Developmental Biology

Mac/Lac-tosylceramide regulates intestinal homeostasis and secretory cell fate commitment by facilitating Notch signaling

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eLife Assessment

This **important** study provides **solid** evidence that glucosylceramide synthase (GlcT), a rate-limiting enzyme for glycosphingolipid (GSL) production, plays a role in the differentiation of intestinal cells. Mutations in GlcT compromise Notch signaling in the Drosophila intestinal stem cell lineage resulting in the formation of enteroendocrine tumors, and preliminary data suggests that a homolog of glucosylceramide synthase also influences Notch signaling in the mammalian intestine. While the outstanding strengths of the initial genetic and downstream pathway analyses are noted, there are weaknesses in the data regarding the potential role of this pathway in Delta trafficking. Nevertheless, this study opens the way for future mechanistic studies addressing how specific lipids modulate Notch signalling activity.

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Abstract

Cell-to-cell communication via Delta-Notch signaling is widely used in various tissues and organs to regulate development and patterning; however, the mechanisms regulating Notch signaling for precise cell fate decisions remain poorly understood. Similar to mammals, the intestinal stem cells (ISCs) in the adult *Drosophila* midgut generate both absorptive and secretory cell progeny, guided by differential levels of Notch activation. Here we performed a forward genetic screen in *Drosophila* and identified glucosylceramide synthase (GlcT), a rate-limiting enzyme for glycosphingolipid (GSL) production, whose mutation causes the development of secretory cell tumors. Genetic analysis of the GSL synthesis pathway, combined with metabolite rescue experiments, revealed that the tumor formation is linked to a deficiency in Mactosylceramide/Lactosylceramide. This deficiency impaired the endocytic recycling of the Delta, subsequently reducing Notch signaling activation. Conditional knockout of *UGCG*, the mammalian ortholog of *GlcT*, in mouse small intestine caused an

excessive differentiation of goblet cells, phenotypes similar to these caused by Notch inhibition. Our study suggests an evolutionarily conserved role for a specific GSL metabolite in modulating Notch signaling during stem cell fate decisions and provides a molecular connection between ceramide metabolism and Notch signaling in regulating tissue homeostasis and tumor formation.

Introduction

Notch signaling is an evolutionarily conserved pathway in metazoans that mediates local cell-cell interactions through the membrane-tethered ligand Delta (Dl) and the membrane receptor Notch. This pathway transduces signals from the cell surface to the nucleus, regulating the transcription of target genes [1,2]. Dl-Notch signaling controls various cell fates and developmental processes, with mutations in the pathway implicated in numerous human diseases, including congenital defects and cancer [3–5]. Given that Notch can transduce, amplify, and consolidate molecular differences to influence cell fate decisions, it is crucial to understand how the strength of the Notch signaling pathway is precisely regulated to ensure proper cell fate determination and tissue development.

Notch signaling is known as a major regulator of cell fate decisions in both mammalian and *Drosophila* intestinal stem cell (ISC) lineages, as its activity determines the binary fate of intestinal progenitor cells. A high level of Notch activity promotes the specification of absorptive enterocytes, while low or absent Notch activity favors the specification of secretory cell types.[6,7]. The *Drosophila* intestinal stem cell (ISC) lineage in the adult midgut is relatively simple, comprising only one type of secretory cell: enteroendocrine cells (EEs). This simplicity makes it an attractive experimental system for studying Notch signaling in cell fate decisions during epithelial renewal [6]. The fly ISCs, which specifically express Dl, periodically generate two types of committed daughter cells: enteroblasts, which experience high levels of Notch activation and are primed for the specification of absorptive enterocytes, and enteroendocrine progenitor cells (EEPs), which receive low levels of Notch activation. Typically, EEPs undergo one round of mitosis before terminal differentiation, resulting in the formation of EE pairs [8–11]. The loss of Notch signaling results in a complete blockage of enteroblast differentiation, leading to the accumulation of ISCs, EEPs and EEs in the intestinal epithelium, producing an “EE tumor” phenotype [8,10]. It is suggested that the expression level of Dl in individual ISCs varies, leading to differential levels of Notch activation in the immediate daughter cells [9]. This variation in Dl expression may result from dynamic bidirectional Dl-Notch signaling between ISCs and EEPs, which in parallel or in conjunction with an intrinsic feedback mechanism in ISCs, guides a periodic activation of an EE fate inducer Scute, thereby periodic generation of EEPs from ISCs [10,12,13]. The transient activation of Scute induces irreversible Prospero expression in EEPs through a Phyl-Sina-Ttk69 regulatory cascade, initiating terminal differentiation into EEs [10,14–16]. During the period of Scute inactivation, ISCs only generate enterocyte-committed enterobalsts by default via Dl-Notch mediated lateral inhibition [8,17]. In newly enclosed flies, increased lipid intake from food can alter the membrane trafficking of Dl, subsequently increasing the frequency of EE specification from ISCs [18]. Thus, both intrinsic and extrinsic mechanisms are involved in regulating Dl-Notch signaling to orchestrate stem cell fate decisions.

To better understand fate regulation in the *Drosophila* ISC lineage, here we conducted a forward mosaic genetic screen in the adult *Drosophila* midgut, focusing on the right arm of the second chromosome to identify genes whose mutations could disrupt ISC fate decisions. From this screen, we identified several complementation groups that result in EE tumor phenotypes. While most of the identified gene loci correspond to known components of the Notch signaling pathway, we discovered one gene locus not previously associated with Notch signaling. Further analyses revealed a specific GSL metabolite that regulates Notch signaling and cell fate decisions in the intestinal epithelium, and this function appears to be conserved from *Drosophila* to mice.

Results

A mosaic genetic screen identifies *GlcT* as a tumor suppressor in the adult *Drosophila* midgut

We performed chemical mutagenesis on chromosome 2R (carrying FRTG13/42B) and used the MARCM system to induce mutant clones in the adult midgut (Supplementary Figure 1). We screened approximately 10,000 lethal lines and identified totally 12 mutations across six complementation groups that exhibited a "small cell tumor" phenotype, characterized by a significant accumulation of diploid cells within the clones (Supplementary Figure 1 and supplementary Table 1). Co-staining with the ISC marker Dl and the EE cell marker Pros revealed that most of these tumors contained an excessive number of ISCs and EEs, the EE tumor phenotype commonly observed in Notch mutant clones [11,19]. Further genetic mapping showed that three complementation groups were associated with genes known to function in the Notch pathway: *mam*, *Gmer*, and *O-fut1* [20–22]. One complementation group, comprising two alleles (*EA30* and *E230*), mapped to the *GlcT* gene (see details in Methods), while the remaining two complementation groups, each consisting of one allele, remain undetermined (Supplementary Table 1). *GlcT* encodes glucosylceramide synthase, an enzyme that catalyzes the formation of glucosylceramide, the core component of glycosphingolipids (GSLs). Both alleles carried one or more missense mutations resulting in the amino acid replacements E292K (for *EA30*) and L395P/S397T (for *E230*, Figure S1D). The two mutations produced a similar tumor phenotype in terms of tumor size and the composition of EEs in the tumors (**Figure 1B-D**). The large polyploid cells were still present in the tumors, indicating that the multipotency of ISCs was retained, but the differentiation of ISCs may have been biased towards the EE fate commitment. Additionally, $GlcT^{\Delta 8}$, a previously characterized null allele of *GlcT* [23], exhibited a similar EE tumor phenotype (**Figure 1B-D**). Expressing a *UAS-GlcT* transgene in the mutant clones fully prevented the tumor phenotype (**Figure 1E, F**). Furthermore, depleting *GlcT* in progenitor cells using RNAi with *esg-GAL4*^{ts}also led to overproliferation of ISCs and excessive generation of EEs in the midgut epithelium (**Figure 1G-I**). Taken together, these data suggest that *GlcT* acts as a tumor suppressor in adult *Drosophila* midgut. Loss of *GlcT* induces ISC over-proliferation and excessive EE differentiation, resulting in an EE tumor or "neuroendocrine tumor (NET)" phenotype.

Genetic analysis of the GSL synthesis pathway components reveals specific tumor-suppressive activity for *egh*, in addition to *GlcT*

GlcT is involved in the initial step in the GSL biosynthesis pathway, which synthesizes glucosylceramide by transferring glucose to ceramide (**Figure 2A**). The loss of *GlcT* could result in the accumulation of ceramide or the absence of glucosylceramide and the downstream GSL metabolites. Ceramide accumulation has been linked to promoting cell apoptosis [24], which could potentially explain the ISC over-proliferation phenotype since apoptotic cells can induce compensatory ISC proliferation [25]. However, we did not observe a significant increase in apoptotic cells within the mutant clones (**Figure 2B**). Moreover, the overexpression of p35, an inhibitor of cell apoptosis, failed to suppress or alleviate the tumor phenotype (**Figure 2B**). Ceramidase (CDase) catalyzes ceramide into shingosine and free fatty acid, and overexpressing *CDase* can in theory downregulate ceramide levels. We therefore introduced *CDase* expression in the mutant clones, but this intervention also did not alleviate the tumor phenotype (**Figure 2B**). Hence, the EE tumors developed from *GlcT* mutant clones is unlikely caused by the accumulation of ceramide.

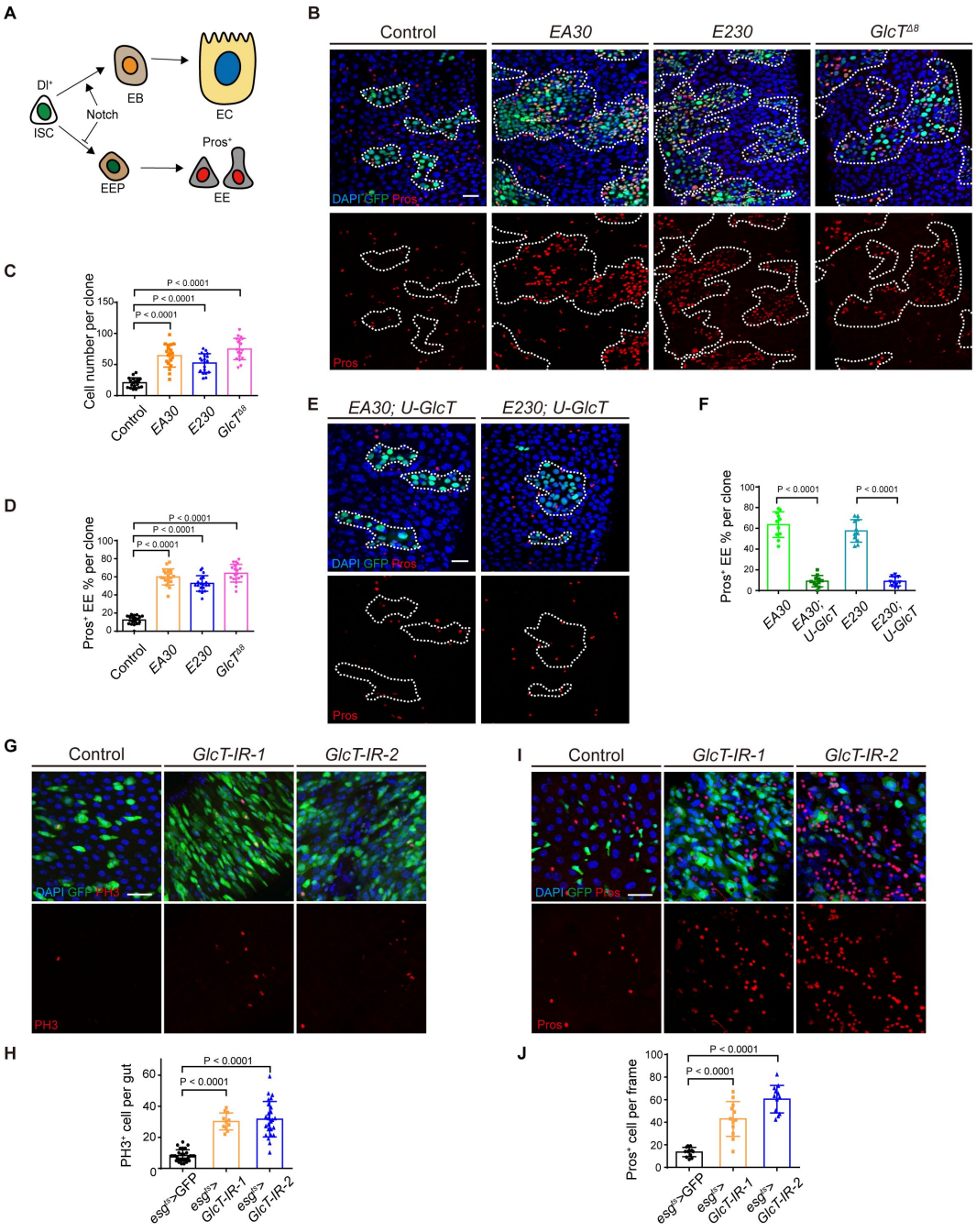

Figure 1.

A mosaic genetic screen identifies *GlcT* as a tumor suppressor in adult *Drosophila* midgut

(A) A diagram showing lineage hierarchy of intestinal stem cells (ISCs) in the *Drosophila* intestine. Abbreviations: EB, enteroblast; EEP, enteroendocrine cell; EC, enterocyte; EE, enteroendocrine cell. (B-D) Compared to control clones (green), the *EA30* and *E230* clones induced at day 5 are larger and contain a higher number of *Pros*$^{+}$ cells within each clone. *GlcT* null allele (*GlcT*$^{\Delta 8}$) mutant clones exhibit a similar phenotype. (E-F) Expression of *UAS-GlcT* in *EA30* or *E230* mutant clones rescues the tumor phenotype. (G-J) Depletion of *GlcT* in progenitor cells using RNAi with *esg-GAL4*^{ts} leads to ISC proliferation (G-H, stained with Phospho-Histone 3) and excessive EEC generation in the midgut (I-J). For G-J, flies were shifted to 29°C for 14 days. Error bars represent the mean ± SEM, with p-values indicated in the figure (two-tailed Student's t-test). Scale bars: 25 μm.

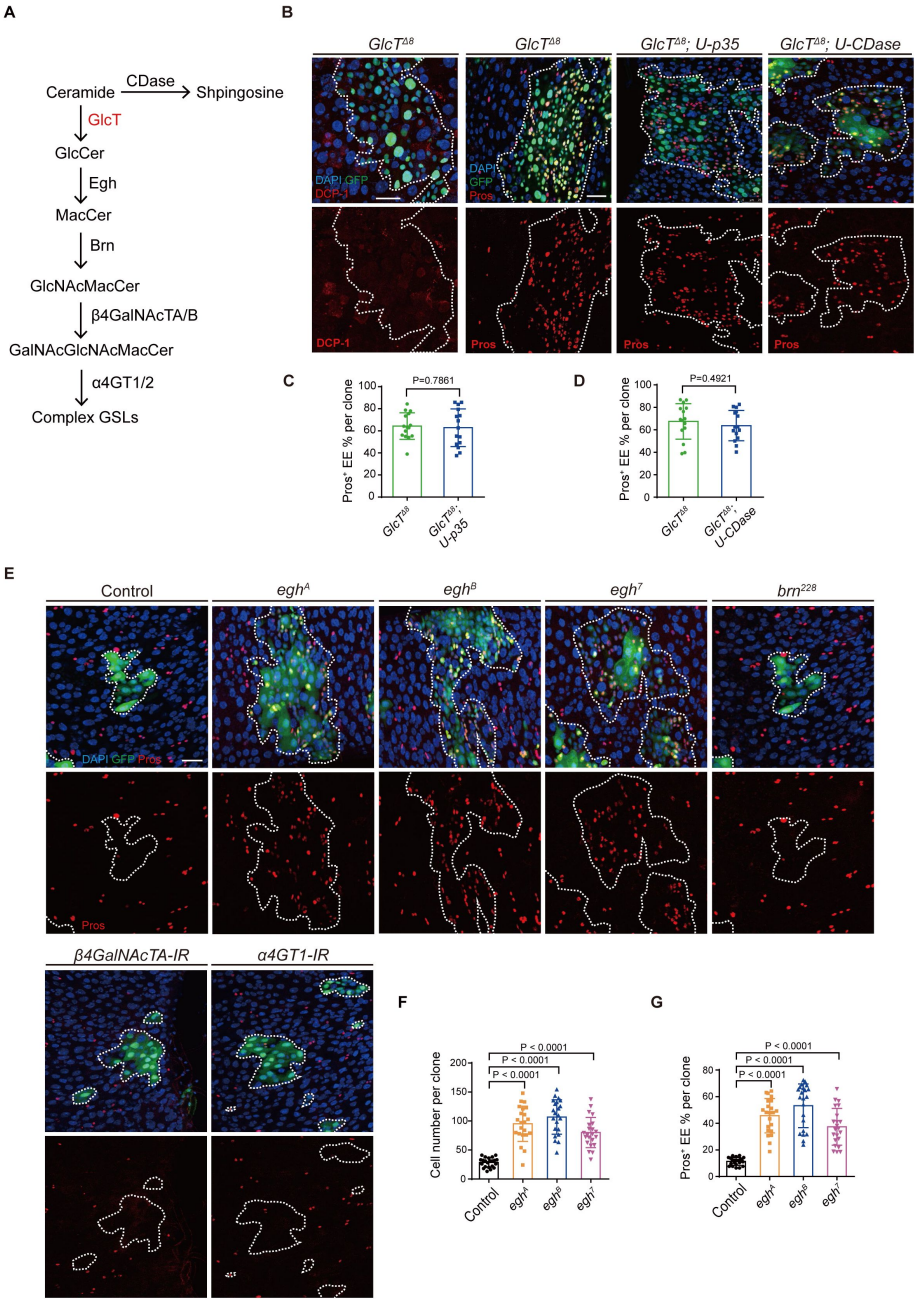

Figure 2.

Genetic analysis of the components of the GSL synthesis pathway reveals specific tumor-suppressive activity for *egh*, in addition to *GlcT*

(A) A diagram illustrating a portion of the metabolic pathways of glycosphingolipids (GSLs) synthesized from ceramide, highlighting the key enzymes involved at each step. (B) $GlcT^{\Delta 8}$ mutant clones without or with co-expression of *p35* or *CDase* stained with anti-DCP-1 or anti-Pros, as indicated. Clones were examined at day 7-10 after clone induction. (C-D) Quantitative analysis of the percentage of $Pros^{+}$ cells in clones of specified genotypes. (E) Genetic analysis of the key enzymes in the GSL synthesis pathway. Note that *egh* mutant clones (egh^{A}, egh^{B}, or egh^{7}) exhibited a phenotype of increased EEs similar to that observed in *GlcT* mutant clones, but Brn^{228}, *β4GalNAcTA-IR*, and *α4GT1-IR* clones did not. (F, G) Quantitative analysis of cell number (F) and the percentage of $Pros^{+}$ cells (G) in clones of specified genotypes. For E-G, clones were examined at day 7 after clone induction. Error bars represent the mean ± SEM, with p-values indicated in the figure (two-tailed Student's t-test). Scale bars: 25 μm.

In the GSL biosynthesis pathway in *Drosophila*, glucosylceramide undergoes sequential modifications catalyzed by Egghead (Egh) and Brainiac (Brn), which add the second (Mactosyl-) and third (GlcNAc-) glycosyl residues, respectively (**Figure 2A**) [26,27]. This leads to the synthesis of Mactosylceramide (MacCer) and GlcNAcβ1-3Manβ1-4Glcβ1Ceramide. Complex GSL biosynthesis involves the participation of β4-N-Acetylgalactosyltransferase (*β4GalNAcTA/TB*) and α1,4-Galactosyltransferase (*α4GT1/2*) in the fourth and fifth steps of GSL sugar chain elongation [28,29]. These enzymes catalyze the formation of GalNAcβ1-4GlcNAcβ1-3Manβ1-4Glcβ1Ceramide and GalNAc-α1-4GalNAcβ1-4GlcNAcβ1-3Manβ1-4Glcβ1Ceramide, respectively.

To understand the consequences of mutations in these GSL biosynthesis pathway genes, we generated mutant MARCM clones for each of the above-mentioned genes and examined their effects. Interestingly, mutations in *egh*, assessed using three different loss-of-function alleles, consistently resulted in cell over-proliferation and excessive EE phenotypes, similar to those caused by the loss of *GlcT* (**Figure 2E-G**). In contrast, none of the mutant clones of *brn*, *β4GalNAcTA*, or *α4GT1* exhibited any noticeable abnormalities in clone size or percentages of EEs, when compared to the control group of wild-type clones (**Figure 2E-G**). Therefore, GlcT and Egh, but not other enzymes in the GSL biosynthesis pathway, exhibit specific tumor suppressive activity in the adult *Drosophila* midgut.

The EEC tumor phenotype is a result of MacCer deficiency

As Egh is necessary for the biosynthesis of MacCer, the specific phenotype resulted from the loss GlcT and Egh but not Brn could be attributed to the absence of MacCer. To test this hypothesis, we fed flies carrying *GlcT* mutant clones with Lactosylceramide (LacCer), an analog of MacCer that functions similarly to MacCer in mammalian cells. Remarkably, LacCer significantly suppressed the overgrowth and excessive EE phenotype in the anterior midgut clones (**Figure 3B, C**). The suppression effect was also significant, though relatively mild, in the posterior midgut (**Figure 3B, C**). This difference may be attributed to substantial absorption or metabolism of LacCer prior to its entry into the posterior midgut. Therefore, among the various GSL metabolites, MacCer and LacCer appear to possess specific tumor-suppressive activities in the *Drosophila* midgut.

Loss of *GlcT* leads to reduced activation of Notch signaling in ISC progenies

In adult *Drosophila* midgut, the proliferation and differentiation of ISCs are regulated by multiple signaling pathways. Among these, the EGFR/Ras/MAPK and JAK/STAT signaling pathways play significant roles in promoting ISC proliferation during intestinal renewal and regeneration following tissue damage or infection [30,31]. Additionally, Notch signaling not only regulates ISC proliferation but also controls fate decisions between EE/EC during ISC differentiation [19,32,33]. Loss of Notch leads to increased ISC proliferation and excessive EE generation, phenotype similar to those caused by *GlcT* mutations. Therefore, we examined whether the loss of *GlcT* affects the activities of these signaling pathways.

Normally, among the intestinal cells in the midgut epithelium, progenitor cells including ISCs and enteroblasts, exhibit low but specific activation of EGFR and JAK/STAT activities [30,31,34]. The growth of *GlcT* mutant clones appeared to cause a general increase of EGFR and JAK/STAT activities within the intestinal epithelium, as many differentiated cells also exhibited EGFR or JAK/STAT signaling activation (Supplementary Figure 2), which is likely due to stress responses induced by tumor growth [35]. Nevertheless, we observed that pERK, a direct indicator of EGFR/Ras/MAPK signaling activity, was not significantly increased in *GlcT* mutant cells compared to cells outside the clones (Supplementary Figure 2). Similarly, the nuclear expression of STAT92E, which reflects JAK/STAT pathway activation, did not show significant changes in *GlcT* mutant cells compared to normal cells outside the clones (Supplementary Figure 2). However, the expression of *NRE-lacZ*, a reporter for Notch pathway activation, was specifically down-regulated in *GlcT* mutant

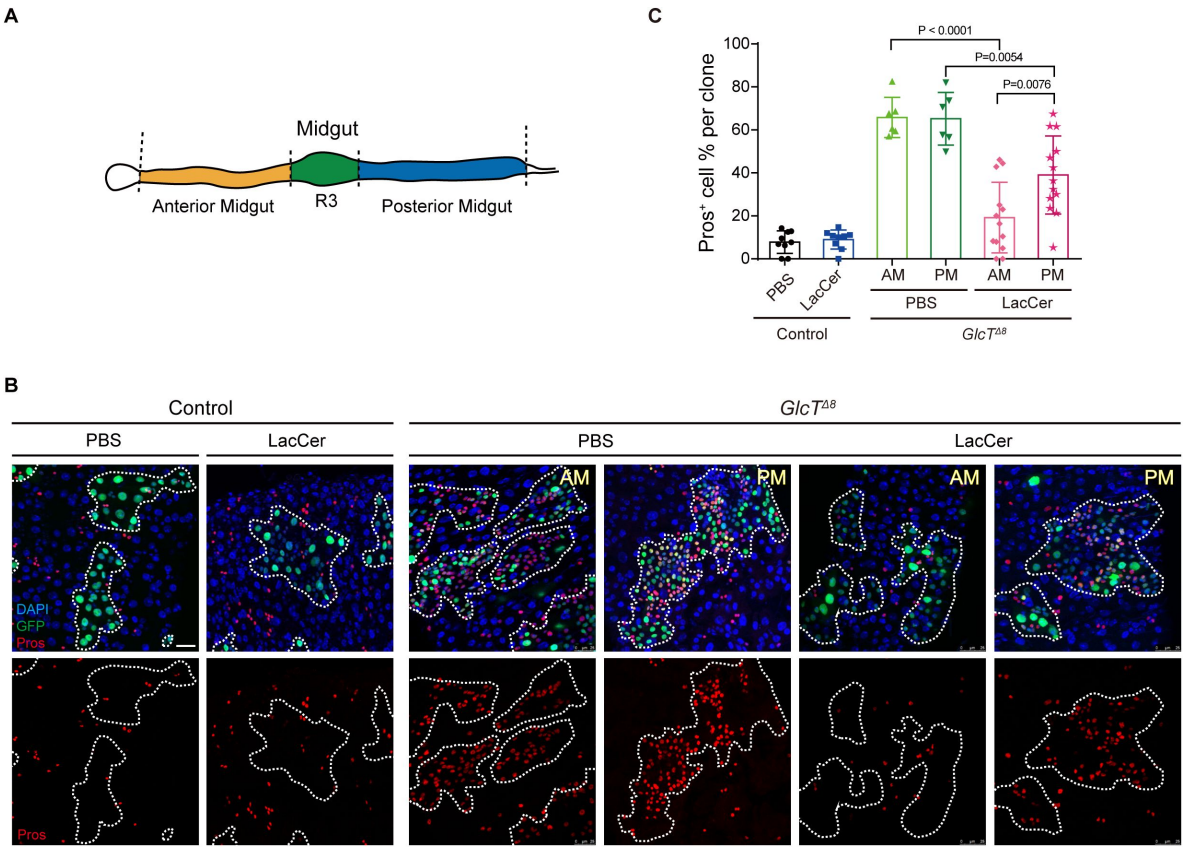

Figure 3.

The EE tumor phenotype is a result of MacCer deficiency

(A) A diagram showing the major compartments along the length of the *Drosophila* midgut, including the anterior (yellow), middle (Region 3, R3, green), and posterior midgut (blue). (B-C) Feeding *GlcT* mutant flies with LacCer alleviated the overgrowth and excessive EE phenotype, which was observed in clones of both the anterior and posterior midgut. Note that the suppressive effect was more pronounced in the anterior midgut compared to the posterior midgut. Error bars represent the mean ± SEM, with p-values indicated in the figure (two-tailed Student's t-test). Scale bar: 25 μm.

clones. Most cells within the clones exhibited lower LacZ expression levels compared to *NRE-lacZ* positive cells outside the clones (**Figure 4A, B**). Previous studies have shown that while the loss of function of Notch completely blocks EC differentiation from ISCs, a reduction in Notch activity does not necessarily hinder EC differentiation from ISCs. Instead, it may lead to a dose-dependent increase in the ratio of differentiated EEs to ECs, depending on the severity of Notch loss [19]. Therefore, the increased EE to EC ratio and the reduced NRE-lacZ expression observed in *GlcT* mutant clones collectively suggest that the mutant phenotype may be caused by a reduction in Notch signaling activity.

In the ISC lineage, Notch signaling not only guides EC/EE fate decisions, but is also participated in regulating EE subtype diversity. Typically, each EEP undergo one round of asymmetric cell division to yield a pair of distinctive EEs, an allatostatin C $(AstC)^+$ class I EE and a tachykinin $(Tk)^+$ class II EE [10]. The loss of *Notch* specifically compromises Mirr-dependent specification of class II EE generation, resulting in all EEs in *Notch* mutant clones becoming $AstC^+$ class I EEs [16,36]. We found that in *GlcT* mutant clones, virtually all EEs were positive for AstC but TK (**Figure 4C**), further supporting the idea that Notch signaling is compromised in these clones.

We next tested whether forced activation of Notch in *GlcT* mutant clones could suppress the EE tumor phenotype. To achieve this, we expressed N^{intra}, an active form of Notch, in *GlcT* mutant clones. As expected, this resulted in the complete suppression of cell proliferation in *GlcT* mutant clones and promoted the differentiation of these cells into ECs, leading all clones to become single-cell clones that often contained a polyploid cell (**Figure 4D**). This finding suggests that N^{intra} is epistatic to *GlcT* in the signaling transduction pathway, indicating that GlcT may regulate Notch signaling at the ligand/receptor level.

GlcT regulates the endocytic trafficking of Delta

The observations above collectively suggest that full activation of Notch signaling in the ISC lineage may require a specific GSL: Mac/Lac-Cer. As transmembrane proteins, the proper internalization and recycling of Dl and Notch are important for the proper activation of Notch signaling [37,38]. Specific lipids have been implicated in the formation of micro-domains on the cell membrane, which play a role in regulating cell-cell signaling [39]. Therefore, we asked whether the loss of MacCer caused by *GlcT* mutation could disrupt Notch signaling by affecting the stability, endocytosis, or recycling of either Dl or Notch.

Knocking down *GlcT* in ISCs using $Dl\text{-}GAL4^{ts}$ cased an increase in EEs in the midgut epithelium (**Figure 5 A, B**). However, when *GlcT* was specifically knocked down in EBs using $NRE\text{-}GAL4^{ts}$, there was no noticeable increase in EEs (**Figure 5 A, B**). This suggests that *GlcT* is specifically required in ISCs and implies its potential role on the regulation of Dl. It is well-known that the internalization of Dl in the signaling-sending cell is necessary for Notch activation in the signaling-receiving cell [37]. To investigate whether the endocytosis process of Dl is altered in *GlcT* mutant ISCs, we performed an *ex vivo* Dl internalization assay, following the previously described protocol [40]. In this assay, we cultured and incubated the freshly dissected gut with anti-Dl antibodies, and the endocytosis of the antibody-labeled Dl was visualized after fixation and immunostaining with secondary antibodies. Interestingly, during the 3-hour period, we observed a significant difference in the subcellular localization pattern of Dl between normal and *GlcT* mutant ISCs (**Figure 5 C**). In *GlcT* mutant ISCs, the majority of labeled Dl had already been internalized, while in wild-type ISCs, it remained on the cell membrane. This observation suggests that the loss of *GlcT* causes either reduced stability of Dl on the cell membrane or accelerated internalization of Dl from the cell membrane.

To further characterize the endocytic trafficking of Dl in *GlcT* mutant ISCs, we performed conditional depletion of *GlcT* and examined the co-localization of internalized Dl with the early endosome marker Rab5 and the late endosome marker Rab7, respectively. In comparison to control ISCs, *GlcT-RNAi* ISCs exhibited a significant increase in early endosomes containing Dl

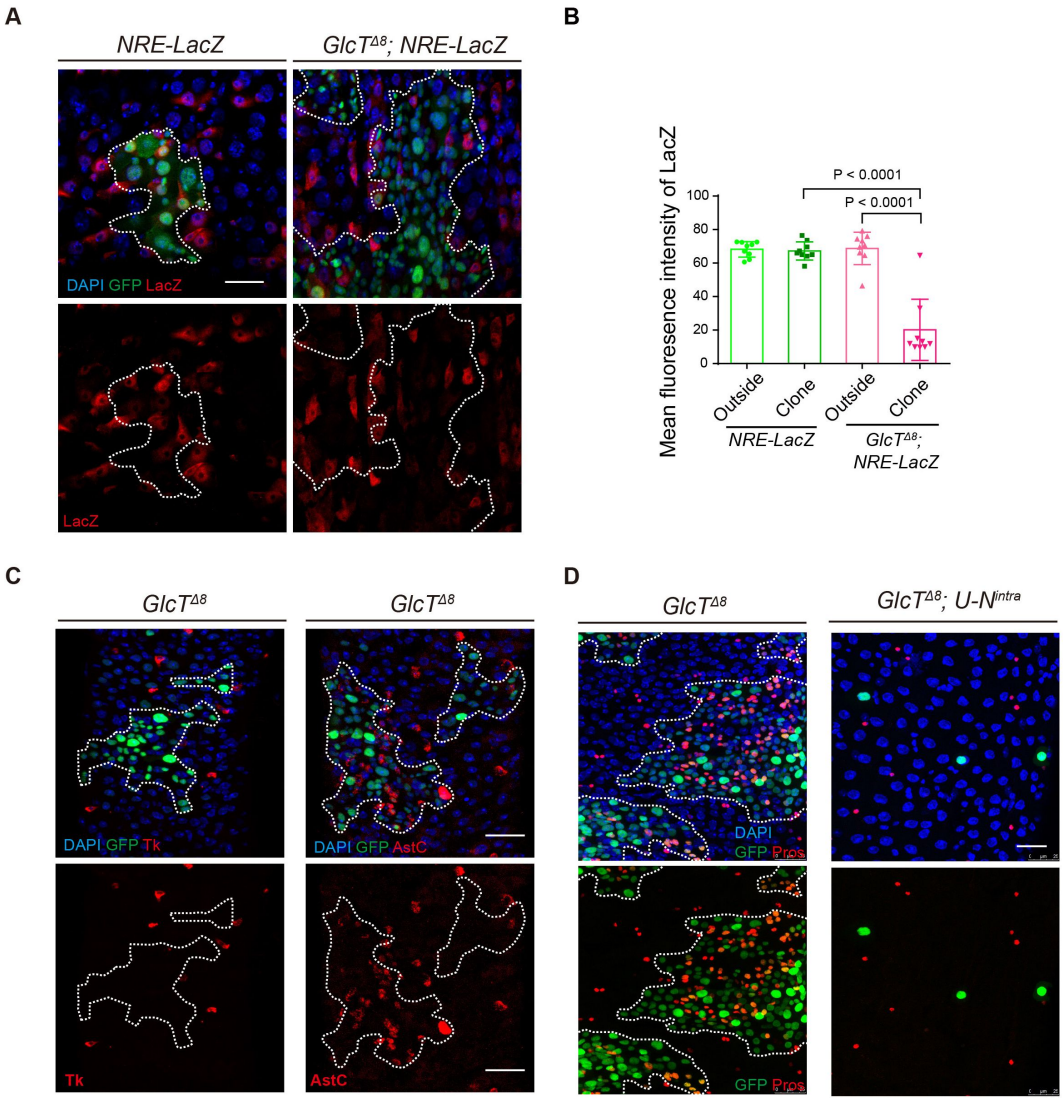

Figure 4.

Loss of *GlcT* leads to reduced activation of Notch signaling in ISC progenies

(A-B) The expression of *NRE-lacZ*, the Notch activity reporter, in *GlcT* mutant clones. Note that the fluorescence intensity of LacZ was significantly reduced within *GlcT* mutant clones compared to the cells outside of the clone. (C) *GlcT* mutant clones in the posterior (R5) region of the *Drosophila* midgut stained with anti-Tk (left) or anti-AstC (right). The two EE subtypes at R5 region are normally present in a 1:1 ratio. Note that $AstC^{+}$ EEs, but not TK^{+} EEs, were present in *GlcT* mutant clones. (D) The effect of forced activation of Notch in *GlcT* mutant clones by expressing N^{intra}. Error bars represent the mean ± SEM, with p-values indicated in the figure (two-tailed Student's t-test). Scale bars: 25 μm.

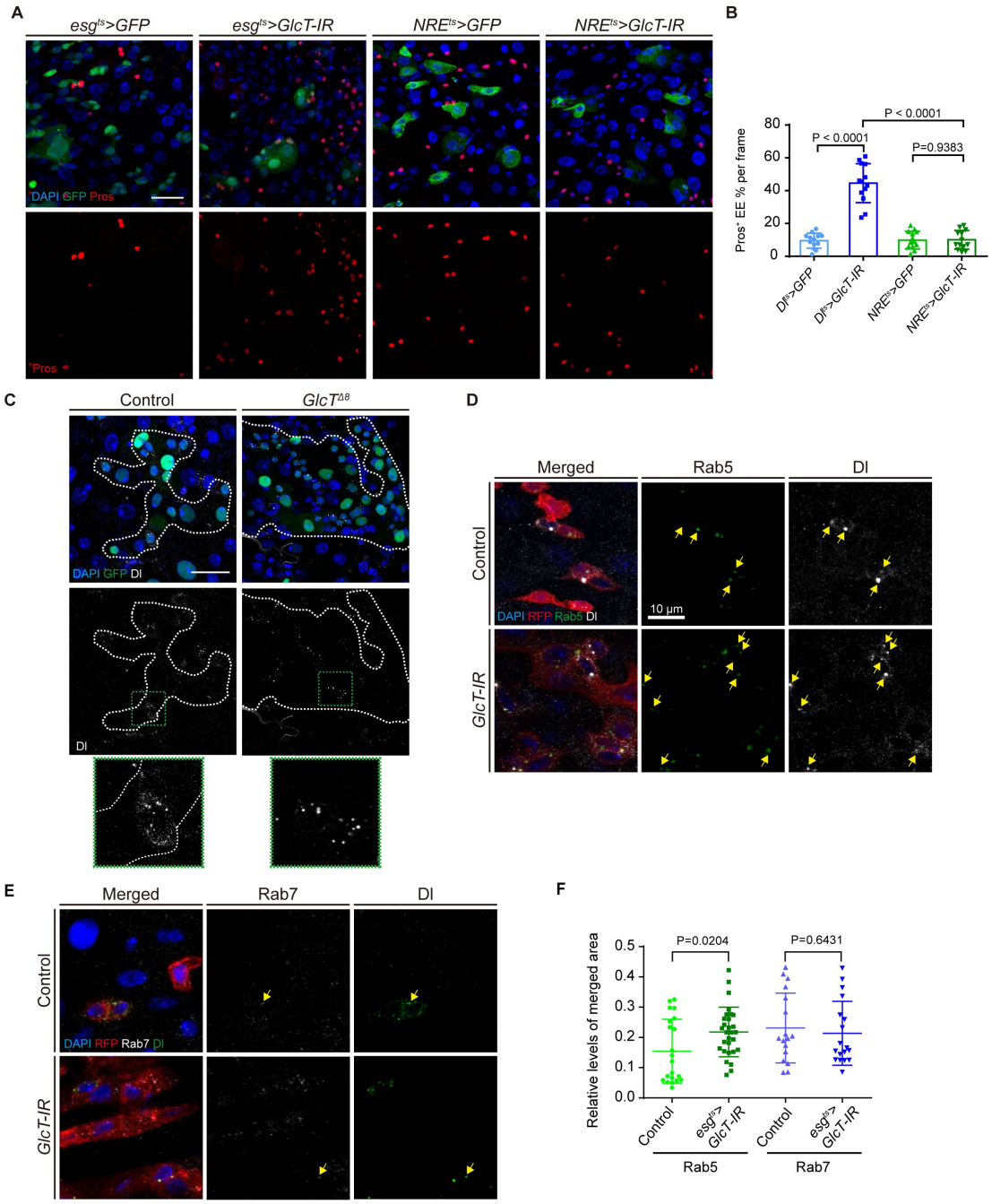

Figure 5.

GlcT regulates the endocytic trafficking of Delta

(A-B) Anti-Pros staining in flies carrying *GlcT-IR* driven by $Dl^{ts}$and NRE^{ts} respectively. Flies were shifted to 29°C for 14 days before analysis. (C) In a 3-hour Dl antibody uptake assay performed in the *Drosophila* midgut, Dl in ISCs of *GlcT* mutant clones (green box) showed reduced membrane localization and increased intracellular fluorescence compared to that in ISCs of control clones (see the enlarged inset boxes). (D, F) ISCs in esg^{ts}>*GlcT-RNAi* flies exhibited increased accumulation of Dl in early endosomes ($Rab5^{+}$, with co-localization indicated by yellow arrows). (E-F) The percentage of Dl^{+} late endosomes ($Rab7^{+}$) was largely unchanged. For D-F, flies were shifted to 29°C for 10 days before analysis. Co-localization analysis was performed using the intensity-based quantification feature in Leica Application Suite X (LAS X) software, with the Overlap Coefficient (ranging from 0 to 1) employed to quantify co-localization (1 indicating perfect co-localization and 0 indicating no co-localization). Error bars represent the mean ± SEM, with p-values indicated in the figure (two-tailed Student's t-test). Scale bar: 25 μm, unless otherwise noted.

(**Figure 5D, F**). However, the percentage of late endosomes containing Dl remained similar between wild-type and *GlcT-RNAi* ISCs (**Figure 5E, F**). These observations suggest that the internalized Dl in *GlcT-RNAi* ISCs experiences a delay in early endosomes, which might cause a delay in Dl recycling and consequently a reduction in Dl-Notch signaling activity (**Figure 7E**).

GlcT shows tissue specificity in regulating Notch signaling activity

Notch signaling has pleiotropic function and is known to be participated in the development of diverse tissue and organs. We therefore asked whether the discovered mechanism above is generally utilized in controlling Notch signaling in diverse tissues and organs. In the wing disc of third instar larvae, Notch signaling is activated by ligands Dl and Serrate at the dorsal-ventral boundary to induce the expression of Cut [41]. We found that in *GlcT* mutant clones spanning the dorsal-ventral boundary region of the wing disc, the expression of Cut was not significantly altered (**Figure 6A**). This indicates that *GlcT* is not essential for regulating Notch-mediated Cut expression in this specific context.

During oogenesis in the *Drosophila* ovary, Notch signaling regulates differentiation and transition of follicle cells from the mitotic cell cycle to the endocycle during stage 6 [42,43]. We found that in the developing egg chambers with germline mutations of *GlcT*, the patterning of the surrounding follicle cells remained largely normal (**Figure 6B**). However, we observed an abnormal accumulation of Dl protein in cytoplasmic vesicles of the mutant germline cells (**Figure 6C**), indicating that there is a defect in Dl trafficking in *GlcT* mutant germline cells. However, this defect is not sufficient to impact Dl-Notch signaling to a degree where follicle cell development is adversely affected. Collectively, these observations suggest that the role for GlcT in Notch signaling does not appear to be a universal mechanism across all developmental contexts. Even in situations where this mechanism is involved, the extent to which GlcT affects Dl-Notch signaling may vary in different developmental scenarios.

Transient loss of *UGCG* in mouse small intestine causes reduced number of ISCs and increased number of goblet cells

As both the Notch signaling and the metabolic pathway of GSLs are conserved from *Drosophila* to mammals, we asked whether this mechanism is conserved in the mouse small intestine to regulate the proliferation and differentiation of ISCs. Notch is known to control the absorptive versus secretory cell fate of mammalian intestinal progenitors as well, and the inhibition of Notch signaling in the intestinal epithelium of mouse small intestine leads excessive number of goblet cells [44,45]. However, unlike in *Drosophila*, Notch positively regulates ISC self-renewal in mice. The inhibition of Notch causes ISC loss, and the excessive activation of Notch promotes the proliferation of intestinal progenitor cells [45,46]. We therefore performed conditional knockout of *UGCG*, which encodes the mammalian glucosylceramide synthase, in the mouse small intestine and examined the consequences.

It has been previously reported that in the new born mice with intestine-specific knockout of *UGCG*, the proliferation of enterocytes appeared largely normal. However, 3-4 days after intestinal-specific knockout of UGCG in adult mice, epithelial phenotypes including epithelial hyperproliferation, impaired lipid absorption and the detachment of enterocytes from the basal lamina were observed, and these phenotypes are considered to be consequences of gut barrier disruption [47]. To separate the primary from the secondary phenotypes, we repeated the experiment by generating $UGCG^{flox/flox}$; *Vil-CreERT2* mice, and examined the phenotype at 48h post tamoxifen administration, an early timepoint with a hope that the secondary phenotypes have not yet developed.

Consistent with the previous findings, the conditional knockout mice showed a severe diarrhea phenotype and died several days following tamoxifen induction. Interestingly, we observed a few phenotypes at the 48h timepoint that was not observed before. We found that in the mutant

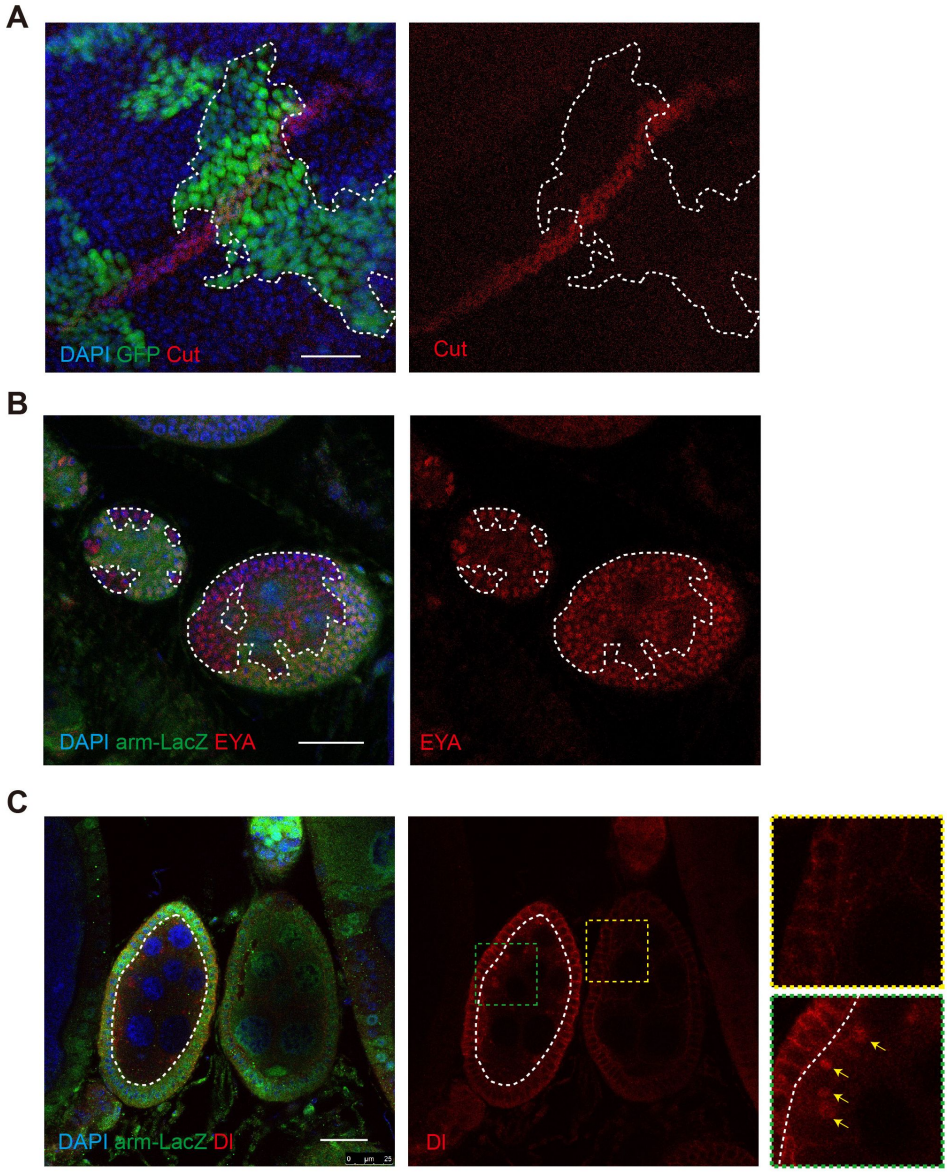

Figure 6.

GlcT shows tissue specificity in regulating Notch signaling activity

(A) The expression of Cut in a *GlcT* mutant clone (green area) in wing disc spanning the dorsal-ventral boundary. (B) The expression of EYA in *GlcT* mutant follicle cell clones (non-green areas). Note that there was no obvious difference in the level of EYA expression inside and outside of the clones. (C) Punctate accumulation of Dl was observed in *GlcT* mutant germ cell clones (non-green area, indicated by arrows in the enlarged inset), but not in the germline of a wild-type developing egg chamber (see enlarged inset with the dashed yellow line). Scale bars: 25 μm.

intestine, there was a marked increase in crypt depth accompanied by a marked reduction in villus length (Supplementary Figure 3). In addition, an excess population of goblet cells was observed, dispersed along the crypt-villus axis (**Figure 7C, D**). By examining the expression of Olfm4, which is transcriptionally regulated by Notch in ISCs [48], we found that the fluorescence intensity of Olfm4 staining was significantly reduced in *UGCG* mutant ISCs (**Figure 7A, B**). It is unclear how the crypt-villus morphology phenotype is developed in *UGCG* mutant epithelium, but the increased goblet cells and the reduced expression level of Olfm4 are consistent with a Notch phenotype, suggesting a conserved role for glucosylceramide synthase in modulating Notch signaling from *Drosophila* to mammals.

Discussion

From an unbiased genetic screen on the right arm of chromosome II, here we have pinpointed four mutant loci that cause an excessive EE phenotype in the intestinal epithelium. Three of them encode known components of the Notch signaling (*mam*, *Gmer*, and *O-fut1*), and the remaining one, *GlcT*, turns out to be a new Notch regulator as well. This work further reinforces the notion that Notch as the key regulator of the secretory versus absorptive cell fate decisions from ISCs, and reveals an evolutionarily conserved link between GSL metabolism and Notch signaling in regulating intestinal homeostasis.

As *GlcT* encodes glucosylceramide synthase, which that catalyzes the formation of glucosylceramide from ceramide, we further analyzed *egh* and *brn*, which encodes enzymes for the subsequent sequential modifications of glucosylceramide by adding the second (Mactosyl-) and third (GlcNAc-) glycosyl residues, respectively. We found that loss of *egh*, but not *brn*, can give rise to a similar excessive EE phenotype. In addition, the phenotype caused by *GlcT* can be suppressed by feeding with LacCer, which is analogous to MacCer. Collectively, these observations demonstrate that the excessive EE phenotype caused by *GlcT* or *egh* mutation is a result of MacCer deficiency.

GSLs constitute a specific category of glycolipids localized on the outer leaflet of eukaryotic cell membranes, and studies have implicated their roles in regulating multiple cellular signaling pathways by serving on the lipid raft to facilitate signaling transductions [49–52]. In particular, there is an evolutionarily conserved phospholipid binding domain (C2 domain) on the Notch ligand that is known to interacts with GSLs, and this interaction facilitates ligand-receptor interactions and thereby promotes robust Notch signaling [53–55]. Our analyses of *GlcT* mutant cells in the *Drosophila* midgut suggest that MacCer may represent a key member of GSLs in facilitating Dl-Notch signaling transduction between intestinal stem cells (ISCs) and their immediate progeny to steer binary cell fate decisions. To do so, MacCer may help to modulate the endocytic trafficking of Dl, an event that is known to be essential for Notch activation. We found that Dl on membrane of *GlcT* mutant ISCs tends be rapidly endocytosed and the resultant endosomes appear to experience a delay in transition from early endosome to late endosome. Prior genetic investigations have linked glycosphingolipids synthesized by α4GT1 to the modulation of Dl ligand activity i*n vivo*. While the loss of α4GT1 does not overtly affect Dl-Notch signaling, it can exacerbate the wing phenotype of haploinsufficient *Notch* mutants. Furthermore, the overexpression of α4GT1 has been shown to counteract signaling and endocytosis dysfunctions of Dl caused by the inhibition of the E3 ligase Mib1 or Neur activity [50]. Therefore, the robust genetic results presented in this study provide definitive evidence for a functional role of MacCer and its downstream derivatives in regulating Notch signaling. Future structural studies focusing on the interactions between GSLs and Dl should offer valuable insights into the role of GSLs in modulating Dl-Notch interactions and membrane trafficking processes.

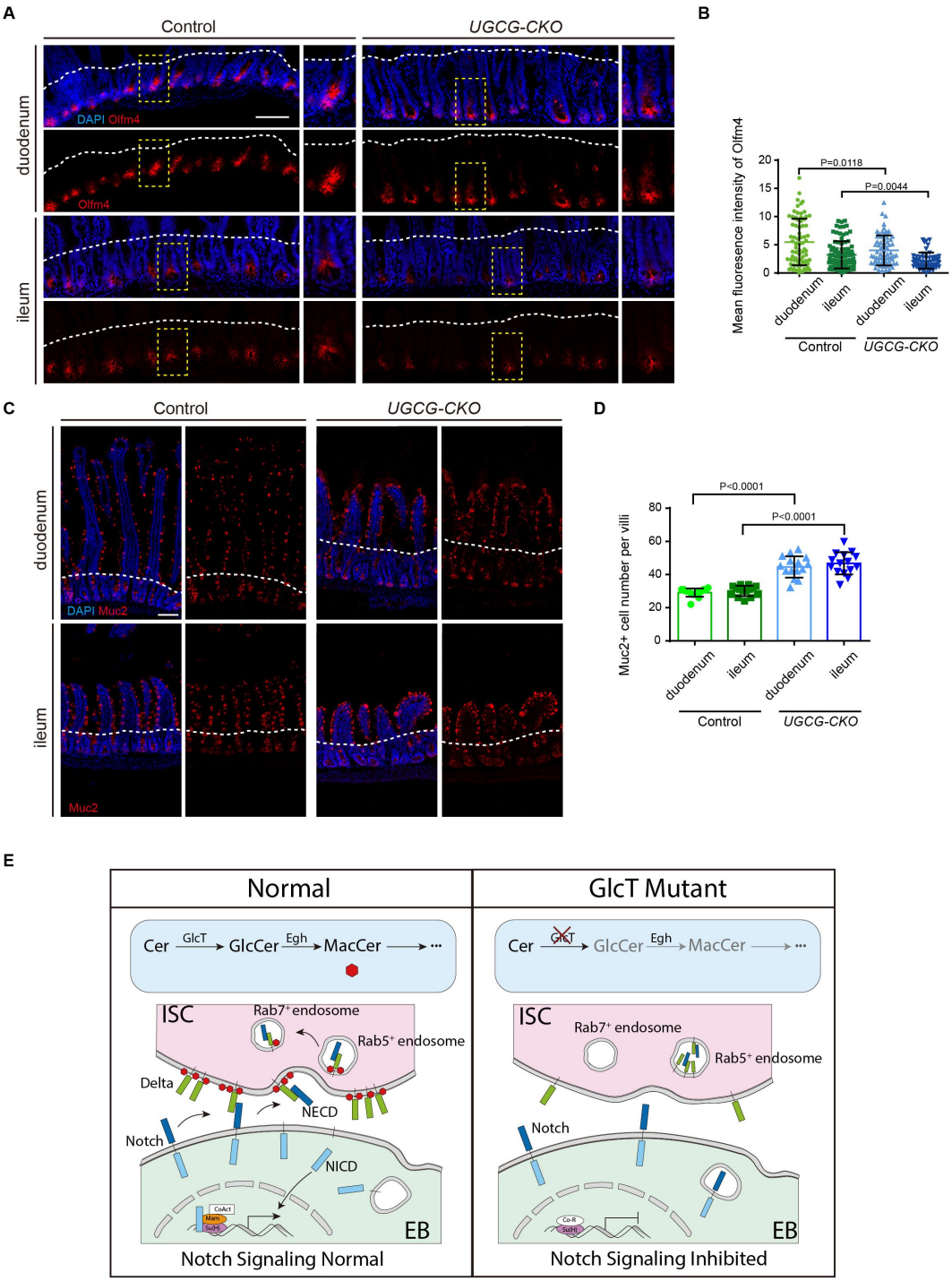

Figure 7.

Transient loss of *UGCG* in mouse small intestine causes reduced number of ISCs and increased number of goblet cells

(A-B) The expression of the Notch target gene O*lfm4* was downregulated in *villin-Cre*^{ERT2}, *UGCG*$^{flox/flox}$ mice 48 hours post-induction (n=3). The *CKO* mice displayed shorter villi (above the dotted line) and elongated crypts (below the dotted line). (C-D) The number of Muc2^{+} goblet cells was significantly increased in the *CKO* mice. (E) A schematic model for the role of MacCer in regulating the Notch signaling pathway. In signaling-sending cells, mutations in *GlcT* result in the loss of the downstream metabolite MacCer, leading to a rapid endocytosis of the membrane Dl and an accumulation of Dl in early endosomes. This causes a reduced level of Notch signaling activation in signaling-receiving cells. Error bars represent the mean ± SEM, with p-values indicated in the figure (two-tailed Student's t-test). Scale bars: 100 μm.

One intriguing observation from our study is the tissue-specific regulatory role of *GlcT* in Notch signaling. Its loss in ISCs significantly impairs Notch signaling, leading to the formation of EE tumors. However, when *GlcT* is lost in the germline cyst within the developing egg chamber, it only modestly disrupts the endocytic trafficking of Dl, without conspicuous effects on the differentiation and patterning of surrounding follicle cells. In the context of imaginal discs, the loss of *GlcT* appears to have no discernible impact on Notch signaling activity at all. This tissue-specific modulation of Notch signaling by *GlcT* suggests a potential variability in membrane lipid composition across different cell types. Such variability could render certain cells more susceptible to alterations in specific GSLs and their influence on Dl-Notch signaling transduction, while others remain unaffected. As Dl trafficking in *Drosophila* ISCs can be modulated by dietary lipid content, which impacts the duration and level of Notch activation to control the production of EEs in newly enclosed flies [18], the sensitivity of ISCs to specific GSL content may serve as a mechanism enabling ISCs to govern the fate of their progeny based on nutrient content.

Taken together, our study identified MacCer/LacCer as a tissue-specific regulator of Dl-Notch signaling by regulating the membrane localization and endocytic trafficking of Dl. As Notch signaling has pleiotropic roles in many developmental processes, diseases and cancer, the findings should contribute to our understanding of Notch signaling transduction and may help to open up new approaches for developing prevention and treatments against human diseases and cancer.

Materials and methods

Drosophila Stocks and mice

Drosophila melanogaster stocks were maintained on standard cornmeal molasses food in a 25°C incubator. Flies for all experiments were maintained at a constant 25°C with 15–25 flies per vial and tossed onto new food every 2–3 days. *Drosophila* stocks used in this study: *hsflp, act-GAL4, UAS-GFP; FRT42B tub-GAL80; hsflp, act-GAL4, UAS-GFP; FRT42D tub-GAL80;* and *hsflp, Tub-GAL80, FRT19A; act-GAL4,UAS-GFP/Cyo* [56]; *hsflp; FRT42D, arm-lacZ/Cyo; esg-GAL4, UAS-GFP* [56]; *Su(H)GBE (NRE)-GAL4;* and *Dl-Gal4* [57]; *Su(H)GBE-lacZ* (NRE-lacZ, gift from Sarah Bray); Glc^{EA30}(generated in this study); Glc^{E230}(generated in this study); $GlcT^{\Delta 8}$ [23]; $Tub\text{-}GAL80^{ts}$ [58]; *UAS-GlcT* (generated in this study); *GlcT-RNAi* #1 (VDRC, id:44912); *GlcT-RNAi* #2 (VDRC, id:108064); *UAS-CDase* (gift from Tao Wang); *UAS-p35* (BDSC, #6298); egh^{A} (BDSC, #52353); egh^{B} (BDSC, #52354); egh^{7} (BDSC, #77889); brn^{228} (BDSC, #7392); *β4GalNAcTA-RNAi* (VDRC, id:4867); *α4GT1-RNAi* (VDRC, id:2608); $UAS\text{-}N^{intra}$ [56]; *UAS-GFP-Rab5* (BDSC, #43336).

Mice used in this study include floxed *UGCG*, which was generated as previously described [47], and villin-CreERT2 [59]. All the research performance underwent strict ethical review and was approved by the Ethics Committee of the National Institute of Biological Sciences, Beijing. Mice were housed at the animal center in the National Institute of Biological Sciences in a Specific Pathogen Free (SPF) facility.

EMS Mutagenesis in *Drosophila*

3~5 day-old, isogenized y w; FRT42B(G13) males were subjected to a 10-hour starvation period before being fed with EMS (30 mM in sucrose solution applied on filter papers) overnight. Following recovery, these males were mated with y w; Sco/Cyo virgin females. In the F1 offspring, y w; FRT42B */Cyo males were individually mated with several y w; Sco/Cyo virgin females. The y w; FRT42B */Cyo males and virgin females in the F2 generation were carefully selected and crossed with each other to establish balanced stocks for further genetic studies. Altogether, around 10,000 stocks harboring lethal mutations were obtained and screened.

Deficiency Mapping

The complete Bloomington Deficiency Kit for chromosome 2R (*https://bdsc.indiana.edu/stocks/df/dfkit-info.html*) was used for initial mapping, which helped to localize both the EA30 and E230 lethal mutations to a cytogenetic location between 58A3 and 58F1. Subsequent mapping with additional deficiency lines encompassing this region allowed the identification of two small deficiency lines, Df(2R)Exel7170 and Df(2R)Excel6078, which failed to complement both mutations. This refinement narrowed the critical region down to 58B2-58C1. There are 13 protein-coding genes within this region, and genomic sequencing data indicated that both lines carried missense mutations on the *GlcT* gene, as described in the text.

Genomic Sequencing and Analysis Method

As all mutants exhibited lethality in the adult stage, homozygous larvae were selected using a green balancer, and the extracted genomic DNAs were sequenced at BGI Genomics using a library with an insert size of ≤800bp. The sequencing platform employed was the HiSeq 2000. For sequence analysis, all mutants were compared to a reference genome. Subsequently, sequencing reads were assigned to each allele based on heterozygote SNPs retained using a custom Perl script. The candidate heterozygous sites were annotated using snpEff.

Mosaic Analysis with a Repressible Cell Marker (MARCM) Analysis

To generate MARCM clones [60], flies of the desired genotype were raised and maintained at 25°C until they reached 7 days of age. Subsequently, they were subjected to a 60-minute heat shock in a water bath at 37°C. Following the heat shock, the flies were fed with standard food supplemented with yeast paste and this feeding regimen was repeated every 2 days before the flies were dissected for further analysis.

GAL4/GAL80^{ts} System

The GAL4/UAS and GAL80ts systems were utilized as previously described [58,61]. To inhibit the GAL4 system, the crosses were sustained at 18°C when employing temperature-sensitive GAL4-mediated RNAi or gene overexpression. Subsequently, F1 adult flies with the correct genotype were transitioned to 29°C to activate the GAL4 system, thereby triggering RNAi or gene overexpression.

Tamoxifen Treatment in Mice

Cre recombinase activity was induced via intraperitoneal injection of 2 mg tamoxifen (Sigma-Aldrich, T5648), and tissues were collected 48 hours post-injection. Cre-negative littermates served as controls.

Immunofluorescent Staining and Imaging

Adult female midguts were dissected in PBS and fixed for 30 minutes at room temperature in 4% paraformaldehyde. The fixed tissues were then dehydrated in methanol for 5 minutes followed by rehydration in a PBT solution (PBS containing 0.1% Triton X-100). The tissues underwent four washes with 0.1% Triton in 1×PBS (PBST) and were subsequently incubated with PBST and primary antibodies overnight at 4°C. After another round of washing, the samples were incubated for 2 hours with secondary antibodies in PBST. Finally, the samples were stained with DAPI for visualization of nuclei.

Mouse small intestine was dissected out and gently flushed with cold 1× PBS to remove fecal content, and fixed overnight at 4°C in 4% paraformaldehyde (PFA) prepared in 1× PBS. The tissue was then incubated in a 30% sucrose solution at 4°C for 24 hours, and embedded in OCT (Tissue-

Tek) before frozen. Frozen sections of 15 μm thickness were prepared for further analysis. For immunostaining, tissue sections were permeabilized with 1% Triton X-100 (Sigma-Aldrich) in PBS for 1 hour at room temperature (RT), followed by blocking with a solution containing 0.2% Triton X-100, 1% bovine serum albumin (BSA), and 3% goat serum in PBS for 1 hour at RT before antibody staining. The sections were mounted in Vectashield mounting media with DAPI (Vector Laboratories) for imaging.

Primary antibodies used in this study: mouse anti-Pros (DSHB #MR1A; 1:300); mouse anti-Dl (DSHB Cat#C594.9B; RRID:AB_528194; 1:300); rabbit anti-AstC (gift from Dr. Dick Nassel; 1:300); rabbit anti-Tk (gift from Dr. Jan-Adrianus Veenstra; 1:300); Rabbit anti-pH3 (CST Cat# 9701; RRID:AB_331535; 1:500); mouse monoclonal anti-β-galactosidase (DSHB, #40-1a; 1:30); mouse anti-Cut (DSHB, #2B10; 1:20); mouse anti-Rab7 (DSHB, 1:300); rabbit anti-pERK (CST, 1:200); rabbit anti-STAT92E (gift from Dr. Zhaohui Wang, 1:300); rabbit anti-DCP-1 (CST Cat#9578S; 1:300); mouse monoclonal anti-EYA (DSHB, #10H6; 1:300); rabbit monoclonal anti-GFP (Invitrogen Cat#G10362; 1:300). Secondary antibodies used in this study include Alexa Fluor 568- or Cy5-conjugated goat anti-rabbit, anti-mouse IgGs (Molecular Probes, A11034-A11036, A10524; 1:300). For nuclei staining, DAPI (Sigma-Aldrich, 1 μg/ml) was used. The preparations were mounted in 70% glycerol, and the slides were stored at -20°C. Imaging was conducted using a Leica SP8 confocal microscope. All captured images were processed and compiled using Adobe Photoshop and Illustrator.

LacCer Treatment

The 2-3 days old female flies of the specified genotype were moved to fresh food layered with a filter paper soaked in a 5% sucrose solution containing 20 μM of LacCer (Sigma-Aldrich CAS#4682-48-8) and this transfer was repeated daily before dissection and analysis.

Antibody Uptake Assay

The anti-Dl antibody Uptake assay was conducted following a previously described protocol [40]. In brief, the *Drosophila* gut was dissected and placed in fresh medium containing anti-Delta. The dissecting dish was then placed in a humidified box and incubated at 25 °C for the necessary duration. Subsequently, the medium containing the antibody was removed, and a single wash with Schneider's *Drosophila* medium was carried out. The midgut was then fixed directly in the dissecting dish for 20 minutes under a chemical fume hood by adding 1 ml of fixative. Following this, samples underwent three washes with PBT and were then incubated for 60 minutes with fluorescently labeled anti-mouse secondary antibodies in PBT on a rotating platform. After another three washes with PBT, the samples were mounted in 50% glycerol in PBS for analysis under microscopy.

Fluorescence Intensity Statistics

The Image J software (downloaded from *https://imagej.nih.gov/ij/*) was utilized to measure the fluorescence intensity of the captured images. The corrected fluorescence intensity is determined as the average fluorescence intensity of each cell's nuclear region minus the average background fluorescence intensity.

Statistical analysis

All quantifications were presented in the form of mean ± SEM. GraphPad Prism 5 software (GraphPad Software Inc.) was used to calculate p-values using an unpaired Student's t-test. The number of intestines used for calculations was labeled on the figures or in the related figure legends.

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Additional files

Supplementary Data. Supplementary Figure 1. Cross scheme for the mosaic genetic screen on the 2R chromosome. *(A) A schematic illustrating the principles of FLPase-induced mitotic recombination for generating homozygous mutant cells in heterozygous animals. (B) The cross scheme and workflow for the forward genetic mosaic screen conducted in this study. Abbreviation: EMS, ethyl methanesulfonate.* ***Supplementary Figure 2. The EGFR/Ras/MAPK and JAK/STAT signaling activities in GlcT mutant clones.*** *GlcT mutant clones stained with pERK (red, upper panels), a direct indicator of EGFR/Ras/MAPK signaling activity, and STAT92E (red, lower panels), whose nuclear localization is indicative of JAK/STAT signaling activity. Note that pERK was not significantly increased in GlcT mutant cells compared to normal cells outside of the clones, and STAT92E activity was also not obviously altered in GlcT mutant cells compared to normal cells outside the clones. Scale bar: 25 μm.* ***Supplementary Figure 3. The Crypt-Villus morphology in UGCG CKO mice. The duodenal structure exhibits significant abnormalities in CKO mice*** *Small intestine (duodenum) from control and villin-creERT2, UGCG$^{flox/flox}$ mice stained with K20, which marks enterocytes and goblet cells, and Ki67, which marks proliferative cells. Note that the mutant intestine has shortened villi and elongated crypts. Dotted lines indicate the boundary between the crypt and the villus. Samples were collected at 48h after tamoxifen injection. Scale bar: 100 μm.*

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Reviewer #1 (Public review):

Summary:

From a forward genetic mosaic mutant screen using EMS, the authors identify mutations in glucosylceramide synthase (GlcT), a rate-limiting enzyme for glycosphingolipid (GSL) production, that result in EE tumors. Multiple genetic experiments strongly support the model that the mutant phenotype caused by GlcT loss is due to by failure of conversion of ceramide into glucosylceramide. Further genetic evidence suggests that Notch signaling is comprised in the ISC lineage and may affect the endocytosis of Delta. Loss of GlcT does not affect wing development or oogenesis, suggesting tissue-specific roles for GlcT. Finally, an increase in goblet cells in UGCG knockout mice, not previously reported, suggests a conserved role for GlcT in Notch signaling in intestinal cell lineage specification.

Strengths:

Overall, this is a well-written paper with multiple well-designed and executed genetic experiments that support a role for GlcT in Notch signaling in the fly and mammalian intestine. I do, however, have a few comments below.

Weaknesses:

(1) The authors bring up the intriguing idea that GlcT could be a way to link diet to cell fate choice. Unfortunately, there are no experiments to test this hypothesis.

(2) Why do the authors think that UCCG knockout results in goblet cell excess and not in the other secretory cell types?

(3) The authors should cite other EMS mutagenesis screens done in the fly intestine.

(4) The absence of a phenotype using NRE-Gal4 is not convincing. This is because the delay in its expression could be after the requirement for the affected gene in the process being studied. In other words, sufficient knockdown of GlcT by RNA would not be achieved until after the relevant signaling between the EB and the ISC occurred. Dl-Gal4 is problematic as an ISC driver because Dl is expressed in the EEP.

(5) The difference in Rab5 between control and GlcT-IR was not that significant. Furthermore, any changes could be secondary to increases in proliferation.

https://doi.org/10.7554/eLife.106184.1.sa3

Reviewer #2 (Public review):

Summary:

This study genetically identifies two key enzymes involved in the biosynthesis of glycosphingolipids, GlcT and Egh, which act as tumor suppressors in the adult fly gut. Detailed genetic analysis indicates that a deficiency in Mactosyl-ceramide (Mac-Cer) is causing tumor formation. Analysis of a Notch transcriptional reporter further indicates that the lack of Mac-Ser is associated with reduced Notch activity in the gut, but not in other tissues.

Addressing how a change in the lipid composition of the membranes might lead to defective Notch receptor activation, the authors studied the endocytic trafficking of Delta and claimed that internalized Delta appeared to accumulate faster into endosomes in the absence of Mac-Cer. Further analysis of Delta steady-state accumulation in fixed samples suggested a delay in the endosomal trafficking of Delta from Rab5+ to Rab7+ endosomes, which was interpreted to suggest that the inefficient, or delayed, recycling of Delta might cause a loss in Notch receptor activation.

Finally, the histological analysis of mouse guts following the conditional knock-out of the GlcT gene suggested that Mac-Cer might also be important for proper Notch signaling activity in that context.

Strengths:

The genetic analysis is of high quality. The finding that a Mac-Cer deficiency results in reduced Notch activity in the fly gut is important and fully convincing.

The mouse data, although preliminary, raised the possibility that the role of this specific lipid may be conserved across species.

Weaknesses:

This study is not, however, without caveats and several specific conclusions are not fully convincing.

First, the conclusion that GlcT is specifically required in Intestinal Stem Cells (ISCs) is not fully convincing for technical reasons: NRE-Gal4 may be less active in GlcT mutant cells, and the knock-down of GlcT using Dl-Gal4ts may not be restricted to ISCs given the perdurance of Gal4 and of its downstream RNAi.

Second, the results from the antibody uptake assays are not clear.: i) the levels of internalized Delta were not quantified in these experiments; ii) additionally, live guts were incubated with anti-Delta for 3hr. This long period of incubation indicated that the observed results may not necessarily reflect the dynamics of endocytosis of antibody-bound Delta, but might also inform about the distribution of intracellular Delta following the internalization of unbound anti-Delta. It would thus be interesting to examine the level of internalized Delta in experiments with shorter incubation time. Overall, the proposed working model needs to be solidified as important questions remain open, including: is the endo-lysosomal system, i.e. steady-state distribution of endo-lysosomal markers, affected by the Mac-Cer deficiency? Is the trafficking of Notch also affected by the Mac-Cer deficiency? is the rate of Delta endocytosis also affected by the Mac-Cer deficiency? are the levels of cell-surface Delta reduced upon the loss of Mac-Cer?

Third, while the mouse results are potentially interesting, they seem to be relatively preliminary, and future studies are needed to test whether the level of Notch receptor activation is reduced in this model.

https://doi.org/10.7554/eLife.106184.1.sa2

Reviewer #3 (Public review):

Summary:

In this paper, Tang et al report the discovery of a Glycoslyceramide synthase gene, GlcT, which they found in a genetic screen for mutations that generate tumorous growth of stem cells in the gut of Drosophila. The screen was expertly done using a classic mutagenesis/mosaic method. Their initial characterization of the GlcT alleles, which generate endocrine tumors much like mutations in the Notch signaling pathway, is also very nice. Tang et al checked other enzymes in the glycosylceramide pathway and found that the loss of one gene just downstream of GlcT (Egh) gives similar phenotypes to GlcT, whereas three genes further downstream do not replicate the phenotype. Remarkably, dietary supplementation with a predicted GlcT/Egh product, Lactosyl-ceramide, was able to substantially rescue the GlcT mutant phenotype. Based on the phenotypic similarity of the GlcT and Notch phenotypes, the authors show that activated Notch is epistatic to GlcT mutations, suppressing the endocrine tumor phenotype and that GlcT mutant clones have reduced Notch signaling activity. Up to this point, the results are all clear, interesting, and significant. Tang et al then go on to investigate how GlcT mutations might affect Notch signaling, and present results suggesting that GlcT mutation might impair the normal endocytic trafficking of Delta, the Notch ligand. These results (Fig X-XX), unfortunately, are less than convincing; either more conclusive data should be brought to support the Delta trafficking model, or the authors should limit their conclusions regarding how GlcT loss impairs Notch signaling. Given the results shown, it's clear that GlcT affects EE cell differentiation, but whether this is via directly altering Dl/N signaling is not so clear, and other mechanisms could be involved. Overall the paper is an interesting, novel study, but it lacks somewhat in providing mechanistic insight. With conscientious revisions, this could be addressed. We list below specific points that Tang et al should consider as they revise their paper.

Strengths:

The genetic screen is excellent.

The basic characterization of GlcT phenotypes is excellent, as is the downstream pathway analysis.

Weaknesses:

(1) Lines 147-149, Figure 2E: here, the study would benefit from quantitations of the effects of loss of brn, B4GalNAcTA, and a4GT1, even though they appear negative.

(2) In Figure 3, it would be useful to quantify the effects of LacCer on proliferation. The suppression result is very nice, but only effects on Pros+ cell numbers are shown.

(3) In Figure 4A/B we see less NRE-LacZ in GlcT mutant clones. Are the data points in Figure 4B per cell or per clone? Please note. Also, there are clearly a few NRE-LacZ+ cells in the mutant clone. How does this happen if GlcT is required for Dl/N signaling?

(4) Lines 222-225, Figure 5AB: The authors use the NRE-Gal4ts driver to show that GlcT depletion in EBs has no effect. However, this driver is not activated until well into the process of EB commitment, and RNAi's take several days to work, and so the author's conclusion is "specifically required in ISCs" and not at all in EBs may be erroneous.

(5) Figure 5C-F: These results relating to Delta endocytosis are not convincing. The data in Fig 5C are not clear and not quantitated, and the data in Figure 5F are so widely scattered that it seems these co-localizations are difficult to measure. The authors should either remove these data, improve them, or soften the conclusions taken from them. Moreover, it is unclear how the experiments tracing Delta internalization (Fig 5C) could actually work. This is because for this method to work, the anti-Dl antibody would have to pass through the visceral muscle before binding Dl on the ISC cell surface. To my knowledge, antibody transcytosis is not a common phenomenon.

(6) It is unclear whether MacCer regulates Dl-Notch signaling by modifying Dl directly or by influencing the general endocytic recycling pathway. The authors say they observe increased Dl accumulation in Rab5+ early endosomes but not in Rab7+ late endosomes upon GlcT depletion, suggesting that the recycling endosome pathway, which retrieves Dl back to the cell surface, may be impaired by GlcT loss. To test this, the authors could examine whether recycling endosomes (marked by Rab4 and Rab11) are disrupted in GlcT mutants. Rab11 has been shown to be essential for recycling endosome function in fly ISCs.

(7) It remains unclear whether Dl undergoes post-translational modification by MacCer in the fly gut. At a minimum, the authors should provide biochemical evidence (e.g., Western blot) to determine whether GlcT depletion alters the protein size of Dl.

(8) It is unfortunate that GlcT doesn't affect Notch signaling in other organs on the fly. This brings into question the Delta trafficking model and the authors should note this. Also, the clonal marker in Figure 6C is not clear.

(9) The authors state that loss of UGCG in the mouse small intestine results in a reduced ISC count. However, in Supplementary Figure C3, Ki67, a marker of ISC proliferation, is significantly increased in UGCG-CKO mice. This contradiction should be clarified. The authors might repeat this experiment using an alternative ISC marker, such as Lgr5.

https://doi.org/10.7554/eLife.106184.1.sa1

Author response:

We would like to express our gratitude to all three reviewers for their time and valuable feedback on the manuscript. Below, we provide our point-by-point responses to their comments. Additionally, we summarize here the experiments we plan to conduct in accordance with the reviewers' suggestions:

Revision plan 1. To include live imaging of Dl/Notch trafficking in normal and *GlcT* mutant ISCs.

We agree that the effect of *GlcT* mutation on Dl trafficking was not convincingly demonstrated in our previous work. Although we attempted live imaging of the intestine using GFP tagged at the C-terminal of Dl, the fluorescent signal was regrettably too weak for reliable capture. In this revision, we will optimize the imaging conditions to determine if this issue can be resolved. Alternatively, we will transiently express GFP/RFP-tagged Dl in both normal and mutant ISCs to investigate the trafficking dynamics through live imaging.

Revision plan 2. To update and improve the presentation of the data regarding the features of early/late/recycling endosomes in *GlcT* mutant ISCs.

Our analysis of Rab5 and Rab7 endosomes in both normal and *GlcT* mutant ISCs revealed that Dl tends to accumulate in Rab5 endosomes in *GlcT* mutant ISCs. To strengthen our findings, we will include additional quantitative data and conduct further analysis on recycling endosomes labeled with Rab11-GFP. We acknowledge that this portion of the data is not entirely convincing, and in accordance with the reviewers' suggestions, we will revise our conclusions to present a more tempered interpretation.

Revision plan 3. To include western blot analysis of Dl in normal and *GlcT* mutant ISCs.

While we propose that MacCer may function as a component of lipid rafts, facilitating the anchorage of Dl on the membrane and its proper endocytosis, it is also possible that it acts as a substrate for the modification of Dl, which is essential for its functionality. To investigate this further, we will conduct Western blot analysis to determine whether the depletion of *GlcT* alters the protein size of Dl.

Please find our detailed point-by-point responses below.

> ***Public Reviews:***
>
> ***Reviewer #1 (Public review):***
>
> *Summary:*
>
> *From a forward genetic mosaic mutant screen using EMS, the authors identify mutations in glucosylceramide synthase (GlcT), a rate-limiting enzyme for glycosphingolipid (GSL) production, that result in EE tumors. Multiple genetic experiments strongly support the model that the mutant phenotype caused by GlcT loss is due to by failure of conversion of ceramide into glucosylceramide. Further genetic evidence suggests that Notch signaling is comprised in the ISC lineage and may affect the endocytosis of Delta. Loss of GlcT does not affect wing development or oogenesis, suggesting tissue-specific roles for GlcT. Finally, an increase in goblet cells in UGCG knockout mice, not previously reported, suggests a conserved role for GlcT in Notch signaling in intestinal cell lineage specification.*
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> *Strengths:*
>
> *Overall, this is a well-written paper with multiple well-designed and executed genetic experiments that support a role for GlcT in Notch signaling in the fly and mammalian intestine. I do, however, have a few comments below.*
>
> *Weaknesses:*
>
> *(1) The authors bring up the intriguing idea that GlcT could be a way to link diet to cell fate choice. Unfortunately, there are no experiments to test this hypothesis.*

We indeed attempted to establish an assay to investigate the impact of various diets (such as high-fat, high-sugar, or high-protein diets) on the fate choice of ISCs. Subsequently, we

intended to examine the potential involvement of GlcT in this process. However, we observed that the number or percentage of EEs varies significantly among individuals, even among flies with identical phenotypes subjected to the same nutritional regimen. We suspect that the proliferative status of ISCs and the turnover rate of EEs may significantly influence the number of EEs present in the intestinal epithelium, complicating the interpretation of our results. Consequently, we are unable to conduct this experiment at this time. The hypothesis suggesting that GlcT may link diet to cell fate choice remains an avenue for future experimental exploration.

> *(2) Why do the authors think that UCCG knockout results in goblet cell excess and not in the other secretory cell types?*

This is indeed an interesting point. In the mouse intestine, it is well-documented that the knockout of Notch receptors or Delta-like ligands results in a classic phenotype characterized by goblet cell hyperplasia, with little impact on the other secretory cell types. This finding aligns very well with our experimental results, as we noted that the numbers of Paneth cells and enteroendocrine cells appear to be largely normal in UGCG knockout mice. By contrast, increases in other secretory cell types are typically observed under conditions of pharmacological inhibition of the Notch pathway.

> *(3) The authors should cite other EMS mutagenesis screens done in the fly intestine.*

To our knowledge, the EMS screen on 2L chromosome conducted in Allison Bardin's lab is the only one prior to this work, which leads to two publications (Perdigoto et al., 2011; Gervais, et al., 2019). We will include citations for both papers in the revised manuscript.

> *(4) The absence of a phenotype using NRE-Gal4 is not convincing. This is because the delay in its expression could be after the requirement for the affected gene in the process being studied. In other words, sufficient knockdown of GlcT by RNA would not be achieved until after the relevant signaling between the EB and the ISC occurred. Dl-Gal4 is problematic as an ISC driver because Dl is expressed in the EEP.*

We agree that the lack of an observable phenotype using NRE-Gal4 might be attributed to a delay in its expression, which could result in missing the critical window necessary for effective *GlcT* knockdown. Consequently, we cannot rule out the possibility that *GlcT* may also play a role in early EBs or EEPs. We will revise our manuscript to present a more cautious conclusion on this issue.

> *(5) The difference in Rab5 between control and GlcT-IR was not that significant. Furthermore, any changes could be secondary to increases in proliferation.*

We agree that it is possible that the observed increase in proliferation could influence the number of Rab5+ endosomes, and we will temper our conclusions on this aspect accordingly. However, it is important to note that, although the difference in Rab5+ endosomes between the control and GlcT-IR conditions appeared mild, it was statistically significant and reproducible. As we have indicated earlier, we plan to further analyze Rab11+ endosomes, as this additional analysis may provide further support for our previous conclusions.

> ***Reviewer #2 (Public review):***
>
> *Summary:*
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As previously mentioned, we acknowledge that a role for GlcT in early EBs or EEPs cannot be completely ruled out. We will revise our manuscript to present a more cautious conclusion and explicitly describe this possibility in the updated version.

> *Second, the results from the antibody uptake assays are not clear.: i) the levels of internalized Delta were not quantified in these experiments; ii) additionally, live guts were incubated with anti-Delta for 3hr. This long period of incubation indicated that the observed results may not necessarily reflect the dynamics of endocytosis of antibody-bound Delta, but might also inform about the distribution of intracellular Delta following the internalization of unbound anti-Delta. It would thus be interesting to examine the level of internalized Delta in experiments with shorter incubation time.*

We thank the reviewer for these excellent questions. In our antibody uptake experiments, we noted that Dl reached its peak accumulation after a 3-hour incubation period. We recognize that quantifying internalized Dl would enhance our analysis, and we will include the corresponding statistical graphs in the revised version of the manuscript. In addition, we agree that during the 3-hour incubation, the potential internalization of unbound anti-Dl cannot be ruled out, as it may influence the observed distribution of intracellular Dl. To address this concern, we plan to supplement our findings with live imaging experiments to capture the dynamics of Dl endocytosis in GlcT mutant ISCs.

> *Overall, the proposed working model needs to be solidified as important questions remain open, including: is the endo-lysosomal system, i.e. steady-state distribution of*

> *endo-lysosomal markers, affected by the Mac-Cer deficiency? Is the trafficking of Notch also affected by the Mac-Cer deficiency? is the rate of Delta endocytosis also affected by the Mac-Cer deficiency? are the levels of cell-surface Delta reduced upon the loss of Mac-Cer?*

Regarding the impact on the endo-lysosomal system, this is indeed an important aspect to explore. While we did not conduct experiments specifically designed to evaluate the steady-state distribution of endo-lysosomal markers, our analyses utilizing Rab5-GFP overexpression and Rab7 staining did not indicate any significant differences in endosome distribution in MacCer deficient conditions. Moreover, we still observed high expression of the NRE-LacZ reporter specifically at the boundaries of clones in *GlcT* mutant cells (Fig. 4A), indicating that GlcT mutant EBs remain responsive to Dl produced by normal ISCs located right at the clone boundary. Therefore, we propose that MacCer deficiency may specifically affect Dl trafficking without impacting Notch trafficking.

In our 3-hour antibody uptake experiments, we observed a notable decrease in cell-surface Dl, which was accompanied by an increase in intracellular accumulation. These findings collectively suggest that Dl may be unstable on the cell surface, leading to its accumulation in early endosomes.

> *Third, while the mouse results are potentially interesting, they seem to be relatively preliminary, and future studies are needed to test whether the level of Notch receptor activation is reduced in this model.*

In the mouse small intestine, olfm4 is a well-established target gene of the Notch signaling pathway, and its staining provides a reliable indication of Notch pathway activation. While we attempted to evaluate Notch activation using additional markers, such as Hes1 and NICD, we encountered difficulties, as the corresponding antibody reagents did not perform well in our hands. Despite these challenges, we believe that our findings with Olfm4 provide an important start point for further investigation in the future.

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> *Weaknesses:*
>
> *(1) Lines 147-149, Figure 2E: here, the study would benefit from quantitations of the effects of loss of brn, B4GalNAcTA, and a4GT1, even though they appear negative.*

We will incorporate the quantifications for the effects of the loss of *brn*, *B4GalNAcTA*, and *a4GT1* in the updated Figure 2.

> *(2) In Figure 3, it would be useful to quantify the effects of LacCer on proliferation. The suppression result is very nice, but only effects on Pros+ cell numbers are shown.*

We will add quantifications of the number of EEs per clone to the updated Figure 3.

> *(3) In Figure 4A/B we see less NRE-LacZ in GlcT mutant clones. Are the data points in Figure 4B per cell or per clone? Please note. Also, there are clearly a few NRE-LacZ+ cells in the mutant clone. How does this happen if GlcT is required for Dl/N signaling?*

In Figure 4B, the data points represent the fluorescence intensity per single cell within each clone. It is true that a few NRE-LacZ+ cells can still be observed within the mutant clone; however, this does not contradict our conclusion. As noted, high expression of the NRE-LacZ reporter was specifically observed around the clone boundaries in MacCer deficient cells (Fig. 4A), indicating that the mutant EBs can normally receive Dl signal from the normal ISCs located at the clone boundary and activate the Notch signaling pathway. Therefore, we believe that, although affecting Dl trafficking, MacCer deficiency does not significantly affect Notch trafficking.

> *(4) Lines 222-225, Figure 5AB: The authors use the NRE-Gal4ts driver to show that GlcT depletion in EBs has no effect. However, this driver is not activated until well into the process of EB commitment, and RNAi's take several days to work, and so the author's conclusion is "specifically required in ISCs" and not at all in EBs may be erroneous.*

As previously mentioned, we acknowledge that a role for *GlcT* in early EBs or EEPs cannot be completely ruled out. We will revise our manuscript to present a more cautious conclusion and describe this possibility in the updated version.

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We thank the reviewer for these insightful comments and suggestions. In our in vivo experiments, we observed increased co-localization of Rab5 and Dl in *GlcT* mutant ISCs, indicating that Dl trafficking is delayed at the transition to Rab7^{+} late endosomes, a finding that is further supported by our antibody uptake experiments. We acknowledge that the data presented in Fig. 5C are not fully quantified and that the co-localization data in Fig. 5F may appear somewhat scattered; therefore, we will include additional quantification and enhance the data presentation in the revised manuscript.

Regarding the concern about antibody internalization, we appreciate this point. We currently do not know if the antibody reaches the cell surface of ISCs by passing through the visceral muscle or via other routes. Given that the experiment was conducted with fragmented gut, it is possible that the antibody may penetrate into the tissue through mechanisms independent of transcytosis.

As mentioned earlier, we plan to supplement our findings with live imaging experiments to investigate the dynamics of Dl/Notch endocytosis in both normal and *GlcT* mutant ISCs. Anyway, due to technical challenges and potential pitfalls associated with the assays, we agree that this part of data is not fully convincing and we will provide a more cautious conclusion in the revised manuscript.

> *(6) It is unclear whether MacCer regulates Dl-Notch signaling by modifying Dl directly or by influencing the general endocytic recycling pathway. The authors say they observe increased Dl accumulation in Rab5+ early endosomes but not in Rab7+ late endosomes upon GlcT depletion, suggesting that the recycling endosome pathway, which retrieves Dl back to the cell surface, may be impaired by GlcT loss. To test this, the authors could examine whether recycling endosomes (marked by Rab4 and Rab11) are disrupted in GlcT mutants. Rab11 has been shown to be essential for recycling endosome function in fly ISCs.*

We agree that assessing the state of recycling endosomes, especially by using markers such as Rab11, would be valuable in determining whether MacCer regulates Dl-Notch signaling by directly modifying Dl or by influencing the broader endocytic recycling pathway. We will incorporate these experiments into our future experimental plans to further characterize Dl trafficking in *GlcT* mutant ISCs.

> *(7) It remains unclear whether Dl undergoes post-translational modification by MacCer in the fly gut. At a minimum, the authors should provide biochemical evidence (e.g., Western blot) to determine whether GlcT depletion alters the protein size of Dl.*

While we propose that MacCer may function as a component of lipid rafts, facilitating Dl membrane anchorage and endocytosis, we also acknowledge the possibility that MacCer could serve as a substrate for protein modifications of Dl necessary for its proper function. Conducting biochemical analyses to investigate potential post-translational modifications of Dl by MacCer would indeed provide valuable insights. To address this, we will incorporate Western blot analysis into our experimental plan to determine whether *GlcT* depletion affects the protein size of Dl.

> *(8) It is unfortunate that GlcT doesn't affect Notch signaling in other organs on the fly. This brings into question the Delta trafficking model and the authors should note this. Also, the clonal marker in Figure 6C is not clear.*

In the revised working model, we will explicitly specify that the events occur in intestinal stem cells. Regarding Figure 6C, we will delineate the clone with a white dashed line to enhance its clarity and visual comprehension.

> *(9) The authors state that loss of UGCG in the mouse small intestine results in a reduced ISC count. However, in Supplementary Figure C3, Ki67, a marker of ISC proliferation, is significantly increased in UGCG-CKO mice. This contradiction should be clarified. The authors might repeat this experiment using an alternative ISC marker, such as Lgr5.*

Previous studies have indicated that dysregulation of the Notch signaling pathway can result in a reduction in the number of ISCs. While we did not perform a direct quantification of ISC numbers in our experiments, our olfm4 staining—which serves as a reliable marker for ISCs—demonstrates a clear reduction in the number of positive cells in UGCG-CKO mice.

The increased Ki67 signal we observed reflects enhanced proliferation in the transit-amplifying region, and it does not directly indicate an increase in ISC number. Therefore, in UGCG-CKO mice, we observe a decrease in the number of ISCs, while there is an increase in transit-amplifying (TA) cells (progenitor cells). This increase in TA cells is probably a secondary consequence of the loss of barrier function associated with the UGCG knockout.

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