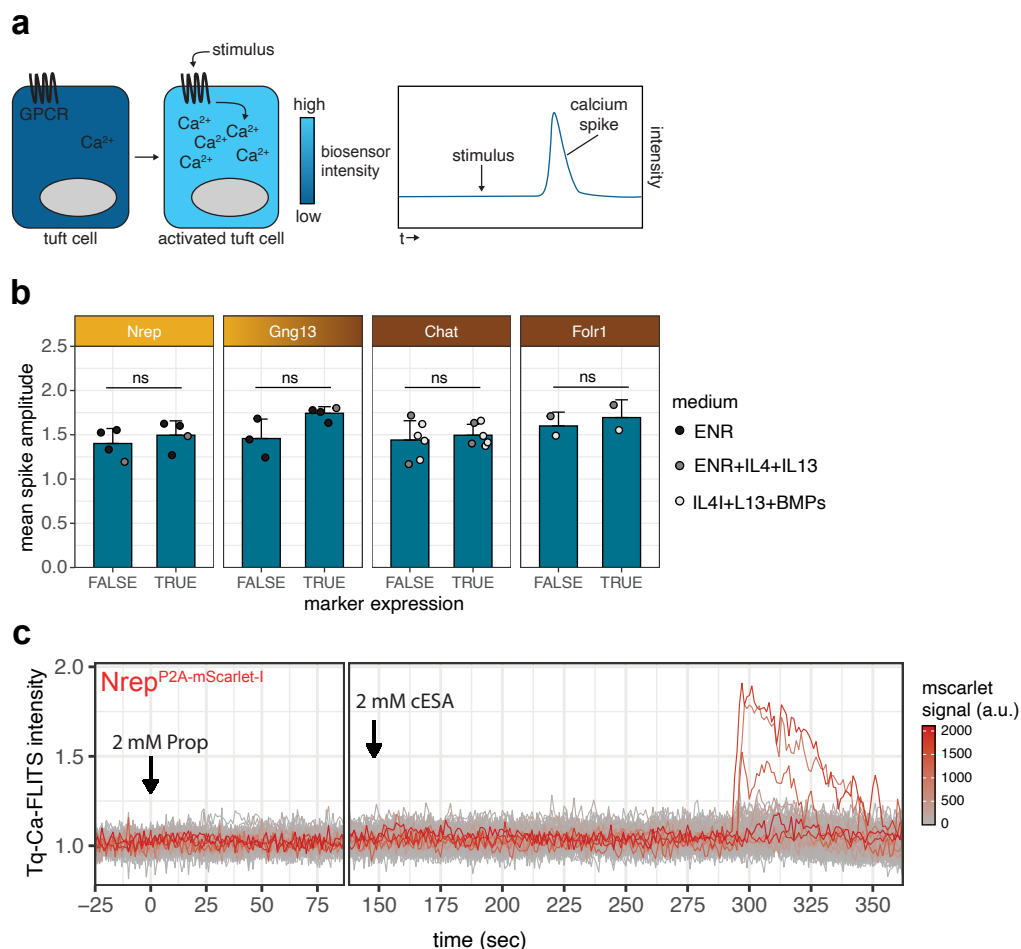
Supplementary Figure 10 | scRNA-seq of organoids in villus-inspired medium Related to Figure 5.

a Transcriptomic analysis of single cells in organoids upon induction with cytokines in ENR or villus-inspired culture conditions. Left, UMAP of organoid cells enriched for tuft cells by FACS. Cell types are annotated by color. Right, same UMAP but cells are colored by medium condition. **b** Violin plots depicting distribution of relative levels of tuft signature scores in tuft-1 cells (left) or tuft-2 cells (right) originating from different culture conditions (ns not significant, ** p-value < 0.01, *** p-value < 0.001, Wilcoxon rank-sum test).



Supplementary Figure 11 | Dynamic tuft cell protrusion analysis Related to Figure 6.

Stills exemplifying automated rotation algorithm used to visualize the position of tuft cell protrusions within the organoid epithelial layer. Protrusions wrap around the basal side of the organoid. Green: *Chat*^{BAC}-eGFP, Red: H2B-mScarlet-I.



Supplementary Figure 12 | Calcium signaling dynamics in organoid monolayers following stimulation with cESA and propionate Related to Figure 6.

a Schematic of tuft cell activation measurements with Tq-Ca-FLITS calcium biosensor. **b** Bar diagram depicting mean amplitude of calcium spike following stimulation with 1.5 mM cESA. Per bar, each point represents a separate experiment and is colored per medium used. Data are represented as mean $\pm$ SD (ns not significant, t-test). **c** Single-cell Tq-Ca-FLITS intensity traces following sequential stimulation with 2 mM propionate, followed by 2 mM cESA. Traces are colored by mScarlet-I signal measured in the corresponding cell.

Supplementary Table 1

List of primers targeting genomic loci of genes selected for tuft subtype-specific reporter knock-ins.

<i>Nrep</i>	untargeted allele	fw	5' CTGCTGGGTTCTGAGGGAGGCT
		rv	5' GTGACTGCACATCCACCAGGGC
<i>Nrep</i>	5' knock-in junction	fw	5' CTGCTGGGTTCTGAGGGAGGCT
		rv	5' CTCGCCCTTGCTCACAGG
<i>Nrep</i>	3' knock-in junction	fw	5' GCAACCTCCCCTTCTACGAG
		rv	5' GTGACTGCACATCCACCAGGGC
<i>Gng13</i>	untargeted allele	fw	5' CACTGGTTCCTTGCGAGCCCTG
		rv	5' TCCCAAGGAGCCACCTGTTGCT
<i>Gng13</i>	5' knock-in junction	fw	5' CACTGGTTCCTTGCGAGCCCTG
		rv	5' CTTCAGCTTGGCGGTCTG
<i>Gng13</i>	3' knock-in junction	fw	5' GCAACCTCCCCTTCTACGAG
		rv	5' TCCCAAGGAGCCACCTGTTGCT
<i>Folr1</i>	untargeted allele	fw	5' CAGTGGTGGGAGGACTGCCAGA
		rv	5' GCCCCTCCCCGCAAACAAAAC
<i>Folr1</i>	5' knock-in junction	fw	5' CAGTGGTGGGAGGACTGCCAGA
		rv	5' CTTCAGCTTGGCGGTCTG
<i>Folr1</i>	3' knock-in junction	fw	5' GCAACCTCCCCTTCTACGAG
		rv	5' GCCCCTCCCCGCAAACAAAAC

References

1. Haber, A. L. *et al.* A single-cell survey of the small intestinal epithelium. *Nature* **551**, 333–339 (2017).
2. Manco, R. *et al.* Clump sequencing exposes the spatial expression programs of intestinal secretory cells. *Nat Commun* **12**, (2021).
3. Lindholm, H. T. *et al.* *BMP Signaling in the Intestinal Epithelium Drives a Critical Feedback Loop to Restrain IL-13-Driven Tuft Cell Hyperplasia*. *Sci. Immunol* vol. 7 <https://www.science.org> (2022).
4. Beumer, J. *et al.* BMP gradient along the intestinal villus axis controls zonated enterocyte and goblet cell states. *Cell Rep* **38**, 110438 (2022).