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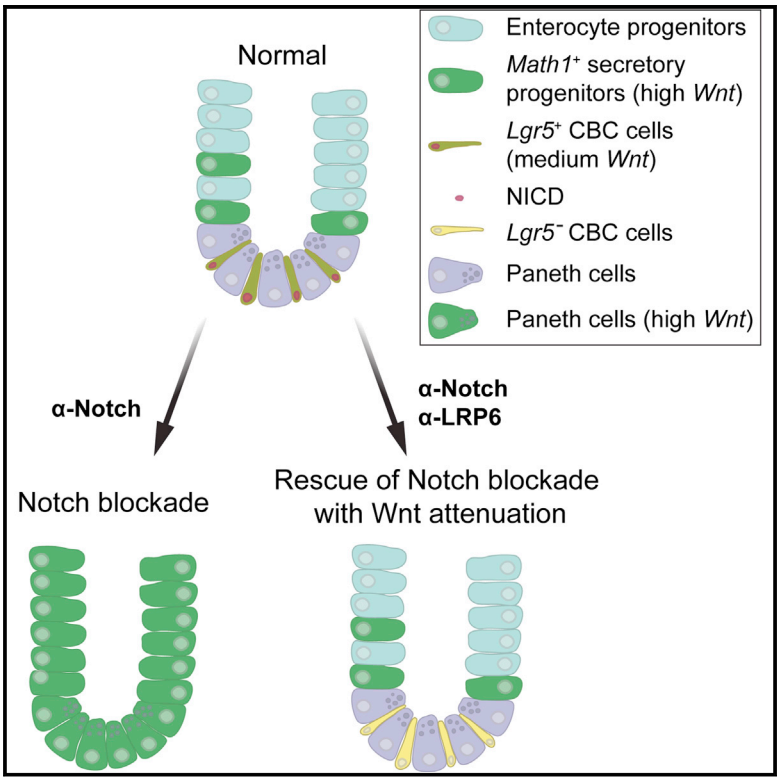
Opposing Activities of Notch and Wnt Signaling Regulate Intestinal Stem Cells and Gut Homeostasis

Graphical Abstract



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In Brief

Notch and Wnt signaling are required both for stem cell maintenance and for a proper balance of differentiation between cell lineages in the small intestine. Tian et al. demonstrate that Notch blockade perturbs intestinal stem cell function by causing a derepression of the Wnt signaling pathway.

Highlights

- A proper balance of Notch and Wnt signaling is required in the intestine
- Notch blockade causes derepression of the Wnt signaling pathway in stem cells
- Attenuation of the Wnt pathway rescues the phenotype associated with Notch blockade

Accession Numbers

GSE66751

Tian et al., 2015, Cell Reports *11*, 33–42
April 7, 2015
http://dx.doi.org/10.1016/j.celrep.2015.03.007

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Opposing Activities of Notch and Wnt Signaling Regulate Intestinal Stem Cells and Gut Homeostasis

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http://dx.doi.org/10.1016/j.celrep.2015.03.007

SUMMARY

Proper organ homeostasis requires tight control of adult stem cells and differentiation through the integration of multiple inputs. In the mouse small intestine, Notch and Wnt signaling are required both for stem cell maintenance and for a proper balance of differentiation between secretory and absorptive cell lineages. In the absence of Notch signaling, stem cells preferentially generate secretory cells at the expense of absorptive cells. Here, we use function-blocking antibodies against Notch receptors to demonstrate that Notch blockade perturbs intestinal stem cell function by causing a derepression of the Wnt signaling pathway, leading to misexpression of prosecretory genes. Importantly, attenuation of the Wnt pathway rescued the phenotype associated with Notch blockade. These studies bring to light a negative regulatory mechanism that maintains stem cell activity and balanced differentiation, and we propose that the interaction between Wnt and Notch signaling described here represents a common theme in adult stem cell biology.

INTRODUCTION

The mouse intestinal epithelium provides an important model for studying tissue renewal. Continuous turnover of the epithelium is supported by intestinal stem cells (ISCs) located near the base of the crypts. Genetic lineage tracing studies have led to the identification of distinct ISC populations, including crypt base columnar cells (CBCs) that are marked by *Lgr5*, a Wnt target gene (Barker et al., 2007). CBCs reside at the bottom of crypts, occupying cell positions +1 through +5 from the base, where they are intercalated between postmitotic Paneth cells, which constitute the stem cell niche (Sato et al., 2011). CBCs contribute to all intestinal cell types, including the secretory and absorptive lineages, through rapidly proliferating intermediates known as transit-amplifying (TA) cells (Barker et al., 2007).

Both development of the small intestine and homeostasis of the adult intestine require canonical Wnt signaling (Fevr et al., 2007; Korinek et al., 1998; van Es et al., 2012). Conversely, misexpression of the Wnt signaling agonists *R-spondin1-4 (Rspo1-4)* leads to enhanced activation of β-catenin and increased proliferation in the small intestine (Kim et al., 2006). *Lgr5* and its homologs *Lgr4* and *Lgr6* associate with *Rspos* to enhance Wnt signaling (de Lau et al., 2011; Ruffner et al., 2012). The central role of Wnt signaling is highlighted by the Wnt-dependent expression of numerous ISC markers, including *Lgr5* (de Lau et al., 2011). Beyond its role in maintaining ISCs, Wnt signaling confers competence for the secretory fate decision. Specifically, Wnt signaling plays a role in Paneth cell differentiation (Andreu et al., 2005; van Es et al., 2005a), and overexpression of the Wnt inhibitor *Dkk1* leads to loss of all secretory cells (Pinto et al., 2003).

The Notch pathway affects intestinal homeostasis by regulating CBCs and by promoting the absorptive cell fate. Compromising Notch signaling in adult mice with the γ-secretase inhibitor DAPT, which blocks conversion of the Notch receptor into a transcriptionally active molecule, causes a loss of proliferating *Lgr5*-positive CBCs and an overall increase in secretory cells (VanDussen et al., 2012). Secretory cell hyperplasia in the gut also occurs with deletion of the Notch effector *Rbp-j* (van Es et al., 2005b). Genetic evidence indicates that Notch signaling negatively regulates secretory cell differentiation through repression of *Math1/Atoh1* (Yang et al., 2001) because conditional deletion of *Math1* rescues the *Rbp-j* loss of function phenotype (Kim and Shivdasani, 2011). However, while *Math1* is upregulated in the absence of Notch (VanDussen et al., 2012), the signal(s) required for positively maintaining normal levels of *Math1* in the small intestine is unknown.

Although Notch and Wnt signaling have been studied individually, how these pathways are integrated to maintain ISCs and to regulate cell fate choices for ISC progeny is unknown. Here, using Notch blocking antibodies, we have found that a principal function of Notch signaling in maintaining ISCs is its ability to dampen Wnt signaling output. Notch blockade caused

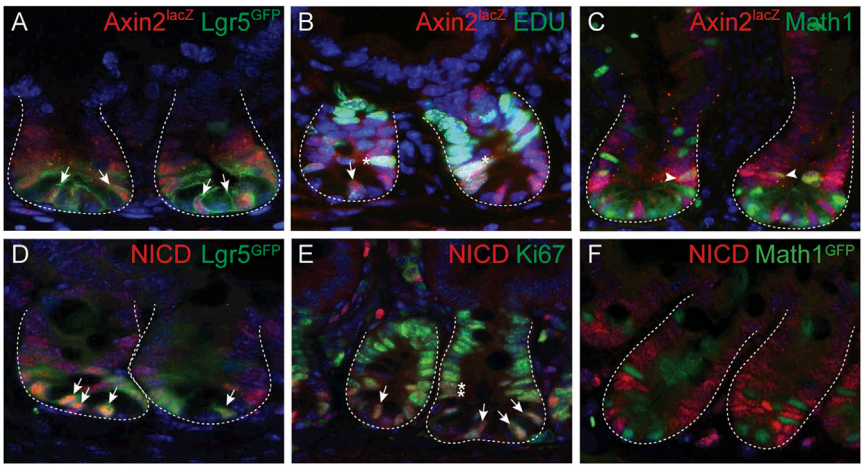

Figure 1. Distribution of Wnt and Notch Signaling in Crypts of the Mouse Small Intestine

(A) *Lgr5*^{GFP} (green) and *Axin2*^{LacZ} (red) expression are co-incident in CBCs (arrows).

(B) *Axin2*^{LacZ} (red) overlaps with Edu incorporation (green) in cells at the base of the crypt (arrows) and in TA cells (asterisks) adjacent to CBCs.

(C) *Axin2*^{LacZ} expression (red) overlaps with secretory cell progenitors marked by *Math1*^{GFP} (green, arrowheads).

(D) The active form of Notch1 (NICD, red) is localized to the nuclei of CBCs (green, arrows).

(E) NICD (red) overlaps with Ki67 staining (green) in CBCs (arrows) and TA cells (asterisks).

(F) Notch signaling (red) is absent from secretory progenitor cells (green).

conversion of *Lgr5*-expressing ISCs to secretory cells, leading to stem cell depletion. This coincided with Wnt pathway upregulation and increased secretory cell differentiation. The attenuation of canonical Wnt signaling rescued the Notch phenotype, demonstrating that the secretory cell metaplasia caused by Notch loss of function was due to upregulation of WNT ligand production.

RESULTS

Localization of Wnt and Notch Signaling in the Intestinal Crypt

To begin to investigate the role of the Notch and Wnt pathways in maintaining crypt homeostasis, we determined which cells within the crypts receive Notch and Wnt signals. We detected expression of *Lgr5*^{GFP} and the Wnt reporter *Axin2*^{LacZ} in CBCs (Figures 1A and 1B), and strong *Axin2*^{LacZ} expression was detected in proliferating cells near the border of the stem cell compartment and TA zone (Figure 1B). The *Atoh1/Math1* gene is required for the specification of secretory cell progenitors in the small intestine (VanDussen and Samuelson, 2010; Yang et al., 2001). Approximately 76% of the crypts that we analyzed showed that *Math1*^{GFP} expression also overlapped with the Wnt reporter in cells near the border of the stem cell compartment and TA zone (Figure 1C, arrowheads; n = 3, ≥100 crypts per mouse analyzed).

Lgr5 is a Wnt target gene and an established marker of CBCs. We found that CBCs marked by *Lgr5*^{GFP} were also positive for the transcriptionally active form of Notch (NICD) (Figure 1D), confirming that the Notch pathway is active in ISCs. Nuclear NICD staining was also detected in TA cells closest to the crypt bottom (Figure 1E, asterisks). NICD staining and the secretory progenitor marker *Math1*^{GFP} never overlapped in these cells (Figure 1F; n = 3, ≥100 crypts per sample analyzed), consistent with the role of Notch signaling in induction of absorptive lineages (Fre et al., 2005; van Es et al., 2005b). These results reinforce the notion that both the Wnt and Notch pathways are active in CBCs. However, the complete lack of NICD in *Math1*^{GFP} and *Axin2*^{LacZ} double-positive progenitors reaffirms that Notch signaling and Wnt signaling also have divergent functions during cell fate specification.

Notch Signaling Blockade Impairs ISC Function

Based on the observations that both the Notch and Wnt signaling pathways are active in ISCs, we set out to test their respective roles by reducing the levels of signaling. To block the activity of NOTCH receptors 1 and 2, we employed therapeutic antibodies that specifically inhibit the activity of NOTCH1 and NOTCH2 in vivo (Wu et al., 2010). Inhibition of NOTCH1 and NOTCH2 together effectively blocked Notch signaling, based on a complete loss of NICD accumulation in ISCs and TA cells (Figures 2A and 2B). NOTCH inhibition also decreased proliferation associated with the TA zone (Figures 2C, 2D, and S2E) and substantially increased expression of the Paneth cell marker lysozyme, an indication of the switch from an absorptive to secretory cell fate (Figures 2E, 2F, and S2F). Thus, our dosing scheme effectively blocked Notch signaling in the small intestine.

We next examined the ISC markers *Lgr5* and *Olfm4* at different time points during the Notch blockade. *Lgr5* and *Olfm4* were downregulated 7 hr after NOTCH antibody treatment (Figures S1A–S1D, S1I, and S1J). Expression of *Olfm4*, a Notch target gene (VanDussen et al., 2012), remained downregulated during the entire Notch blockade (Figures S1J–S1L). *Lgr5* expression, along with proliferating CBCs, was greatly reduced 24 hr after Notch blockade (Figures 3C, 3K, S1E, and S1F). Surprisingly, *Lgr5* expression recovered 72 hr later, became ectopically expressed throughout the crypt base, and extended upward to the region normally occupied by TA cells (Figures S1G and S1H). This finding is consistent with the activation of *Lgr5* in newly formed Paneth cells (Buczacki et al., 2013). By day 6, *Lgr5*^{GFP} and lysozyme staining were strongly upregulated in crypts (Figures 2D and 2F).

We next examined the effect of partial Wnt inhibition on small intestine homeostasis by treating mice with an antibody designed to block the activity of the Wnt co-receptor LRP6 (Experimental Procedures). Treatment of mice with anti-LRP6 antibody led to a marked reduction in *Axin2*^{LacZ} staining (Figures 2G and

2H), consistent with the functional role of LRP6 in transducing Wnt signaling in the gut (Zhong et al., 2012). Wnt signaling is required for secretory cell differentiation (Farin et al., 2012; Pinto et al., 2003), and we focused our attention on the specification of the secretory lineage in anti-LRP6-treated mice. Consistent with our observation that *Axin2*^{LacZ} overlaps with *Math1*^{GFP} expression, anti-LRP6 treatment also resulted in loss of these *Math1*^{GFP}-positive secretory progenitor cells (Figures 2G, 2H, and S2G). Paneth cells, which are long lived (Ireland et al., 2005), were maintained in the presence of anti-LRP6 treatment (Figure S2J). We observed a decrease in the average number of short-lived goblet cells per villus compared with controls (6.5 compared with 10.5 goblet cells per villus; n = 3, $\geq$49 villi per sample analyzed). Anti-LRP6 treatment also led to the loss of *Lgr5*^{GFP} expression in CBCs while maintaining normal crypt proliferation in the intestine (Figures 2I, 2J, and S2H). This result indicates that the anti-LRP6 blocking antibody inhibits but does not abrogate Wnt signaling, and it reinforces the idea that different Wnt signaling thresholds are required to maintain *Lgr5* expression versus maintaining proliferation. Interestingly, we observed a near complete downregulation of *Axin2*^{LacZ} levels in mice treated with anti-LRP6 blocking antibody (Figure 2H), suggesting that the levels of Wnt signaling remaining during anti-LRP6 treatment are not sufficient to drive *Axin2*^{LacZ} expression. We did not observe an effect on NICD staining in the anti-LRP6-treated mice (Figures 2K, 2L, and S2I).

To definitively determine whether CBCs require Notch and Wnt signaling to give rise to differentiated cells, we performed genetic lineage tracing experiments using *Lgr5*$^{CreER/+}$; *Rosa*$^{RFP/+}$ mice. First, mice were given Notch blocking antibodies before induction of recombination with Tamoxifen (TAM). Compared with control mice (Figure 2M), treatment with Notch blocking antibodies before TAM induction completely prevented lineage tracing from *Lgr5*-expressing cells (Figure 2N). RFP-marked cells were present at the crypt base (Figure 2N, inset) and expressed the secretory marker lysozyme (Figure S2B), indicating that CBCs had converted into Paneth cells during Notch blockade. In addition, we injected Notch blocking antibodies following Tam-induced recombination. Under these conditions, lineage tracing from the base of the crypts was almost completely absent, with patchy lineage tracing remaining in cells near the top of villi (Figure 2O). RFP marked cells were also detected at the crypt base (Figure 2O, inset) and expressed lysozyme (Figure S2D), indicating that a small population of CBCs had given rise to Paneth cells during Notch blockade. Together, these results show that Notch signaling is required for CBC stem cell activity.

Treating mice with LRP6 blocking antibody before induction with Tam caused a complete loss of *Lgr5* lineage tracing events (Figure 2P). To differentiate between a defect in stem cell activity and suppression of *Lgr5* expression through Wnt signaling attenuation, we next induced recombination in CBCs followed by LRP6 blocking antibody treatments. This led to uninterrupted lineage tracing similar to that observed in control mice (Figure 2Q), indicating that CBC stem cells function normally under LRP6 signaling attenuation and that the loss of lineage tracing seen in the pre-Tam treatments was likely due to loss of *Lgr5* expression. These results, together with the presence of proliferating CBCs and *Olfm4* expression in the anti-LRP6-treated animals (Figures 2J and S1M), highlight the ability of the anti-LRP6 treatment to uncouple *Lgr5* expression from CBC stem cell activity.

Notch Blockade Leads to Wnt Signaling Upregulation, which Promotes Secretory Cell Hyperplasia

The downregulation of *Math1*^{GFP} expression by administration of the LRP6 blocking antibody is consistent with a role for Wnt signaling in promotion of secretory cell fate decisions. We detected downregulation of *Math1*^{GFP} expression as early as 24 hr after anti-LRP6 injection (Figure 3N). This led us to examine Wnt signaling readouts during Notch blockade. By 7 hr after injection with the Notch blocking antibodies, intestinal crypts showed a pronounced increase in *Axin2*^{LacZ} expression (Figures 3A and 3E) without an apparent increase in *Math1*^{GFP} expression (Figures 3B and 3F). Notch signaling represses *Math1* in the intestine (Fre et al., 2005; van Es et al., 2005b), and we detected a substantial increase in *Math1*^{GFP} expression by 24 hr post treatment (Figures 3J and 3Y). Interestingly, at this time point, *Math1*^{GFP}-positive cells overlapped with the increased *Axin2*^{LacZ} expression (Figure 3J), indicating that the Notch secretory hyperplasia phenotype correlated with cells that were actively undergoing high levels of Wnt signaling.

These observations prompted us to perform gene expression analysis using microarrays followed by quantitative PCR (qPCR) validation on intestinal crypts from mice treated with Notch blocking antibodies for 24 hr (Figure 3Y; Tables S2, S3, and S4). The top differentially expressed genes were bona fide Notch targets (*Olfm4* (log2[fold change] = −4.07)) and *Neurog3* (log2 [fold change] = 3.83) (Fre et al., 2005; VanDussen et al., 2012). In addition, we also detected upregulation of *Wnt3* as well as several known Wnt target genes and regulators of secretory cell differentiation. Within the set of anti-Notch upregulated genes, two groups were distinguished based on responsiveness to co-treatment with anti-LRP6. One group showed a greater than 2-fold decrease in expression and included *Wnt3* and *Atoh1/Math1* (Table S4), whereas the other showed minimal responsiveness to anti-LRP6 treatment and included *Pax4* (<2-fold; Table S5). Several of the minimally responsive genes have been identified previously in screens for Wnt target genes (de Lau et al., 2011), suggesting that treatment with the anti-LRP6 antibody represents an incomplete Wnt signaling blockade. In addition, other established Wnt targets, *Sox9* and *EphB3*, were also increased during the early Notch blockade (Figures 3D, 3H, and 3L). Thus, immunofluorescence and microarray results indicated that Notch signaling attenuates Wnt signaling, preventing secretory differentiation in the intestinal epithelium. Next, we set out to address the function of Notch and the mechanism of Wnt inhibition.

Wnt Signaling Upregulation Occurs Independently of Paneth Cell Hyperplasia

Paneth cells are a major source of *Wnt3* in the small intestine, which led us to ask if Paneth cell hyperplasia represents the sole mechanism by which Wnt signaling upregulation is achieved during Notch blockade. Levels of the Paneth cell marker *Defa1* (data not shown) and lysozyme staining in the small intestine

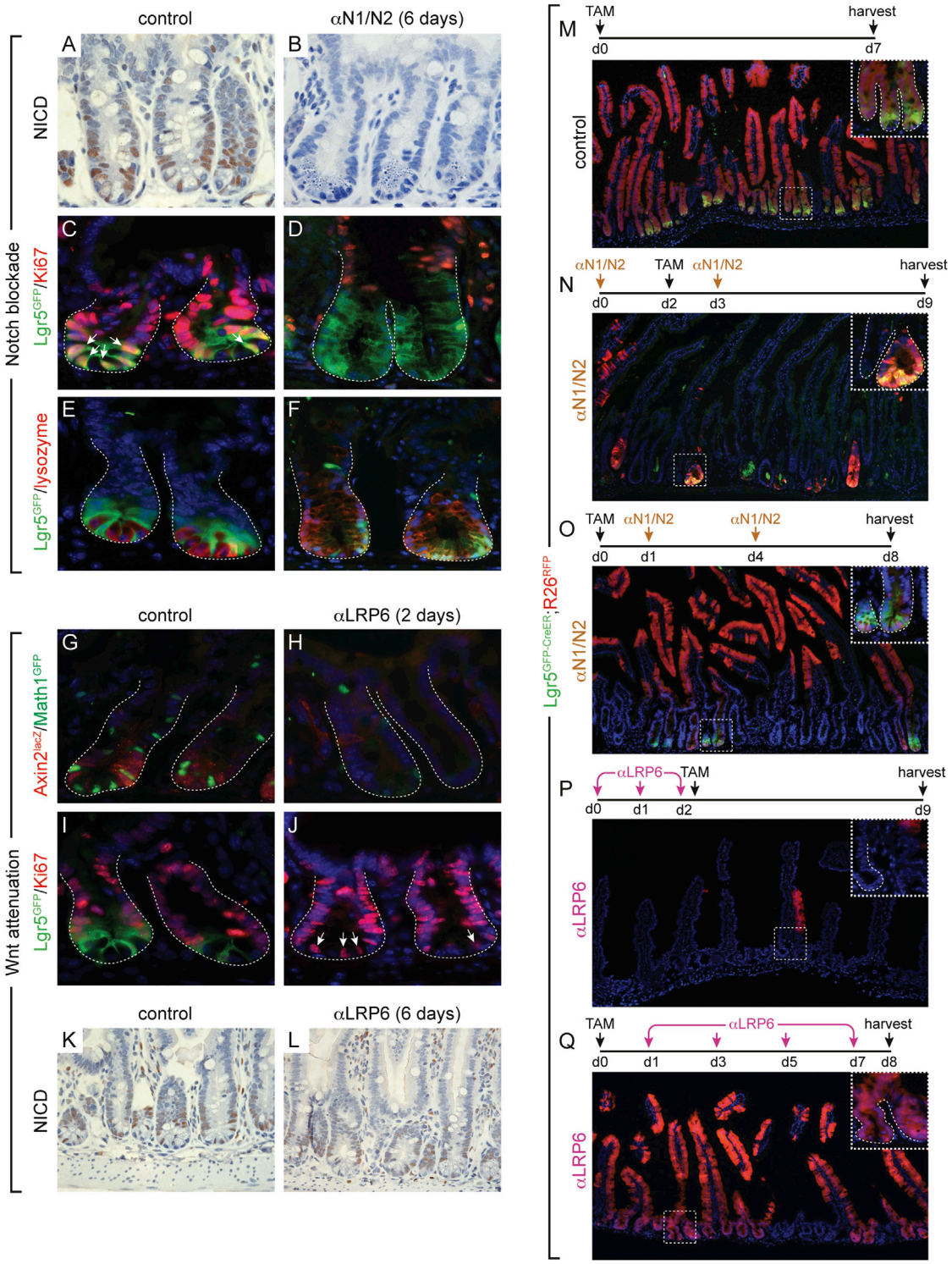

Figure 2. Loss of Notch Signaling Perturbs the Function of *Lgr5*-Positive Stem Cells

(A) Control crypts show a normal distribution of NICD staining in CBCs and TA cells.

(B) NICD immunostaining is absent from crypts treated with Notch1 and Notch2 (αN1/N2) blocking antibodies over 6 days.

(C) Control crypts show a normal distribution of $Lgr5^{GFP}$ expression in proliferating CBCs and a subset of TA cells (arrows).

(D) Notch blockade causes an increase in $Lgr5^{GFP}$ expression (green) and a decrease in proliferating cells (red) in the TA zone.

(E) *Lgr5*-positive CBCs (green) are largely non-overlapping with lysozyme-positive Paneth cells (red).

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(Figures S3A and S3B) were not significantly increased at the 24-hr time point. To test whether Paneth cells are required for a Wnt response, we blocked Notch signaling in *VillinCre;Math1*$^{fl/fl}$ mice that lack both secretory progenitors as well as differentiated secretory cells, including Paneth cells. *Villin Cre;Math1*$^{fl/fl}$ mice had significantly reduced levels of *Defa1* that did not change with Notch blockade (Figure S3C). Tissue samples from the same region of intestine had increased Ki67 and SOX9 staining (Figures S3D–S3J), which indicated that Wnt signaling was upregulated even in the absence of the *Wnt3* contribution from Paneth cells. To test for alternative sources of Wnt signaling activation, we examined the levels of Wnt target genes, Wnt isoforms, and *Rspondin1-4* in the intestines of *Villin Cre;Math1*$^{fl/fl}$-treated mice. Although *Wnt3* was not upregulated in treated mice lacking Paneth cells (Figure S3K), we observed a consistent upregulation of several Wnt target genes (Figure 3Z), as well as mesenchyme-derived *Wnt5a* and epithelium-derived *Wnt9b* (Figure 3Z'). In addition, the Wnt signaling agonist *Rspondin-4* (*Rspo4*) was significantly increased in both WT and *Villin-Cre;Math1*$^{fl/fl}$ mice treated with NOTCH blocking antibodies (Figures S3M and 3Z'). Thus, although the Paneth cells are an important source of Wnt proteins when Notch signaling is blocked in control mice, the small intestine can still mount a Wnt response in the absence of Paneth cells. Moreover, activation of the Wnt pathway due to loss of Notch signaling in control mice results at least in part from an initial amplification of Wnt signaling through upregulation of the canonical ligand *Wnt9b* and Wnt signaling agonist *Rspo4*. *Wnt3* production stemming from ongoing Paneth cell hyperplasia may then lock in place the secretory cell fate decision.

Cotreatment with Wnt Blocking Antibodies Rescues Secretory Cell Metaplasia Induced by Anti-Notch

To functionally test whether Wnt attenuation could prevent the secretory cell metaplasia induced by Notch inhibition, we analyzed secretory cell differentiation and stem cell activity while simultaneously blocking Notch signaling and attenuating Wnt signaling. We observed that the increased goblet cell content and reduced crypt proliferation seen in Notch antibody-treated animals were restored to normal levels in mice co-treated with LRP6 blocking antibody (Figures 4A–4D). Thus, co-blockade of Wnt and Notch signaling prevents secretory metaplasia and restores intestinal homeostasis. As a definitive test of CBC stem cell activity, we treated *Lgr5*^{CreER};*Rosa*^{RFP} mice with both Notch and LRP6 blocking antibodies after induction of recombination with Tam and found that co-treatment led to a dramatic rescue of CBC proliferation (Figure 3S) and stem cell activity (Figure 4F). Importantly, CBCs in this context no longer express the stem cell markers *Lgr5* (inset, Figure 4F) and *Olfm4* (Figure S1N), indicating that these markers are dispensable for stem cell activity. Together, these data implicate upregulated Wnt signaling as the mechanism that underlies secretory metaplasia when levels of Notch signaling are reduced.

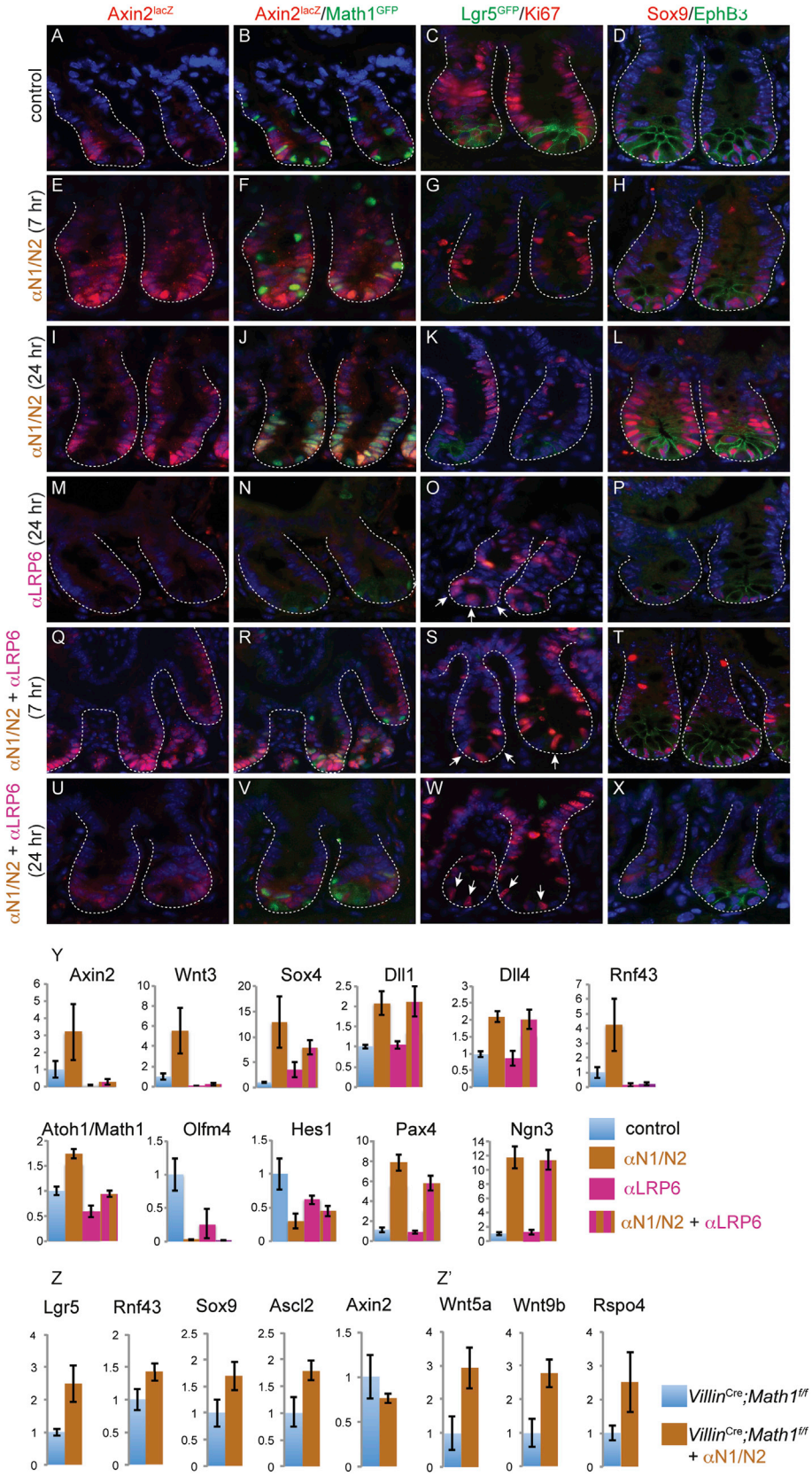
To complement the above experiments, we also tested whether a decoy Wnt receptor, Frizzled 8 CRD (F8CRD) (Experimental Procedures), could similarly rescue the effect of Notch antibody blockade. As with single agent anti-LRP6 treatment, F8CRD treatment led to downregulation of Wnt signaling, as assessed by *Axin2*^{LacZ}, and secretory differentiation, as assessed by *Math1*^{GFP} (Figure S4). Combined treatment with Notch antibodies and F8CRD led to complete rescue of the secretory metaplasia phenotype (Figures 4G and 4H), reinforcing the notion that Notch blockade leads to secretory conversion in large part through driving an increase in Wnt signaling via upregulation of *Wnt* ligand expression. Importantly, this treatment combination also rescued the weight loss and lethality induced by Notch blocking antibodies (Figure S4S). Additional experiments will be required to fully understand whether rescue of the weight loss associated with Notch blockade is due specifically to a shift in the balance of differentiated cells toward the secretory fate.

DISCUSSION

Notch Signaling Antagonizes Wnt Signaling to Maintain Stem Cell Activity

Previous studies have found that Notch activity plays an essential role in maintaining CBCs and TA cell proliferation while preventing secretory differentiation (van Es et al., 2005b; VanDussen et al., 2012). Our data indicate that Notch signaling is active in CBCs and TA cells and is absent from all secretory progenitors and their differentiated progeny. Using function blocking antibodies against Notch receptors, we found an upregulation of Wnt signaling shortly after Notch blockade, indicating that Notch signaling antagonizes Wnt signaling in the intestinal epithelium. This finding has surprising implications for how Wnt signaling normally regulates the maintenance and activity of CBC stem cells. First, when Wnt signaling is elevated due to Notch blockade, CBC activity and proliferation in the crypt are severely compromised. These phenotypes are likely mediated through increased Wnt ligand expression, particularly *Wnt3*, and not other downstream components of the Wnt signaling

(F) Increased lysozyme-expressing cells (red) are present after 6 days αN1/N2 treatment.
(G) Control crypts showing distribution of Wnt signaling (*Axin2*^{LacZ}, red) and secretory cell progenitors (*Math1*^{GFP}, green).
(H) αLRP6 treatment downregulates *Axin2*^{LacZ} (red) and *Math1*^{GFP} (green).
(I) Control crypts showing *Lgr5*^{GFP} (green) and proliferating Ki67-positive cells (red).
(J) Wnt attenuation with αLRP6 blocking antibody downregulates *Lgr5*^{GFP} expression (absence of green staining) without affecting proliferating CBCs (red, arrows).
(K and L) Treatment with αLRP6 does not affect the distribution of NICD.
(M) Lineage tracing experiments using *Lgr5*$^{CreER/+}$;*Rosa*$^{RFP/+}$ mice show widespread labeling of crypts and villi 7 days post induction with TAM.
(N) Treatment with αN1/N2 before and after induction with TAM causes decreased lineage tracing from *Lgr5*-positive cells.
(O) *Lgr5*-positive stem cells were first induced to undergo recombination with TAM and then treated with αN1/N2 on days 1 and 4.
(P) αLRP6 treatment causes a loss of lineage tracing from *Lgr5*-positive cells if provided before induction with TAM.
(Q) αLRP6 treatment does not affect lineage tracing if provided after induction with TAM.



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pathway, as CBC activity and TA cell proliferation could be rescued by attenuating Wnt signaling at the ligand and receptor level with either anti-LRP6 or F8CRD. Second, attenuation of normal Wnt signaling levels with anti-LRP6 or F8CRD had no effect on stem cell activity, such that diminished levels of Wnt signaling were sufficient for normal stem cell function. Together, these results indicate that Notch activity is required for maintaining the proper level of Wnt signaling in the crypt that allows for the simultaneous maintenance and activity of ISCs as well as crypt proliferation.

Our experiments with the anti-LRP6 antibody point to a differential requirement for Wnt signaling in ISC self-renewal and secretory differentiation, which may reflect functional differences between the LRP6 and LRP5 receptors. Wnt signaling attenuation in our studies caused downregulation of secretory differentiation, while leaving CBC stem cell activity intact. This points to a model in which lower levels of Wnt signaling are needed for stem cell maintenance and higher levels for secretory cell differentiation, which is similar to the gradient of Wnt activity seen in other systems, such as in hair follicle stem cell maintenance and differentiation (Blanpain and Fuchs, 2006). However, ISCs are embedded in a WNT-rich environment (Gregorieff et al., 2005), which implies that CBCs should receive the highest levels of Wnt signaling. Our analysis of Wnt activity, as assessed by the *Axin2*^{lacZ} reporter, shows that cells near the border of the stem cell compartment express *Axin2*^{lacZ} as strongly as or even higher than CBCs (Figures 1A–1C). Based on these observations and together with our findings that the highest intensity of nuclear NICD occurs in CBCs and that high expression of the Notch target gene *Olfm4* also occurs in CBCs, we propose that the lower level of Wnt signaling needed for ISC activity is achieved through the antagonistic activity of Notch signaling. In line with this hypothesis, Notch blockade caused an increase in *Axin2*^{LacZ} expression throughout the crypt, with a particular increase in intensity detected at the crypt base (Figures 3E and 3I), along with aberrant secretory cell differentiation and a complete loss of ISC activity. The exact mechanism underlying the collaboration between Notch and Wnt signaling in maintenance of ISCs is still unknown, but our rescue experiments suggest that Notch signaling is dispensable for ISC activity as long as the Wnt signaling output approximates normal levels (Figure 4F).

Anti-LRP6 or F8CRD treatment alone did not cause a reduction in CBC activity and TA proliferation while suppressing Wnt target genes. Potential explanations for why these reagents do not completely eliminate Wnt signaling include technical reasons, such as an inability of F8CRD to completely titrate all WNT3 ligand, or partial blockade of Wnt signaling by the anti-LRP6 antibody; alternatively, signaling events initiated by compensating Wnt pathway components may play a role.

Notch and Wnt Signaling Interplay Controls Cell Differentiation

Math1/*Atoh1*, which is negatively regulated by Notch signaling, is the key mediator of secretory metaplasia after Notch loss of function (Kazanjian et al., 2010). In addition to rescuing secretory cell metaplasia, *Math1* deletion also restored proliferation after Notch blockade, suggesting that *Math1*-mediated cell cycle exit is a factor in maintaining crypt homeostasis. We found that attenuation of the Wnt pathway by treatment with anti-LRP6 restored the normal distribution of *Math1*-expressing cells, and this treatment rescued the Notch phenotype, including restoration of proliferation. Importantly, the notion that increased Wnt

Figure 3. Notch Blockade Leads to Wnt Signaling Upregulation

(A) Control crypts showing *Axin2*^{LacZ} staining (red).
(B) Combined *Axin2*^{LacZ} (red) and *Math1*^{GFP} (green) staining.
(C) Control crypts showing expression of *Lgr5*^{GFP} (green) and proliferating cells (Ki67, red).
(D) Control crypts showing antibody staining of the Wnt targets SOX9 (red) and EPHB3 (green) at the base of the crypt.
(E) The 7-hr time point during Notch blockade shows an increase in the Wnt reporter *Axin2*^{LacZ}.
(F) The 7-hr time point shows a normal distribution of *Math1*^{GFP} relative to controls.
(G) At 7-hr time point during Notch blockade, *Lgr5*^{GFP}-positive CBCs are still present but have stopped proliferating (arrows).
(H) The 7-hr time point during Notch blockade shows increasing intensity of SOX9 positive nuclei at the base of the crypt (red).
(I) The 24-hr time point during Notch blockade shows an increased in *Axin2*^{LacZ} staining.
(J) The 24-hr time point during Notch blockade shows an increased distribution of *Math1*^{GFP} staining (green).
(K) At the 24-hr time point during Notch blockade, *Lgr5*^{GFP} (green) and proliferating cells (red) are largely absent from the base of the crypt.
(L) The 24-hr time point during Notch blockade shows increased staining and distribution of the Wnt targets SOX9 and EPHB3.
(M and N) The 24-hr time point during LRP6 blockade shows absence of *Axin2*^{LacZ} (M, red) and loss of *Math1*^{GFP} expression (N) in treated crypts. Arrows in (N) point to residual *Math1*^{GFP} expression.
(O) The 24-hr time point during LRP6 blockade shows an absence of *Lgr5*^{GFP} (green) and a normal distribution of Ki67 staining in CBCs (arrows).
(P) The 24-hr time point during LRP6 blockade shows near complete downregulation of the Wnt targets SOX9 and EPHB3.
(Q) The 7-hr time point during combined Notch/LRP6 blockade shows a reduced distribution of *Axin2*^{LacZ} relative to Notch blockade alone (E).
(R) The 7-hr time point during combined Notch/LRP6 blockade shows rescued of *Math1*^{GFP} expression pattern.
(S) The 7-hr time point during combined Notch/LRP6 blockade shows a loss of *Lgr5*^{GFP} expression and a rescued distribution of Ki67-positive cells at the base of the crypt.
(T) The 7-hr time point during combined Notch/LRP6 blockade shows the Wnt target genes Sox9 and EphB3 remain downregulated.
(U) The 24-hr time point during combined Notch/LRP6 blockade shows that *Axin2*^{LacZ} expression is reduced relative to (I).
(V) The 24-hr time point during combined Notch/LRP6 blockade shows that *Math1*^{GFP} expression is reduced relative to (J).
(W) The 24-hr time point during combined Notch/LRP6 blockade shows a rescued distribution of Ki67-positive cells including CBCs (arrows).
(X) The 24-hr time point during combined Notch/LRP6 blockade shows that the Wnt target genes Sox9 and EphB3 remain downregulated.
(Y) Fold changes in gene expression relative to controls after antibody treatments. mRNA was purified from isolated crypts. Results are mean ± SEM.
(Z) Relative levels of Wnt target gene expression in *Villin Cre;Math1*$^{fl/fl}$ mice treated with Notch blocking antibodies.
(Z') Levels of *Wnt5a*, *Wnt9b*, and *Rspo4* increase in *Villin Cre;Math1*$^{fl/fl}$ mice treated with Notch blocking antibodies.

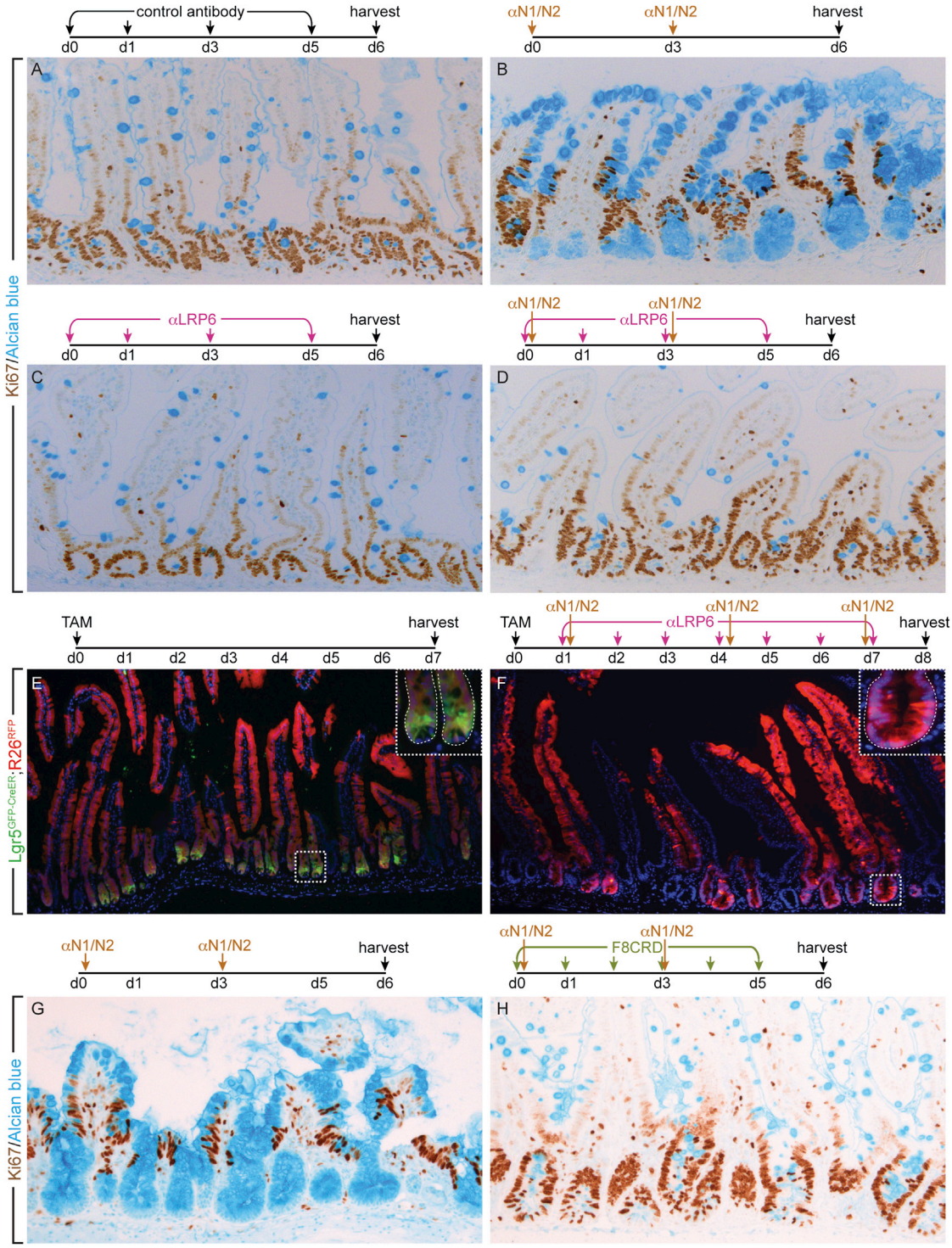

Figure 4. Cotreatment with Notch and Wnt Blocking Antibodies Rescues Secretory Cell Metaplasia and ISC Activity

(A) Control ileum showing proliferating cells (Ki67, brown) and Alcian Blue stained Goblet cells.

(B) Notch blockade causes goblet cell metaplasia.

(C) αLRP6 treatment leads to no significant changes in the distribution of Goblet cells or proliferating cells.

(D) Combined αN1/N2 and αLRP6 treatment rescues the proliferation defects and Goblet cell metaplasia associated with Notch blockade alone.

(E) Lineage tracing experiments using *Lgr5*$^{CreER/+}$;*Rosa*$^{RFP/+}$ mice with fully labeled crypts and villi at 7 days postinduction with TAM. Inset shows expression of *Lgr5*^{GFP} at crypt base.

(legend continued on next page)

signaling mediates the Notch phenotype through upregulation of *Math1* is substantiated by the observation that LRP6 blockade represses *Math1* expression on its own and that Wnt/β-catenin signaling directly regulates *Math1* expression in other contexts (Shi et al., 2010). We also found that the upregulation of *Axin2*^{lacZ} and other Wnt target genes preceded the activation of *Math1* and secretory cell metaplasia. During Notch blockade, the small intestine increases the production of Wnts as well as the agonist *Rspo4* in the absence of *Math1* and the secretory cell lineage. This strongly suggests that the trigger for secretory cell metaplasia in Notch-deficient mice is an immediate hyperactivation of the Wnt pathway. Our data, together with previous studies, indicate that Notch activity is required for maintaining the correct balance of Wnt signaling in the crypt, which allows for simultaneous maintenance of ISCs, proliferation, and differentiation.

Notch/Wnt Interaction in Other Contexts

The interaction between Wnt and Notch signaling described here may be a common theme in stem cell biology. For example, deletion of the *Notch1* receptor in mouse skin causes inappropriate activation of Wnt/β-catenin signaling in the epidermis and impaired differentiation in primary keratinocytes, as well as excess β-catenin accumulation in the eye epithelium leading to Wnt-dependent hyperproliferation (Nicolas et al., 2003). Because Notch signaling plays diverse roles in organ homeostasis and Notch/Wnt interactions may be prevalent in other systems, we cannot conclude that rescue of the Notch blockade induced weight loss and lethality by F8CRD is the result of secretory cell metaplasia suppression alone. In fact, anti-LRP6 treatments in combination with Notch antibodies gave similar results in terms of secretory cell metaplasia suppression; however, these mice continued to lose weight at a similar rate compared with treatment with anti-Notch alone. We speculate that the F8CRD acts less broadly than the LRP6 blockade, and therefore, this reagent is able to rescue the effects in the intestine without causing other problems for the animal. Our work indicates that Notch signaling normally serves as a natural brake on the Wnt pathway and that attenuation of Notch signaling releases the brake and allows for high levels of Wnt signaling. Thus, in certain contexts, it may be beneficial to attenuate Notch signaling in order to assist Wnt-mediated injury repair and stem cell-fueled regeneration.

Finally, the interaction between Wnt and Notch signaling in ISCs has important implications for the use of Notch pathway inhibitors. Intestinal goblet cell metaplasia is a major challenge in the development of therapies that block Notch signaling, such as the γ-secretase inhibitors that hold promise for the treatment of Alzheimer disease or of cancers that are caused by mutations in the NOTCH pathway. The intestinal metaplasia disrupts nutrient absorption, and animals succumb due to severe weight loss. By modulation of Wnt signaling, it may be possible to overcome the intestinal metaplasia toxicity and the lethality associated with Notch blockade. We propose that layering gentle attenuation of Wnt signaling on top of γ-secretase inhibition or other modalities that block Notch signaling can potentially overcome the intestinal toxicity associated with such treatments, thus allowing for long-term dosing with such agents.

EXPERIMENTAL PROCEDURES

Mice

All procedures were performed while observing University of California, San Francisco regulations and guidelines. Please see Supplemental Information for details about mouse use.

Immunohistochemistry and Immunofluorescence

Immunohistochemistry was performed using Dako Envision+ system-HRP polymer detection kit. For immunofluorescence staining, samples were blocked with Dako protein-free blocking solution. Primary and secondary antibodies were diluted in Dako antibody diluent, and staining conditions are summarized in Table S1. Secondary antibodies were from Jackson Immunology.

Isolation of Crypts for qRT-PCR and Microarray Analysis

Four groups of C57Bl/6 mice were injected intraperitoneally with 1-mg/kg Notch1 and Notch2 blocking antibodies, 30-mg/kg LRP6 blocking antibody, 1-mg/kg Notch1, Notch2 and 30-mg/kg LRP6 blocking antibodies, or 30-mg/kg anti-ragweed antibody. At 24 hr later, isolated small intestines were opened longitudinally and washed with cold PBS. The tissue was then chopped into 5-mm pieces and incubated in cold chelation buffer (2-mM EDTA, 0.5-mM DL-DTT in PBS) for 30 min on ice. Chelation buffer was then removed, and tissue fragments were vigorously resuspended in cold PBS using a 10-ml pipette. The process was repeated until individual crypts were released from the tissue chunks. The crypt suspension fractions were pooled and strained through a 70-micron filter. Crypts were pelleted, and RNA was extracted using the QIAGEN RNeasy mini kit. See Supplemental Experimental Procedures for array procedures.

ACCESSION NUMBERS

The accession number for the data reported in this paper is GSE66751. The data can be accessed at http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE66751.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and five tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.03.007.

AUTHOR CONTRIBUTIONS

H.T., B.B., F.J.d.S., and O.D.K. designed experiments and analyzed data. H.T. and B.B. performed experiments. B.B., F.J.d.S. and O.D.K. wrote the manuscript. C.C., C.S., Y.W., and M.C. contributed reagents. All authors read and discussed the manuscript.

ACKNOWLEDGMENTS

We thank Sarah Alto, Asoka Rathnayake, Jose Diaz, Brad Friedman, Zora Modrusan, Adriane Joo, and members of the Genetic Analysis lab for assistance. Members of the de Sauvage and Klein laboratories provided insightful suggestions. This work was funded by the California Institute for Regenerative

(F) Combined αN1/N2 and αLRP6 treatment rescues stem cell activity, as indicated by a recovery of lineage tracing events from *Lgr5*-positive stem cells. Inset shows representative fully labeled crypt with suppressed *Lgr5*^{GFP} expression.
(G) Notch blockade causes goblet cell metaplasia.
(H) Combined αN1/N2 and αF8CRD treatment rescues the proliferation defects and Goblet cell metaplasia.

Medicine (RN3-06525) and by the NIH through the NIH Director's New Innovator Award Program (DP2-OD007191), both to O.D.K. B.B., C.C., C.S., Y.W., M.C., and F.J.d.S. are employees of Genentech, Inc. and own shares of Roche.

Received: October 16, 2014
Revised: January 10, 2015
Accepted: February 27, 2015
Published: March 26, 2015

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