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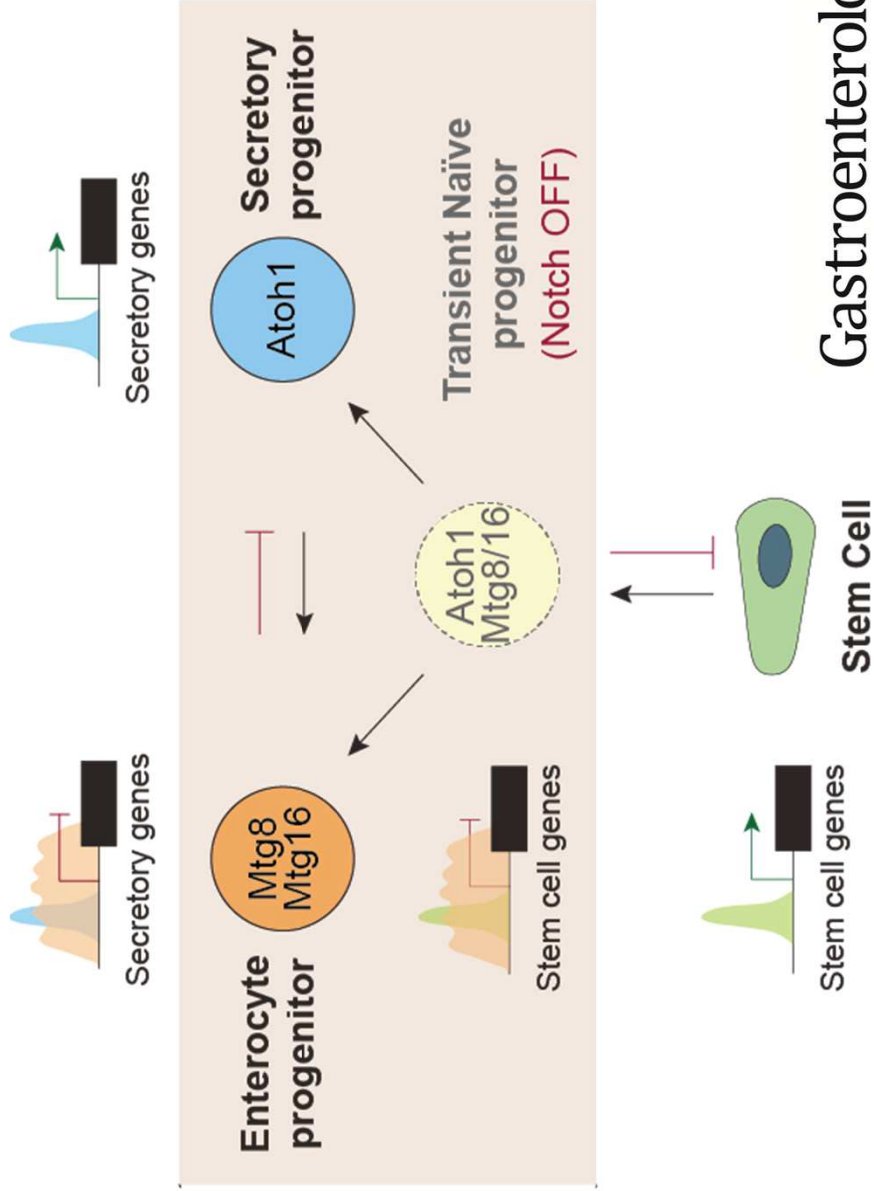
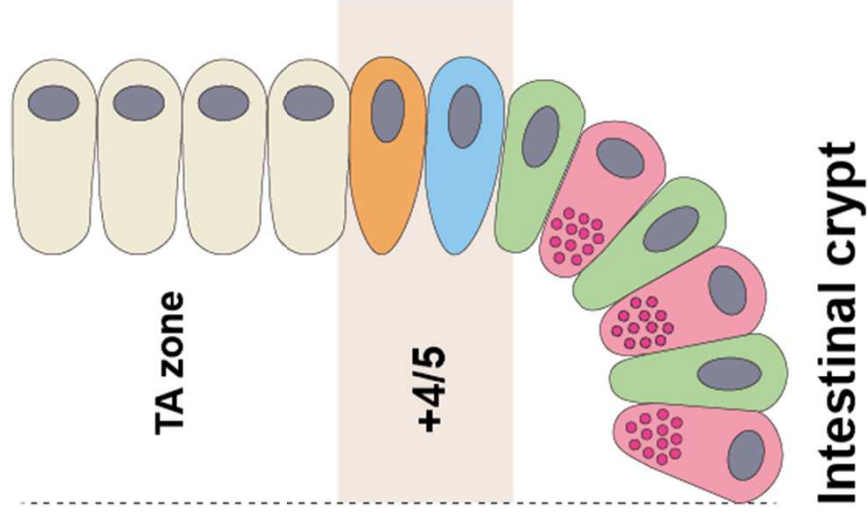
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Gastroenterology

The Transcription co-Repressors MTG8 and MTG16 Regulate Exit of Intestinal Stem Cells From Their Niche and Differentiation into Enterocyte vs Secretory Lineages

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Author contributions

V.S.W.L. conceived the project. V.S.W.L., A.B. and N.A. designed the experiments and analyzed data. A.B., N.A., T.D., V.F., L.N. and A.K. performed the experiments. A.B., N.A. and T.D. performed ChIP-seq and RNA-seq experiments. A.B., N.A. and H.P. analyzed the ChIP-seq and RNA-seq data. P.D.C collected human intestinal tissues and provided scientific advice. V.S.W.L and A.B. wrote the manuscript.

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Abstract

BACKGROUND & AIMS: Notch signaling maintains intestinal stem cells (ISCs). When ISCs exit the niche, Notch signaling among early progenitor cells at position +4/5 regulates their specification toward secretory vs enterocyte lineages (binary fate). The transcription factor ATOH1 is repressed by Notch in ISCs; its de-repression, when Notch is inactivated, drives progenitor cells to differentiate along the secretory lineage. However, it is not clear what promotes transition of ISCs to progenitors and how this fate decision is established.

METHODS: We sorted cells from Lgr5-Gfp knock-in intestines from mice and characterized gene expression patterns. We analyzed Notch regulation by examining expression profiles (by quantitative reverse transcription PCR and RNAscope) of small intestinal organoids incubated with the Notch inhibitor DAPT, intestine tissues from mice given injections of the γ -secretase inhibitor dibenzazepine, and mice with intestine-specific disruption of Rbpj. We analyzed intestine tissues from mice with disruption of the RUNX1 translocation partner 1 gene (Runx1t1, also called Mtg8) or CBFA2/RUNX1 partner transcriptional co-repressor 3 (Cbfa2t3, also called Mtg16), and derived their organoids, by histology, immunohistochemistry, and RNA sequencing. We performed chromatin immunoprecipitation and sequencing analyses of intestinal crypts to identify genes regulated by MTG16. **RESULTS:** The transcription co-repressors MTG8 and MTG16 were highly expressed by +4/5 early progenitors, compared with other cells along crypt–villus axis. Expression of MTG8 and MTG16 were repressed by Notch signaling via ATOH1 in organoids and intestine tissues from mice. MTG8- and MTG16-knockout intestines had increased crypt hyperproliferation and expansion of ISCs, but enterocyte differentiation was impaired, based on loss of enterocyte markers and functions. Chromatin immunoprecipitation and sequencing analyses showed that MTG16 bound to promoters of genes that are specifically expressed by stem cells (such as Lgr5 and Ascl2) and repressed their

transcription. MTG16 also bound to previously reported enhancer regions of genes regulated by ATOH1, including genes that encode delta-like canonical Notch ligand and other secretory-specific transcription factors. **CONCLUSIONS:** In intestine tissues of mice and human intestinal organoids, MTG8 and MTG16 repress transcription in the earliest progenitor cells to promote exit of ISCs from their niche (niche exit) and control the binary fate decision (secretory vs enterocyte lineage) by repressing genes regulated by ATOH1.

KEY WORDS: niche exit, lineage specification, chromatin remodeling, lateral inhibition

Introduction

The intestinal epithelium renews every five days, a process that is driven by the ISCs located at the crypt base. ISCs divide and give rise to early progenitor populations at the +4/5 cell position, where lineage specifications take place (Extended Data Figure 1a)^{1,2}. Notch signaling plays a key role in lineage commitment and plasticity. Activation of Notch drives enterocyte differentiation, while Notch inactivation de-represses the transcription factor ATOH1, a master regulator of all secretory lineages: Paneth, goblet and enteroendocrine cells³⁻⁶. This binary fate decision is believed to be driven by the emerging expression of the Notch ligand Delta-like (Dll) family on early secretory progenitors, which activates Notch in surrounding progenitor cells. This instructs these 'naïve' neighbors to take the opposite (enterocyte) fate. This process is termed 'lateral inhibition' and is proposed to be under ATOH1 regulation⁷⁻¹⁰. Dll1+ secretory progenitors exert plasticity, i.e. they can revert to stem cells upon stem cell loss¹¹. Although the signaling pathways regulating ISC fate are well-defined, the underlying mechanism of how stem cells commit to differentiation and undergo the subsequent binary fate decision remains largely uncharacterized. Importantly, transcriptional control and molecular markers of enterocyte progenitors remain largely undefined. Very recent studies propose that chromatin accessibility plays a crucial role in fate decisions and plasticity at the early progenitor stage^{8,12,13}.

To delineate the early stem cell-daughter cell transition, we studied transcriptional control directly at the earliest progenitor cell at the +4/5 position upon niche exit. In this study, we identified two transcriptional co-repressor homologues, MTG8 and MTG16, that are expressed in these early progenitors. MTG8 and MTG16 were repressed by Notch signaling both in *ex vivo* organoids and *in vivo*. We further showed that the two co-repressors play central roles in early fate decision of ISCs by repressing the stem cell gene expression program and *Dll* expression for lateral inhibition. Previous studies have demonstrated that

MTG8 and MTG16 recruit chromatin-modifying enzymes for transcriptional regulation¹⁴. Together, our findings indicate a critical role for MTG8 and MTG16 in niche exit and in early fate decision of ISCs by regulating chromatin accessibility of the target genes.

Materials and Methods

Please refer to the online Supplementary Materials for detailed additional Methods.

Animals and drug administration

All animal regulated procedures were carried out according to Project License constraints (PEF3478B3) and Home Office guidelines and regulations. *Lgr5-EGFP-IRES-CreERT2*¹⁵ mice were used for FACS sorting experiments. *Rbpj*^{fl/fl} mice¹⁶ were crossed with *VillinCreER*¹⁷ mice for inducible intestinal-specific deletion. *Mtg16*^{-/-}, *Mtg8*^{-/-} and *Mtgr1*^{-/-} mice were kind gift from Scott W. Hiebert. *Lgr5DTR-EGFP* mice (kind gift from Genentech, hereafter named as *Lgr5-GFP* mice since only the GFP reporter element was used in this study) were crossed with *Mtg16*^{-/-} mice to generate *Mtg16*^{-/-}; *Lgr5-GFP* animals. *VillinCreER*; *Rbpj*^{fl/fl} or *VillinCreER* animals were injected with tamoxifen (Acros-Organics 416545000) intraperitoneally at 1.6 mg per 10g of mice and collected at the indicated time points. For proliferation analysis, animals were injected intraperitoneally with 30mg/kg EdU 2h before tissue collection. Edu treatment in newborn pups was performed the same as in adults except that the pups were culled 20 min after injection. Dibenazepine (DBZ, Tocris, 4489) was administered to wild-type (WT) animals as described previously⁸. Briefly, mice were injected intraperitoneally twice the same day, 6h apart, with a dose of 100umol/kg DBZ suspended in 0.5% (w/v) hydroxypropylmethyl-cellulose (Methocel E4M, Sigma-Aldrich, 94378) and 0.1% (w/v) Tween 80 (Sigma-Aldrich, P1754) in water or only the vehicle as a control. Mice were collected at the indicated time points after the first injection.

Statistical analysis

Results are expressed as mean \pm standard error of the mean (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). Statistical significance of mean values was assessed using unpaired Student t-test or analysis of variance, 1- or 2-way, followed by Tukey's or Sidak's Multiple Comparison Post-test respectively. The corresponding number of N and experiments are indicated in the figure legends. Statistics were performed using GraphPad Prism 7 software (La Jolla, CA).

Results

Expression of *Mtg8* and *Mtg16* in a sub-population of +4/5 cells of intestinal crypt

To investigate the transcriptional regulation of stem cell fate and early lineage commitment at the +4/5 progenitor stage, we analyzed the expression profile of sorted LGR5-GFP cells (GSE36497)¹⁸. Rather than focusing on the GFP-high (LGR5-GFP 5+) stem cell population, we examined the GFP-low (LGR5-GFP 2+, 3+ and 4+) fractions that represent immediate daughter cells. This allowed us to identify +4/5 cell-enriched genes in the absence of a specific molecular marker. Hierarchical clustering analysis revealed 525 genes that were enriched in GFP-low populations (Supplementary Figure 1B and Supplementary Table 1). These included *Atoh1*, *Dll1* and *Dll4* that have previously been reported to be expressed in the early secretory progenitors^{8,11,19}. Among the 525 genes, we further screened for transcription factors that were enriched in LGR5-GFP-low and absent in LGR5-GFP-high stem cell populations. This resulted in the identification of the two related transcriptional regulators *Mtg8* and *Mtg16*.

MTG8, MTG16 and MTGR1 (also known as RUNX1T1, CBFA2T3 and CBFA2T2 respectively) are transcriptional corepressors that comprise the myeloid translocating gene (MTG) family^{14,20}. Quantitative reverse transcription (RT)-PCR confirmed the enriched

expression of *Mtg8* and *Mtg16* in LGR5-GFP-low early progenitors in the crypts, similar to *Atoh1* (Figure 1A and Supplementary Figure 1C). RNAscope analysis further demonstrated enriched expression of *Mtg8* and *Mtg16* at +4/5 cell positions directly above the LGR5+ ISC compartment (Figure 1B-D), while *Mtgr1* was expressed throughout the crypt (Figure 1A, B). Quantification of the RNAscope signal confirmed that majority of *Mtg8*+ cells were present at positions 4 and 5 (31.5% and 25.3% respectively), while *Mtg16*+ cells were distributed throughout the lower crypt with a peak frequency at positions 4 and 5 (24.7% and 12.6% respectively) (Figure 1C). RNAscope co-staining further revealed that expression of *Mtg16* was mostly exclusive with *Lgr5* but co-localized with *Atoh1* at the crypt bottom, indicating that *Mtg16* is also expressed by Paneth cells (Figure 1D). It is worth noting that the RNAscope signal of *Mtg16* was significantly stronger at positions 4 and 5 than in Paneth cells (Figure 1F, Supplementary Figure 1D), indicating that *Mtg16* is indeed enriched in early crypt progenitors. Expression of *Mtg8* was mostly exclusive from *Atoh1* (Figure 1E), while *Mtg16* was expressed in both *Atoh1*+ and *Atoh1*- populations in the early progenitors in roughly equal proportions (Figure 1D, G). *Mtg8* was also found to co-localize with *Mtg16* (Supplementary Figure 1E). In addition, co-staining of *Mtg16* and Muc2 showed that *Mtg16* was also expressed in a small subset of goblet cells that were mainly localized at the crypt-villus junction (red arrows in Supplementary Figure 1F). We also observed stromal expression of *Mtg16* in the intestine (Figure 1B, white arrows in Supplementary Figure 1F). Since *MTG16* is required for hematopoietic progenitor cell fate decision²¹, it is highly likely that the mesenchymal expression of *Mtg16* is localized to hematopoietic cells. Taken together, our data suggest that a subpopulation of +4/5 cells express *Mtg8/16* and are negative for *Atoh1*. Of note, *Mtg8* transcript levels were very low in abundance throughout the intestinal tissue, suggesting that its expression might be transient and dynamic under strict regulation at the +4/5 cells.

Mtg8 and Mtg16 are negatively regulated by Notch signaling

Since *Mtg16* expression partially overlaps with *Atoh1* expression in +4/5 cells, we asked whether the co-repressors are regulated by Notch signaling. Mouse small intestinal organoids were treated with the Notch inhibitor DAPT followed by qRT-PCR analysis. As expected, DAPT-treated organoids showed significant suppression of Notch signaling and de-repression of *Atoh1* and *Dll1*, while secretory lineage markers were upregulated (Figure 2A). Remarkably, both *Mtg8* and *Mtg16*, but not *Mtgr1*, were significantly upregulated upon DAPT treatment (Figure 2B). We observed similarly upregulated expression of *MTG8* and *MTG16* in DAPT-treated human intestinal organoids, indicating that the Notch-controlled expression of the two genes is conserved in human (Supplementary Figure 2A).

To confirm the Notch regulation *in vivo*, we depleted the Notch downstream transcription factor *Rbpj* for 3-6 days. Loss of *Rbpj* resulted in a progressive drift of differentiation towards the secretory lineage, concurrent with increased *Atoh1* expression (Figure 2C; Supplementary Figure 2B, C). Consistent with the organoid data, qRT-PCR analysis showed robust upregulation of *Mtg8* and *Mtg16* in the intestinal crypts upon *Rbpj* loss *in vivo*, while *Mtgr1* expression was unchanged (Figure 2C). Interestingly, expression of *Mtg8* and *Mtg16* was also significantly increased at day 4 post-*Rbpj* deletion *in vivo*, when most of the differentiation markers remained unchanged (Figure 2C and Supplementary Figure 2C, D). RNAscope staining confirmed upregulation of *Mtg8* and *Mtg16*, and loss of the ISC marker *Lgr5* upon *Rbpj* deletion (Figure 2D, E). We further validated the findings by treating WT animals with the γ -secretase inhibitor, dibenzazepine (DBZ), as an alternative Notch inactivation model, which again resulted in a progressive shift towards the secretory lineage (Supplementary Figure 2E). Similar to the *Rbpj* deletion data, expression of *Atoh1*, *Mtg8* and *Mtg16* was significantly upregulated immediately after treatment (1-2 days) (Figure

2F and Supplementary Figure 2F), confirming that MTG8 and 16 are repressed by Notch signaling. Previous data have suggested regional differences in Notch signaling in the intestine, where proximal duodenum shows higher Notch signaling than distal parts of ileum²². In agreement, higher expression of *Atoh1* and *Mtg16* was observed in the distal ileum (Supplementary Figure 2G).

Since the secretory progenitor marker ATOH1 is also repressed by Notch signaling in +4/5 cells, we asked whether the Notch-regulated *Mtg8* and *Mtg16* expression is dependent on ATOH1 using *Atoh1* floxed organoids. Indeed, the DAPT-induced expression of *Mtg8* and *Mtg16* was abrogated upon *Atoh1* deletion, indicating that *Mtg8* and *Mtg16* expression is mediated by Atoh1 (Figure 2G). Furthermore, ectopic expression of ATOH1 in HEK293T cells was able to induce *MTG8* and *MTG16* expression (Supplementary Figure 2H), indicating that the Atoh1-mediated MTG expression is independent of change of cell fate. To verify the hierarchical regulation of ATOH1 and MTG8/16, we further examined their expression dynamics in a short time-course DAPT treatment of organoids. The results demonstrated that *Atoh1* was significantly upregulated 12hr after DAPT induction, whereas *Mtg16* was only upregulated 15hrs after induction (Supplementary Figure 2I). *Mtg8* remained unchanged during the first 16hrs of DAPT treatment. The data suggest that the expression of *Mtg8* and *Mtg16* is likely to be driven by Atoh1 at different dynamics. We conclude that MTG8 and MTG16, but not MTGR1, are repressed by Notch signaling indirectly via ATOH1 in the intestine.

Loss of *Mtg8* and *Mtg16* induces hyperproliferation and expansion of ISCs

The Notch regulated expression of *Mtg8* and *Mtg16* in +4/5 cells led us to investigate whether the co-repressors play a role in ISC fate decision. We analyzed the *Mtg8*^{-/-} and *Mtg16*^{-/-} animals. *Mtg16*^{-/-} mice were healthy and viable, while *Mtg8*^{-/-} and double

knockout (DKO) animals died shortly after birth, in accordance with previous findings^{21,23}. We then proceeded to analyze *Mtg16*^{-/-} and DKO newborn pups. The intestine obtained from DKO pups was significantly shorter than from WT, which was consistent with the *Mtg8*-null phenotype (Supplementary Figure 3A)²³. Both *Mtg16*^{-/-} and DKO animals showed significantly increased proliferation in the inter-villus regions that would later give rise to crypts (Figure 3A and Supplementary Figure 3B). Notably, Edu⁺ cells were also detected in the villi of the DKO intestine, indicating that epithelial cell proliferation was extended beyond inter-villus regions to the villi. Next, we analyzed whether the increase in the inter-villus epithelial cell proliferation is accompanied by upregulated ISC gene expression. RNAscope and qRT-PCR demonstrated that the ISC marker *Lgr5* and its transcriptional activator *Ascl2* were both significantly upregulated in the mutants (Figure 3B). Similarly, significant increases in crypt proliferation and ISC markers (*Lgr5* and *Olfm4*) were also observed in *Mtg16*^{-/-} adult intestine (Figure 3C, D and Supplementary Figure 3C, D). Co-staining of *Lgr5* and *Atoh1* confirmed significantly increased *Lgr5* expression in the trans-amplifying region above the *Atoh1*⁺ progenitors in the mutant intestines (Supplementary Figure 3E). We further generated *Mtg16*^{-/-};*Lgr5*-GFP mice to label the endogenous LGR5⁺ ISCs. In agreement with the RNAscope observations, an increased number of GFP⁺ ISCs were detected in the *Mtg16*^{-/-} adult intestine, confirming the ISC expansion phenotype (Supplementary Figure 3F).

To test whether the stem cell-repressive role of MTG16 is cell-intrinsic, we further examined the colony formation capacity of WT and mutant organoids *ex vivo* in the absence of any stromal niche. We confirmed that *Mtg16*^{-/-} organoids grew faster than the WT controls, suggesting that the stem cell repressive role of MTG16 is indeed cell-intrinsic (Figure 3E). Similarly, organoids derived from *Mtg16*^{-/-} and DKO newborn intestine also grew significantly faster than the WT counterparts (Figure 3F and Supplementary Figure 3G).

We further challenged the organoids by reducing the Wnt agonist R-spondin (RSPO) concentration. Neither WT nor MTG mutant organoids survived in the absence of RSPO. However, *Mtg16*^{-/-} and DKO organoids grew significantly better in the low-RSPO (2%) condition with fewer collapsed organoids and more healthy branching organoids compared with WT (Figure 3G). The results suggest that MTG KO organoids have a growth advantage in the low-RSPO condition but are still dependent on exogenous Wnt signaling for ISC survival. Since MTG16 is expressed in Paneth cells, we asked whether the increase in crypt proliferation and stem cell markers in *Mtg16*^{-/-} intestine is caused by dedifferentiation of Paneth cells, which has recently been reported to occur upon injury²⁴. However, immunostaining of WT and *Mtg16*^{-/-} tissue did not show any co-localization of Paneth cell marker lysozyme and proliferation marker Ki67 (Supplementary Figure 3H), indicating that the increase in stem cell gene expression upon MTG16 loss is caused by stem cell derepression in the early progenitors rather than Paneth cell plasticity. Together, we tentatively conclude that MTG8 and MTG16 regulate niche exit at the +4/5 cells by repressing the ISC fate and proliferation.

Mtg8 and Mtg16 deletion impairs intestinal lineage specification

MTGR1 has previously been shown to be required for intestinal secretory cell differentiation in adult tissue²⁵. We therefore asked whether loss of MTG8/16 would alter lineage selection in the intestine. Since intestinal differentiation is incomplete in newborn animals, we decided to focus on analyzing *Mtg16*^{-/-} adult intestine. Reduced goblet cell numbers (AB-PAS) were observed in *Mtg16*^{-/-} adult intestine (Supplementary Figure 4A, B). This is in concordance with the previously reported phenotype of *Mtg16* null animals²⁶. Similarly, there was a tendency towards reduction of enterocyte markers (Villin and Alkaline Phosphatase) in the mutant intestines (Supplementary Figure 4A). We believe that the

moderate alteration of terminal differentiation might be due to the redundant role of Mtg8. To provide a global, unbiased picture of gene expression changes in the mutant animals, we further performed transcriptional profiling on the WT and *Mtg16*^{-/-} intestine. RNA-seq analysis revealed 478 genes that were differentially expressed upon *Mtg16* deletion (Supplementary Figure 4C, D, Supplementary Table 2). Consistent with the increased crypt proliferation observed in Figure 3, Wnt and stem cell signatures^{18,27} were both significantly upregulated upon loss of MTG16 (Figure 4B). Interestingly, we further observed significant reduction of enterocyte markers and upregulation of secretory markers such as Paneth cells and enteroendocrine cells in the *Mtg16* mutant intestine (Figure 4A). Of note, RNA-seq data did not reveal significant alteration of goblet cell markers. Comparison of various enterocyte markers with the previously published single-cell RNA-seq data (GSE92332) revealed differential expression of the markers between mature and immature enterocytes²⁸. In particular, *Alpi* expression did not distinguish between mature and immature enterocytes, while other markers such as *Apoa4*, *Fabp1* and *Fabp2* were preferentially expressed in mature enterocytes (Supplementary Figure 4E). Our RNA-seq data suggested that deletion of *Mtg16* results in a loss of mature enterocyte markers. Indeed, a clear reduction of FABP1 and APOA4 proteins was observed in the *Mtg16*^{-/-} intestine, indicating that loss of MTG16 inhibits enterocyte differentiation and maturation (Figure 4C). Gene set enrichment analysis (GSEA) further confirmed the loss of absorptive signatures (Figure 4D) and enrichment of secretory signatures^{8,29} (Figure 4E) in the *Mtg16*^{-/-} intestine. To further demonstrate the functional defect of the MTG mutant intestine, we examined the disaccharidase (brush border enzyme) activity in the WT and *Mtg16*^{-/-} organoids (Figure 4F). Downregulated expression of mature enterocyte markers was confirmed in *Mtg16*^{-/-} organoids (Figure 4G). Consistent with our observation of impaired enterocyte differentiation *in vivo*, the disaccharidase activity

of the adult *Mtg16*^{-/-} organoid was significantly reduced when compared to WT control organoids (Figure 4H).

Taken together, our data support the notion that MTG16 represses stem cell proliferation and promotes enterocyte over secretory lineage differentiation. Interestingly, we also noted enrichment of chromatin remodeling and epigenetic regulatory genes in the *Mtg16* mutant intestine (Supplementary Figure 4F), suggesting that the co-repressor MTG16 may regulate gene expression by chromatin remodeling.

Mtg16 binds to ISC signature genes and *Atoh1*-targets for niche exit and fate decision

To investigate how the co-repressors regulate ISC gene expression program and lineage selection, we then performed chromatin immunoprecipitation coupled-deep sequencing (ChIP-seq) to identify the MTG targetome. To capture the physiological binding targets *in vivo*, intestinal crypts were isolated 4 days after *Rbpj*-depletion to enhance endogenous MTG16 expression, while most of the differentiation markers remained unaltered (Figure 2C, D and Supplementary Figure 2C, D). MTG16 ChIP-seq identified 7843 reproducible binding sites (Figure 5A, Supplementary Figure 5A and Supplementary Table 3). Gene ontology (GO) analysis of MTG16 targets revealed enrichment of genes associated with Wnt and Notch signaling, as well as histone modifying genes (Figure 5B and Supplementary Table 4). Comparison between the ChIP-seq and RNA-seq data showed that 35% of the genes differentially expressed upon *Mtg16* deletion harbored MTG16-binding sites within 5kb of the transcription start site (TSS) (Supplementary Figure 5B), where the odds of genes being differential were observed to be increased by a factor of 2.4 ($p < 2e-16$, hypergeometric test). In particular, we observed clear MTG16 binding signals over the key ISC genes *Lgr5* and *Ascl2* (Figure 5C). These sites coincided with the previously reported regulatory regions in these genes^{30, 31}. MTG16 also bound to the promoter regions of other

Wnt targets such as *Axin2*, *Myc* and *Sox9*, suggesting that MTG16 represses ISC signature genes and Wnt targets through direct binding to their regulatory sequences (Supplementary Figure 5C). This result was consistent with our observation that *Lgr5* and *Ascl2* are upregulated upon MTG8 and MTG16 loss (Figure 3B, D).

Since MTG8 and MTG16 are repressed by Notch signaling in +4/5 cells, we asked whether they play a role in lineage selection, similar to ATOH1. We compared our MTG16 ChIP-seq data with the previously reported ATOH1 ChIP-seq and DNase I hypersensitivity (DHS, a measure of chromatin accessibility) data on secretory- or enterocyte-progenitors (SP, EP)⁸. A striking overlap (84.08%) between MTG16 and ATOH1 binding sites was observed between the two datasets, suggesting that MTG16 may also be involved in fate decision (Figure 5D, E).

ATOH1 has previously been reported to drive lateral inhibition and to set the secretory fate by regulating expression of the *Dll* Notch ligands^{8,29}. We analyzed the ChIP-seq profiles of the Notch ligands *Dll1* and *Dll3*. Remarkably, we found that MTG16 bound to the previously reported ATOH1-enhancer regions of both *Dll1* and *Dll3* (Figure 5F). Interestingly, loss of or reduced levels of DHS were observed in enterocyte progenitors compared to secretory progenitors at the regions where MTG16- and ATOH1-binding overlapped (Figure 5F). These results suggest that MTG16 binds to the ATOH1-bound loci to reduce chromatin accessibility of *Dll* genes in enterocyte progenitors for lateral inhibition and early fate decision. Similar to *Dll* ligands, MTG16 also occupied most of the reported ATOH1-binding sites in all secretory signature genes including *Spdef*, *Gfi1* and *Neurog3* with reduced DHS levels in EP (Figure 5F). Given that MTG proteins repress gene transcription by recruiting various chromatin-modifying enzymes (e.g. histone deacetylases)¹⁴, we propose that MTG8/16 regulate lateral inhibition and binary fate decisions of +4/5 progenitors by repressing ATOH1-mediated *Dll* ligands and secretory signature gene transcription. Indeed,

de novo motif analysis identified an MTG16 consensus motif that was matched to the reported ATOH1 motif, suggesting that MTG16 occupies ATOH1-bound enhancers to regulate lineage specification (Figure 5G and Supplementary Table 5). Co-immunoprecipitation analysis further confirmed the physical binding of ATOH1 with both MTG8 and MTG16 (Supplementary Figure 5D). Consistently, ATOH1-mediated *DLL1* expression was significantly downregulated by MTG8 or MTG16 expression (Supplementary Figure 5E). In addition to ATOH1, MTG family members have previously been shown to interact with TCF4 for transcriptional suppression³². Together, our findings support the notion that the MTG co-repressors bind to the transcription factors TCF4/ β -catenin and ATOH1 to repress the stem cell program and *Dll* expression for lateral inhibition. We further noted that MTG16 bound strongly to its own locus as well as to the promoter regions of *Mtgr1* and *Atoh1* (Supplementary Figure 5F), while MTG16 has recently been reported as an ATOH1 target²⁹. The data imply that ATOH1 and the MTG family together contribute to a ‘cross-over’ feedback loop in +4/5 cells to regulate rapid, dynamic fate decisions.

Discussion

Extensive studies in the past have focused on characterizing the signaling pathways regulating ISCs, yet it has remained elusive how the tightly regulated ISC fate remains restricted to a fixed number of proliferative cells at the crypt base. Paneth cells have been shown to constitute the essential niche to define ISC identity^{9,33-35}, yet functional ISCs can be maintained upon Paneth cell ablation^{36,37}. Therefore, it remains unclear how Paneth cells contribute to ISC homeostasis. The undifferentiated cells immediately above the ISC compartment (+4/5 progenitors) are heterogeneous in terms of marker gene expression. ATOH1 marks a sub-population of +4/5 cells that have entered the secretory lineage differentiation and mediate lateral inhibition^{3,8}, while molecular markers of the remaining

+4/5 progenitors entering the absorptive enterocyte differentiation have not yet been identified. Here we report that the Notch-repressed transcriptional co-repressors MTG8 and MTG16 are expressed in +4/5 progenitors to switch off the stem cell expression program. Our current findings provide insights into the underlying mechanism of ISC fate decisions (Figure 6A). Our data support the notion that the ‘Notch-off’ state is the first “priming” step to drive ISC-daughter cell transition by committing to transient bi-potent progenitors, which is consistent with the recently proposed “multi-lineage progenitor” population as the earliest progeny of LGR5+ stem cells³⁸. When an ISC occupies the +4/5 cell position and loses its contact with *Dll*-expressing Paneth cells (niche exit), Notch is switched off as a consequence, thereby de-repressing ATOH1, MTG8 and MTG16. The co-repressors then drive differentiation by switching off the Wnt-mediated ISC gene expression program in the immediate progenitors, leading to transient activation of the whole differentiation program. This is consistent with the data obtained from our time-course DAPT-treated organoids, where downregulation of ISC markers was accompanied by transient upregulation of both absorptive and secretory lineage markers upon early Notch inhibition (Figure 6B). Subsequently, ATOH1 and MTG8/16 work together in these naïve bi-potent progenitors to control lateral inhibition and binary fate decision (Figure 6A). Our findings uncover a novel role of MTG8/16 in promoting enterocyte differentiation by direct repression of ATOH1-mediated secretory differentiation and *Dll* ligands expression. The differential expression dynamics of *Atoh1*, *Mtg8* and *Mtg16* and their potential negative feedback network may perhaps explain the heterogeneity within the early progenitor population. MTG16 is initially co-expressed with ATOH1 immediately after niche exit and Notch inhibition. Subsequently, MTG8 and MTG16 expression starts to dominate and repress ATOH1 expression, resulting in MTG8/16+ATOH1- cells. It is conceivable that the fate decision at these progenitors is dependent on the expression dynamics of ATOH1 and MTG. Interestingly, two recent studies

showed direct binding of HES1 to the promoter of *Mtg16*^{39,40}, suggesting that MTG8/16 may also be actively repressed by Notch directly via HES1 at the ISCs. It is also worth noting that all *Dll* ligands are transcriptional targets of ATOH1 and MTG16, including *Dll3* that has previously been reported to function exclusively as cis-inhibition rather than trans-activation of Notch signalling⁴¹. This may imply a previously underappreciated role of DLL3 in the dynamic lateral inhibition and fate decision in the early progenitors, where DLL1/4 trans-activate Notch in the neighboring cells and DLL3 inhibits Notch cell-autonomously.

Previous studies have shown that MTG16 is required for injury-induced epithelial cell survival and regeneration in the intestine^{26,42}. Interestingly, increased proliferation of *Mtg16*-depleted intestine has been demonstrated, although the underlying mechanism remained uncharacterised²⁶. More recently, increased β -CATENIN staining has also been observed in MTG16-deleted colitis-associated tumours⁴³, suggesting a potential Wnt inhibitory role of MTG16. In the current study, we focused on characterizing the mechanistic role of MTG in normal intestinal stem cell homeostasis. Beyond the increase in crypt proliferation as previously reported, we further observed a significant increase in stem cell number in the MTG mutants. Global genomic and transcriptomic analysis further revealed that MTG16 binds to the gene loci of stem cell and Wnt signature genes for transcriptional repression. Our data on the Notch-regulated MTG expression at +4/5 progenitor cells provide mechanistic insight into how MTG regulates stem cells and the Wnt transcriptional program under normal stem cell homeostasis, which will help understand the tumor suppressive role of MTG in colorectal cancer⁴⁴.

Regulation of chromatin accessibility has recently been reported in these highly dynamic progenitors for fate decision and plasticity^{8,12,13}. It is believed that dynamic reorganization of chromatin remodeling controls the rapid, dynamic lineage specifications of early progenitors, as well as permitting dedifferentiation of progenitors into stem cells upon

damage. However, the underlying mechanism of how chromatin remodeling is regulated remains unknown. The discovery of the co-repressors Mtg8/16 in the +4/5 cells offers a compelling explanation for this epigenetic regulation by recruiting various chromatin-modifying enzymes to stem cell- and lineage-specific genes for dynamic fate decisions. Controlling the expression of MTG8 and MTG16, via Notch signaling upon damage could allow the early progenitors to reacquire multipotency by de-repressing the ISC gene expression program. It is interesting to note that MTGR1 is not regulated by Notch signaling despite the previously reported role in secretory lineage differentiation²⁵. Since our ChIP-seq data revealed that MTGR1 is a transcriptional target of MTG16, it is conceivable that the MTG family function together with ATOH1 to drive fate decision via transcription activator-repressor network and chromatin remodeling. Our findings provide a direct link between Notch signaling and chromatin remodeling for ISC fate decision. Further characterization of MTG8, MTG16 and MTGR1 targetomes will help understand their transcriptional regulation of ISC fate as homodimer or heterodimer.

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Figure Legends

Figure 1. Expression of *Mtg8* and *Mtg16* in the +4/5 early progenitor cells. (A) FACS isolation of GFP high and GFP low cells from the *Lgr5-EGFP-IRES-CreERT2* intestinal crypts. qRT-PCR analysis of the indicated genes in the two populations. Data represent mean \pm s.e.m. from biologically independent animals (n=3). *P < 0.05, **P < 0.01, ***P < 0.001, two-sided t-test. (B) RNAscope brown staining of *Mtg8*, *Mtg16* and *Mtgr1* in intestinal crypts from WT mice. (C) Quantification of *Mtg8* and *Mtg16* RNAscope staining in (B) along the crypt. Data represent mean \pm s.e.m. from biologically independent animals (n=3). (D, E) RNAscope duplex staining of *Mtg16* (D) or *Mtg8* (E) (blue) with *Atoh1* or *Lgr5* (red) in WT intestinal crypts. Empty arrows indicate exclusive staining, black arrows indicate co-localized staining. (F) Quantification of *Mtg16* RNAscope signal (area of dots) in Paneth cells and progenitor cells. Data represent mean \pm s.e.m. from biologically independent animals (n=3). **P < 0.01, two-sided t-test. (G) Quantification of *Mtg16*+*Atoh1*+ and *Mtg16*+*Atoh1*- cell populations in early progenitors (+3-5 positions) from the RNAscope staining in (D). Scale bars, 20 μ m.

Figure 2. *Mtg8* and *Mtg16* are regulated by Notch signaling. (A, B) qRT-PCR analysis of WT mouse intestinal organoids treated with Notch inhibitor DAPT for 2 and 3 days. Data represent mean \pm s.e.m. from biologically independent small intestinal organoid isolations (n=3). The experiment was performed twice. *P < 0.05, **P < 0.01, ***P < 0.001, two-sided t-test. (C) qRT-PCR analysis of intestinal epithelium from *Villin CreER* and *Villin CreER;Rbpjfl/fl* mice collected at indicated days after tamoxifen induction. Data represent mean \pm s.e.m. from biologically independent animals (n=4 per group). Three independent experiments were performed. *P < 0.05, **P < 0.01, ***P < 0.001, two-way ANOVA. (D) RNAscope brown staining of *Mtg8*, *Mtg16* and *Mtgr1* in intestinal tissue obtained from *Villin CreER* and *Villin CreER;Rbpjfl/fl* mice collected at day 4 post-tamoxifen induction. Arrows indicate *Mtg8*+ cells. Scale bars, 50 μ m. (E) RNAscope duplex staining of *Mtg16* (blue) and *Lgr5* (red) in intestinal tissues of the indicated genotypes at day 6 post-tamoxifen induction. (F) qRT-PCR analysis of intestinal epithelium from WT mice collected at the indicated days after DBZ or vehicle treatment. Data represent mean \pm s.e.m. from biologically independent animals (n=3 per group). *P < 0.05, **P < 0.01, ***P < 0.001, two-way ANOVA (G) qRT-PCR analysis of DAPT-treated *Villin CreER* and *Villin CreER;Atoh1fl/fl* organoids induced with 4-OHT. Data represent mean \pm s.e.m. The experiment was performed 4 times. *P < 0.05,

P < 0.01, *P < 0.001, compared to untreated control group; #P < 0.05, ##P < 0.01, compared to DAPT treated control group, two-sided t-test.

Figure 3. Loss of *Mtg8* and *Mtg16* increases ISC numbers and proliferation. Intestinal tissues were collected from newborn (P0) (n=4-5 for each genotype) (A, B) or adult mice (n=3-6 mice per group) (C-E) for analysis. (A) EdU staining showing increased proliferation in *Mtg16*^{-/-} and *Mtg8*^{-/-} *Mtg16*^{-/-} animals compared to WT. Graphs showing EdU⁺ cells distribution along the crypt and quantitation of EdU⁺ cells per inter-villus region in WT, *Mtg16*^{-/-} and *Mtg8*^{-/-}*Mtg16*^{-/-} intestine. Data represent mean ± s.e.m. of three independent experiments. At least 10 representative crypts per animal have been analyzed. (B) *Lgr5* RNAscope staining and qRT-PCR showing increased ISC gene expression in newborn *Mtg16*^{-/-} and *Mtg8*^{-/-}*Mtg16*^{-/-} tissues. Data represent mean ± s.e.m. of three independent experiments (n=4 per group). *P < 0.05, **P < 0.01, ***P < 0.001, 1-way ANOVA. (C) EdU staining in WT and *Mtg16*^{-/-} adult intestine. (D) *Lgr5* and *Olfm4* RNAscope brown staining in small intestinal tissue from WT and *Mtg16*^{-/-} adult mice. (E) Colony formation assay of small intestine organoids isolated from WT and *Mtg16*^{-/-} adult mice. Data represent mean ± s.e.m. of two independent experiments (n=6 mice per group). *P < 0.05, **P < 0.01, ***P < 0.001, two-sided t-test. Scale bars, 100µm (B-D), 1000µm (E). (F) Colony formation assay of small intestine organoids derived from WT, *Mtg16*^{-/-} and *Mtg16*^{-/-}*Mtg8*^{-/-} newborn pups. Data represent mean ± s.e.m. of three independent experiments (n=2-4 mice per group). ***P < 0.001, 1-way ANOVA. (G) Representative images showing newborn organoids of the indicated genotypes cultured in 5% or 2% RSPO conditions for 3-4 days. Scale bar, 1000µm. Right, quantification of the organoids health status maintained in 2% of RSPO condition. Data represent mean ± s.e.m. of two independent experiments (n=2-4 mice per group). **P < 0.01, ***P < 0.001 compared to WT, 1-way ANOVA. #P < 0.05, ##P < 0.01 compared to *Mtg16*^{-/-}, 1-way ANOVA.

Figure 4. *Mtg8* and *Mtg16* deletion impairs intestinal lineage specification. Intestinal tissues were collected from newborn (P0) (n=4-5 for each genotype) (A) Heatmap showing genes differentially expressed in WT and *Mtg16*^{-/-} intestine. (B, D, E) Gene Set Enrichment Analysis (GSEA) probing (B) Wnt/Stem cell signature genes, (D) intestinal absorption and digestion and (E) secretory signature genes. (C) FAPB1 and APOA4 immunostaining in adult WT and *Mtg16*^{-/-} intestinal tissue. (F) Scheme showing the disaccharidase assay performed in organoids. (G) qRT-PCR of mature enterocyte markers in adult WT and *Mtg16*^{-/-}

organoids. (H) Glucose levels detected in the supernatant of intestinal organoids of the indicated genotypes after 1hr sucrose incubation. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, two-sided t-test.

Figure 5. MTG16 binds to ISC- and secretory lineage-signature gene loci. (A) Genome-wide distribution of 7843 Mtg16-binding sites. (B) GO analysis identified ontology terms associated significantly with MTG16 targetome, including Wnt, Notch and Ephrin pathways as well as in histone modifying genes. (C) ChIP-seq data showing MTG16 binding signal (per million reads) to ISC gene loci (*Lgr5* and *Ascl2*). (D, E) Composite profile (D) and heatmap (E) showing striking overlap between ATOH1 and MTG16 binding sites (7843 sites). (F) ChIP-seq data showing MTG16 binding signal (per million reads) to previously reported ATOH1-enhancer regions¹² (asterisk) of the indicated gene. Reduced levels of DHS in enterocyte progenitors (EP) versus secretory progenitors (SP) are indicated by dotted box. (G) MTG16 *de novo* motif matches with previously reported ATOH1 and ASCL1/2 motif.

Figure 6. Proposed model for intestinal stem cell hierarchy. (A) Updated ISC fate model. See text for details. (B) qRT-PCR analysis of the indicated genes after 1, 2 or 3 days of DAPT treatment. On the right, illustration of expression kinetics of the stem cell, secretory and enterocyte markers upon time-course Notch inhibition. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, two-sided t-test.

Figure 1

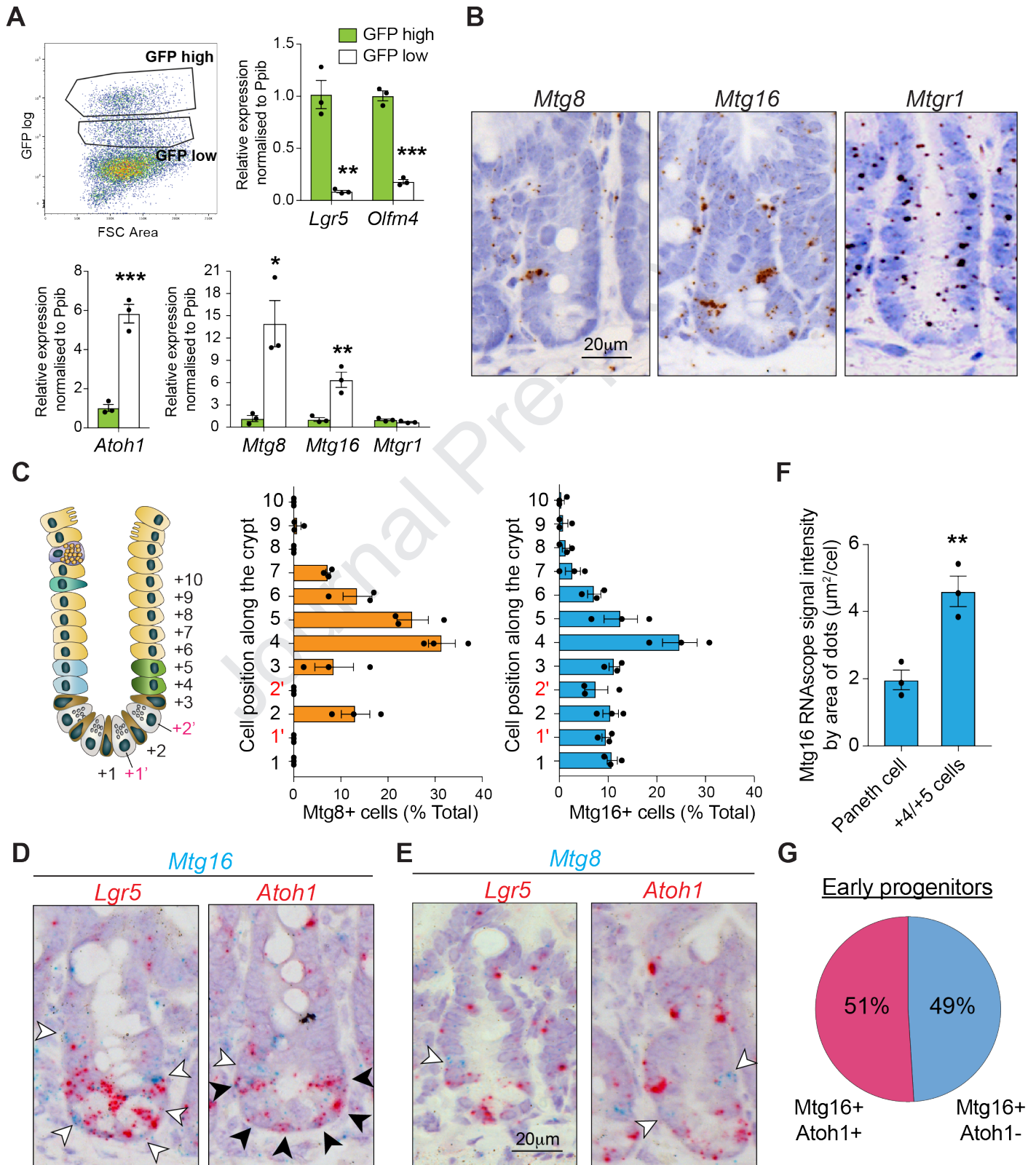


Figure 2

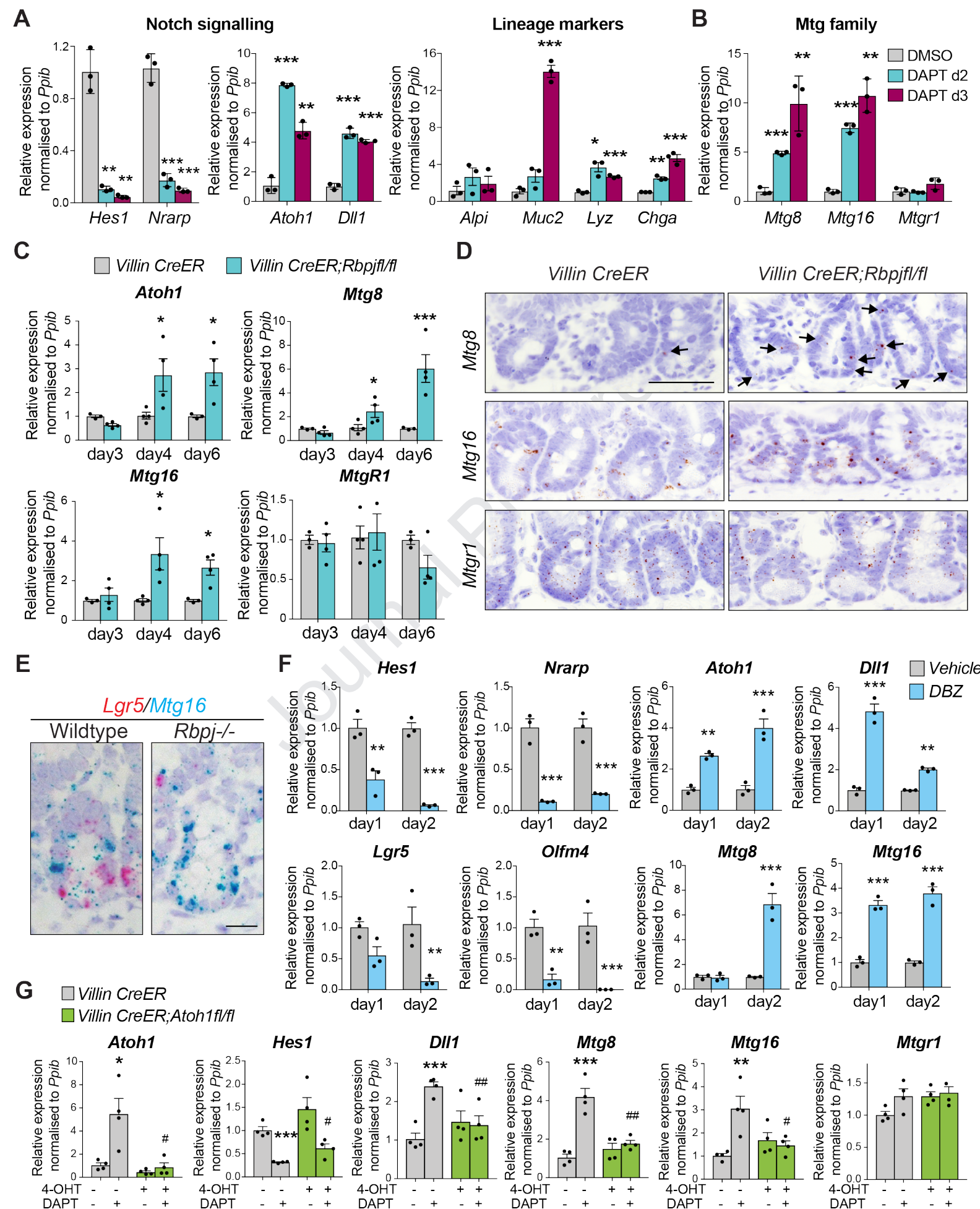


Figure 3

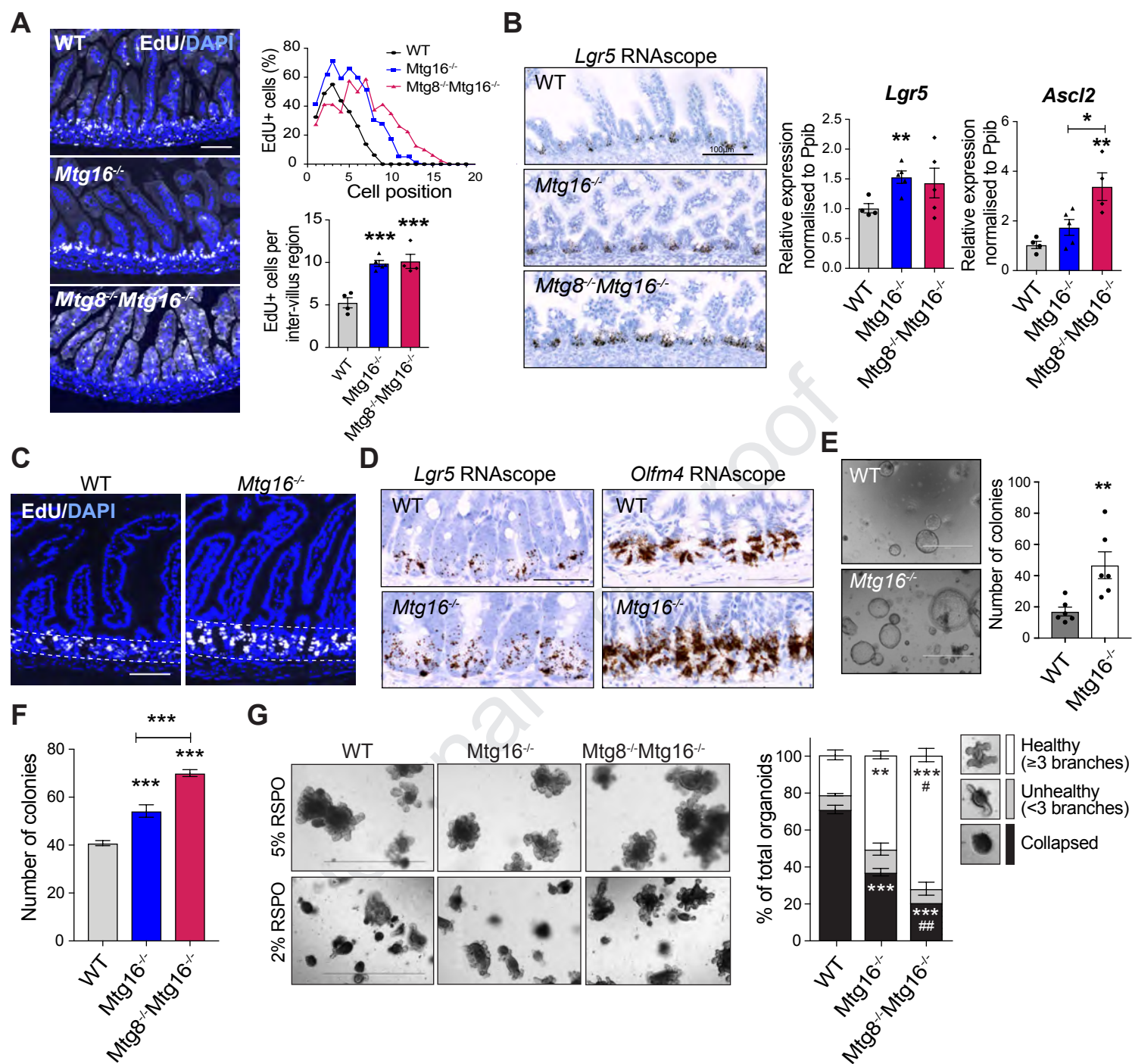


Figure 4

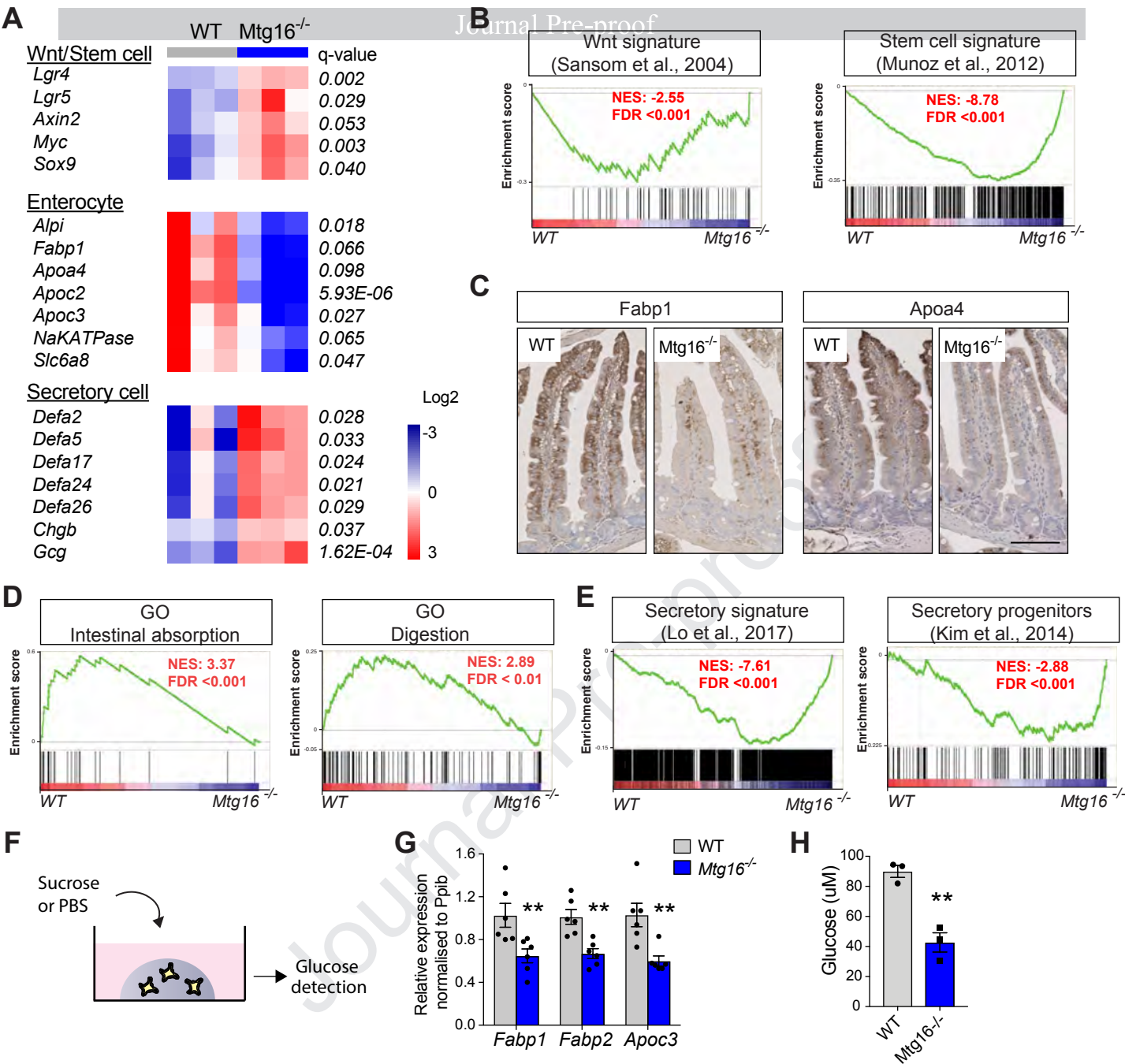


Figure 5

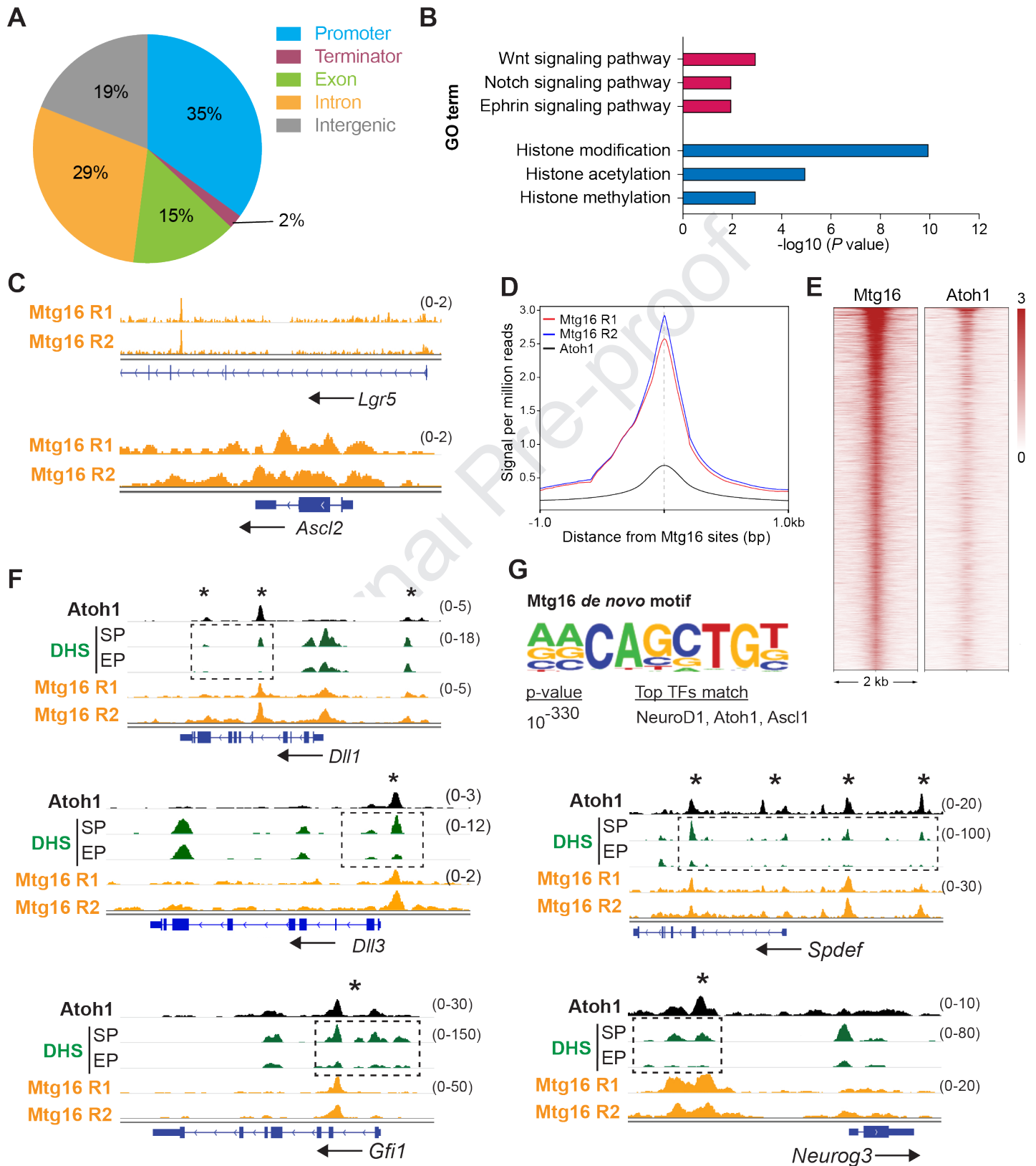
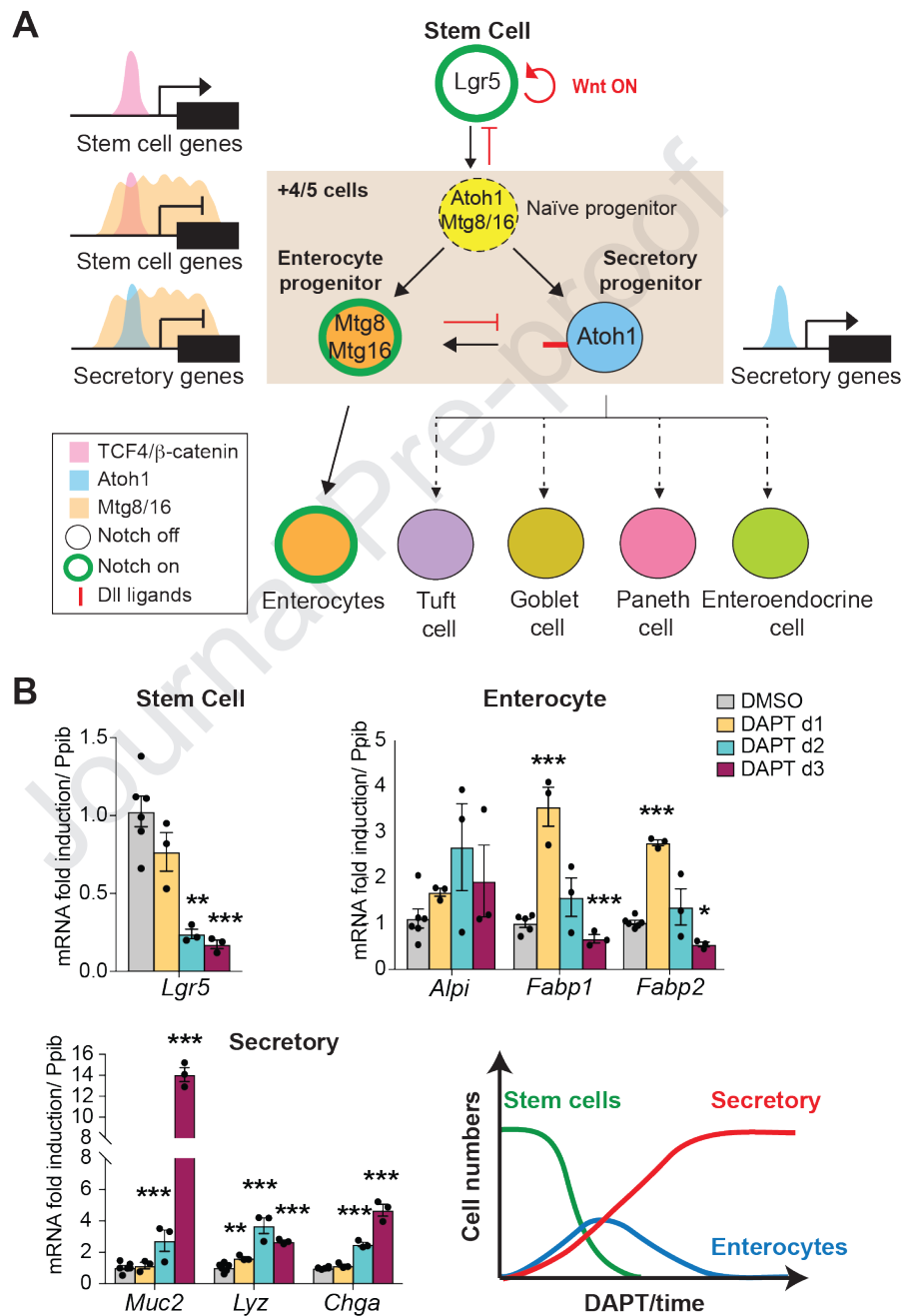
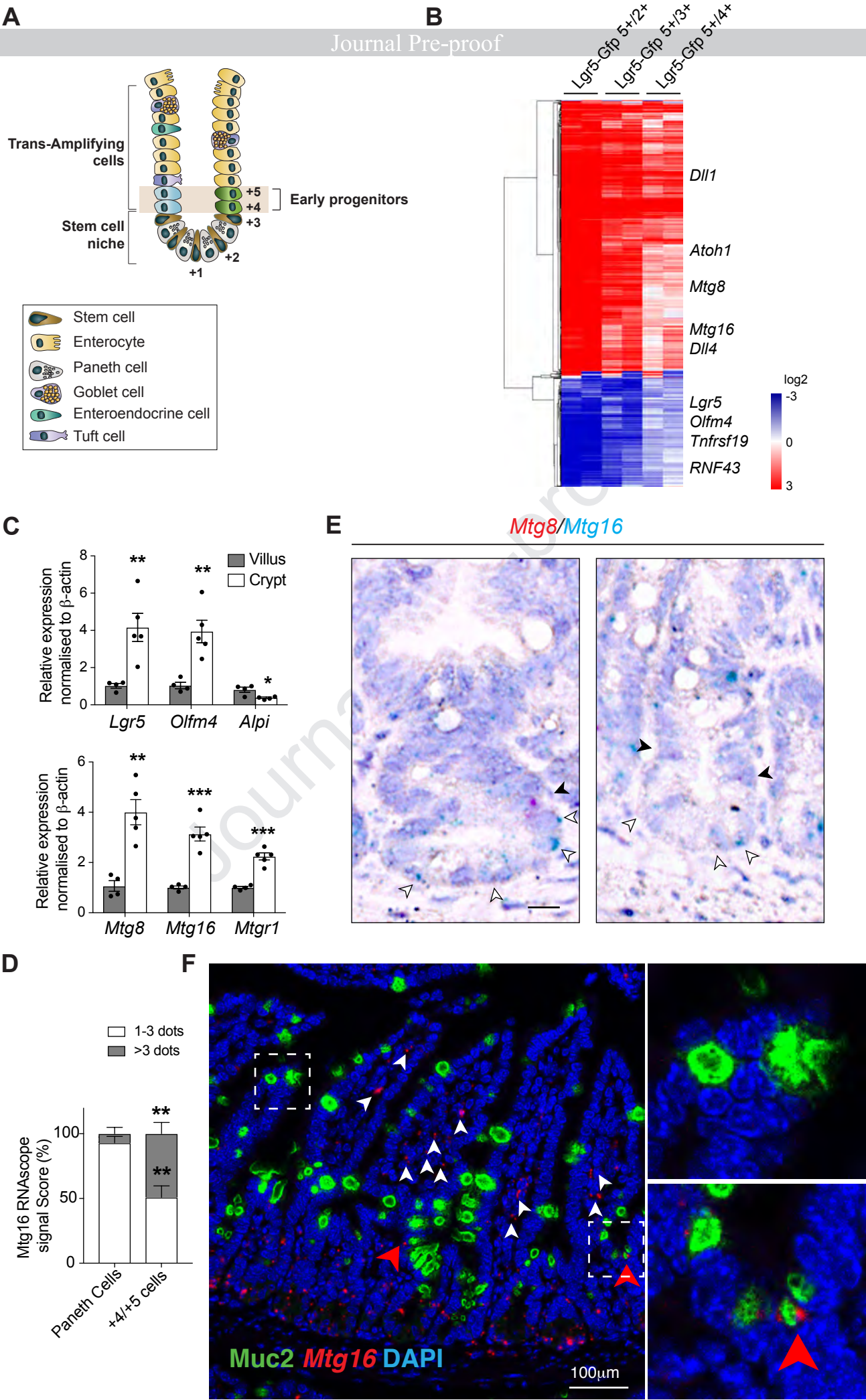


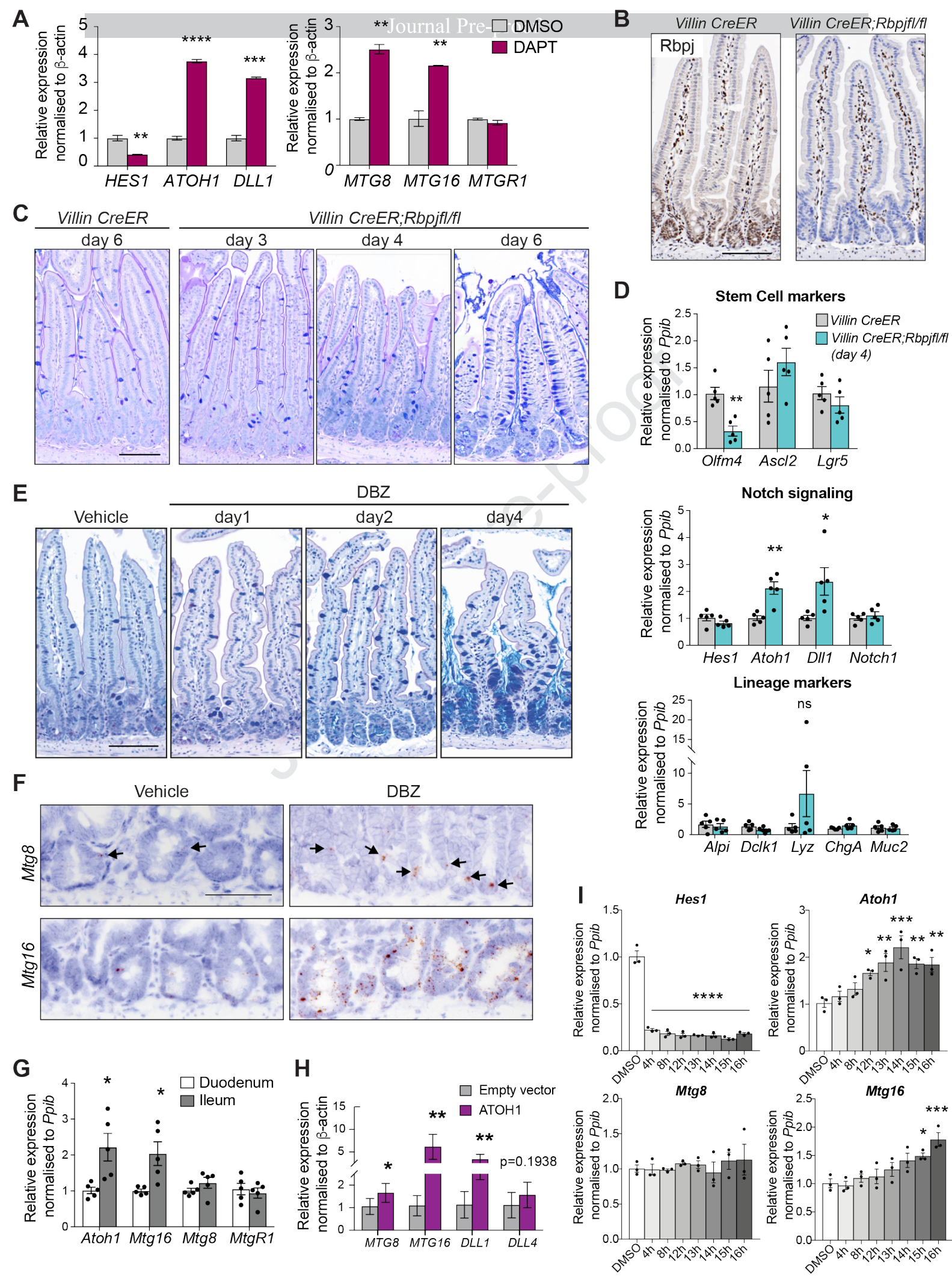
Figure 6



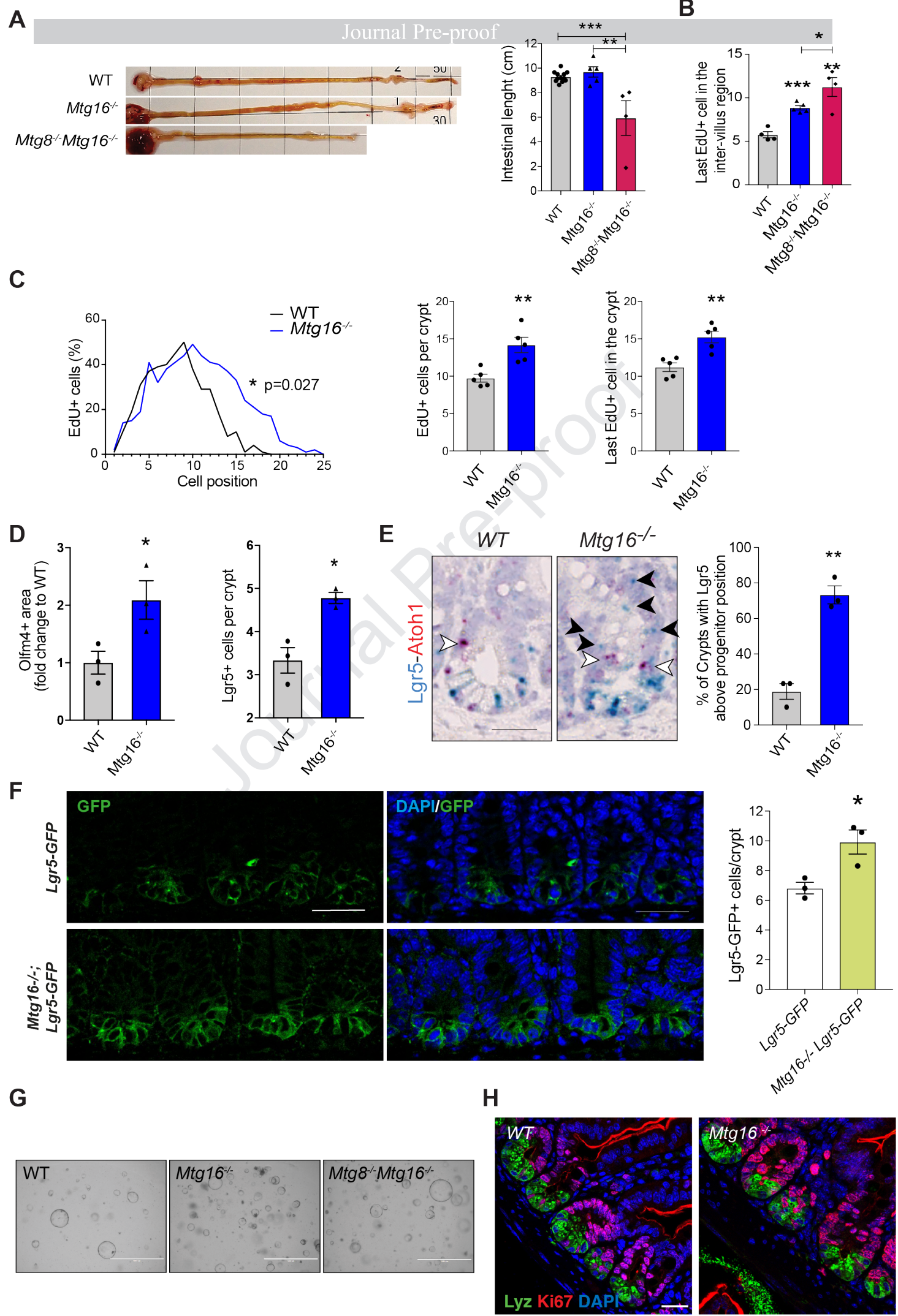
Supplementary Figure 1



Supplementary Figure 2

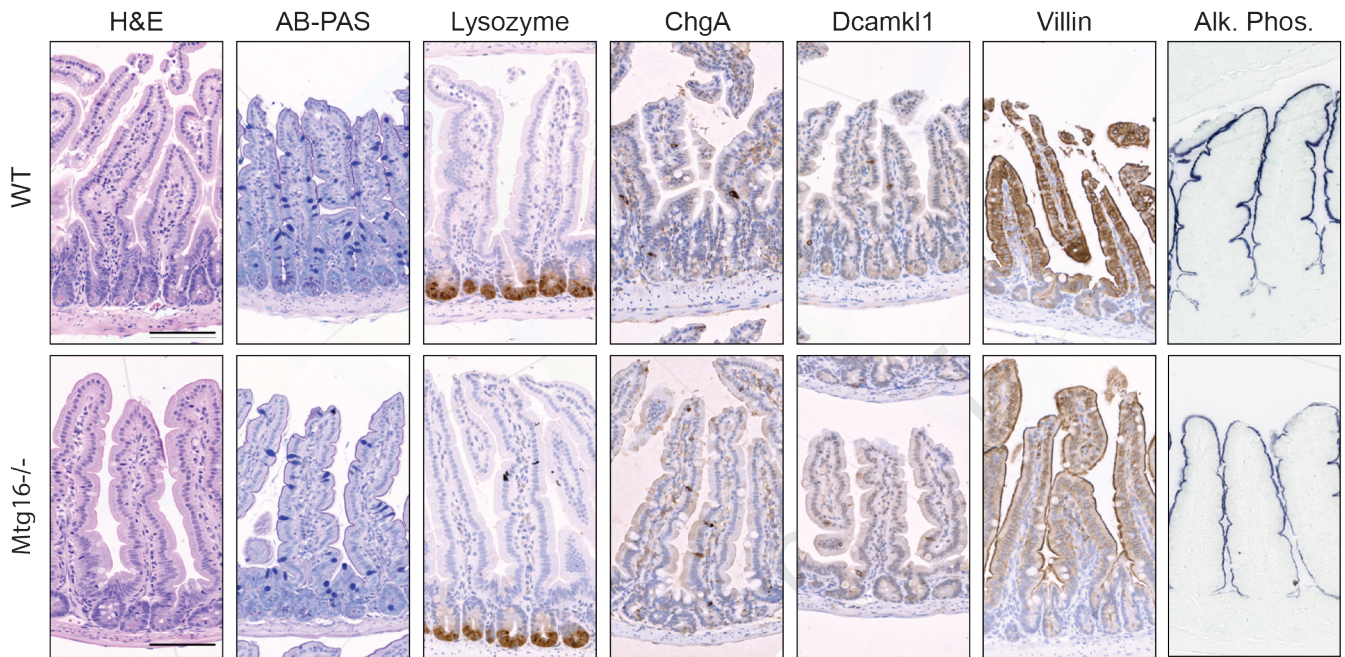


Supplementary Figure 3

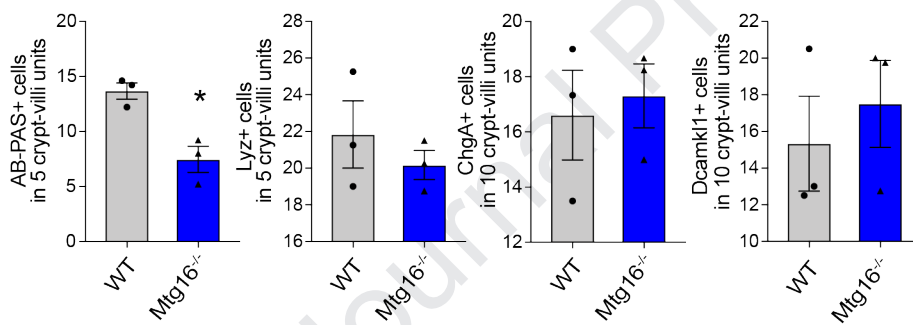


Supplementary Figure 4

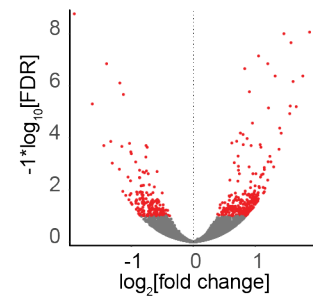
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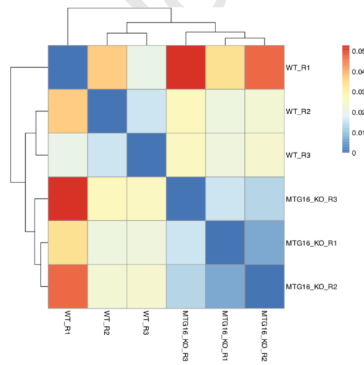
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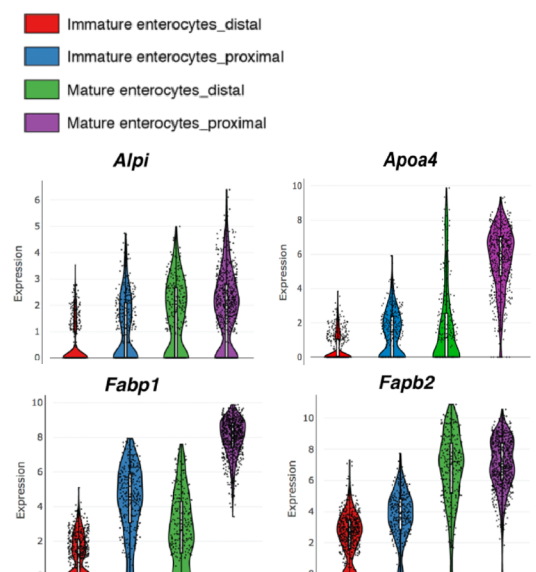
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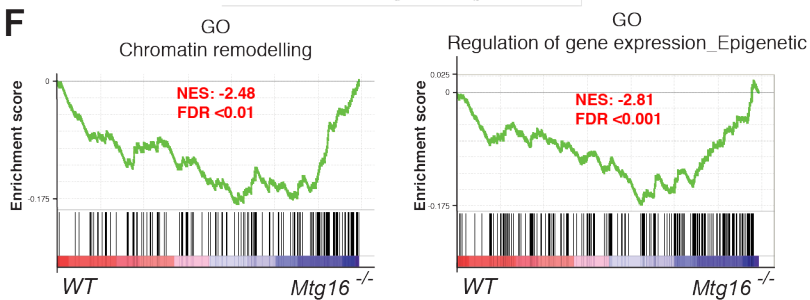
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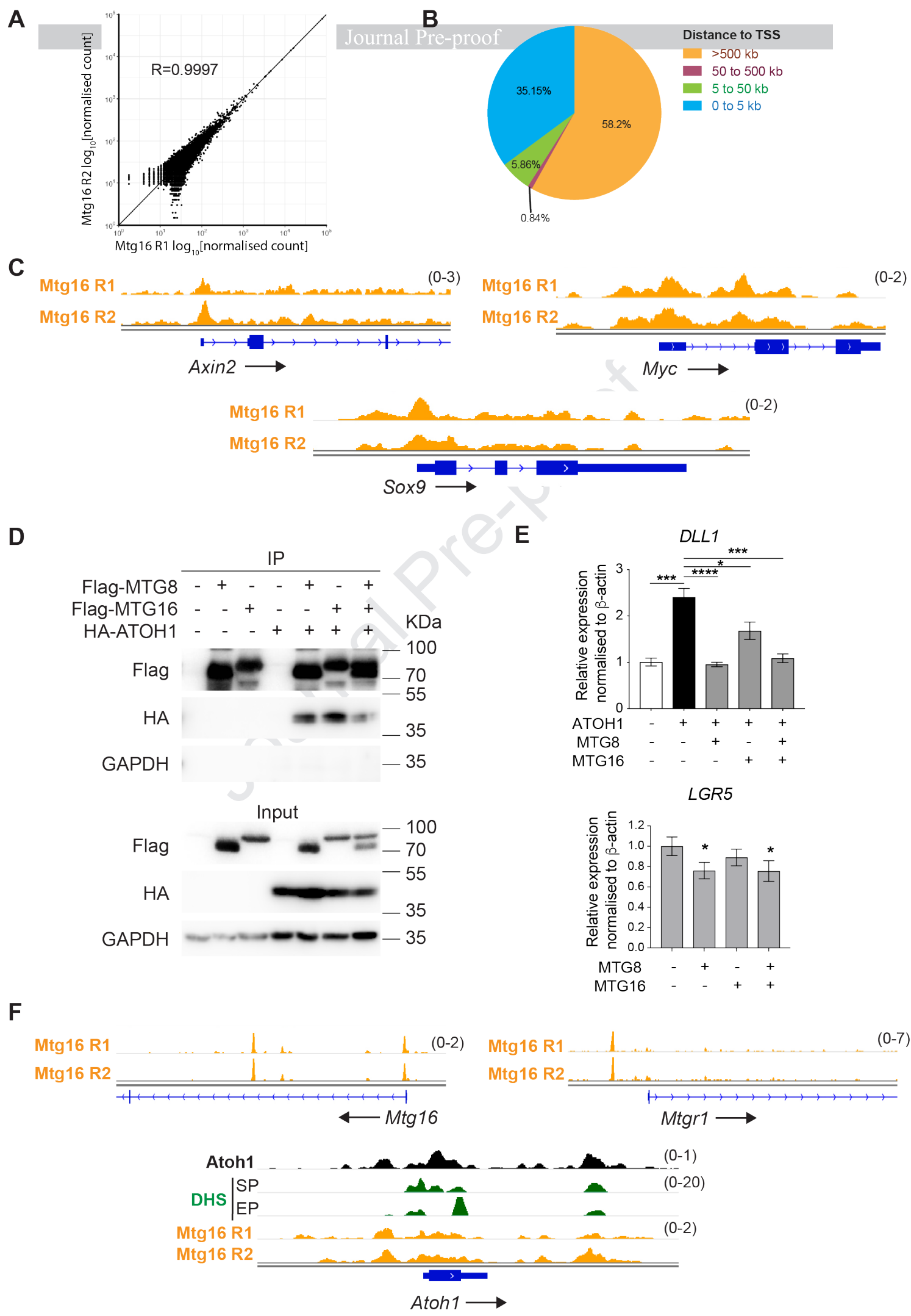
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Supplementary Figure 5



What you need to know:

Background and Context: Notch signaling maintains intestinal stem cells (ISCs) and determines whether early progenitor cells develop along the secretory or enterocyte lineage. The transcription factor ATOH1 is repressed by Notch in ISCs; its de-repression causes precursor cells to differentiate along the secretory lineage.

New Findings: In the intestine, MTG8 and MTG16 are repressed by Notch signaling indirectly, via ATOH1; this promotes exit of ISCs from their niche and regulates progenitor lineage specification, by repressing ATOH1-target genes.

Limitations: This study was performed in mice and human intestinal organoids. Studies of mice with intestine-specific knockout of MTG8 are needed, determine phenotypes of adult intestine.

Impact: MTG8 and MTG16 are chromatin modulators that regulate differentiation of ISCs into secretory vs enterocyte lineages.

Lay Summary: This study identified chromatin modulators, named MTG8 and MTG16, that regulate differentiation of intestinal stem cells and might be important for studies of intestine tissue regeneration.