Epigenetic modifier balances Mapk and Wnt signalling in differentiation of goblet and Paneth cells

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Introduction

The small intestinal epithelium consists of absorptive enterocytes and secretory cells including Paneth cells, mucus-secreting goblet cells, hormone-producing enteroendocrine cells, and tuft cells, which are organized in crypt-villus structures. Actively proliferating stem cells at the crypt base constantly renew the epithelium (Crosnier et al, 2006; Clevers, 2013). The intestinal stem cells, which are characterized by the expression of the stem cell marker Lgr5 (Barker et al, 2007), give rise to highly proliferative transit-amplifying (TA) progenitor cells, which are located above the stem cell niche in the crypt. Moving upwards towards the crypt-villus junction, the TA cells differentiate into absorptive enterocytes or secretory cells. The differentiating cells migrate into the villi, except for the Paneth cells which remain at the bottom of the crypts, interspersed between the Lgr5+ stem cells, secrete antimicrobial peptides such as defensins and lysozyme, and provide Wnt ligands and other growth factors for stem cell maintenance (Sato et al, 2011).

In recent years, research generated insights into a network of transcription factors and signalling pathways that guide cell type specification in the TA zone (Beumer & Clevers, 2021). Active Wnt signalling is critical for intestinal homeostasis, stem cell maintenance, and the formation of secretory progenitor cells (Pinto et al, 2003; Fevr et al, 2007). The transcription factor Math1 (Atoh1) is essential for specification towards the secretory lineage (Yang et al, 2001). Gh1, a transcriptional repressor of enteroendocrine specification, acts downstream of Math1 to select Paneth/goblet cell fates versus the enteroendocrine fate (Shroyer et al, 2005). The ETS transcription factor Spdef further directs secretory maturation in Paneth and goblet cells and deletion of Spdef in the mouse intestine leads to an accumulation of immature secretory progenitor cells (Gregorieff et al, 2009; Noah, 2010). Persisting high Wnt activity in secretory progenitor cells promotes the differentiation of Paneth cells (van Es et al, 2005). Paneth cells are absent in mice deficient for Tcf4, the transcriptional mediator of Wnt signalling (van Es et al, 2005). In addition, high Wnt activity prevents goblet cell differentiation, as goblet cells are absent in Apc-mutant intestine (Sanson et al, 2004). We have previously shown that Mapk signalling impedes the Wnt-induced maturation of Paneth cells and shifts the differentiation of common Paneth-goblet progenitors towards a goblet cell fate (Heuberger et al, 2014).

Besides the role of transcription factors, epigenetic regulation of gene expression has emerged as a powerful determinant of cell type identity as well as stem cell maintenance (Jadhav et al, 2016; Piunti & Shilatifard, 2016; Brand et al, 2019; Grinat et al, 2020; Goveas et al, 2021). We have recently reported a crucial role of the histone methyltransferase Mll1 in development of colorectal...
cancer (Grinat et al., 2020; Heuberger et al., 2021). Mll1 promotes a highly proliferative regenerative cell state which renders intestinal epithelial cells susceptible for tumorigenesis (Heuberger et al., 2021), maintains intestinal cancer stem cells, and promotes Wnt-induced tumorigenesis by antagonizing the polycomb repressive complex 2 (PRC2)–mediated repression of stem cell genes (Grinat et al., 2020). Ablation of Mll1 caused differentiation of Wnt-activated cancer stem cells by increasing a secretory gene expression profile (Grinat et al., 2020). Further work showed that Mll1 is required for intestinal stem cell maintenance at homeostasis (Goveas et al., 2021).

We here addressed the role of Mll1 in secretory cell fate determination in the adult intestinal epithelium at homeostasis. Using mouse genetics and intestinal organoid cultures, we show that Mll1 sustains the progenitor cell state and controls Wnt/Mapk-driven secretory cell specification into Paneth and goblet cells.

Results

Ablation of Mll1 causes aberrant secretory differentiation in intestinal epithelial crypts

To assess the role of Mll1 in intestinal cell fate determination, we ablated the expression of Mll1 in Lgr5+ stem cells by conditional mutagenesis. Lgr5-EGFP-ires-CreERT2 mice, which express eGFP and a tamoxifen-inducible Cre recombinase under the control of the Lgr5 promoter, were crossed with Mll1lox/lox mice to generate Lgr5-EGFP-ires-CreERT2, Mll1lox/− and Lgr5-EGFP-ires-CreERT2, Mll1lox/lox mice (referred to as Mll1+/− and Mll1−/−, respectively) (Barker et al., 2007; Denissov et al., 2019; Chen et al., 2019; Grinat et al., 2020). For lineage tracing, we further crossed in the Rosa26-LacZ reporter strain (Soriano, 1999). Mutagenesis was induced by intraperitoneal injections of tamoxifen. To test whether production of progeny from stem cells is affected by loss of Mll1, we first performed lineage tracing. Conditional mutagenesis using the Lgr5-CreERT2 caused a mosaic of recombined LacZ− and adjacent non-recombined (wild-type) crypts (Fig S1A), as previously described (Schuijers et al., 2014; Grinat et al., 2020). We traced mutant cells post induction and observed that both Mll1+/− and Mll1−/− Lgr5+ stem cells gave rise to LacZ-positive progeny, which populated mutant crypts and villi starting from day 10 after induction of mutagenesis (Fig S1A). Ablation of Mll1 using the Lgr5-CreERT2 did not affect crypt cell proliferation, as seen by similar numbers of BrDU-incorporating cells in Mll1+− and Mll1−/− crypts (Fig S1B, quantification on the right). Neither did Mll1-deficient cells undergo apoptosis, as shown by the absence of positive cleaved Caspase-3 staining in the crypts (Fig S1C). Hence, Lgr5-CreERT2-driven ablation of Mll1 did not affect production of intestinal epithelial cells (Fig S1D, left panel). Heterozygous ablation of Mll1 (Mll1+/− mice) did not alter crypt cell morphology and cell type composition compared with wild-type control mice (Fig S1D, upper and middle right panel). Mll1−/− crypts, however, exhibited an increased number of cells with goblet cell-like features (Fig S1D, lower right panel, quantification below). By Alcian blue staining we identified these cells as mucus-containing goblet-like cells (Fig 1A, quantification on the right), as also previously described (Goveas et al., 2021). To exclude transient effects, we analysed mutant intestinal epithelia at 30–50 d after mutagenesis. Periodic acid–Schiff (PAS) staining of duodenum, jejunum, ileum, and colon epithelial sections revealed increases in mucus-producing goblet cells in all parts of Mll1-deficient intestinal epithelia with the exception of the ileum (Fig S1E). Increased goblet cell specification was most prominent in the jejunum. Using immunofluorescence, we observed an increased number of secretory cells in the upper parts of Mll1−/− jejunal crypts, which were double-positive for the goblet cell marker intestinal trefoil factor (ITF) (van der Sluis et al., 2006) and the Paneth cell marker lysozyme (Lyz) (Porter et al., 2002) (Fig 1B, quantification on the right). At the base of Mll1−/− crypts, Lyz-positive Paneth cells acquired Alcian blue–positive staining (compare Fig 1A with Figs 1B and S1F). In agreement with our previous characterisation of Mll1-deficient intestines (Goveas et al., 2021), the loss of Mll1 did not alter the number of chromogranin A (ChroA)-positive enteroendocrine cells per crypt–villus axis (Fig S1G, quantification on the right).

To explore the mechanistic role of Mll1 in secretory cell fate determination, we explored small intestinal organoids from villinCreERT2, Mll1lox/− and villinCreERT2, Mll1lox/lox mice (el Marjou et al., 2004; Denissov et al., 2014; Chen et al., 2019) and induced
mutagenesis in culture by addition of 4-OHT tamoxifen. We tested for markers of Wnt activity and Paneth cell positioning (Batlle et al., 2002). Mll1 ablation neither changed the level of Axin2 expression (Lustig et al., 2002; Grinat et al., 2020) (Fig S1H, right) nor did it alter the expression of EphB2 and EphB3 (Fig S1H, left), indicating that the diminished Paneth cell identity is not a result of decreased Wnt activity or an altered EphB2/3 gradient. Together, these findings suggest that the loss of Mll1 impairs Paneth/goblet cell specification and results in an accumulation of Paneth-goblet double-positive secretory cells, which appears to be caused by an intrinsic switch in cell fate rather than by decreased Wnt activity or an incorrect positioning of Paneth cells.

**Mll1-deficient intestinal organoids show increased expression of secretory Paneth and goblet cell genes**

In the initial days after induction of mutagenesis, the loss of Mll1 did not alter organoid morphology (Fig 2A). However, RT–PCR analysis of Mll1-deficient organoids (Mll1−/−) revealed an up-regulation of the expression of the goblet cell–specific genes Gob5 (Leverkoehne & Gruber, 2002), Muc2 (van der Sluis et al., 2006) and the Kruppel-like factor Klf4, which is required for terminal goblet cell differentiation (Katz et al., 2002), as well as the Paneth cell–specific genes Mmp7 and Lyz (Porter et al., 2002) (Fig 2B, light blue bars) compared with control and Mll1+− organoids (grey and light green bars). In agreement, immunofluorescence stainings revealed that Mll1-deficient organoids exhibited increased numbers of IFI-positive goblet cells and Lyz-positive Paneth cells compared to non-induced control organoids (Fig S2A). To promote the production of Wnt-primed secretory progenitor cells, we treated the organoids with Wnt3a (Pinto et al., 2003; Heuberger et al., 2014), which resulted in increased expression of the secretory transcription factor Math1 (Fig 2B, right, dark green bar). Wnt3a stimulation of Mll1-deficient organoids caused enhanced Math1 expression (Fig 2B, right, blue bars) and further increased the expression of the goblet cell genes Gob5, Klf4, Muc2, the Paneth cell genes Mmp7 and Lyz, and the secretory genes Gfi1 (Shroyer et al., 2005), Spdef (Gregorieff et al., 2009), and Ift (Mashimo et al., 1996) (Figs 2B and S2B, blue bars). The Notch-dependent transcription factor Hes1, which represses Math1 induction (Yang et al., 2001; Fre et al., 2005), was not significantly altered, indicating that Mll1 acts downstream of Notch-mediated repression to specify secretory cell differentiation (Fig S2C).

**Mll1-deficient Wnt-high crypt cells exhibit goblet cell features**

To consolidate our findings in vivo, we activated Wnt signalling by genetic stabilization of β-catenin through Cre-mediated deletion of the exon 3 of the β-catenin locus in Lgr5-EGFP-IRES-CreERT2, β-cateninΔE3/ΔE3 mice (Harada et al., 1999; Barker et al., 2007) (hereafter called β-catGOF) (Fig S3A, see nuclear location of β-catenin). High Wnt signalling in secretory progenitors induces differentiation into Paneth cells and prevents the maturation of goblet cells (Sansom et al., 2004; Andreu et al., 2005; van Es et al., 2005). Indeed, the high Wnt activity imposed a Paneth-like identity on the epithelial cells: β-catGOF, Mll1−/− intestines showed high numbers of Mmp7− and Lyz-positive cells, and few ITF-positive goblet cells (Fig S3B). Alcian blue staining of control and β-catGOF intestines confirmed reduced numbers of goblet cells in Wnt-high epithelium (Fig S3C). Ablation of Mll1 in β-catGOF, Mll1−/− crypts caused accumulation of secretory cells double-positive for Paneth and goblet cell markers (Fig S3D), as it was observed in Mll1−/− crypts (see Fig 1). We isolated Paneth cells from β-catGOF, Mll1−/− and β-catGOF, Mll11−/− mice at 10 d after the induction of mutagenesis by fluorescence-activated cell sorting (FACS) and analysed transcriptomic changes by RNA sequencing. Immuno-histochemistry (IHC) for Mll1 on intestinal crypts of β-catGOF, Mll1−/− mice con

**Figure 2.** Mll1-deficient organoids show aberrant Paneth/goblet cell differentiation. (A) Brightfield images of non-induced (control) and villinCreERT2; Mll11−/− intestinal organoids at 5 d after 4-OHT-induced mutagenesis, scale bars 100 μm. (B) mRNA expression of Mll1 and secretory cell genes in non-induced control (grey), villinCreERT2; Mll11−/− (green) and villinCreERT2; Mll11+/− organoids (blue) at 7–12 d after 4-OHT-induced mutagenesis, n = 3 independent experiments, two-tailed unpaired t test. Data are presented as mean values ± SEM. Treatment with 500 ng/ml recombinant Wnt3a for 72 h.

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RT–PCR analysis of Paneth cells isolated from β-catGOF; Mll1+/− and β-catGOF; Mll1−/− mice at 10 d after induction of mutagenesis showed a decrease in the expression of the Paneth cell genes Mmp7 and Lyz upon loss of Mll1, whereas the expression of the early goblet-specific gene Gob5 was increased (Fig 3B). The expression of the canonical Wnt target gene Axin2 was not changed (Fig S3F).

We established β-catGOF intestinal organoids with heterozygous and homozygous deletion of Mll1 from Lgr5-EGFP-ires-CreERT2; β-catdeletex3; Mll1flox mice (Harada et al, 1999; Barker et al, 2007; Denissov et al, 2014; Chen et al, 2019) and induced mutagenesis in culture to obtain β-catGOF, Mll1+/− and β-catGOF, Mll1−/− organoids (Fig S3G). As observed in vivo, the ablation of Mll1 in β-catGOF; Mll1−/− organoids increased the expression of secretory genes specific for both Paneth (Mmp7) and goblet cells (Klf4, Muc2, and Gob5) (Fig S3H), substantiating enhanced secretory differentiation and a switch towards a goblet cell fate upon loss of Mll1. The Paneth cell marker Lyz was expressed in Mll1-deficient organoids, but its expression was not increased (Fig S3H). β-catGOF; Mll1−/− organoids exhibited increased numbers of goblet cells and strong mucinous secretion towards the inside of the organoid, as assessed by Alcian blue staining (Fig 3C, quantification on the right). Inhibition of the Mll1 methyltransferase activity in β-catGOF organoids by MI-2 (Grembecka et al, 2012; Heuberger et al, 2021) reduced the...
expression of the Paneth cell gene Mmp7 and reverted the Wnt-induced decrease in Gob5 and by tendency Muc2 expression (Fig 3D), suggesting that the function of Mll1 in secretory differentiation depends on its methyltransferase activity. Immunostaining revealed unchanged expression of β-catenin in β-catGOF; Mll1+/− and β-catGOF; Mll1−/− organoids (Fig S3I), revealing a role of Mll1 in fine-tuning Wnt-driven secretory specification independent of a global change in Wnt activity.

Loss of Mll1 unleashes Mapk signalling in Wnt-activated crypt cells

In previous work we had shown that intestinal goblet cell differentiation depends on active Mapk signalling (Heuberger et al, 2014). We therefore investigated whether changes in Mapk signalling occur in Mll1-deficient and Wnt-activated crypt cells. IHC staining on sections of β-catGOF; Mll1+/− and β-catGOF; Mll1−/− organoids revealed a strong increase in phospho-Erk1/2 levels upon loss of Mll1 (Fig 4A upper panel). Western blotting for phospho-Erk1/2 and phospho-Mek1/2 confirmed an increased activity of the Mapk pathway in β-catGOF; Mll1−/− organoids compared with β-catGOF; Mll1+/− organoids (Fig 4B, quantification on the right). Increased phospho-Erk1/2 levels were also detected in the mutant crypts of β-catGOF;Mll1−/− mice at 10 d after induction, compared with β-catGOF; Mll1+/− mice and adjacent non-recombined crypts (Fig S4A). This global increase in phospho-Erk1/2 levels was not detectable in the mutant crypts of Mll1−/− mice with loss of Mll1 alone (Fig S4B, compare phospho-Erk1/2 levels in two Mll1-deficient crypts to non-recombined crypt on the right). Inhibition of Mapk signalling by treatment with the Mek inhibitor U0126 potently inhibited the growth of β-catGOF; Mll1−/− organoids (Fig S4C). These data suggest that Mll1 prevents goblet cell differentiation of Wnt-activated crypt cells.

Figure 4. Loss of Mll1 unleashes Mapk signalling and goblet cell differentiation in Wnt-high crypt cells and β-catGOF organoids.
(A) Immunohistochemistry stainings for phospho-Erk1/2 on sections of β-catGOF; Mll1+/− and β-catGOF; Mll1−/− organoids, nuclei counterstained with haematoxylin, scale bars 50 μm. (B) Western blot for phospho-Erk1/2 and Erk1/2, phospho-Mek1/2 and Mek1/2, and β-catenin in β-catGOF; Mll1+/− (+/−) and β-catGOF; Mll1−/− (−/−) intestinal organoids. Right: Quantification of phospho-Erk1/2 relative to total Erk1/2 levels in β-catGOF; Mll1+/− (+/−) and β-catGOF; Mll1−/− (−/−) organoids, n = 2 independent organoid lines. Tubulin as loading control for β-catenin. (C) Left: Immunofluorescence for Lyz (green) and ITF (red) on sections of Mek1GOF; Mll1+/− and Mek1GOF; Mll1−/− mouse small intestine at 10 d after induction. Recombined mutant crypts are surrounded by dashed lines. Middle: Immunofluorescence staining for Mmp7 (green) on serial sections. E-cadherin (yellow) stains cell borders, nuclei in blue (DAPI), scale bars 20 μm. Right: Immunohistochemistry for phospho-Erk1/2 and Alcian blue staining for goblet cells, nuclei counterstained with haematoxylin, scale bars 20 μm. Stainings were performed in five independent mice per genotype. (D) Number of Lyz-positive, Mmp7-positive, ITF-positive, and Alcian blue-positive cells in Mek1GOF; Mll1+/− and Alcian blue staining for goblet cells, nuclei counterstained with haematoxylin, scale bars 20 μm. Stainings were performed in five independent mice per genotype. Mann–Whitney U test, ***p < 0.0001, *p = 0.012, **p = 0.002, ***p = 0.0002. Box plot indicates median (red line) and 25th, 75th percentile (box) with Tukey whiskers.
cells by suppressing Mapk signalling, which is unleashed upon ablation of Mll1.

**Mll1 restricts Mapk-driven goblet cell differentiation**

To further examine the role of Mll1 in the Wnt/Mapk-driven differentiation of secretory Paneth and goblet cells, we crossed in the Mek1DD allele (Cowley et al., 1994; Srinivasan et al., 2009). Co-mediating recombination removes a transcription stop cassette and activates the expression of a constitutively active gain-of-function variant of Mek1 (Mek1DD) from the Rosa26 locus. We analysed Mek1DD mice with heterozygous and homozygous ablation of Mll1, Lgr5-EGFP-ires-CreERT2; Mek1DD; Mll1lox/lox and Lgr5-EGFP-ires-CreERT2; Mek1DD; Mll1lox/lox mice (hereafter called Mek1GOF, Mll1+/− and Mek1GOF; Mll1−/−, respectively) at 10 d after the induction of mutagenesis. Activation of Mapk signalling by Mek1GOF in Mek1GOF; Mll1−/− mice increased the proliferation of crypt cells, as seen through an increase in the incorporation of BrdU in mutant eGFP-positive crypts compared with adjacent non-recombined crypts (Fig S4D upper panel, quantification on the right). The eGFP is co-expressed with Mek1GOF from an IRES-EGFP linked to the Rosa26 locus, Mek1DD mice the eGFP also identifies Lgr5−/− stem cells (Barker et al., 2007). The homozygous ablation of Mll1 in Mek1GOF; Mll1−/− mice prevented the Mek1GOF-induced hyper-proliferation and restricted proliferation to the base of the crypts (Fig S4D lower panel, quantification on the right). In accordance with the observed increase in cell proliferation, Mek1GOF; Mll1−/− intestines exhibited elongated villi and crypts (Fig S4E and F). The ablation of Mll1 reduced the Mek1GOF-driven elongation of crypts and villi (Fig S4E and F). High Mapk signalling in the crypts decreased the number of Paneth cells at the crypt bottom, as shown by immunostaining for the Paneth cell marker Lyz (green) on serial sections of adjacent recombined and non-recombined crypts (Fig 4C upper left, quantification in Fig 4D). Mmp7 staining (green) revealed the presence of secretory Paneth-like cells further up in the crypts (Fig 4C upper middle, quantification in Fig 4D). Mek1GOF; Mll1−/− crypts did not show a strong increase in crypt-based goblet cells, as revealed by IFT (red) and Alcian blue staining compared with adjacent non-recombined crypts (Fig 4C upper panel). In contrast, Mek1GOF; Mll1+/− cells were filled with secretory cells, which were positive for the Paneth cell marker Mmp7 (green) and exhibited a strong expression of goblet cell markers (ITF, Alcian blue) (Fig 4C lower panel, quantification in Fig 4D). To note, loss of Mll1 did not impede the Mek1DD-induced activation of Mapk signalling, as shown by IHC for phospho-Erk1/2 in the mutant crypts of Mek1GOF; Mll1−/− mice compared with Mek1GOF; Mll1+/− mice (Fig 4C right panel). Furthermore, Mek1GOF, Mll1+/− and Mek1GOF; Mll1−/− crypts both exhibited nuclear β-catenin (Fig S4G, left panel) and in situ hybridisation revealed equal expression levels of the Wnt target gene Axin2 (Fig S4G, right panel), demonstrating that Mek1 activation and loss of Mll1 did not change the global Wnt activity at the crypt base.

The data demonstrate that loss of Mll1 in the background of activated Mapk signalling has a dual effect, whereas loss of Mll1 prevents the Mek1DD-induced crypt hyper-proliferation and villus elongation, it on the other hand synergizes with Mapk signalling in promoting generation of goblet cells. In other words, the presence of Mll1 promotes Mapk-induced progenitor cell proliferation and restricts goblet cell differentiation. Mll1-deficient secretory cells in Mek1GOF; Mll1−/− crypts were double-positive for Paneth and goblet cell markers and did not fully mature into goblet cells, as we had also observed in β-catenin−/−, Mll1−/− crypts. These data indicate that Mll1 is critical for specifying Paneth and goblet cell fates. It safeguards the Wnt/Mapk-driven differentiation of secretory cells by preserving lineage-specific maturation.

**Mll1 controls the Wnt/Mapk-driven specification of Paneth and goblet cells**

We established intestinal organoids from Lgr5-EGFP-ires-CreERT2; β-catenin−/−, Mek1DD; Mll1lox/lox and Lgr5-EGFP-ires-CreERT2; β-catenin−/−, Mek1DD; Mll1lox/lox mice (hereafter called β-catenin−/−, Mek1GOF; Mll1+/− and β-catenin−/−, Mek1GOF; Mll1−/− mice, respectively) and induced mutagenesis in culture by in vitro administration of 4-OH tamoxifen. The β-catenin−/−, Mek1GOF; Mll1+/− organoids were highly proliferative, as revealed by staining for the proliferation marker Ki67 (Fig S5A, middle panel). The loss of Mll1 prevented the β-catenin−/−, Mek1GOF-driven proliferation (Fig S5A, lower panel). We previously proposed opposing Wnt and Mapk signalling activities in goblet and Paneth cell lineage specification (Heuberger et al., 2014). In accordance, the simultaneous genetic activation of Wnt and Mapk signalling deregulated maturation of both Paneth (Lyz and Mmp7) and goblet cells (Alcian blue) (Fig 5A upper and middle panel, quantification in Fig 5B). These findings indicate the production of a proliferative non-specified cell population by simultaneous activation of Wnt and Mapk signalling. Remarkably, Paneth and goblet cells re-appeared in organoids with homozygous ablation of Mll1, largely as double-positive Paneth-goblet entities, as shown by immunostaining for Lyz, Mmp7, and ITF (Fig 5A lower panel, quantification in Fig 5B). RT-PCR analysis of β-catenin−/−, Mek1GOF; Mll1+/− and β-catenin−/−, Mek1GOF; Mll1−/− organoids confirmed the role of Mll1 in the control of Wnt/Mapk-driven secretory cell fate: The simultaneous activation of Wnt and Mapk signalling in the presence of Mll1 in β-catenin−/−, Mek1GOF; Mll1+/− organoids abrogated the expression of the Paneth cell genes Mmp7 and Lyz (Fig 5C, dark green bars). The expression of the goblet cell gene Klf4 was not decreased, which corresponded with the presence of ITF-positive goblet cells in β-catenin−/−, Mek1GOF; Mll1+/− organoids (Fig 5A middle panel, Fig 5C). Upon ablation of Mll1, the expression of Lyz was partly re-established and Mmp7 expression strongly increased, as did the levels of the goblet cell-specific Klf4 (Fig 5C, light green bars). These data from organoid culture support the role of Mll1 in controlling secretory cell specification induced by Wnt and Mapk.

To further corroborate the regulatory function of Mll1 downstream of Wnt and Mapk signalling we induced the production of stabilized β-catenin, Mek1GOF and the loss of Mll1 in intestinal epithelia by intraperitoneal injections of tamoxifen in β-catenin−/−, Mek1GOF; Mll1+/− and β-catenin−/−, Mek1GOF; Mll1−/− mice. The intestines of β-catenin−/−, Mek1GOF; Mll1+/− mice became dysplastic and exhibited elongated villi (Fig S5B, quantification in Fig 5C). The mice did not survive beyond 20 d after the induction of mutagenesis. Homozygous ablation of Mll1 attenuated the β-catenin−/−, Mek1GOF−/−-induced hyperplastic phenotype. The villi were of normal (wild-type) length.
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Mll1 regulates Gata4/6 expression to restrict Mapk signalling and segregation of secretory Paneth and goblet cell fates.

The loss of Mll1 unleashes Mapk signalling and cell-intrinsically perturbs induced goblet cell fate and thereby promotes Paneth cell specification. In Wnt-high crypt cells, Mll1 suppresses a Mapk-specific regulation of Spdef secretory cell differentiation in vivo, as demonstrated by up-regulation of Spdef and the Paneth cell-specific Mmp7, and by expression of the goblet cell markers Ift, Muc2, and Gob5 (Fig S5D).

In summary, these data demonstrate that Mll1 keeps intestinal epithelial cells in an immature state while impeding secretory cell specification, and a decrease in Mll1 expression is necessary to allow for secretory differentiation. Our data further show that Mll1 also plays a role in specifying the Wnt/Mapk-driven differentiation of Paneth and goblet cells. In Wnt-high crypt cells, Mll1 suppresses a Mapk-induced goblet cell fate and thereby promotes Paneth cell specification. The loss of Mll1 unleashes Mapk signalling and cell-intrinsically perturbs segregation of secretory Paneth and goblet cell fates.

Mll1 regulates Gata4/6 expression to restrict Mapk signalling and goblet cell fate

We previously reported that Mll1 regulates the expression of the transcription factor Gata4 to sustain stemness and restrict secretory goblet cell differentiation of β-catGOF intestinal cancer stem cells (Grinat et al., 2020). Mll1-deficient crypts exhibited a decreased expression of Gata4 (Fig S6A). Gata4 expression was reduced in villinCreERT2, Mll1+/− organoids compared with non-induced control and tamoxifen-induced β-catGOF, MekGOF, Mll1+−/− and β-catGOF, MekGOF, Mll1−−/− intestinal organoids. Middle: immunofluorescence staining for Lyz (green) and ITF (red) on sections of organoids of the three genotypes, white arrows indicate cells with weak ITF expression. E-cadherin (yellow) stains cell borders and nuclei in blue (DAPI). Right: Alcian blue staining for goblet cells, nuclear fast red counterstaining, black arrows indicate Alcian blue-positive goblet cells. Scale bars 50 μm.

(B) Quantifications of the numbers of Lyz-, Mmp7-, ITF-, and Alcian blue-positive cells per organoid in non-induced (control) and tamoxifen-induced β-catGOF, MekGOF, Mll1+/−, and β-catGOF, MekGOF, Mll1−−/− cultures, quantified from two independent organoid lines, Mann–Whitney U test, ****P = 0.0001, Mmp7 ****P = 0.0001, ITF ****P = 0.00006 (control-Mll1−−/−), ****P = 0.0002, Alcian blue ****P = 0.0008, *P = 0.001. Box plots indicate median (middle line) and 75th and 25th percentile (box) with Tukey whiskers. (C) mRNA expression of Mll1 and the secretory Paneth/goblet cell genes Klf4, Mmp7, and Lyz in non-induced control and 4-OHT-induced β-catGOF, MekGOF, Mll1−−/− (n = 4) and β-catGOF, MekGOF, Mll1+/− (n = 6) organoids, two-tailed unpaired t test, Mll1: ****P < 0.0001, ***P < 0.0015, **P < 0.0001, Mmp7: *P = 0.017, *P = 0.004 (control-Mll1−−/−), **P = 0.003 (Mll1−−/−/−−/−), Lyz: ***P < 0.00013 (control-Mll1−−/−), ****P < 0.00015 (control-Mll1−−/−). Data are presented as mean ± SEM.
Here we report a role of the epigenetic regulator Mll1 in Wnt- and Mapk-driven cell fate specification of secretory progenitors in the intestinal epithelium. We show that Mll1 promotes proliferation and the progenitor cell state, and impedes secretory differentiation into goblet and Paneth cells. Our data reveal an Mll1-dependent regulation of the dual role of Mapk signalling in progenitor cells of the secretory lineage.

Discussion

In β-catenin−/− organoids, CRISPR/Cas9-mediated knockout of GATA6 in Ls174T cells increased the expression of the secretory transcription factor MATH1 (Fig 6D). Vice versa, transient overexpression of GATA6 in Ls174T cells decreased MATH1 expression (Fig 6E). IHC staining for GATA6 on control and shMll1 Ls174T spheres revealed a strong decrease in GATA6 levels upon depletion of Mll1 (Fig 6F). Immunohistochemistry for GATA6 on sections of wild-type control organoids and Gata4-negative areas of sgGata4 organoids, counted in at least five organoids from two sgGata4 organoid lines in three independent rounds of Cas9 induction, Mann–Whitney U test. Box plot indicates median (middle line) and 25th, 75th percentile (box) with Tukey whiskers.

Figure 6. Mll1 controls Mapk signalling and goblet cell fate through regulation of Gata4/6 expression. (A) Immunofluorescence for Mll1 (red) and Gata4 (green) on sections of 4-OHT−/−-induced β-catGOF and β-cat−/− organoids. Nuclei in blue (DAPI), scale bars 20 μm. (B) mRNA expression of Gata4 and Math1 in β-catGOF and β-cat−/− organoids, n = 3 independent experiments, two-tailed unpaired t test. Data are presented as mean values ± SEM. (C) Immunohistochemistry for Gata4 (upper panel) and phospho-Erk (lower panel) on serial sections of non-induced control and doxycycline (Dox)-induced sgGata4 organoids at day 8 after Cas9 induction, nuclei counterstained with haematoxylin. Alcian blue stains mucus and goblet cells, scale bars 50 μm. Right: quantification of the number of goblet cells in non-induced control organoids and Gata4-negative areas of sgGata4 organoids, counted in at least five organoids from two sgGata4 organoid lines in three independent rounds of Cas9 induction, Mann–Whitney U test. Box plot indicates median (middle line) and 25th, 75th percentile (box) with Tukey whiskers. (D) mRNA expression of Gata4 and Math1 in control and two independent sgGATA6 Ls174T cell clones, n = 3 experiments, two-tailed unpaired t test. Data are presented as mean values ± SEM. (E) mRNA expression of GATA6 and MATH1 in Ls174T cells at 72 h after transfection with GATA6 cDNA (blue bars) compared to control cells (grey bars), n = 5 replicates from four independent experiments, two-tailed unpaired t test. Data are presented as mean values ± SEM. (F) Immunohistochemistry for GATA6 on sections of control and shMll1 Ls174T spheres, scale bar 50 μm, inset 25 μm. (G) mRNA expression of GATA6 in control and shMll1 Ls174T sphere cells, n = 4 independent experiments with three biologically independent samples, two-tailed unpaired t test. Data are presented as mean values ± SEM. (H) CHIP for Mll1 in control (light grey columns) and 6d doxycycline-induced shMll1 Ls174T cells (black columns), binding at the GATA6 promoter, represented as % input, n = 5 replicates from two biologically independent cell clones over four independent experiments, two-tailed unpaired t test, significance calculated for control versus shMll1 and IgG. **P = 0.002, ***P = 0.007. Data are presented as mean values ± SD. (I) CHIP for H3K4me3 and H3K27me3 at the transcriptional start site of GATA6 in control (grey columns) and 1td doxycycline-induced shMll1 Ls174T cells (black columns), represented as % input, n = 5 replicates from two biologically independent cell clones over four independent experiments, two-tailed unpaired t test, significance calculated for control versus shMll1, ***P = 0.007, **P = 0.004. Data are presented as mean values ± SEM. (J) Immunofluorescence for Mll1 (red) and Gata4 (green) on sections of 4-OHT−/−-induced β-catGOF and β-cat−/− organoids, n = 3 independent experiments, two-tailed unpaired t test. Data are presented as mean values ± SEM. (K) mRNA expression of Gata4 and Math1 in β-catGOF and β-cat−/− organoids, n = 3 independent experiments, two-tailed unpaired t test. Data are presented as mean values ± SEM.
TA zone, promoting cell proliferation in Mll1-high TA cells and instructing goblet cell differentiation in Mll1-low progenitor cells. Ablation of Mll1 resulted in cells double-positive for Paneth and goblet cell markers, revealing a role of residual Mll1 in segregating Wnt- and Mapk-instructed Paneth and goblet cell fates. Our data provide evidence for a dual role of Mll1 in progenitor cell maintenance and cell fate determination in the intestinal epithelium.

Cell fate choice in the TA zone is guided by an interplay of various signalling pathways. Notch signalling controls the specification of absorptive versus secretory lineages. Inhibition of Notch signalling up-regulates the expression of Math1 and causes accumulation of secretory cells double-positive for the Paneth and goblet cell markers Mmp7 and Muc2 (van Dussen et al, 2012). The differentiation of the secretory Paneth and goblet cells critically depends on Wnt and Mapk activities, respectively (Heuberger et al, 2014). We observed that simultaneous activation of Wnt/β-catenin and Mek1/Mapk signalling abrogated secretory cell maturation in intestinal organoids and imposed an immature precursor state, as we had previously suggested (Heuberger et al, 2014). Whereas β-catGOF-induced Wnt activation promoted a Paneth cell fate and prevented Mapk-induced goblet cell differentiation, the ablation of Mll1 in β-catGOF and in β-catGOF; Mek1GOF cells led to re-appearance of goblet cells, largely as mixed Paneth–goblet entities. This suggests that Mll1 restricts Mapk signalling and goblet cell specification of secretory progenitor cells. Of note, the loss of Mll1 results in increased goblet cell specification in the wild-type intestine but a global increase in Mapk activity is not visible, which we ascribe to the transient nature and small population of secretory progenitor cells, in which this regulatory circuit is active. Consistent with the role of Wnt signalling for Math1-positive secretory progenitors (Tian et al, 2015), Wnt activation by Wnt3a treatment or β-catGOF expands the secretory progenitor pool and hence allows to detect molecular and signalling effects. In Mll1-competent Mek1GOF crypts, Mapk signalling promoted cell proliferation. The ablation of Mll1 shifted the effect of Mapk signalling from pro-proliferative to induction of goblet cell differentiation. Mek1GOF; Mll1−/− crypts exhibited increased numbers of goblet cells. The presence of Mll1 thus maintains a proliferative progenitor state and restricts goblet cell differentiation. This closely fits our observation of high levels of Mll1 in TA cells, in which active Mapk signalling promotes cell proliferation (Heuberger et al, 2014; Grinat et al, 2020).

The accumulating secretory cells in Mek1GOF; Mll1−/− crypts were double-positive for Paneth and goblet cell markers despite high Mapk activity and absence of Mll1, indicating that Mll1 is essential for preserving the lineage specification of Paneth and goblet cells. Like Mll1, polycomb PRC2 complexes have been implicated in the maintenance of intestinal stem and progenitor cells: their genetic ablation causes loss of stem cells and aberrant secretory differentiation (Chiacchiera et al, 2016; Jadhav et al, 2016). Deletion of PRC2 has been shown to disrupt the intestinal differentiation program and cause accumulation of mixed Paneth/goblet cells in intestinal crypts (Koppens et al, 2016). In the light of our data, this highlights the importance of a proper balance of Mll1 and PcG activities for establishing and maintaining cell identity in Paneth/goblet cell progenitors. Our data suggest that Mll1−/−PRC2—exerts a dual role in intestinal homeostasis: it sustains intestinal progenitor cells and needs to be down-regulated to enable terminal differentiation (Grinat et al, 2020; Goveas et al, 2021), but also plays a role in controlling cell fate specification of secretory Paneth and goblet cells.

The transcription factors Gata4 and Gata6 are required for secretory cell differentiation and lineage maturation in the epithelium of the small intestine and colon (Beuling et al, 2011). Gata4 is expressed throughout the small intestinal epithelium of the jejunum, but is absent in goblet and enteroendocrine cells (Bosse et al, 2006). In line with our data, the ablation of Gata4 promotes maturation of goblet cells (Beuling et al, 2011). However, we did not observe a strong effect of Mll1 and Gata4 depletion on the enteroendocrine cell fate. Mll1−/− organoids showed a slightly reduced expression of the enteroendocrine progenitor marker Neurop3, which may further promote the acquisition of a goblet cell fate (Li et al, 2021), but the number of differentiated enteroendocrine cells was unchanged in Mll1−/− intestine.

A recent study reported a negative regulation of Mapk signalling by Gata4 in the developing stomach epithelium through Gata4-mediated regulation of negative regulators such as Spry2, Dusp4, and Dusp6 (Sankoda et al, 2021). In the intestine, loss of Dusp6, which would correspond to a loss of Mll1/Gata4, results in increased numbers of goblet cells without affecting enteroendocrine cells (Beaudry et al, 2019). This might also happen in the TA cells of the intestinal epithelium, where Mapk activity must be balanced between promoting cell proliferation and inducing goblet cell differentiation. Loss of negative Mapk regulator expression in Gata4-low cells increases Mapk activity and promotes goblet cell differentiation. Our data reveal Mll1 as an epigenetic regulatory hub in the crosstalk of Wnt and Mapk signalling in the context of secretory cell specification. By sustaining the expression of Gata4/6 transcription factors, Mll1 balances opposing Wnt and Mapk activities to suppress goblet cell specification and promote the alternate Paneth cell fate in mixed-lineage secretory progenitors. A similar mechanism has been described in the immune system, where Mll1 regulates Gata3 to specify and maintain memory Th2 cells (Yamashita et al, 2006). In intestinal development, a suppressive function of Mapk signalling on Wnt signalling has been reported (Wei et al, 2020). In lineage specification in the adult intestinal epithelium, Mapk signalling suppresses Wnt-driven Paneth cell specification (Heuberger et al, 2014). Crosstalk of Wnt and Mapk signalling is also frequently observed in cancer, where both pathways regulate each other either negatively or positively dependent on the cancer type (Guardavaccaro & Clevers, 2012). In oncogenic Wnt-activated intestinal cancer cells, the loss of Mll1 promotes the Mapk-dependent goblet cell fate (Grinat et al, 2020). We here show that loss of Mll1 in Mapk-activated cells results in the production of mixed Paneth/goblet cells. Concomitant genetic activation of Wnt and Mek1 results in non-specified cell populations that differentiate upon ablation of Mll1. Thus, Mll1 emerges as a regulatory module of Wnt and Mapk signalling.

Altogether, our study illustrates the interplay of epigenetic and signalling cues in the control of cell fate specification in adult tissues. Our data unravel Mll1 as an epigenetic factor in the regulation of secretory lineage specification in the intestinal epithelium. Mll1 coordinates Wnt and Mapk signalling to sustain progenitor cell proliferation and specify secretory Paneth and goblet cell fates through Gata4/6 transcription factors.
Materials and Methods

Mice

Mice were bred in pathogen-free conditions, and care and use of animals were performed according to the European and national regulations, published in the Official Journal of the European Union L 276/33, 22 September 2010. Transgenic mouse lines used have been previously described: Lgr5-EGFP-IRESCreERT2 (Barker et al, 2007), villin-CreERT2 (el Marjou et al, 2004), Mitfllox (Denissov et al, 2014; Chen et al, 2019), Rosa26-lacZ (Soriano, 1999), β-catGOF (Harada et al, 1999), and Mek1DD (Cowley et al, 1994; Srinivasan et al, 2009). Mutagenesis was induced in 4–6 wk-old mice by intraperitoneal injections of tamoxifen (50 mg/kg; Sigma-Alrich, diluted 1:10 in sunflower oil) on three consecutive days. Mice were analysed at the indicated time points after the last tamoxifen injection. Mice were given i.p. injections of BrdU (Millipore), final concentration 50 μg/g of body weight in PBS at 2 h before euthanasia. Both females and males were analysed.

Isolation of Paneth cells and RNA sequencing

Lgr5-EGFP-IRESCreERT2, Mitfllox/+ and Lgr5-EGFP-IRESCreERT2, Mitfllox/fllox littermates (n = 4) were given tamoxifen via i.p. injections for three consecutive days and were dissected 10 d later. For isolation of Paneth cells, crypts were dissociated into single cells with TrypLE Express (Thermo Fisher Scientific) for 30 min at 37°C. Dissociated cells were passed through 70 μm cell strainer and washed with 5% FBS/PBS. Cells were stained with PE-conjugated anti-CD24 (1:100 dilution, Cat. no. 12-0242-81; eBioscience) antibody, APC-conjugated anti-326 epithelial cell adhesion molecule (EpCAM) antibody (1:50 dilution, 560693; BD) for 45 min on ice. Stained cells were collected by centrifugation and resuspended in SYTOX blue containing 2 μl of nuclease-free H2O with 0.2% Triton-X 100 and 4 μM U0126 (Cat. no. 662005; Calbiochem) treatment for 72 h in EGF-free crypt medium supplemented with Noggin and R-spondin1, 15 μM MI-2 (Cat. no S7618; Selleckchem) treatment for 48 h.

Cell culture

DLD1 and Ls174T human colon cancer cell lines were cultured in 1x DMEM supplemented with 10% FBS and 1% Penicillin/Streptomycin (PenStrep) at 37°C and 5% CO2. Cell line identity was confirmed by Multiplex human Cell line Authentication (Multiplexion). Inducible plnd11-shMLL1 knockdown cell lines were generated as previously described (Meerbrey et al, 2011; Fellmann et al, 2013; Grinat et al, 2020). For GATA6 overexpression, Ls174T cells were transiently transfected with pcDNA3.1-GATA6 (clone OHu27933; GenScript) or pcDNA3.1 empty vector control using Lipofectamine 2000 (Invi- trogen) and harvested for RNA isolation at 72 h after transfection.

CRISPR/Cas9 genome editing

Generation of lentiviral particles

For CRISPR/Cas9-mediated genome editing, the TLCV2 vector system was used (Addgene plasmid #87360, a gift from Adam Karpf). sgRNAs targeting mouse Gata4 (sgGata4-#1: 5’-GTCATCAAAACA-TACTCACGT-3’ and sgGata4-#3: 5’-AGAACATGCTCTGCTGTT-3’; sgGata4-#4_fwd: 5’-GACAGACTGATCTATAATCG-3’; sgGata4-#4_rev: 5’-GACAGACTGATCTATAATCG-3’) or human GATA6 (sgGATA6-#1: 5’-TTTCTAGCCTTCATCACGG-3’) were individually cloned into TLCV2. For production of lentiviral particles, 293TN cells were co-transfected with 10 μg psPAX2, 2.5 μg psMD2.G, and 10 μg TLCV2 containing the respective sgRNA by transfection with PEI (Sigma-Aldrich). Lentiviral particle-containing supernatants were collected at 24 and 48 h post transfection, passed
through a 0.45-μm filter and concentrated with Lenti-X Concentrator (Takara).

### Transduction of intestinal organoids

Intestinal organoids were cultured in ENR medium supplemented with 3 μM CHIR99021 and 500 ng/ml Wnt3a for 96 h before lentiviral transduction. Organoids were dissociated with TrypLE (Gibco) for 2 min in a 37°C water bath, washed once with 0.1% BSA/PBS, resuspended in ENR medium supplemented with 500 ng/ml Wnt3a, 10 μM Y27632 (#A9165; Sigma-Aldrich), and Transducex (#631231; System Bioscience) and mixed 1:1 with the lentiviral particles (1:1 mix of TLCV2-sgGata4-#1 and -#3 and TLCV2-sgGata4-#4fwd and -#4rev, respectively). Spinoculation was performed in PolyHEMA-coated 48-well plates at 1,000 g for 1 h at RT, followed by overnight incubation at 37°C. Transduced cells were then washed with 0.1% BSA/PBS, seeded in Matrigel, and cultured in ENR medium supplemented with 500 ng/ml Wnt3a and 10 μM Y27632 for 2 d. The medium was changed to basic ENR crypt medium and transduced organoids were selected with 1 μg/ml puromycin for 3 d starting from day 4 after transduction. Cas9 activity in stable organoids was induced with 600 ng/ml doxycycline (D5897; LKT Laboratories) for 3 d. Gata4 knockout organoids were analysed 5 d after mutagenesis.

### Transduction of Ls174T cells

For lentiviral transduction, lentiviral particles (TLCV2-sgGATA6#1 or TLCV2-sgGATA6#2) were mixed 1:1 with fresh growth medium containing 8 μg/ml polybrene. Ls174T cells were spinoculated at 300 g for 1 h at RT, followed by overnight incubation at 37°C. Transduced cells were selected with 0.1% BSA/PBS, seeded in Matrigel, and cultured in ENR medium supplemented with 500 ng/ml Wnt3a and 10 μM Y27632 for 2 d. The medium was changed to basic ENR crypt medium and transduced organoids were selected with 1 μg/ml puromycin for 3 d starting from day 4 after transduction. Cas9 activity in stable organoids was induced with 600 ng/ml doxycycline (D5897; LKT Laboratories) for 3 d. Induced cells were sorted by FACS for GFP, which is co-expressed with Cas9 from TLCV2 and cultured as single cell clones to establish Ls174T sgGATA6 cell lines. One representative cell clone per sgRNA was selected for further analysis and compared with non-induced parental control.

### Histology, immunofluorescence staining, and analysis

Murine tissue was fixed in 4% formaldehyde/PBS, and histological analyses were performed on 5–7-μm paraffin sections. Organoids were fixed in 4% formaldehyde/PBS for 1 h and embedded in 15% agarose/PBS. For immunostaining on paraffin sections, antigen retrieval was carried out by boiling in 10 mM sodium citrate, pH 6.0, or 1 mM EDTA 20 mM Tris, pH 8.5. For IHC, sections were incubated in 4% H2O2/PBS for 5 min before 1-h incubation with blocking solution (0.1% Tween 20, 5–10% horse serum, 1% BSA in PBS) followed by incubation of the primary antibody diluted in blocking solution overnight at 4°C. For Mll1 staining, antigen retrieval was performed by boiling in 10 mM sodium citrate, pH 6.0, for 15 min, and sections were permeabilized in ice-cold methanol for 10 min before incubation in blocking solution. Fluorochrome-conjugated or HRP-coupled secondary antibodies and DAPI were incubated in blocking solution for 1–2 h at RT. IHC was developed with the DAB chromogenic substrate (DAKO), dehydrated and mounted with non-aqueous mounting medium (Entellan). Fluorochrome-conjugated or HRP-coupled secondary antibodies were incubated for 1 h at RT. IHC was developed with the DAB chromogenic substrate (DAKO). For H&E staining, tissue sections were incubated with haematoxylin solution for 1 min and stained with eosin for 5 min before dehydration and mounting with non-aqueous mounting medium. Alcian blue solution (pH 2.5 in 3% acetic acid) was incubated for 30 min, and sections were counterstained with nuclear fast red for 5 min or haematoxylin for 30 s. PAS staining was performed using the PAS staining kit (#101646; Millipore).

### In situ hybridization

5-μm-thick intestinal paraffin sections were dehydrated and treated with 0.2 N hydrochloric acid and proteinase K. Slides were then post-fixed with 4% formaldehyde/PBS, demethylated with acetic anhydride, and prehybridized. Hybridization with RNA probes was performed in a humid chamber with 1 μg/ml digoxigenin-labelled Axin2 RNA probe for 24 h at 63°C (Klaus et al., 2007). Slides were washed, blocked, and incubated with anti-digoxigenin-alkaline phosphatase conjugate overnight at 4°C. The stainings were washed with BM purple and counterstained with Pyronin G.

### Light microscopy and data analysis

Representative z-stacks were acquired with inverted laser scanning microscopes LSM710 and LSM700 using 405, 488, 561, and 633-nm lasers and a PlanApochromat 60× NA 1.3 objective (Zeiss) or a spinning disc confocal microscope CSU-W1 (Nikon/Andor) equipped with an iXON888 camera, using PlanApo 20× NA 0.75 and Apo LWD 40× NA 1.15 objectives. Maximal intensity projections of z-stacks were performed with ImageJ.

### ChIP

ChIP of histone modifications was performed from plnd11-shMll1 Ls174T cell lines induced with 300 ng/ml doxycycline for 11 d, following the instructions of the iDEAL ChIP-seq kit for histones (Diagenode), as described in Grinat et al (2020). For ChIP of Mll1, plnd11-shMll1 Ls174T and DL1 cell lines were induced with 300 ng/ml doxycycline for 6 d and chromatin was prepared using the ChIP-IT Express kit (Active Motif). Cells were grown to 80% confluency, trypsinized for 3 min at 37°C, fixed in 1% formaldehyde for 10 min, and quenched in glycerol for 5 min at RT. Chromatin was sheared with a Branson Sonifier 450 (3 min shearing time, duty cycle 60, output control 6, sonified 10× for histone ChIPs, 4× for Mll1 ChIPs, and 1 min pause between each sonication round). Shearing efficiency was checked on a 1% agarose gel. 10 μg of sheared chromatin were used for Mll1 ChIPs. ChIP-qPCR analysis was performed in a total volume of 20 μl SYBR green reaction mix (Roche Diagnostics) containing 0.25 μM of forward and reverse primers each in a CFX96-C1000T thermal cycler (Bio-Rad): 2 min at 50°C and 2 min at 95°C followed by 42 cycles of 15 s at 95°C and 1 min at 60°C. Ct values of precipitated DNA were calculated relative to input DNA (% input). ChIP-qPCR primers were designed using H3K4 methylation profiles available in the UCSC genome browser (human reference genome GRCh37/hg19) and the Mll1 ChIP-seq UCSC genome browser dataset from Active
Motif (https://www.activemotif.com/catalog/details/61295/mll-hxr-antibody-pab). Primer sequences used for ChIP-qPCR are given in Table S1.

Western blotting

Organoids were harvested and washed once in ice-cold 0.1% BSA-PBS, and cells were washed twice in PBS before lysis in ice-cold RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.1% SDS, 1% NP40, and 0.5% sodium deoxycholate) containing protease inhibitors (Complete Mini EDTA-free; Roche) and phosphatase inhibitor cocktails 2 and 3 (Sigma-Aldrich). Total cell extracts were separated on polyacrylamide gels and transferred to a nitrocellulose membrane via semidy transfer for 1 h 15 min at 90 mA. Membranes were blocked with 5% BSA or 5% skim milk in 0.1% Tween 20/TBS and probed with primary antibody diluted in blocking solution overnight at 4°C. HRP-conjugated secondary antibodies were incubated for 1 h at RT. Immunoblots were developed with Western Lightning Plus ECL (PerkinElmer) for 3 min and imaged with a Vilber Lourmat imaging system FUSION SL-3.

Antibodies

The following antibodies were used in this study (dilutions given for immunostaining): anti-Mll1 (D6G8N, #14197; Cell Signaling Technology, 1:100; 150 for ChIP), anti-E-cadherin (610181; BD, 1:200), anti-ChroA (Abcam, 1,300), anti-Mmp7 (Santa Cruz Biotechnology, 1:100), anti-GFP (ab6673; Abcam, 1:500), anti-Ki67 (MA5-14520; Thermo Fisher Scientific, 1:300), anti-BrdU (ab6326; Abcam, 1:100), anti-cleaved Caspase-3 (#9661; Cell Signaling Technology, RRID:AB_2341188, 1:400), anti-β-catenin (610153; BD, 1:300 for IHC, 1:10,000 for Western blot), anti-Lyz (A0099; DAKO, 1:500), anti-ITF (sc-18272; Santa Cruz Biotechnology, 1:300), anti-phospho-Erk1/2 (#4370; Cell Signaling Technology, RRID:AB_2315112, 1:200 for IHC), anti-phospho-Erk1/2 (M8159; Sigma-Aldrich, RRID:AB_572245, 13,000 for Western Blot), anti-Erk1/2 (9102; Cell Signaling Technology, RRID:AB_23074/4, 1:1,000 for Western Blot), anti-phospho-Mek1/2 (9154; Cell Signaling Technology, RRID:AB_2138017, 1:1,000 for Western Blot), anti-Mek1/2 (4694; Cell Signaling Technology, RRID:AB_10695868, 1:1,000 for Western Blot), anti-α tubulin (Sigma-Aldrich, 1:10,000 for Western Blot), anti-GATA4 (sc-25310; Santa Cruz Biotechnology, RRID:AB_627667, 1:200), anti-H3K4me3 (#9727; Cell Signaling Technology, RRID:AB_561095, 1:50 for ChIP), anti-H3K27me3 (#07-449; Millipore, RRID:AB_310624, 5 μg for ChIP), and rabbit monoclonal IgG control (#3900; Cell Signaling Technology, RRID:AB_1550038). For immunofluorescence and IHC, cyanine-labelled secondary antibodies (Jackson Immunoresearch) and HRP-conjugated polymer and DAB reagent (DAKO) were used.

RNA preparation for RT–PCR analysis

Total RNA from organoids and snap-frozen tissue was isolated by Trizol extraction (Invitrogen) or with the NucleoSpin RNA isolation kit (Macherey-Nagel). DNA contaminations were removed by DNaseI digestion (Invitrogen) in the presence of RNase inhibitor (RNase Out; Invitrogen), and RNA was purified via phenol/chloroform extraction. For qRT-PCR, up to 5 μg of total RNA were reverse-transcribed with random hexamer primers (Invitrogen) and MMLV reverse transcriptase (200 U/μl; Promega). qRT-PCR was performed in a total volume of 20-μl SYBR green reaction mix (Roche Diagnostics) containing 0.25 μM of forward and reverse primers each in a CFX96-C1000T thermal cycler (Bio-Rad): 2 min at 50°C and 2 min at 95°C followed by 42 cycles of 15 s at 95°C and 1 min at 60°C. All reactions were performed as duplicates. Expression of target genes in treated versus control samples relative to the endogenous reference GAPDH was calculated using the ΔΔCt method. Primer sequences used for qRT-PCR are listed in Table S2.

Quantification and statistical analysis

All data are presented as mean ± SEM unless otherwise indicated. Statistical details of the experiments can be found in the figure legends. Graphs and statistics were generated with GraphPad Prism software. Tests for normal distribution were performed with D’Agostino-Pearson and Shapiro-Wilk tests. Significance (P-values) was determined with Mann–Whitney U test (two-tailed), two-tailed t-test or ordinary one-way ANOVA. No statistical method was used to estimate sample size, and no specific randomization or blinding protocol was used. N indicates the numbers of independent biological replicates per experiment unless otherwise indicated. P-values ≤ 0.05 were considered statistically significant (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001).

Data Availability

The dataset produced in this study is available in the following database: RNA seq data: Gene Expression Omnibus GSE177047.

Supplementary Information

Supplementary Information is available at https://doi.org/10.26508/lsa.202101187.

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Author Contributions

J. Grinat: formal analysis, validation, investigation, visualization, methodology, and writing—original draft, review, and editing. F. Kosel: investigation and methodology. N. Goveas: conducted the Paneth cell sort.
A Kranz: conducted the Paneth cell sort.
D Alexopoulou: performed bioinformatic analyses.
K Rajewsky: resources.
M Sigal: resources.
AF Stewart: resources.
J Heuberger: conceptualization, formal analysis, supervision, validation, investigation, visualization, methodology, project administration, and writing—original draft, review, and editing.

Conflict of Interest Statement
The authors declare that they have no conflict of interest.

References

Andreu P, Colnot S, Godard C, Gad S, Chafey P, Niwa-Kawakita M, Laurent-Puig P, Kahn A, Robine S, Perret C, et al (2005) Crypt-restricted proliferation and commitment to the Paneth cell lineage following Apc loss in the mouse intestine. Development 132: 1443–1451. doi:10.1242/dev.01700

Barker N, van Es JH, Kuipers J, Kujala P, van den Born M, Cozijnsen M, Beaudry K, Langlois MJ, Montagne A, Cagnol S, Carrier JC, Rivard N (2019) Dual-synthesis and differentiation of intestinal stem cells in small intestine and colon by marker gene Lgr5. Nature 564: 274–278. doi:10.1038/s41586-019-0168-5

Bosse T, Piascecky CM, Burghard F, Fialkovich JJ, Rajagopala S, Pu WT, Krasinski SD (2006) Gata4 is essential for the maintenance of jejunal-ileal identities in the adult mouse small intestine. Mol Cell Biol 26: 9060–9070. doi:10.1128/MCB.00124-06

Brand M, Nakka K, Zhu J, Dilworth FJ (2019) Polycylic/trithorax antagonism: Cellular memory in stem cell fate and function. Cell Stem Cell 24: 518–533. doi:10.1016/j.stem.2019.03.005

Cowley S, Paterson H, Kemp P, Marshall CJ (1994) Activation of MAP kinase is necessary and sufficient for PC12 differentiation and for transformation of NIH 3T3 cells. Cell 77: 841–852. doi:10.1016/0092-8674(94)90133-3

Crosnier C, Stamatakis D, Lewis J (2006) Organizing cell renewal in the intestine: Stem cells, signals and combinatorial control. Nat Rev Genet 7: 349–359. doi:10.1038/nrg1840

Denissov S, Hofmeester H, Marks H, Kranz A, Cotta G, Singh S, Anastassiadi K, Stunnenberg HG, Stewart AF (2014) MLL2 is required for H3K4 trimethylation on bivalent promoters in embryonic stem cells, whereas MLL1 is redundant. Development 141: 526–537. doi:10.1242/dev.106831

El-Marjou F, Janssens KP, Chang BH, Li M, Hindle V, Chan L, Louvard D, Chambon P, Metzger D, Robine S (2004) Tissue-specific and inducible Cre-mediated recombination in the gut epithelium. Genesis 39: 186–193. doi:10.1002/gene.200422

Fellmann C, Hoffmann T, Snidhar V, Hofgartner B, Murah M, Roth M, Lai DY, Barbosa IA, Kron S, Guan Y, et al (2013) An optimized microRNA backbone for effective single-copy RNAi. Cell Rep 5: 1704–1713. doi:10.1016/j.celrep.2013.11.020

Feve T, Robine S, Louvard D, Huelsken J (2007) Wnt/beta-catenin is essential for intestinal homeostasis and maintenance of intestinal stem cells. Mol Cell Biol 27: 7551–7559. doi:10.1128/MCB.01034-07

Fre S, Huyghe M, Mourikis P, Robine S, Louvard D, Artavanis-Tsakonas S (2005) Notch signals control the fate of immature progenitor cells in the intestine. Nature 435: 964–968. doi:10.1038/nature03589

Goveas N, Waskow C, Arndt K, Heuberger J, Zhang Q, Alexopoulou D, Dahl A, Bircher M, Anastassiadi K, Stewart AF, et al (2021) MLL1 is required for maintenance of intestinal stem cells. PLoS Genet 17: e1009250. doi:10.1371/journal.pgen.1009250

Gregorieff A, Stange DE, Kujala P, Begthel H, van den Born M, Kranz J, Stewart AF, et al (2009) The Wnt-signalling domain factor Spdef promotes maturation of goblet and paneth cells in the intestinal epithelium. Gastroenterology 137: 1333–1345. doi:10.1053/j.gastro.2009.06.044

Grembecka J, He S, Shi A, Purohit T, Muntean AG, Sorenson RJ, Showalter HD, Murai MJ, Belcher AM, Hartley T, et al (2012) Menin-MLL inhibitors reverse oncogenic activity of MLL fusion proteins in leukemia. Nat Chem Biol 8: 277–284. doi:10.1038/nchembio.773

Grinat J, Heuberger J, Vidal RO, Goveas N, Kranz A, Berenguer-Llengoa Kranz A, Wulf-Goldenberg A, Behrens D, Melcher B, et al (2020) The epigenetic regulator MLL1 is required for Wnt-driven intestinal tumorigenesis and cancer stemness. Nat Commun 11: 6422. doi:10.1038/s41467-020-20222-z

Guadavacarco D, Clevers H (2012) Wnt/beta-catenin and MAPK signaling: Allies and enemies in different battlefields.Wnt/beta-catenin and MAPK signaling: allies and. Sci Signal 5: pe15. doi:10.1126/scisignal.2002921

Haber AL, Biton M, Rogel N, Herbst RH, Shekhar K, Smillie C, Burgin G, Delorey TM, Howitt MR, Katz Y, et al (2017) A single-cell survey of the small intestinal epithelium. Nature 551: 333–339. doi:10.1038/nature24849

Harada N, Tamai Y, Ishikawa T, Sauer B, Takaku K, Oshima M, Taketo MM (1999) The intestine is regulated by homeobox gene CDX1. Proc Natl Acad Sci U S A 96: 18.21.5931

Heuberger J, Grinat J, Kosel F, Rajewsky K, Birchmeier W (2014) Epigenetic control of secretory cell fates. Grinat et al. https://doi.org/10.26508/lsa.202101187 vol 5 | no 4 | e20210187

Heuberger J, Grinat J, Kosel F, Liu L, Kunz S, Guan Y, et al (2013) An optimized microRNA backbone for effective single-copy RNAi. Cell Rep 5: 1704–1713. doi:10.1016/j.celrep.2013.11.020

Heuberger J, Grinat J, Kosel F, Liu L, Kunz S, Vidal RO, Keil M, Haybaeck J, Robine S, Louvard D, Chambon P, Metzger D, Robine S (2004) Tissue-specific and inducible Cre-mediated recombination in the gut epithelium. Genesis 39: 186–193. doi:10.1002/gene.200422

Cowley S, Paterson H, Kemp P, Marshall CJ (1994) Activation of MAP kinase is necessary and sufficient for PC12 differentiation and for transformation of NIH 3T3 cells. Cell 77: 841–852. doi:10.1016/0092-8674(94)90133-3

Crosnier C, Stamatakis D, Lewis J (2006) Organizing cell renewal in the intestine: Stem cells, signals and combinatorial control. Nat Rev Genet 7: 349–359. doi:10.1038/nrg1840
Katz JP, Perreault N, Goldstein BG, Lee CS, Labosky PA, Yang VW, Kaestner KH (2002) The zinc-finger transcription factor Klf4 is required for terminal differentiation of goblet cell lineages in the colon. Development 129: 2619–2628. doi:10.1242/dev.129.11.2619

Klaus A, Saga Y, Taketo MM, Tzahor E, Birchmeier W (2007) Distinct roles of Wnt/beta-catenin and BMP signaling during early cardiogenesis. Proc Natl Acad Sci U S A 104: 18531–18536. doi:10.1073/pnas.0703113104

Kohlnhofer BM, Thompson CA, Walker EM, Battle MA (2016) GATA4 regulates epithelial cell proliferation to control intestinal growth and development in mice. Cell Mol Gastroenterol Hepatol 2: 189–209. doi:10.1016/j.jcmgh.2015.11.010

Koppens MA, Bounova G, Gargiulo G, Tanger E, Janssen H, Cornelissen-Steijger P, Sansom OJ, Reed KR, Hayes AJ, Ireland H, Brinkmann H, Newton IP, Batlle E, Sankoda N, Tanabe W, Tanaka A, Shibata H, Woltjen K, Chiba T, Haga H, Sakai Y, Piunti A, Shilatifard A (2016) Epigenetic balance of gene expression by Polycomb and COMPASS families. Cell 165: 1098–1113. doi:10.1016/j.cell.2016.06.020

Li HJ, Ray SK, Kucukural A, Gradwohl G, Leiter AB (2021) Reduced Neurog3 gene dosage shifts enteroendocrine progenitor towards goblet cell lineage in the mouse intestine. Cell Mol Gastroenterol Hepatol 11: 433–448. doi:10.1016/j.jcmgh.2020.08.006

Leverkoehne I, Gruber AD (2002) The murine mCLCA3 (alias gob-5) protein is located in the mucin granule membranes of intestinal, respiratory, and uterine goblet cells. J Histochem Cytochem 50: 829–838. doi:10.1177/002215540205000609

Li Hy, Ray SK, Kucukural A, Gradwohl G, Leiter AB (2021) Reduced Neurog3 gene dosage shifts enteroendocrine progenitor towards goblet cell lineage in the mouse intestine. Cell Mol Gastroenterol Hepatol 11: 433–448. doi:10.1016/j.jcmgh.2020.08.006

Lustig B, Jerchow B, Sachs M, Weiler S, Pietsch T, Karsten U, van de Wetering M, Lustig B, Jerchow B, Sachs M, Weiler S, Pietsch T, Karsten U, van de Wetering M, Blom M, Song JY, Wessels LF, van Lohuizen M (2016) Deletion of polycomb repressive complex 2 from mouse intestine causes loss of stem cells. Cell Mol Gastroenterol Hepatol 21: 1184–1193. doi:10.1016/jcmgh.2016.06.020

Masunova L, Sasaki Y, Calado DP, Zhang B, Paik JH, DePinho RA, Kukolj Z, Keary JF, Otipoby KL, Rajewsky K (2009) PI3 kinase signals BCR-dependent mature B cell survival. Cell 139: 573–586. doi:10.1016/j.cell.2009.08.041

Tian H, Biene B, Chiu C, Siebel CW, Wu Y, Costa M, de Sauvage FJ, Klein OD (2015) Opposing activities of Notch and Wnt signaling regulate intestinal stem cells and gut homeostasis. Cell Rep 11: 33–42. doi:10.1016/j.celrep.2015.03.007

van der Flier LG, Sabatier-Bellver J, Oving I, Haegebarth A, de Palo M, Anti M, van Gijn ME, Suijkerbuijk S, van der Wetering M, Marr A, et al (2007) The intestinal Wnt/TCF signature. Gastroenterology 132: 628–632. doi:10.1053/j.gastro.2006.08.039

van der Sluis M, de Koning BA, de Bruijn AC, Velchic A, Meijerink JP, van Gouwdeeb J, Bittler HA, Dekker J, van Seuningen I, Renes IB, et al (2006) Muc2-deficient mice spontaneously develop colitis, indicating that MUC2 is critical for colonic protection. Gastroenterology 131: 117–129. doi:10.1053/gastro.2006.04.020

van de Wetering M, Sancho E, Verweij C, de Lau W, Oving I, Hurstone A, van der Horst K, Battle E, Coudreuse D, Hararis AP, et al (2002) The beta-catenin/TCF-4 complex imposes a crypt progenitor phenotype on colorectal cancer cells. Cell 111: 241–250. doi:10.1016/s0018-6919(02)00757-3

van Dussen KL, Carulli AJ, Keely TM, Patel SR, Puthoff BJ, Magness ST, Tran IT, Maillard I, Siebel C, Kosterud A, et al (2012) Notch signaling modulates proliferation and differentiation of intestinal crypt base columnar stem cells. Development 139: 488–497. doi:10.1242/dev.070763

van Es JH, Jay P, Gregoireff A, van Gijn ME, Jonkheer S, Hatzis P, Thiele A, van den Born M, Begthel H, Brambilla MT, et al. (2005) Wnt signalling induces maturation of Paneth cells in intestinal crypts. Nat Cell Biol 7: 381–386. doi:10.1038/ncb1240

Wei G, Gao N, Chen J, Fan L, Zeng Z, Gao G, Li L, Fang G, Hu K, Pang X, et al. (2020) Erk and MAPK signaling is essential for intestinal development through Wnt pathway modulation. Development 147: doi:10.1242/dev.185678

Whessell G, Montagni E, Martinelli P, Hernando-Mombloña X, Sveillano M, Jung P, Cortina C, Calon A, Abuli A, Castells A, et al. (2014) The transcription factor GATA6 enables self-renewal of colon adenoma stem cells by repressing BMP gene expression. Nat Cell Biol 16: 695–707. doi:10.1038/ncb2992

Wiedemann B, Waltherr B, Ruhr H, Hille A, Rosa P, Huttner WB (1988) Identification of gastroenteropancreatic neuroendocrine cells in normal and neoplastic human tissue with antibodies against synaptophysin, chromogranin A, secretogranin I (chromogranin B), and secretogranin II. Gastroenterology 93: 1364–1374. doi:10.1016/0016-5085(88)90374-5

Yamashita M, Hirahara K, Shinnakasu R, Hosokawa H, Norikane S, Kimura MY, Hasegawa A, Nakayama T (2006) Crucial role of MLL for the maintenance of memory T helper type 2 cell responses. Immunity 24: 611–622. doi:10.1016/j.immuni.2006.03.017

Yang Q, Bermingham NA, Finegold MJ, Zoghbi HY (2001) Requirement of Math1 for secretory cell lineage commitment in the mouse intestine. Science 294: 2155–2158. doi:10.1126/science.1065718