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ADAM10 Regulates Notch Function in Intestinal Stem Cells of Mice

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Abstract

BACKGROUND & AIMS—ADAM10 is a cell surface sheddase that regulates physiological processes including Notch signaling. ADAM10 is expressed in all intestinal epithelial cell types but the requirement for ADAM10 signaling in crypt homeostasis is not well defined.

METHODS—We analyzed intestinal tissues from mice with constitutive (Vil-Cre;Adam10^{f/f} mice) and conditional (Vil-CreER;Adam10^{f/f} and Lgr5-CreER;Adam10^{f/f} mice) deletion of ADAM10. We performed cell lineage tracing experiments in mice that expressed a gain-of-function allele of Notch in the intestine (Rosa26^{NICD}) or mice with intestine-specific disruption of

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Disclosures

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None.

Author Contributions

Y-H.T, K. L.V, C.K, S.R, R.G.B and P.J.D performed experiments and prepared data, E.T.S, A.S, A.W.W, H.C.C and P.J.D performed characterization of *Adam10^{f/f}* mice, I.M provided reagents, Y-H.T, K.L.V, I.M, L.C.S, H.C.C and P.J.D. analyzed data and edited manuscript, P.J.D wrote the manuscript and supervised the project.

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Notch (*Rosa26^{DN-MAML}*), to examine the effects of ADAM10 deletion on cell fate specification and intestinal stem cell maintenance.

RESULTS—Loss of ADAM10 from developing and adult intestine caused lethality associated with altered intestinal morphology, reduced progenitor cell proliferation, and increased secretory cell differentiation. ADAM10 deletion led to the replacement of intestinal cell progenitors with 2 distinct, post-mitotic, secretory cell lineages: intermediate-like (Paneth/goblet) and enteroendocrine cells. Based on analysis of *Rosa26^{NICD}* and *Rosa26^{DN-MAML}* mice, we determined that ADAM10 controls these cell fate decisions by regulating Notch signaling. Cell lineage tracing experiments showed that ADAM10 is required for survival of *Lgr5⁺* crypt-based columnar cells. Our findings indicate that Notch-activated stem cells have a competitive advantage for occupation of the stem cell niche.

CONCLUSIONS—ADAM10 acts in a cell autonomous manner within the intestinal crypt compartment to regulate Notch signaling. This process is required for progenitor cell lineage specification and crypt-based columnar cell maintenance.

Keywords

CBC; intestinal epithelium; development; differentiation

INTRODUCTION

The intestinal epithelium undergoes continuous renewal to preserve tissue integrity. Wnt and Notch signaling have distinct roles in controlling proliferation and cell lineage specification within the crypt compartment that must be precisely integrated to maintain intestinal homeostasis¹. Cell lineage tracing studies have shown that Notch signaling is active in multipotent intestinal stem cells (ISCs)^{2–4} and that Notch activation during intestinal development leads to increased proliferation and amplification of intestinal progenitors⁵. Notch stimulation of this mitogenic response requires functional Wnt signaling⁶. Our previous studies demonstrated that Notch directly targets the *Lgr5⁺* crypt base columnar cell (CBC) and is required for stem cell proliferation and survival⁷. However, the mechanism of Notch regulation and the extent to which Notch contributes to ISC replenishment within the crypt stem cell niche has not been clearly defined.

Notch controls cell fate decisions of short-lived, bipotent progenitors by regulating the key transcription factor *Atoh1*⁶. Notch1 and Notch2 receptors and delta-like (Dll) 1 and Dll4 ligands control these events^{8,9}. Upon Notch activation, *Atoh1* expression is repressed in progenitors which drives differentiation into the enterocyte lineage¹⁰. Conversely, in the absence of Notch signaling, progenitors express *Atoh1* and are fated into the secretory lineage. *Atoh1* target genes such as *Gfi1*, *Spdef* and *Neurog3* are responsible for later specification events in the secretory lineage^{11–14}. Some evidence suggests that goblet and Paneth cells have a shared lineage⁷ but it is unclear how multipotent secretory progenitors are allocated and give rise to the major secretory cell types.

Canonical Notch receptor signaling is controlled by sequential processing, which requires extracellular (S2) cleavage by an α -secretase followed by intramembrane (S3) cleavage by a

presenilin-dependent γ -secretase to release the Notch intracellular domain (NICD)¹. The disintegrin-metalloproteinase ADAM10 was proposed to be a candidate Notch α -secretase because ADAM10^{-/-} mice show an embryonic lethal phenotype that resembles Notch-deficient mice¹⁵. More recent analysis of conditional ADAM10-deficient mice and studies using transformed ADAM10^{-/-} mouse embryonic fibroblasts have shown that ADAM10 is required for ligand-induced Notch activation during development¹⁶. However, the absolute dependency of Notch signaling on ADAM10 *in vivo* remains controversial, as other ADAMs (e.g. ADAM17) and metalloproteinases (e.g. MMP7) have been implicated in Notch activation within other contexts^{16–18}. Here, using *loss-of-function* studies, we show that ADAM10 is required for Notch activation in the intestine and reveal that cell-autonomous ADAM10 signaling is essential for cell lineage specification and intestinal stem cell survival. Our findings also suggest a competitive advantage for Notch-activated stem cells to replenish the stem cell compartment.

EXPERIMENTAL PROCEDURES

Mice

All animal procedures were approved by the UCUCA at University of Michigan. The following mouse strains were used: *Adam10*^f¹⁹, *Villin-Cre*²⁰, *Villin-CreERT2*²⁰, *Lgr5-EGFP-ires-CreERT2*²¹, *Rosa26*^{YFP22}, *Atoh1*^{LacZ23}, *Neurog3*^{EYFP24}, *Rosa26*^{NICD-ires-nEGFP25} and *Rosa26*^{DNMAML-GFP26}.

Protocols for all procedures are provided in Supplementary Materials

RESULTS

ADAM10 inactivation leads to reduced viability, altered intestinal morphology and loss of progenitor cell proliferation

ADAM10 is abundantly expressed on the basolateral surface of all epithelial cell types in the intestine, including *Lgr5*⁺ CBCs (Figure 1A and Supplementary Figure 1A). To determine the role of ADAM10 in developing and adult intestine, we analyzed constitutive *Villin-Cre;Adam10*^{f/f} (termed *Vil-Cre;Adam10*^{f/f}) and tamoxifen (TX)-inducible *Villin-CreERT2;Adam10*^{f/f} (termed *Vil-CreER;Adam10*^{f/f}) mice. *Vil-Cre;Adam10*^{f/f} pups were born at the correct Mendelian frequency, had normal body weights and intestinal length, however, no ADAM10-deficient pups survived beyond post-natal day 1 (Supplementary Figure 1B–D, Supplementary Table 1, data not shown). Because of this perinatal lethality, we used TX-inducible *Vil-CreER;Adam10*^{f/f} mice to examine the effect of ADAM10 loss in the adult intestine. Adult *Vil-CreER;Adam10*^{f/f} mice treated with TX (100 mg/kg *i.p.*) for 5 consecutive days achieved near complete ADAM10 deletion from intestinal epithelium (Supplementary Figure 1E–F). These mice rapidly lost weight, became moribund and did not survive beyond 7–9 days from the initial TX treatment.

Histological analysis of the small intestine from newborn *Vil-Cre;Adam10*^{f/f} mice revealed that the epithelium was less cellular and villi were blunted with more goblet cells (Figure 1B). Importantly, the intervillus zone (IVZ) showed a marked reduction in the number of proliferating cells with only a few Ki67⁺ cells located at the villus boundary (Figure 1B).

Similar morphological and proliferative changes were observed in TX-treated adult *Vil-CreER;Adam10^{ff}* mice with a significant reduction in BrdU⁺ cells throughout the crypt (Figure 1C). This was associated with a marked increase in active caspase-3 staining (Supplementary Figure 1F), indicating that apoptosis accompanied the loss of cell proliferation. These results demonstrate that loss of ADAM10 in either the immature or adult intestinal epithelium leads to diminished viability associated with altered intestinal morphology and reduced proliferation.

ADAM10 deficiency leads to increased secretory cell differentiation

Further investigation into the differentiation status of newborn small intestine from *Vil-Cre;Adam10^{ff}* mice revealed dramatic increases in secretory cell marker expression for goblet, (PAS/AB⁺, Muc2⁺), Paneth (MMP7⁺, lysozyme⁺) and enteroendocrine (chromogranin A, CHGA⁺) cells (Figure 2A, data not shown). Analogous increases in secretory cell differentiation were found in TX-treated adult *Vil-CreER;Adam10^{ff}* mice, but here, an expanded crypt compartment was observed in which the mid/upper crypt regions were completely filled with differentiated secretory cells (Figure 2D). Conversely, the enterocyte marker, alkaline phosphatase, was markedly reduced in both ADAM10-deficient models (data not shown). Morphometric and qPCR analyses confirmed the dramatic increase in secretory cell differentiation observed in both ADAM10-deficient models (Figure 2B,C,E,F). Together, these results indicate that ADAM10 plays an important role in cell fate specification of the small intestine and that ADAM10 loss leads to increased secretory cell differentiation.

ADAM10 deficiency produces distinct intermediate-like (Paneth/goblet) and endocrine cell populations

In γ -secretase inhibitor (GSI)-treated mice, we previously showed that increased intestinal secretory cell differentiation is associated with the appearance of distinct intermediate-like cells that co-express Paneth and goblet cell markers⁷. Similarly, in both ADAM10-deficient models, co-staining for MMP7 and MUC2 detected intermediate-like cells throughout the intestine, including the colon (Figure 2 and Supplementary Figure 2A). In newborn *Vil-Cre;Adam10^{ff}* mice, the intermediate-like cells were restricted to the IVZ (Figure 2A) whereas in TX-treated adult *Vil-CreER;Adam10^{ff}* mice, the intermediate-like cells were located primarily in the mid/upper crypt region. Ultrastructural analysis confirmed that mature Paneth cells were present at the crypt base. By contrast, the intermediate-like cells located in the mid-crypt region of ADAM10-deficient mice showed ultrastructural features of both Paneth and goblet cells, including mucus granules and variably-sized electron-dense granules characteristic of Paneth cells (Supplementary Figure 2B). Co-staining of the endocrine-specific marker CHGA with MMP7 showed that the intermediate-like cells did not overlap with endocrine cells in either ADAM10-deficient model (Figure 2A,D). Together, these data demonstrate that crypt progenitor cells are fated towards distinct intermediate-like (Paneth/goblet) or endocrine cell lineages in the absence of ADAM10 signaling.

ADAM10 controls Notch signaling associated with cell fate decisions

Notch signaling controls cell lineage specification in the intestine. In the absence of Notch signaling, the default programming of transit-amplifying (TA) progenitors is towards secretory cell differentiation¹. The similarity of this intestinal phenotype with that observed in ADAM10-deficient mice led us to examine whether ADAM10 directly regulates Notch signaling. In both ADAM10-deficient models, gene expression analysis showed no significant changes in *Notch1*, *Notch2*, *Dll-1* and *Dll-4* mRNA levels suggesting that expression of Notch signaling components was not compromised (data not shown). However, qPCR analysis revealed a 2-fold reduction in expression of Notch target genes *Hes1* and *HeyL* (Figures 3A,D and Supplementary Figure 3A). Consistent with a loss of Notch signaling, IHC analysis confirmed that nuclear Hes1 staining was completely absent from the IVZ of newborn *Vil-Cre;Adam10^{fl/fl}* mice (Supplementary Figure 3B). Moreover, examination of Notch downstream effectors revealed a dramatic increase in the expression of key transcription factors required for secretory lineage specification, including *Atoh1*, *Gfi1*, *Spdef*, *Neurog3* and *Sox9*. (Figure 3A,D). To examine *Atoh1* and *Neurog3* expression at a cellular level, *Atoh1^{lacZ}* and *Neurog3^{YFP}* reporter mice^{23,24}, respectively, were bred to both ADAM10-deficient models. Consistent with the expansion of secretory cell lineages, abundant X-gal staining representing *Atoh1*⁺ cells was observed throughout the IVZ and crypt compartment (Figure 3B,E). Similarly, more *Neurog3*⁺ cells representing early endocrine progenitors were found in newborn and adult intestine that partially co-localized with the endocrine marker, CHGA. Moreover, the expanded *Neurog3*⁺ cell population was distinct from *MUC2*⁺-expressing intermediate cells (Figure 3C,F and Supplementary Figure 3B,C). Together, these results demonstrate that Notch and its downstream signaling events are compromised after ADAM10 inactivation in both developing and adult intestine.

Notch is the dominant signaling pathway regulated by ADAM10

To verify that Notch signaling is the principal pathway controlled by ADAM10 in the intestine, we tested whether over-expression of constitutively active Notch could override ADAM10 deficiency. For this experiment, we used the Notch gain-of-function allele *Rosa26^{NICD-ires-nGFP}* (termed *Rosa26^{NICD}*), in which cDNAs encoding the mouse Notch1-ICD and nuclear GFP have been targeted to the *Rosa26* locus²⁵. Consistent with previous reports, *Vil-Cre;Rosa26^{NICD/+}* mice displayed a loss of secretory cell differentiation⁵. Importantly, the compound *Vil-Cre;Adam10^{fl/fl};Rosa26^{NICD/+}* mice, despite the loss of ADAM10 (data not shown), had an intestinal phenotype that was indistinguishable from *Vil-Cre;Rosa26^{NICD/+}* mice, indicating that activated Notch could override the loss of ADAM10 (Figure 4A). Next, we examined the Notch loss-of-function allele, *Rosa26^{DNMAML/+}*, in which a cDNA encoding a GFP-tagged dominant-negative Mastermind-like construct is targeted to the *Rosa26* locus²⁶. In newborn *Vil-Cre;Rosa26^{DNMAML/+}* mice, co-staining of cell lineage markers revealed the appearance of intermediate-like cells within the IVZ and increased goblet and endocrine cell populations that closely resembled the phenotype found in *Vil-Cre;Adam10^{fl/fl}* mice (Figure 4A and Supplementary Figure 4A). Furthermore, progenitor cell proliferation and nuclear Hes1 immunostaining mirrored the changes in cellular differentiation observed in the different Notch mutants (Figure 4B and Supplementary Figure 4B,C). Similar results were obtained

when the same Notch mutants were examined in the adult intestine using the TX-inducible *Vil-CreER* line (Supplementary Fig 5). Together, these results demonstrate that Notch signaling is the dominant pathway regulated by ADAM10 in the developing and adult intestine.

Mosaic ADAM10 deletion produces crypt degeneration that can be rescued by activated Notch

The pronounced changes in intestinal differentiation and rapid morbidity observed upon ADAM10 deletion prevented long-term fate analysis of adult ADAM10-deficient ISCs. To circumvent this problem, the TX treatment was modified to produce a mosaic pattern of ADAM10 recombination within crypts (Supplementary Figure 6). *Vil-CreER;Adam10^{ff}* mice given a single TX dose (100 mg/kg, *i.p.*) displayed reduced ADAM10 deletion but animals still lost weight, became moribund and had to be sacrificed at day 9 (Figure 5A). Histological evaluation of the small intestine revealed striking changes, with villus atrophy and crypt degeneration (Figure 5B). All degenerating crypts were ADAM10-deficient, had reduced proliferation and numerous apoptotic cells were detected within the gut lumen and in degenerating crypts (Figure 5C and data not shown). By contrast, adjacent wild-type crypts contained tall, pseudostratified epithelial cells that were ADAM10⁺ and highly proliferative (BrdU⁺) suggesting that these crypts were involved in an intestinal repair response (Figure 5B,C).

Next, we tested whether Notch activation could prevent crypt degeneration by examining *Vil-CreER;Adam10^{ff};Rosa26^{NICD/+}* and genotype control mice under the same experimental conditions. Strikingly, TX-treated *Vil-CreER;Adam10^{ff};Rosa26^{NICD/+}* mice were healthy, maintained weight and had normal intestinal histology (Figure 5A,B). Co-staining for nGFP (a surrogate marker of NICD) and ADAM10 showed that NICD(nGFP)⁺ ADAM10-deficient crypts were readily detected and had comparable proliferation to wild-type controls (Figure 5C). Similar results were obtained when recombination was induced with 4-hydroxytamoxifen (4OH-TX) in crypt organoid cultures derived from the same mice. 4OH-TX-treated organoids from *Vil-CreER;Adam10^{ff}* mice showed reduced cell proliferation and growth failure whereas 4OH-TX-treated organoids from *Vil-CreER;Adam10^{ff};Rosa26^{NICD/+}* mice displayed robust cell proliferation and organoid growth similar to wild-type organoids (Figure 6A). Together, these results demonstrate that Notch activation could protect ADAM10-deficient crypts from degeneration and restore organoid growth suggesting that ADAM10 is required for maintenance of ISCs. Consistent with this interpretation was the observed loss of the Notch transcriptional target and stem cell marker *Olfm4* from ADAM10-deficient organoids (Figure 6B) and from ADAM10-deficient immature and adult intestine (Supplementary Figure 7).

ADAM10-mediated Notch signaling is required for long-term maintenance of Lgr5⁺ CBCs

To formally test the fate of ADAM10-deficient ISCs, lineage tracing was performed using *Vil-CreER;Adam10^{ff};Rosa26^{YFP}* and genotype control mice given a single TX dose (50 mg/kg, *i.p.*) and then chased over time. At day 5 of chase, an equivalent % of YFP⁺ crypts were found in both genotypes. In TX-treated *Vil-CreER;Adam10^{ff};Rosa26^{YFP}* crypts, both YFP⁺/ADAM10⁻ and YFP⁻/ADAM10⁻ cells were detected due to the stochastic nature of

recombination (Supplementary Figure 8A,B). However, by day 28 of chase, selection of monoclonal lineage tracing events had occurred. In TX-treated genotype control mice, ADAM10⁺/YFP⁺ crypt-villus units were detected. By contrast, in TX-treated ADAM10-deficient mice, there was a marked reduction in the number of YFP⁺ lineage tracing events and all of these had “escaped” ADAM10 deletion, indicating that ADAM10-deficient cells observed at day 5 had not survived (Figure 7A). Similarly, no YFP⁺ ADAM10-deficient crypt-villus units were detected when lineage tracing was performed using *Lgr5-CreER;Adam10^{fl/f};Rosa26^{YFP}* mice (Figure 7C). The failure to detect any YFP⁺ ADAM10-deficient crypt-villus units in either genetic model, together with the inability to detect DN-MAML⁺ crypt-villus units when the same experiments were performed with *Rosa26^{DNMAML}* allele (data not shown) indicate that ADAM10-deficient and Notch-deficient *Lgr5*⁺ CBCs were not capable of maintaining crypt homeostasis.

Notch-activated ADAM10-deficient *Lgr5*⁺ CBCs have a competitive advantage to occupy the stem cell niche

To test whether activated Notch could rescue ADAM10-deficient ISCs, lineage tracing was performed using *Vil-CreER;Adam10^{fl/f};Rosa26^{NICD/+}* and genotype control mice. At day 5 of chase, NICD(nGFP)⁺ cells were found in both genotypes. However, the *Vil-CreER;Adam10^{fl/f};Rosa26^{NICD/+}* mice had a significant increase in the % of NICD(nGFP)⁺ crypts and these crypts primarily expressed NICD(nGFP)⁺/ADAM10⁻ cells (Supplementary Figure 8C). By day 28 of chase, NICD(nGFP)⁺ ADAM10-deficient crypt-villus units were readily detected indicating that activated Notch had rescued the ability of ADAM10-deficient ISCs to populate the crypt compartment (Figure 7B). Moreover, there was a six-fold increase in the percentage of NICD(nGFP)⁺ lineage tracing events observed in *Vil-CreER;Adam10^{fl/f};Rosa26^{NICD/+}* mice compared to genotype controls suggesting that there was a selective advantage in the ADAM10-deficient state for NICD(nGFP)⁺ ADAM10-deficient ISCs to occupy the stem cell niche (Figure 7B). When this same experiment was repeated using the *Lgr5-CreER* line, a five-fold increase in the number of NICD(nGFP)⁺ ADAM10-deficient lineage tracing events was observed in *Lgr5-CreER;Adam10^{fl/f};Rosa26^{NICD/+}* mice compared to genotype controls (Figure 7D). Notch signaling was clearly active in NICD(nGFP)⁺ ADAM10-deficient crypts as demonstrated by the loss of secretory differentiation and the restoration of crypt cell proliferation (Supplementary Figures 8D and 9). Interestingly, although there was no significant difference in progenitor cell proliferation between NICD(nGFP)⁺/ADAM10⁻ and NICD(nGFP)⁺/ADAM10⁺ crypts, NICD(nGFP)⁺ ADAM10-deficient crypt villus units represented the majority (>97%) of lineage tracing events in ADAM10-deficient state. This enhanced NICD(nGFP)⁺ lineage tracing was not due to an expansion of EGFP⁺-expressing *Lgr5*⁺ crypts in the ADAM10-deficient state as the total number of these crypts remained constant in both genotypes (see Supplementary Methods). These results demonstrate that activated Notch can rescue ADAM10-deficient *Lgr5*⁺ CBCs. While the precise mechanism(s) for how ADAM10-deficiency leads to enhanced NICD(nGFP)⁺ lineage tracing is still unclear, the results clearly demonstrate that Notch-activated ADAM10-deficient stem cells have a competitive advantage to occupy the stem cell niche. We conclude that cell-autonomous AD-AM10-mediated Notch signaling plays an important role in the maintenance of *Lgr5*⁺ CBCs and crypt homeostasis.

DISCUSSION

The progenitor cell compartment of the small intestine is comprised of slowly and actively cycling intestinal stem cells (ISCs) located within the crypt base²⁷. While the interplay between these different ISC populations and transit-amplifying progenitors is crucial for crypt homeostasis, the signaling pathways required for ISC function within the stem cell niche are not well defined. In this study, we demonstrate that cell-autonomous ADAM10 activity functions within the crypt compartment to regulate Notch signaling essential for maintenance of ISCs and progenitor cell lineage specification. We also demonstrate that Notch is the dominant substrate regulated by ADAM10 within the crypt compartment, consistent with ADAM10 deficiency in other organ systems *in vivo*^{19,28}. In addition, we show that activated Notch can rescue ADAM10-deficient *Lgr5*⁺ CBCs and enhance their ability to re-populate the crypt compartment, indicating that Notch-activated stem cells have a competitive advantage to occupy the stem cell niche and re-establish ISC homeostasis.

It is well-established that Notch signaling plays a crucial role in controlling cell lineage specification of intestinal progenitors¹. Earlier Notch loss-of-function studies showed that Notch inhibition resulted in goblet cell hyperplasia^{8,9,29} while more recent reports have demonstrated robust differentiation of all secretory cell lineages^{7,30,31}. Recently, VanDussen et al., showed that Notch inhibition with a γ -secretase inhibitor induces accumulation of a distinct “intermediate” cell population that expresses both Paneth and goblet cell markers throughout the intestine including the colon⁷. Consistent with a loss of Notch signaling, morphological and transcriptional analysis of the ADAM10-deficient intestine showed robust secretory cell differentiation in the immature and adult intestine. Moreover, analysis of Notch loss-of-function (*Rosa26*^{DN-MAML}) and gain-of-function (*Rosa26*^{NICD}) mutants provided direct genetic evidence that cell-autonomous AD-AM10 activity regulates Notch signaling in these cell fate decisions.

Currently, the mechanism(s) by which secretory progenitors give rise to the major secretory cell lineages remain controversial. In one model, it has been proposed that multipotent secretory progenitors must rapidly commit to a specific, secretory cell fate³² whereas recent analysis of different *Atoh1*-, *Neurog3*- and *Gfi1*-deficient mouse models suggests that multipotent *Atoh1*⁺ progenitors give rise to committed enteroendocrine progenitor and bipotent goblet/Paneth progenitor populations^{10–13}. In the ADAM10-deficient crypts, the progenitor cell population was completely replaced by *Atoh1*⁺ cells, which is indicative of cells undergoing secretory differentiation. In addition, we confirmed using the *Neurog3*^{YFP} reporter line that the “intermediate” (Paneth/goblet) cells were distinct from *Neurog3*⁺ progenitors required for enteroendocrine lineage specification. A similar accumulation of distinct intermediate and endocrine cell populations was observed upon expression of DN-MAML, demonstrating that cell-autonomous Notch inhibition was directly responsible for the appearance of these different secretory cell types.

Notch plays an essential role in crypt cell proliferation^{5,29} but it is only recently that lineage tracing and Notch inhibition studies have demonstrated that active Notch signaling is present^{2–4,33} and required for maintenance of ISCs^{7,34}. Notch inhibition led to reduced proliferation, loss of the stem cell marker, *Olfm4*, and apoptosis of CBCs, indicating that

active Notch signaling is required for survival of Lgr5⁺ CBCs. In the immature and adult intestine, intestine-specific ADAM10 inactivation closely recapitulated these findings, including reduced progenitor proliferation and loss of *Olfm4*. Further analysis of ADAM10-deficient crypts *in vivo* or cultured organoid *in vitro* revealed severe crypt degeneration and organoid failure associated with a loss of progenitor proliferation and increased apoptosis. In addition, our data from organoid experiments clearly demonstrates that ADAM10 acts in a cell autonomous manner and does not require any mesenchymal signaling to fulfill its functions. Importantly, the ability of activated Notch to completely restore progenitor proliferation and cell viability in both these settings demonstrates that ADAM10-mediated Notch signaling is essential for long-term crypt survival. Interestingly, a recent study using mice constitutively-expressing dominant-negative ADAM10 under the cryptdin-2 promoter reported mis-localized Paneth cells, a phenotype linked to a loss of Eph signaling³⁵. By contrast, our results clearly demonstrate that ADAM10 deficiency in the stem/progenitor cell compartment produces a dominant Notch loss-of-function phenotype. While ADAM10 likely regulates other substrates in the crypt compartment, the loss of stem/progenitor cell proliferation and rapid crypt degeneration observed in our current ADAM10-deficient models has not permitted analysis of ADAM10 signaling in post-mitotic intestinal cells including Paneth cells and this will be an important area for further investigation.

Mosaic recombination epigenetically found in the *Lgr5-EGFP-ires-CreERT2* line or produced by reduced tamoxifen dosing in the *Villin-CreERT2* line allowed long-term lineage tracing of ADAM10-deficient ISCs. In both models, ADAM10-deficient ISCs failed to populate the crypt compartment and no dormant AD-AM10-deficient ISCs were detected, indicating that ADAM10-deficient ISCs did not survive long-term. Similar results were found when lineage tracing was performed with the Notch loss-of-function mutant DN-MAML. The demise of AD-AM10-deficient ISCs was clearly illustrated by the ability of activated Notch to rescue ADAM10-deficient ISCs and restore their capacity to populate the crypt-villus axis. Together, these results demonstrate that ADAM10-mediated Notch signaling is crucial for the maintenance of these ISC populations.

It has been proposed that neutral drift and symmetric cell divisions are critical elements that control ISC homeostasis. ISCs within the same crypt form an equi-potent population, in which the loss of one stem cell can be replaced stochastically by another stem cell^{36,37}. In addition, lineage tracing after injury or ablation of Lgr5⁺ CBCs demonstrates that the Lgr5⁺ stem cell compartment can be repopulated through mobilization of quiescent stem cell populations^{38,39} or by de-differentiation of crypt progenitors^{40,41}. Analogous to these Lgr5⁺ cell injury/ablation studies, our data suggest that ADAM10 deletion within Lgr5⁺ CBCs leads to stem cell loss and an imbalance within the stem cell niche that promotes permissive signals for wild-type ISCs and/or progenitor populations to reestablish ISC homeostasis. Unexpectedly, lineage tracing in the presence of activated Notch revealed a 5-6-fold increase in the total number of NICD⁺ lineage tracing events within the ADAM10-deficient state compared to genotype control mice. Our data suggest that loss of ADAM10-deficient CBCs creates permissive conditions in which Notch-activated stem cells have a competitive advantage to replenish the ISC compartment. A possible explanation based on the kinetics of ADAM10 deletion and functional NICD expression is that the immediate progeny of

NICD⁺/ADAM10-deficient Lgr5⁺ CBCs efficiently re-populate the stem cell compartment. This result is consistent with the plasticity of secretory progenitors to revert to a stem-cell state^{40,41}. It will be interesting to test whether this enhanced compensatory mechanism observed with active Notch signaling can occur in other regenerative processes in which imbalances of ISC homeostasis are found.

In summary, Notch is the dominant substrate regulated by ADAM10 in the crypt compartment. ADAM10-mediated Notch signaling is essential for cell lineage specification and maintenance of Lgr5⁺ CBCs. Importantly, loss of ADAM10-deficient Lgr5⁺ CBCs created a competitive advantage for Notch-activated stem cells to re-populate the stem cell niche and restore ISC homeostasis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CBCs	crypt based columnar cells
DAPI	4'-6-diamidino-2-phenylindole
Lgr5	Leucine-rich repeat-containing GPCR5
qPCR	quantitative polymerase chain reaction
MMP7	matrix metalloproteinase-7
Neurog3	Neurogenin-3
GSI	gamma-secretase inhibitor

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Figure 1. Reduced progenitor cell proliferation upon ADAM10 deletion

(A), ADAM10 staining in adult small intestine of wild-type and *Lgr5-EGFP-ires-CreER* mice. *Right*, Co-staining with GFP (red). (B), H&E (upper) and Ki67⁺ staining (lower, arrowheads) of small intestine from newborn *Adam10^{ff}* and *Vil-Cre;Adam10^{ff}* mice. Quantification of Ki67⁺ cells per field (n=4). (C), H&E (upper) and BrdU⁺ staining (lower, arrowheads) of adult small intestine from *Vil-CreER;Adam10^{ff}* mice treated with TX (100 mg/kg i.p.) or vehicle for 5 consecutive days and analyzed the next day. Quantification of BrdU⁺ cells in crypts (n=3). **p*<0.05, ***p*<0.01, ****p*<0.001 and *****p*<0.0001. Scale bars (μ m) = A, left, 200 and right, 35; B, upper, 100 and lower, 50; and C, 200 (inset, 65).

Figure 2. ADAM10 deletion converts intestinal crypt progenitors to a secretory cell fate (A–C), Newborn small intestine from *Adam10^{ff}* and *Vil-Cre;Adam10^{ff}* mice (n=4). (D–E), Adult small intestine from *Vil-CreER;Adam10^{ff}* mice treated with TX as described in Figure 1 (n=5–7). (A,D), PAS/Alcian Blue staining (upper) and immunofluorescence analysis of goblet (MUC2), Paneth (MMP7) and enteroendocrine (CHGA) cell markers (lower). Intermediate (Paneth/goblet) and CHGA⁺ cells indicated by arrowheads. (B,E), Morphometric analysis of secretory cell types. (C,F), qPCR analysis of cell lineage markers. **p*<0.05, ***p*<0.01, ****p*<0.001 and *****p*<0.0001. Scale bars (μm) = All 100 except C, upper, 200.

Figure 3. ADAM10 deletion leads to a loss of Notch signaling
 (A–C), Perinatal small intestine from *Adam10*^{f/f} and *Vil-Cre;Adam10*^{f/f} mice. (D–F), Adult small intestine from *Vil-CreER;Adam10*^{f/f} mice treated with TX as described in Figure 1. (A,D), qPCR analysis of Notch gene targets (upper) and cell lineage-specific transcription factors (n=3–7). (B,E), X-Gal staining of *Atoh1*^{LacZ/+} reporter mice. (C,F), Immunostaining of *Neurog3*^{YFP/+} reporter mice. Arrowheads mark *Neurog3*⁺ cells. **p*<0.05, ***p*<0.01 and ****p*<0.001. Scale bars (μm) = B, 100; C, 65; E, 200; F, 135 and (insets 65).

Figure 4. ADAM10 mediated Notch signaling is required for cell lineage specification and progenitor cell proliferation

(A), Immunofluorescence analysis of cell lineage markers in the newborn small intestine from *Vil-Cre;Adam10^{ff}* and in different Notch mutants. (B), Quantification of Ki67⁺ cells per field (n=3–4). *p* values of group comparisons between *Adam10^{ff}* and other genotypes are shown. **p*<0.05, ***p*<0.01, *****p*<0.0001. Scale bars (μm) = 100.

Figure 5. ADAM10 loss leads to crypt degeneration that can be overridden by activated Notch (A–C). Adult mice were given a single TX (100 mg/kg, *i.p.*) dose and analyzed at day 9 post-treatment (n=3). **(A)**, Body weight analysis. **(B)**, H&E analysis. Black boxes are shown at high magnification (lower). In $Vil-CreER; Adam10^{f/f}; Rosa26^{YFP/+}$ mice, crypt degeneration (black asterisks) is observed adjacent to crypts with increased cellularity (orange asterisk). **(C)**, Immunofluorescence analysis of ADAM10 with BrdU (upper) and YFP or NICD(nGFP) (lower). In $Vil-CreER; Adam10^{f/f}; Rosa26^{YFP/+}$ mice, degenerating crypts are ADAM10-deficient and lack cell proliferation (white asterisks) whereas adjacent regenerating crypts express ADAM10 and are highly proliferative (orange asterisk). In $Vil-CreER; Adam10^{f/f}; Rosa26^{NICD/+}$ mice, NICD(nGFP)⁺ ADAM10-deficient crypts (white

asterisks) are filled with NICD(nGFP)⁺ cells (*arrows*) and maintain cell proliferation (*arrowheads*). *p* values from group comparisons of genotypes *Vil-CreER;Adam10^{f/+};Rosa26^{YFP/+}* vs *Vil-CreER;Adam10^{f/f};Rosa26^{YFP/+}* (red) and *Vil-CreER;Adam10^{f/f};Rosa26^{YFP/+}* vs *Vil-CreER;Adam10^{f/f};Rosa26^{NICD/+}* (blue) are shown in A. **p*<0.05; ***p*<0.01, ****p*<0.001, *****p*<0.0001.

Figure 6. Activated Notch restores proliferation and *Olfm4* expression in AD-AM10-deficient organoids

(A), Organoid cultures from adult mice treated with 4-OH-TX (5 μ M) for 12 h and then analyzed at day 5 post-treatment. Whole mount analysis of morphology (*upper*) and EdU staining (*mid-upper*). Co-staining of ADAM10, EdU and YFP or NICD(nGFP) (*lower panels*). Arrows indicate NICD(nGFP)⁺ cells. Scale bars (μ m) = B, 200 (inset, 65); C, 65; D, all 200. (B), qPCR analysis of ISC markers in 4-OH-TX treated organoid cultures (n=3). * p <0.05, *** p <0.001, **** p <0.0001.

Figure 7. ADAM10 is required for maintenance of adult *Lgr5*⁺ CBCs
 (A–B), Lineage tracing of *Vil-CreER;Adam10^{ff};Rosa26^{YFP/+}*, *Vil-CreER;Adam10^{ff};Rosa26^{NICD/+}* and genotype control mice given a single TX (50 mg/kg *i.p.*) dose and then chased for 28 days (n=3). Co-staining of ADAM10 and YFP or NICD(nGFP). (A), In *Vil-CreER;Adam10^{ff};Rosa26^{YFP/+}* mice, no ADAM10-deficient cells were detected. Arrowheads indicate individual YFP⁺ Paneth cells (Supplementary Figure 8A). (B), In both genotypes, NICD(nGFP)⁺ cells are detected (arrowheads). Asterisks mark NICD(nGFP)⁺ ADAM10-deficient crypt-villus units. (C–D), Lineage tracing of *Lgr5-CreER;Adam10^{ff};Rosa26^{YFP/+}*, *Lgr5-CreER;Adam10^{ff};Rosa26^{NICD/+}* and genotype control mice given a single TX (400 mg/kg *o.g.*) dose and then chased for 28 days (n=3). Immunofluorescence analysis of ADAM10 and YFP or NICD(nGFP). Note that EGFP⁺-*Lgr5* cells can co-express YFP or NICD(nGFP). (C), In *Lgr5-CreER;Adam10^{ff};Rosa26^{YFP/+}* mice, no ADAM10-deficient cells were detected. Strong

YFP expression from the *Rosa26^{YFP}* allele masks the weaker EGFP⁺-Lgr5 cells. (**D**), NICD(nGFP)⁺ cells (*arrows*), EGFP⁺-Lgr5 cells displaying stronger cytoplasmic staining (*arrowheads*) and NICD(nGFP)⁺ ADAM10-deficient crypt-villus unit (*asterisk*) are shown. (**AD**), *Lower panels*, Quantification of each lineage tracing event. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, **** $p < 0.0001$; na, not applicable. Scale bars (μm) = 100.