**RAL GTPases Drive Intestinal Stem Cell Function and Regeneration through Internalization of WNT Signalosomes**

**Graphical Abstract**

**Highlights**

- RAL GTPases are widely expressed in the intestinal epithelium
- RAL GTPases activate Wnt signaling through internalization of Wnt receptors
- RAL GTPase signaling is required for optimal stem cell numbers
- RAL GTPases drive regeneration of fruit fly and mouse intestine following damage

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**In Brief**

RAL GTPases are central players in intestinal biology. RALs are required to activate canonical Wnt signaling in *Drosophila*, mice, and human cell lines. Within intestinal stem cells, RALs are essential to sustain tissue homeostasis and regeneration. RAL small GTPases modulate Wnt pathway activity through internalization of cell-surface Wnt receptors.
RAL GTPases Drive Intestinal Stem Cell Function and Regeneration through Internalization of WNT Signalosomes

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SUMMARY

Ral GTPases are RAS effector molecules and by implication a potential therapeutic target for RAS mutant cancer. However, very little is known about their roles in stem cells and tissue homeostasis. Using Drosophila, we identified expression of RalA in intestinal stem cells (ISCs) and progenitor cells of the fly midgut. RalA was required within ISCs for efficient regeneration downstream of Wnt signaling. Within the murine intestine, genetic deletion of either mammalian ortholog, Rala or Ralb, reduced ISC function and Lgr5 positivity, drove hypersensitivity to Wnt inhibition, and impaired tissue regeneration following damage. Ablation of both genes resulted in rapid crypt death. Mechanistically, RALA and RALB were required for efficient internalization of the Wnt receptor Frizzled-7. Together, we identify a conserved role for RAL GTPases in the promotion of optimal Wnt signaling, which defines ISC number and regenerative potential.

INTRODUCTION

RAL GTPases (RALs) are RAS effectors critical for tumor initiation (Lim et al., 2005) and anchorage independent growth of colorectal cancer (CRC) cell lines (Martin et al., 2011). Consequently, RALs are potential therapeutic targets in this setting (Neel et al., 2011; Yan et al., 2014). The mammalian genome encodes for two RALs, RALA and RALB, with non-redundant functions spanning development (Peschard et al., 2012), exocyst formation (Bodemann and White, 2008; Chen et al., 2007; Chien et al., 2006), and endocytosis. Specifically, the RAL effector RALBP1 drives clathrin-mediated endocytosis (Julien-Flores et al., 2000), while RALA promotes caveolar endocytosis (Jiang et al., 2016). RAL activity is potentiated by RAL guanine nucleotide exchange factors (RALGEFs) and negatively regulated by RAL GTPase-activating proteins (RALGAPs) (Neel et al., 2011). Multiple RALGEF molecules, such as RALGDS, contain RAS-binding domains and are activated by association with oncopgenic RAS (Koyama and Kikuchi, 2001). Indeed, RALGDS has a reported role in tumor initiation and growth (González-García et al., 2005; Rodriguez-Viciana and McCormick, 2005). However, the precise role of RALs or associated effectors in intestinal biology remains unknown.

Wnt signaling is critical in intestinal health and disease. Impaired signaling leads to crypt death (Ireland et al., 2004; Kuhnert et al., 2004), while pathway hyperactivation drives cancer (Clevers, 2006). Balanced pathway activity is essential to maintain tissue homeostasis while preventing tumorigenesis. High Wnt signaling is found at the crypt base (Gregoriiff et al., 2005), where intestinal stem cells (ISCs) and Paneth cells exhibit nuclear β-catenin and transcriptional signatures associated with Wnt pathway activation. A subset of ISCs also express Lgr5, which potentiates Wnt signaling by binding to the agonist R-Spondin (R-Spo) (Barker et al., 2007; Sato et al., 2009). Lgr5⁺ ISCs are redundant for homeostasis but required for regeneration following damage (Metcalfe et al., 2014). Wnt ligands are expressed in numerous cell lineages within the intestinal epithelium and its microenvironment, including Paneth and mesenchymal cells (Shoshkes-Carmel et al., 2018; Valenta et al., 2016; Zou et al., 2018). Removal of Paneth cell-derived Wnt affects outgrowth of crypts as organoids, whereas the mesenchymal source is enough to sustain crypts in vivo (Degirmenci et al., 2018; Kim et al., 2012; Sato et al., 2011; Shoshkes-Carmel et al., 2018). The redundant Wnt stem cell niche is conserved in the Drosophila melanogaster intestine. Drosophila Wnt/Wg produced by uncommitted progenitor cells called enteroblasts (EBs) is specifically required for ISC proliferation upon stress and regeneration, while the mesenchyme-derived ligand is sufficient to maintain tissue homeostasis (Cordero et al., 2012b; Lin et al., 2008).

The Wnt signalosome is composed of a cluster of Frizzled receptors, Lrp5/6 co-receptors, and Dishevelled (Dvl) at the plasma membrane (Bilic et al., 2007; Chung et al., 2012; McCrea et al., 2007). Multiple RALGEF molecules, such as RALGDS, contain RAS-binding domains and are activated by association with oncopgenic RAS (Koyama and Kikuchi, 2001). Indeed, RALGDS has a reported role in tumor initiation and growth (González-García et al., 2005; Rodriguez-Viciana and McCormick, 2005). However, the precise role of RALs or associated effectors in intestinal biology remains unknown.
Figure 1. Ral Small GTPase Signaling Controls the Regenerative Capacity of the Drosophila Midgut through Wnt Signaling

(A) Co-labeling of RalA>GFP (green) with markers for ISCs/EBs (Arm; red membrane staining), EEs (Pros; red nuclear staining), EBs (Su(H)GBE-LacZ; red); RalA> nRFP (green pseudo colored), or ISCs (Delta::GFP; red). Scale bar, 5 μm.

(B) RalA transcript levels relative to rpl32 in whole midgut samples following indicated cell-type-specific RalA knockdown (RalA-RNAi(1)) normalized to 1. Student’s t test; ISC/EB, EB n = 3, ISC n = 4; error bars, ± SEM.

(B') RalA transcript levels in sorted ISCs relative to whole midgut samples. Delta is used to confirm ISC enrichment. n = 3; error bars, ± SEM.

(C) Representative confocal images of mock-treated or regenerating (Ecc15) adult posterior midguts from control animals or upon adult-specific RalA or RalBP knockdown by RNAi (RalA-RNAi(1) and RalBP-RNAi, respectively) within stem and progenitor cells (ISCs/EBs; green) using the escargot-gal4, UAS-gfp driver. Scale bar, 20 μm.

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An important step in the assembly of the Wnt signalosome involves polymerization of Dvl and interaction with frizzled receptors during ligand-induced endocytosis of the receptor complex (Gammons et al., 2016). Once assembled, the Wnt signalosome immobilizes the β-catenin destruction complex and stabilizes β-catenin, activating transcription of Wnt target genes (Kishida et al., 1999; MacDonald et al., 2009). Altogether, evidence suggests that the regulation of Wnt activity through ligand availability and cell-surface receptor internalization is critical in control of ISC proliferation and differentiation states.

Here, we used Drosophila and mouse models to address the biological function of RALs in the adult intestine. Our results demonstrate a conserved in vivo role for RALs in ISC function during tissue homeostasis and regeneration. ISCcs lacking RALs were at a disadvantage compared to wild-type neighbors. Importantly, we show that constitutive β-catenin activation through APC deletion rescued the suppression of Wnt signaling following RAL loss, and that RALs promote Wnt signaling through control of Wnt signalosome internalization.

RESULTS

RalA Is Required for Intestinal Regeneration and Wnt Signaling Activation in Drosophila

The epithelium of the adult Drosophila midgut, homologous to the mammalian small intestine (Snaï et al. and Batlle, 2009; Le-maitre and Miguel-Aliaja, 2013), consists of ISCs, undifferentiated progenitors called EBs, secretory enteroendocrine cells (EEs), and absorptive enterocytes (ECs). ISCs proliferate to self-renew the midgut epithelium in homeostatic conditions as well as to drive tissue regeneration following damage by pathogenic bacteria or other toxic stimuli, through activation of conserved signaling pathways (Micchelli and Perrimon, 2006; Nászai et al., 2015; Ohlstein and Spradling, 2006).

Expression of the single Ral GTPase fly ortholog, RalA, appears enriched in the adult Drosophila midgut (http://flyatlas.org/atlas.cgi). A gal4 insertion within the endogenous gene locus (RalA-gal4) (Bourbon et al., 2002) in combination with a gal4-responsive UAS-GFP transgene (RalA-GFP) confirmed RalA expression throughout the adult fly midgut and in enteric neuronal projections (Figure S1A). Co-labeling experiments in the posterior midgut epithelium showed RalA expression in ISCs/EBs, marked by Armadillo (Arm), but not in EEs, labeled with nuclear Prospero (Pros) (Figure 1A). Combining the EB-specific transgenic reporter Su(H)GBe-LacZ with RalA>GFP and an endogenous GFP-tagged form of the ISC marker Delta with RalA > nRFP showed RalA co-labeling with ISCs and EBs (Figure 1A). Consistently, reverse transcription quantitative polymerase chain reaction (RT-qPCR) revealed significant reduction of RalA transcript levels in whole midguts following targeted RalA knockdown by RNA interference (RalA-RNAi(1)) in ISCs and EBs or individually in each cell population (Figure 1B). RT-qPCR confirmed RalA expression in sorted ISCs (Figure 1B). However, RalA is not enriched in ISCs (Figure 1B), which can be partly explained by reporter expression in the visceral muscle (VM) (Figure S1A) and correlates with data from Flygut-seq (http://flygutseq.buchonlab.com).

To assess the role of RalA in homeostatic ISC self-renewal, we performed lineage tracing experiments from control and RalA-RNAi-expressing ISCs using two independent GFP+ve ISC lineage tracing systems. The “escargot flip out” (ISC/EB Flipout) system (Jiang et al., 2009), resulting in transgene expression and lineage tracing of every ISC/EB (Figures S1B and S1C), and the MARCM system, which generates clones following Flipase (FLP)/FRT, mediated mitotic recombination, leading to transgene expression and labeling of a discrete subset of ISCs (Lee and Luo, 2001) (Figures S1D and S1E). Results from both lineage tracing systems showed no difference in the size of control and RalA-RNAi-expressing clones (Figures S1B–S1E). MARCM clones also revealed no change in ISC lineage differentiation following RalA knockdown (Figures S1F and S1G).

We next assessed whether RalA was required to drive the proliferative response of ISCs following intestinal epithelial damage

(D) Quantification of pH3+ cells in posterior midguts as in (C). Two-way ANOVA is shown, followed by Sidak’s multiple comparisons test; n = number of posterior midguts quantified; error bars, ± SEM.

(E) Representative confocal images of control or wild-type RalA ISC/EB-overexpressing posterior midguts (RalAwt(2)) (ISC/EB; green). Scale bar, 50 μm.

(F) Quantification of pH3+ cells in posterior midguts overexpressing independent RalA constructs (RalAwt(1) and RalAwt(2)). One-way ANOVA is shown, followed by Sidak’s multiple comparisons test; n = number of posterior midguts quantified; error bars, ± SEM.

(G) Representative confocal images of Wnt pathway activity reporter Fz3-RFP (red or gray) in mock-treated or regenerating (Ecc15) adult posterior midguts from control animals or animals overexpressing wild-type RalA in ISCs/EBs. Scale bar, 20 μm.

(H) Quantification of the average Fz3-RFP staining intensity within the ISC/EB compartment in posterior midguts as in (G), normalized to 1. Two-way ANOVA is shown, followed by Sidak’s multiple comparisons test; each dot represents values from a z stack confocal image from a posterior midgut; error bars, ± SD.

(I) Quantification of the average nuclear dMyc staining intensity within the ISC/EB compartment in mock-treated or regenerating (Ecc15) adult posterior midguts from control or RalA knockdown (RalA-RNAi(1)) normalized to background staining. Two-way ANOVA is shown, followed by Sidak’s multiple comparisons test; each dot represents values from a z stack confocal image from a posterior midgut; error bars, ± SD.

(J) Representative confocal images of Wnt pathway activity reporter Fz3-RFP (red or gray) from control animals or animals overexpressing wild-type RalA in ISCs/EBs. Scale bar, 20 μm.

(K) Quantification of the average Fz3-RFP staining in posterior midguts as in (J), normalized to 1. Student’s t test is shown; each dot represents a z stack confocal image from a posterior midgut; error bars, ± SD.

(L) Quantification of the average nuclear dMyc staining intensity within the ISC/EB compartment in control or RalA-overexpressing midguts normalized to background staining. Student’s t test is shown; each dot represents values from a z stack confocal image from a posterior midgut; error bars, ± SD.

(M) Representative confocal images of Senseless staining (Sens; green) in third-instar larval wing discs upon RalA knockdown (RalA-RNAi(1)) in the posterior compartment (RFP positive) using the engrailed-RFP driver. Scale bar, 50 μm.

(N) Quantification of Senseless staining intensity perpendicular to the line Senseless expression in the control (RFP positive) versus the RalA knockdown (RFP positive) compartment in larval wing discs. Multiple t tests, false discovery rate [FDR] = 0.01; n = 4 wing discs; error bars, ± SEM.

Where indicated, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
Figure 2. Drosophila Ral Is Required in ISCs for Wnt Signaling Activation, Upstream of the β-Catenin Destruction Complex

(A) Representative confocal images of control, Wg-overexpressing, Apc heterozygous (Apc+/−), and Apc mutant (Apc−−) posterior midguts with or without RalA knockdown (RalA-RNAi(1)) in ISCs/EBs. Scale bar, 50 μm.

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by oral bacterial infection with the pathogen Enwinia carotovora carotovora (Ecc15) (Basset et al., 2000). We quantified ISC proliferation through scoring of pH3 positive cells in posterior midguts from control animals and animals following RalA-RNAi expression within ISCs/EBs using the escargot-gal4 driver (Michelli and Perrimon, 2006; Figures 1C and 1D). We observed approximately 50% decrease in damage-induced ISC proliferation in RalA knockdown animals (Figure 1D). This phenotype was recapitulated with two additional, independent RalA-RNAis (Figure S2A) and upon knockdown of the RAL GTPase effector RalBP1 (Figures 1C and 1D). These results show that RNAi-mediated knockdown of RalA does not alter gut homeostasis in Drosophila but impairs ISC proliferation during damage-induced regeneration of the intestine. Reciprocally, overexpression of RalA in ISCs/EBs using two independent constructs was sufficient to induce ISC proliferation in the posterior midgut (Figures 1E and 1F).

Inducible Wnt ligand (Wingless; Wg) secretion from progenitor cells (EBs) is required for intestinal regeneration in Drosophila but is dispensable for steady-state tissue maintenance (Cordero et al., 2012b). We therefore hypothesized that the damage-specific role of RalA in the intestine might be mediated through regulation of Wnt signaling. To investigate this further, we combined RalA knockdown with a transgenic reporter of Wnt signaling (Fz3-RFP) (Olson et al., 2011). Control animals showed Fz3-RFP significantly upregulated within ISCs/EBs of the posterior midgut following damage, while RalA knockdown impaired Fz3-RFP upregulation in damaged tissues (Figures 1G and 1H). A similar result was observed for dMyc, which is a known target of Wnt signaling during regeneration and aging (Cordero et al., 2012a; Figures 1I and 1J). Consistent with our gain-of-function data (Figures 1E and 1F), overexpression of RalA within ISCs/EBs was sufficient to induce upregulation of Fz3-RFP and dMyc expression (Figures 1J–1L and S2C). Importantly, the role of RalA on Wnt signaling was not restricted to the midgut, as evidenced by downregulation of the Wnt signaling target Senseless in the larval wing disc, following domain-specific RalA knockdown (Figures 1M, 1N, and S2F–S2H). Together, these data suggest that RalA is necessary and sufficient for Wnt signaling activation in vivo.

**RalA Regulates Wnt Signaling Upstream of the β-Catenin Destruction Complex in Drosophila ISCs**

We next assessed how Wnt signaling pathway is regulated by RalA in the Drosophila gut. Ectopic activation of the Wnt pathway through overexpression of Wg or following deletion of Apc drives ISC hyperproliferation (Cordero et al., 2012b; Figures 2A and 2B). Remarkably, only ISC proliferation induced by overexpression of Wg was suppressed by concomitant RalA knockdown (Figures 2A, 2B, S2D, and S2E). This suggests the role of RalA on Wnt signaling lies upstream of the β-catenin destruction complex and downstream of the Wg ligand.

We next investigated whether RalA knockdown impaired production or secretion of Wnt ligands. Critically, knockdown of RalA did not affect damage-induced Wg production (Figures 2C and 2D; Cordero et al., 2012b). Similarly, RalA status had no impact upon levels of extracellular Wg (eWg) in the larval Drosophila wing disc (Figures 2E, 2F, and S2I–S2K; Strigini and Cohen, 2000). These data suggest that RalA functions within cells receiving the signal from the Wg ligand. In response to damage, Wg mainly secreted by EBs activates Wg signaling within ISCs (Cordero et al., 2012b). Consistently, RalA knockdown in EBs alone had no effect on fly midgut regeneration (Figures 2G, 2H, and S2L), while RalA knockdown in ISCs only significantly impaired midgut regeneration using two independent RNAi constructs (Figures 2I, 2J, and S2M). These results suggest a cell-autonomous role for RalA regulating Wnt signaling activation within ISCs during regeneration. Accordingly, RalA knockdown in ISCs but not EBs impaired Fz3-RFP and dMyc upregulation upon damage (Figures 2K, 2L, and S2N–S2Q).

Taken together, these results strongly suggest that RalA has a stem cell-autonomous role in controlling of Wnt signaling activation upstream of the β-catenin destruction complex in the adult fly midgut.

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(B) Quantification of pH3+ cells in posterior midguts as in (A). Two-way ANOVA is shown, followed by Sidak’s multiple comparisons test; n = number of posterior midguts quantified; error bars, ± SEM.

(C) Representative confocal images of Wg staining intensity (red or gray) within the ISC/EB compartment in mock-treated or regenerating (Ecc15) control or RalA knockdown (RalA-RNAi(1)) posterior midguts. Scale bar, 20 μm.

(D) Quantification of the average Wg staining intensity within the ISC/EB compartment in posterior midguts as in (C). Two-way ANOVA is shown, followed by Sidak’s multiple comparisons test; each dot represents a z stack confocal image from a posterior midgut; error bars, ± SD.

(E) Representative confocal images of extracellular Wg staining (eWg; green) in imaginal discs upon RalA knockdown (RalA-RNAi(1)) in the posterior compartment (RFP positive) using the engrailed-RFP driver. Scale bar, 50 μm.

(F) Quantification of extracellular Wg staining intensity perpendicular to the line of Wg secretion in control (RFP negative) versus RalA knocked down (RFP positive) larval wing discs. Multiple t tests, FDR = 0.01; n = 4 wing discs; error bars, ± SEM.

(G) Representative confocal images of mock-treated and regenerating (Ecc15) adult posterior midguts from control animals or animals subject to adult-specific RalA knockdown within EBs (green) using the Su(H)-gale4, UAS-gtp driver. Scale bar, 50 μm.

(H) Quantification of pH3+ cells posterior midguts as in (G). Two-way ANOVA is shown, followed by Sidak’s multiple comparisons test; n = number of posterior midguts quantified; error bars, ± SEM.

(I) Representative confocal images of mock-treated and regenerating (Ecc15) adult posterior midguts from control animals or animals subject to adult-specific RalA knockdown within ISCs (green) using the esg-gale4;Su(H)-gale40 driver. Scale bar, 50 μm.

(J) Quantification of pH3+ cells in posterior midguts as in (I). Two-way ANOVA is shown, followed by Sidak’s multiple comparisons test; n = number of posterior midguts quantified; error bars, ± SEM.

(K) Representative confocal images of the Wnt pathway activity reporter Fz3-RFP (red or gray) in mock-treated or regenerating (Ecc15) adult posterior midguts from control animals or animals subject to adult-specific RalA knockdown (RalA-RNAi(2)) within ISCs (green). Scale bar, 20 μm.

(L) Quantification of the average Fz3-RFP staining intensity within the ISC compartment in posterior midguts as in (K), normalized to 1. Two-way ANOVA is shown, followed by Sidak’s multiple comparisons test; each dot represents a z stack confocal image from a posterior midgut; error bars, ± SD.

Where indicated, **p < 0.01 ****p < 0.0001.
Figure 3. Loss of Either RALA or RALB Suppresses Wnt Signaling in the Murine Intestine

(A) Representative in situ hybridization (ISH) images of Wnt target gene expression; Axin2, Ascl2, Lgr5, and Olfm4 in the small intestine after Rala and Ralb deletion (red). Ppib used as positive control. Scale bar, 50 μm.

(B) Quantification of ISH staining in 25 crypts per mouse, WT = 5, Rala/+/ = 5, Ralb/+/ = 4 or 5 for Axin2, Lgr5, Olfm4, and Ascl2. Error bars, ± SD.

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Loss of RALA or RALB Suppresses Wnt Signaling in the Murine Intestine

Given the impact of Rala deletion in Drosophila, we predicted that loss of Rala or Ralb would reduce Wnt signaling in the murine intestine. As both isoforms are expressed within the intestinal crypt, we generated intestinal-specific conditional knockout mice for Rala or Ralb to test this hypothesis (Figure S3A). Genetic deletion of Rala was confirmed by immunohistochemistry (IHC) for RALA and transcriptionally by qPCR (Figures S3B and S3D), and for Ralb through both qPCR and RNA in situ hybridization (RNA-ISH) targeting the deleted exon (Figures S3C and S3D). VillinCreER Ralafl/fl and VillinCreER Ralbfl/fl intestines were examined 4 days post-induction (PI) of recombination and exhibited no significant alterations in gross intestinal morphology or epithelial proliferation (Figure S3E). Moreover, analysis of periodic acid-Schiff (PAS) or Alcian blue stains and IHC for LYSOZYME indicated that goblet and Paneth cell lineages, respectively, were also unaffected (Figure S3G). These observations suggest Rala and Ralb have redundant functions in mammalian intestinal homeostasis.

While the intestinal epithelium was viable following deletion of Rala or Ralb, this did not preclude alterations in ISC number or Wnt pathway activation. It is known that inhibition rather than ablation of Wnt signaling can reduce expression of Wnt target genes and ISC markers, rather than perturb intestinal homeostasis (Flanagan et al., 2015; Huels et al., 2018; Metcalfe et al., 2014; Yan et al., 2017). To gain a spatial overview of RNA expression of Wnt regulated genes in the SI, we performed RNA in situ hybridization (RNAscope). This indicated reduced expression of the canonical Wnt target genes Axin2 and Ascl2 in the intestinal crypts of Ralafl/fl and Ralbfl/fl mice. Similarly, the ISC markers Lgr5 and Oflm4 were decreased following deletion of either isoform (Figures 3A and 3B), as were the number of intestinal crypt cells expressing these markers (Figure S3H). This was confirmed by reduced expression of the stem cell marker CD44 following Rala or Ralb deletion (Figure 2C). To directly test Wnt pathway activation, we scored for nuclear β-catenin, finding significantly fewer positive nuclei of the intestinal crypts in Ralafl/fl and Ralbfl/fl mice (Figures 3C and 3D). These data indicate that, as with RalA in Drosophila, loss of Rala or Ralb reduces Wnt signaling in the murine intestinal epithelium. As maintenance of the Lgr5+ ISC pool requires high levels of Wnt signaling, this is consistent with a marked reduction in the size of this population.

Since intestinal regeneration following damage is Wnt ligand dependent (Ashton et al., 2010; Saha et al., 2016), and considering Rala activity in the fly intestine, we sought to determine the impact of Rala or Ralb deletion upon intestinal regeneration (Figure 3E). Exposure of mice in which intestinal Rala or Ralb had been deleted (VillinCreER Ralafl/fl and VillinCreER Ralbfl/fl) to 10 Gy irradiation impaired regenerative capacity when compared to controls (Figure 3F), shown by reduced number and size of regenerating crypts (Figure 3H). Proliferation was also decreased in Rala- and Ralb-deficient regenerating crypts (Figures 3F and 3H). These data confirmed the conserved role for RALs in regeneration following damage in the adult Drosophila and murine intestine.

Next, we investigated whether RAL depletion in the Lgr5+ ISC population could block regeneration (Figures 2G–2J). Ralafl/fl and Lgr5-EGFP-CreER (Lgr5CreER; Barker et al., 2007) mice were interbred to specifically delete Rala in the Lgr5+ population. Since the Lgr5CreER transgene exhibits mosaic expression and drives recombination with incomplete penetrance (Leushacke et al., 2017), we employed a robust, 4 day induction regime prior to irradiation (Figure 3E). Use of a R26-LSL-ttdTomato (tdTom) reporter strain indicated that recombination occurred in approximately 60% of crypts under this regime (Figure S3I). When Lgr5-EGFP-CreER; Rala+/− and Lgr5-EGFP-CreER; Ralb+/− mice were harvested 72 h post-irradiation, it was observed that Lgr5-EGFP-CreER Rala+/− exhibited significantly fewer regenerating crypts than control animals (Figures 3I and 3J). Importantly, of regenerating crypts within Lgr5-EGFP-CreER Rala+/− intestines, those that had escaped recombination and were Rala proficient (Figure 3I) and significantly larger than Rala-deficient crypts (Figure 3K).

These experiments confirm a conserved role for RALs in intestinal regeneration, a role directly related to RAL function in Lgr5-expressing ISCs.

RAL GTPases Affect Wnt Signaling Activity Upstream of the β-Catenin Destruction Complex in Mice

Given strong evidence of an in vivo role for the RALs in regulation of Wnt signaling in Drosophila and mice, we investigated which aspect of Wnt signaling was RAL dependent. Initially, since Rala or Ralb depletion affected the ISC pool in the murine intestine, we predicted that reduction of Wnt ligand would cooperate with RAL depletion to markedly reduce Wnt signaling. To this end, we impaired Wnt ligand secretion using an inhibitor of the O-palmitoyltransferase Porcupine (WNT974) (Figure 4A; Jiang et al., 2013). We have recently shown that this compound has the effect of reducing ISC number in vivo (Huels et al., 2018). We observed no significant impact upon normal intestinal viability following continuous treatment for 8 days in VillinCreER Rala+/− Ralb+/− mice, but a striking cooperation of RAL deficiency with Porcupine inhibition, whereby Rala or Ralb-deficient intestines lost intestinal crypts and exhibited reduced proliferation after...
4 days of treatment with WNT974 when compared to controls (Figures 4B and 4C).

Next, we examined whether loss of the destruction complex could rescue the impact of RAL deletion in the context of genetic ablation of the tumor suppressor gene Apc, through generation of VillinCreER; Apcfl/fl; Ralbfl/fl mice. Deletion of Apc in the entire intestinal epithelium results in a “crypt-progenitor phenotype” characterized by increased proliferation (Sansom et al., 2004), and expression of ISC markers such as Lgr5 and Wnt target genes, alongside perturbed migration and differentiation. Critically, deletion of Apc rescued loss of normal homeostasis and protected Ralb-deficient intestines from Porcupine inhibition (Figures 4B and 4C). This indicates that RAL proteins are important for Wnt signaling at the level of the ligand or receptor rather than the destruction complex or transcriptional activation of Wnt target genes in both Drosophila and murine intestines.

To expand upon the link between RALs and Wnt signaling, we utilized intestinal organoid culture. Organoids are readily prepared from the intestinal epithelium of mice and grow in a Wnt-dependent manner (Sato et al., 2009). We first cultured organoids from VillinCreER; Ralafl/fl or VillinCreER; Ralbfl/fl mice, sampled at 4 days PI. We found that, after 3 days in culture, organoids isolated from Ralafl/fl or Ralbfl/fl mice exhibited impaired seeding and growth in culture medium supplemented with standard 50 ng/mL R-Spo1 (Figures 4D and 4E). Furthermore, these organoids were inefficient in colony outgrowth (Figure S4A),
implying a loss of stem cell function. To distinguish between establishment and sustained growth of organoids, we next deleted Ralb through addition of 1 μM 4-OHT to the culture medium of organoid cultures derived from uninduced VillinCreER<sup>R+</sup>; Ralb<sup>fl/fl</sup> mice. These organoids rapidly lost the expression of the stem cell marker Cd44 and lost viability at 48 h PI (Figure S4B). Thus, RALs are required for establishment and maintenance of intestinal organoids in vitro. Given that the Rala- and Ralb-deficient crypts were rapidly lost in culture, we questioned whether this was due to ISC differentiation or apoptosis. IHC against cleaved caspase-3 indicated that equivalent levels of apoptosis were present in intestinal crypts of Rala- or Ralb-deficient mice compared to wild-type (WT) controls in vivo (Figures S4C and S4D). This suggests that the loss of ISCs and lack of crypt viability in culture arises from differentiation caused by Rala or Ralb loss. Given data from Drosophila, we predicted that phenotypes driven by Ralb depletion in murine intestinal organoids were unlikely to result from impaired production or secretion of Wnt ligands. To test this, we sought to rescue growth of Ralb-deficient organoids with exogenous Wnt ligand. Growth was not rescued, suggesting the impact of Ralb depletion is not mediated by ligand availability (Figure 4E). In support of in vivo observations, Apc deficiency restored growth of Ralb-deficient organoids (Figure 4E). We also sought to rescue growth by increasing the R-spo1 concentration, which again had no effect on growth (Figure 4E). Interestingly, removing R-spo1 from culture medium impaired growth but did not reduce number of WT organoids, while Rala<sup>fl</sup> and Ralb<sup>fl</sup>-derived organoids almost completely failed to seed and grow (Figures 4D and 4E).

**RAL GTPase Signaling Mediates Internalization of Frizzled Receptors and Wnt Pathway Activation in HEK293T Cells**

RAL depletion had no impact upon Wnt ligand secretion/production in Drosophila, and Wnt phenotypes in the fly and mouse were rescued by Apc loss, indicating that RALs may regulate receptor complex activation. Given the link between RALs and endocytosis (Jiang et al., 2016; Jullien-Flores et al., 2000), we hypothesized that their role in Wnt signaling may involve regulation of receptor localization. To address this, we deleted RALA, RALB, and their effector RALBP1 using CRISPR/Cas9 in HEK293T cells, a well-established model that allows biochemical assessment of Wnt signaling in vitro (Figure S5A).

Through analysis of the internalization of biotinylated Frizzled-7 and LRP6 over time, we found that internalization of these Wnt-signalosome receptors was significantly decreased following RALA, RALB, or RALBP1 deletion (Figure 5A). This was corroborated through high-resolution imaging of internalization of fluorescently labeled Frizzled-5 or -7 over time (Figure S5B). While the kinetics of Frizzled-5 and -7 internalization differ, with Frizzled-7 internalized more rapidly under control conditions, internalization of both was reduced following depletion of RALA, RALB, or RALBP1 (Figures 5B and 5C). Furthermore, internalization of SNAP-Frizzled-7 was reduced in serum-free medium supplemented with 100 ng/mL Wnt-3a in these lines (Figure 5D), and internalization of biotinylated Frizzled-7 was impaired following RALA knockout under the same conditions (Figure 5E). This suggests that RAL GTPases and RALBP1 are involved in regulating Frizzled-7 receptor internalization.

We next assessed canonical Wnt signaling in this setting through accumulation of nuclear β-catenin in nuclear protein fractions as a surrogate. RALA, RALB, and RALBP1 depletion resulted in a reduction of nuclear β-catenin when compared to control (Figure 5F). Furthermore, Wnt-3a treatment resulted in a reduction of β-catenin in the cytoplasm of cells with RAL expression (Figure 5F). Moreover, baseline Wnt activity and response to Wnt-3a ligand in RALA, RALB, and RALBP1-depleted lines were reduced as measured by TCF/LEF transcriptional activity using a Super TopFlash construct (Figure 5G).

Given that APC loss rescued lethality caused by RAL depletion in intestinal organoid cultures, APC-deficient organoids can be used to assess the impact of RAL deficiency upon Frizzled receptor internalization. We examined endogenous Frizzled-7 localization through immunofluorescent staining and fluorescence-activated cell sorting (FACS) analysis of cell-surface Frizzled-7 on living disaggregated organoids. Critically, we observed elevation of cell-surface Frizzled-7 expression upon knockdown of Apc and Ralb in organoids when compared to Apc deficiency alone (Figures 5H and 5I). This is supported by higher levels of Frizzled-5, -6, and -7 detected on the cell surface on Ralb GTPase knockout HEK293T cells in pull-down experiments (Figure S5D). These data support a role for RAL signaling in activation of Wnt signaling through promotion of internalization of Frizzled-7, and the Wnt signalosome.

**RAL GTPases Regulate Stem Cell Function in the Intestine**

Next, we explored whether reduced ISC marker expression associated with RAL deletion impacted stem cell fitness when compared to wild-type neighbors. In homeostasis, ISC replace-ment in the mammalian intestine is a stochastic process, where the presence of competing stem cells eventually results in repopulation of the entire crypt from an individual stem cell (Lopez-García et al., 2010; Snippert et al., 2010). This process is known as neutral drift and is a mechanism for removal of stem cells carrying oncogenic or deleterious mutations (Vermeulen et al., 2013). Indeed, we have shown that reducing the ISC pool increases the likelihood that an oncogenic mutation might overtake the crypt (Huels et al., 2018). These observations suggest that, if Wnt activation in a RAL-deficient stem cell was reduced compared to wild-type neighbors, it would be more likely to be lost within an intestinal crypt over time.

We interbred Lgr5-EGFP-Cre<sup>ER</sup> (Lgr5CreER<sup>ER</sup>) mice (Barker et al., 2007) to Ralb conditional knockout, and R26-LSL-tTomato (tdTom<sup>fl</sup>) mice (Madsen et al., 2010), allowing us to visualize expansion of Ralb<sup>fl</sup> and Ralb<sup>fl</sup>-ISC clones (Figures 6A and 6B). We then induced Ralb deletion within individual stem cells per crypt using a low dose of tamoxifen (0.15 mg, in line with established protocols) (Figure 6A). It is notable that, in this system, the induction of Ralb deletion in the Lgr5<sup>+</sup> compartment takes place prior to any impact that Ralb has on Wnt-target expression, in particular, Lgr5, while expression of TdTomato from the Rosa26 locus as the readout for the experiment is agnostic of any stem cell marker. Consistently, we observe similar labeling with tdTom<sup>fl</sup> in the presence or absence of Ralb at a time point 4 days PI, indicating equal recombination in

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Figure 5. RAL Small GTPase Signaling Mediates Internalization of Frizzled Receptors and Wnt Pathway Activation in HEK293T Cells

(A) HEK293T cells were labeled with NHS-S-S-Biotin at 4 °C and internalization allowed to proceed at 37 °C. Error bars, ± SEM (3 independent experiments); 1-way ANOVA Tukey’s multiple comparisons test.

(B) Representative maximal projection of confocal images of immunofluorescently labeled SNAP-FZD7 after internalization in HEK293T cells. Scale bar, 5 μm.

(C) Quantification of SNAP-FZD7 internalization experiments. Error bars, ± SEM (4 independent experiments); 1-way ANOVA Dunnett’s multiple comparisons test.

(D) Quantification of SNAP-FZD7 internalization in serum-free media supplemented with 100 ng/mL Wnt-3a. Error bars, ± SEM (4 independent experiments). 1-way ANOVA Dunnett’s multiple comparisons test.

(E) Internalization of NHS-S-S-Biotin-labeled HEK293T in serum-free conditions with Wnt-3a supplemented media. Error bars, ± SEM (4 independent experiments); unpaired t test.

(F) Representative western blots of cytosolic and nuclear β-catenin in HEK293T cell lines following Wnt-3a treatment. β-actin and H3 were used as loading controls. H3 was marked nuclear enrichment.

(G) Wnt activity measured as Super TopFlash luciferase signal normalized to β-galactosidase levels in HEK293T cells following RALA, RALB, and RALBP1 knockout and Wnt-3a treatment. Error bars, ± SEM (4 independent experiments). 1-way ANOVA Dunnett’s multiple comparisons test.

(H) Immunofluorescence (IF) staining of Fzd-7 in organoids derived from Apc\(^{+/-}\) = 5, and Apc\(^{+/-}\), Rab\(^{+/-}\) = 5 mice. Scale bar, 50 μm.

(I) Quantification of cell-surface Fzd-7 in organoids using FACS (Apc\(^{+/-}\) = 5 and Apc\(^{+/-}\), Rab\(^{+/-}\) = 5). Non-parametric Mann-Whitney U test. Error bars, ± SD. Where indicated, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
both cases (Figures 6C–6E). However, at later time points (days 10–30) there was a shift to labeled crypts being more sparsely populated in Ralbfl/fl mice when compared to Ralb+/fl mice, where a substantial proportion of crypts were fully fixed (Figure 6D). Importantly, at day 30 we could confirm that Ralb was specifically deleted in fixed tdTomato positive crypts (Figure S6 A). Quantification indicated that loss of Ralb reduced clonogenicity (Figure 6E) and profoundly reduced the numbers of fully fixed crypts at days 14 and 30 (Figure 6D). Importantly, this resulted in significantly fewer labeled clones in the SI of Ralbfl/fl mice (Figure 6F), indicating that depletion of Ralb puts ISCs at a disadvantage when compared to wild-type neighbors.

**Complete Ablation of RAL GTPase Causes Crypt Death, which Can Be Rescued by APC Loss**

Given that our mammalian studies thus far were limited to reduction of global RAL activity through single-gene deletion, we addressed the consequences of ablation of both homologs. We interbred VillinCreER Ralafl/fl and VillinCreER Ralbfl/fl strains to generate double VillinCreER Ralafl/fl and VillinCreER Ralbfl/fl conditional mice and induced highly penetrant recombination as before. Deletion of both genes was confirmed by qPCR (Figure S7A). The deletion of both Rala and Ralb resulted in a dramatic phenotype. At 2 days PI, there was a significant reduction in proliferation (as assessed by bromodeoxyuridine [BrdU] incorporation) (Figures 7A and 7B) and expression of stem cell markers and Wnt target...
genes (Figures 7C, 7D, and S7B), albeit with no significant increase in cleaved caspase-3 positive cells (Figures S7C and S7D). At 3 days PI, crypt structures were absent, indicating functional loss of stem cells within the intestine (Figure 7A), while goblet and Paneth cell numbers remained unaffected (Figure S7C), although Paneth cell localization shifted toward the intestinal lumen as crypts were lost. Given the promiscuous nature of RAL signaling, we sought to confirm whether crypt loss following complete genetic ablation of Rala and Ralb was related to suppression of Wnt signaling, again by assessing whether
deletion of Apc could rescue the phenotype. Remarkably, crypt architecture was maintained in intestines from VillinCreER; Apc<fl/fl>, Ralb<fl/fl>, Rala<fl/fl> mice, and intestinal crypts exhibited significant proliferation (Figures 7A and 7B), alongside expression of Wnt targets and ISC markers such as Lgr5, Olfm4, Axin2, Ascl2, β-catenin, and Cd44 (Figures 7C–7E).

**DISCUSSION**

We present a conserved in vivo role for RAL signaling in regulating ISC number, which impacts intestinal homeostasis and regeneration. RALs do so by promoting internalization of the Wnt pathway receptor complex at the cell surface, and activating canonical Wnt signaling.

**RAL GTPases and the Potentiation of Wnt Signaling in the Intestine**

Studies have demonstrated that Wnt and Rspo interact to drive ISC expansion and maintenance in the intestine (Yan et al., 2017). Our work suggests that loss of RAL expression reduces Wnt pathway activity and results in differentiation of Lgr5+ stem cells, no longer agonized by Rspo. The result would be an intestine with significantly fewer functional ISCs. It should be noted we see a dramatic impact on Frizzled-7 internalization in vitro, and that this receptor is also highly expressed in ISCs. Loss of frizzled 7 also causes specific loss of Lgr5+ ISC (Flanagan et al., 2015).

The non-redundant role of mammalian Rala and Ralb (Peschard et al., 2012) is consistent with our previous observations of single-gene knockdown having essential functions during regeneration of the intestine, while redundant for normal tissue homeostasis (Ashton et al., 2010; Cordero et al., 2014). This implies that the threshold of Wnt signaling required for homeostatic stem cell renewal may differ from that required for intestinal regeneration. Consistently, during regeneration, where Wnt signaling and stem cell function must be potentiated, the effect of the loss of either RAL isoform is more apparent. Similarly, further reduction in Wnt ligand through Porcupine inhibition strongly enhances the effects of partial Ral loss of function in the intestine, resulting in a complete loss of stem cell function and ultimately crypt death. Critically, total ablation of Rala and Ralb is likely to reduce Wnt signaling below a threshold level required for intestinal survival, mimicking the effect of β-catenin deletion or DKK overexpression (Ireland et al., 2004; Kuhnert et al., 2004).

**RAL Proteins Function Upstream of β-Catenin Activation in the Intestine**

Our data provide robust in vivo evidence of the impact of altered Wnt signaling beyond direct control of β-catenin stability by the destruction complex (Figure 3). More precisely, it emphasizes the value of restricting the cellular localization of pathway receptors (Figure 4). The robust rescue of both fly and murine RAL loss of function phenotypes through depletion of Apc suggests that within ISCs, control of Wnt signaling by RAL is a dominant event. This mirrors the phenotype observed following combined deletion of GSK3α and GSK3β in the intestine, which mimics that of Apc loss despite the non-Wnt-related functions of these kinases (Huels et al., 2015).

**RAL GTPases and the Regulation of Signaling Pathways through Receptor Internalization**

Our study strongly suggests that endocytosis driven by RAL and RALBP1 is critical for Wnt activity in vivo. A previous study suggested that the active Wnt signalosome resides within Clathrin-mediated pits (Gammons et al., 2016), while others indicate that caveolar endocytosis is important for Wnt pathway activation (Blitzer and Nusse, 2006; Yamamoto et al., 2006). These differences in part seem to be determined by cell type (Feng and Gao, 2015; Kim et al., 2013). Previously, RALBP1 has been associated with Clathrin-mediated endocytosis (Julien-Flores et al., 2000), while the RALs have also been linked to caveolar endocytosis (Jiang et al., 2016). These studies indicate that RALs have general role controlling endocytic processes. Recently, the Wnt-regulatory tumor suppressor APC has also been postulated to inhibit Clathrin-mediated endocytosis, whereby impairment of APC allows cells to use Clathrin-mediated endocytosis to drive ligand independent activation of the receptors (Saito-Diaz et al., 2018). In addition to a better understanding of the specific role of RAL signaling in endocytosis, there is a need to elucidate the contribution of clathrin and non-clathrin-mediated endocytosis on Wnt signaling in both ISCs and CRC.

**A Conserved Role of RAL GTPases in the Intestine**

Most RAL-dependent intestinal phenotypes analyzed in our studies are conserved between Drosophila and mice. One difference is the role of RAL in homeostasis, which appears redundant in the fly, at least in the context of partial knockdown by RNAi. This may relate to differences in homeostatic proliferative state between the two model systems. In the fly midgut, basal stem cell proliferation is low and there is no transit amplifying proliferative zone (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006), while Wnt signaling pathway activity within ISCs/EBs in the fly midgut is barely detectable in homeostatic conditions. In contrast, there is very high basal Wnt signaling in mammalian Lgr5+ ISCs, which are as a result, rapidly impacted following Rala or Ralb depletion. Our previous studies have shown that Wg is significantly induced during regeneration in the adult Drosophila midgut, where we also see the dramatic impact of Ral knockdown (Cordero et al., 2012b; Figure 1). Despite differences in the two model systems, our results clearly demonstrate the power of combining Drosophila and mouse models to understand fundamental principles of ISC biology.

We detail a conserved functional role for the RALs for ISC regeneration. Importantly, our work points to the regulation of cell-surface receptor complex internalization as a mechanism to maintain Wnt signaling and stem cell responses to the proliferative demands of the intestine.

**STAR METHODS**

Detailed methods are provided in the online version of this paper and include the following:

- **KEY RESOURCES TABLE**
- **CONTACT FOR REAGENT AND RESOURCE SHARING**
- **EXPERIMENTAL MODEL AND SUBJECT DETAILS**
  - Experimental Animals
  - Drosophila breeding and maintenance
Supplemental Information can be found with this article online at https://doi.org/10.1016/j.stem.2019.02.002.

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AUTHOR CONTRIBUTIONS

J.J., M.N., K.A.P., B.W.M., Y.Y., and R.R. performed experiments. J.J., M.N., M.C.H., R.R., B.W.M., P.P., S.B., A.D.C., J.B.C., and O.J.S. designed experiments and analyzed the data. J.J., M.N., A.D.C., J.B.C., and O.J.S. wrote the manuscript. J.B.C. and O.J.S. directed the study.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

Ahmed, Y., Hayashi, S., Levine, A., and Wieschaus, E. (1998). Regulation of armadillo by a Drosophila APC inhibits neuronal apoptosis during retinal development. Cell 93, 1171–1182.

Ashton, G.H., Morton, J.P., Myant, K., Phesse, T.J., Ridgway, R.A., Marsh, V., Wilkins, J.A., Athineos, D., Muncan, V., Kemp, R., et al. (2010). Focal adhesion kinase is required for intestinal regeneration and tumorigenesis downstream of Wnt/cAMP signaling. Dev. Cell 19, 259–269.

Barker, N., van Es, J.H., Kuipers, J., Kujaia, P., van den Born, M., Cozijnsen, M., Haegearth, A., Koning, J., Begthel, H., Peters, P.J., and Clevers, H. (2007). Identification of stem cells in small intestine and colon by marker gene Lgr5. Nature 449, 1003–1007.

Basset, A., Khush, R.S., Braun, A., Gardan, L., Boccard, F., Hoffmann, J.A., and Lemaitre, B. (2000). The pyrophagotrophic bacteria Erwinia carotovora infects Drosophila and activates an immune response. Proc. Natl. Acad. Sci. USA 97, 3376–3381.

Bilic, J., Huang, Y.L., Davidson, G., Zimmermann, T., Cruciat, C.M., Bienz, M., and Niehrs, C. (2007). Wnt induces LRP6 signalosomes and promotes disease-mediated LRP6 phosphorylation. Science 316, 1619–1622.

Blitzer, J.T., and Nusse, R. (2006). A critical role for endocytosis in Wnt signaling. BMC Cell Biol. 7, 28.

Bodmann, B.O., and White, M.A. (2008). Raf GTPTases and cancer: linchpin support of the tumorigenic platform. Nat. Rev. Cancer 8, 133–140.

Bourbon, H.M., Gonzy-Treboul, G., Peronnet, F., Alin, M.F., Ardourel, C., Benassayag, C., Cribsb, D., Deutsch, J., Ferrer, P., Haenlin, M., et al. (2002). A P-insertion screen identifying novel X-linked essential genes in Drosophila. Mech. Dev. 110, 71–83.

Casali, A., and Ballie, E. (2009). Intestinal stem cells in mammals and Drosophila. Cell Stem Cell 4, 124–127.

Chen, X.W., Leto, D., Chiang, S.H., Wang, Q., and Saitlie, A.R. (2007). Activation of Ra1A is required for insulin-stimulated Glut4 trafficking to the plasma membrane via the exocyst and the motor protein Myo1c. Dev. Cell 13, 381–404.

Chen, Y., Kim, S., Bumeister, R., Luo, Y.M., Kwon, S.W., Johnson, C.L., Balakireva, M.G., Romeo, Y., Kopelovich, L., Gale, M., Jr., et al. (2006). RaB GTPTase-mediated activation of the IkapabB family kinase TBK1 couples innate immune signaling to tumor cell survival. Cell 127, 157–170.

Chung, S.S., Lee, J.S., Kim, M., Ahn, B.Y., Jung, H.S., Lee, H.M., Kim, J.W., and Park, K.S. (2012). Regulation of Wnt/beta-catenin signaling by CCAAT enhancer binding protein ß during adipogenesis. Obesity (Silver Spring) 20, 482–487.

Clevers, H. (2006). Wnt/beta-catenin signaling in development and disease. Cell 127, 469–480.

Cordero, J.B., Stefanatos, R.K., Myant, K., Vidal, M., and Sansom, O.J. (2012a). Non-autonomous crosstalk between the Jak/Stat and Egfr pathways mediates Apc1-driven intestinal stem cell hyperplasia in the Drosophila adult midgut. Development 139, 4524–4535.

Cordero, J.B., Stefanatos, R.K., Scopelliti, A., Vidal, M., and Sansom, O.J. (2012b). Inducible progenitor-derived Wingless regulates adult midgut regeneration in Drosophila. EMBO J. 31, 3901–3917.

Cordero, J.B., Ridgway, R.A., Valeri, N., Nixon, C., Frame, M.C., Muller, W.J., Vidal, M., and Sansom, O.J. (2014). c-Src drives intestinal regeneration and transformation. EMBO J. 33, 1474–1491.

Degirmenci, B., Valenta, T., Dimitrieva, S., Hausmann, G., and Basler, K. (2018). GLI1-expressing mesenchymal cells form the essential Wnt-secreting niche for colon stem cells. Nature 558, 449–453.

Dutta, D., Buchon, N., Xiang, J., and Edgar, B.A. (2015). Regional Cell Specific RNA Expression Profiling of FACS Isolated Drosophila Intestinal Cell Populations. Curr. Protoc. Stem Cell Biol. 34, 1–14.

el Marjou, F., Janssen, K.P., Chang, B.H., Li, M., Hindie, V., Chan, L., Louvard, D., Chambon, P., Metzger, D., and Robin, S. (2004). Tissue-specific and inducible Cre-mediated recombination in the gut epithelium. Genesis 39, 186–193.

Feng, Q., and Gao, N. (2015). Keeping Wnt signaling in check by vesicular traffic. J. Cell. Physiol. 230, 1170–1180.

Flanagan, D.J., Phesse, T.J., Barker, N., Schwab, R.H., Amin, N., Malaterre, J., Stange, D.E., Nowell, C.J., Currie, S.A., Saw, J.T., et al. (2015). Frizzled7
functions as a Wnt receptor in intestinal epithelial Lgr5(+) stem cells. Stem Cell Reports 4, 759–767.

Furriols, M., and Bray, S. (2001). A model Notch response element detects Suppressor of Hairless-dependent molecular switch. Curr. Biol. 11, 60–64.

Gammons, M.V., Renko, M., Johnson, C.M., Rutherford, T.J., and Bienza, M. (2016). Wnt Signaling System by DEP Domain Swapping of Dishevelled. Mol. Cell 64, 92–104.

González-García, A., Pritchard, C.A., Paterson, H.F., Mavria, G., Stanton, G., and Marshall, C.J. (2005). RaGDS is required for tumor formation in a model of skin carcinogenesis. Cancer Cell 7, 219–226.

Goto, S., and Hayashi, S. (1999). Proximal to distal cell communication in the Drosophila egg provides a basis for an intercalary mechanism of limb patterning. Development 126, 3407–3413.

Gregoireff, A., Pinto, D., Begthel, H., Destrée, O., Kielman, M., and Clevers, H. (2005). Expression pattern of Wnt signaling components in the adult intestine. Gastroenterology 129, 626–638.

Herranz, H., Perez, L., Martin, F.A., and Milan, M. (2008). A Wingless and Notch double-repression mechanism regulates G1-S transition in the Drosophila wing. EMBO J. 27, 1633–1645.

Huels, D.J., Ridgway, R.A., Radulescu, S., Leushacke, M., Campbell, A.D., Biswas, S., Leedham, S., Serra, S., Chetty, R., Moreaux, G., et al. (2015). E-cadherin can limit the transforming properties of activating β-catenin mutations. EMBO J. 34, 2321–2333.

Huels, D.J., Bruens, L., Hodder, M.C., Cammarena, P., Campbell, A.D., Ridgway, R.A., Gay, D.M., Solar-Abbadou, M., Faller, W.J., Nixon, C., et al. (2018). Wnt ligands influence tumour initiation by controlling the number of intestinal stem cells. Nat. Commun. 9, 1132.

Ireland, H., Kemp, R., Houghton, C., Howard, L., Clarke, A.R., Sansom, O.J., and Winton, D.J. (2004). Inducible Cre-mediated control of gene expression in the murine gastrointestinal tract: effect of loss of beta-catenin. Gastroenterology 126, 1236–1246.

Jiang, H., Patel, P.H., Kohlmaier, A., Grenley, M.O., McEwen, D.G., and Edgar, B.A. (2009). Cytokine/Jak/Stat signaling mediates regeneration and homeostasis in the Drosophila midgut. Cell 137, 1343–1355.

Jiang, X., Hao, H.X., Grommey, J.D., Woolfenden, S., Bottiglio, C., Ng, N., Lu, B., Hsieh, M.H., Bagdasarian, L., Meyer, R., et al. (2013). Mdm2 activates the p53 tumor suppressor pathway required for PtdIns(4,5)P2-mediated tumorigenesis in human cells. J. Cell Biol. 199, 2063–2073.

Julien-Flores, V., Mahé, Y., Mirey, G., Leprince, C., Meunier-Bisceuil, B., Simons, B.D., and Winton, D.J. (2010). Intestinal stem cells are indispensable for radiation-induced intestinal regeneration. Cell Stem Cell 14, 149–159.

Kishida, S., Yamamoto, H., Hino, S., Ikeda, S., Kishida, M., and Kikuchi, A. (2006). The digestive tract of Drosophila melanogaster. Annu. Rev. Genet. 40, 1–30.

Koo, B.K., Jinek, M., Zhang, F., Han, C., Doudna, J.A., and Chang, H.A. (2013). Cas9 as a versatile tool for genome engineering. Cell 154, 1187–1195.

Lee, T., and Luo, L. (1999). Mosaic analysis with a represible cell marker for studies of gene function in neuronal morphogenesis. Neuron 22, 451–461.

Lee, T., and Luo, L. (2001). Mosaic analysis with a represible cell marker (MARCM) for Drosophila neural development. Trends Neurosci. 24, 251–254.

Lemaitre, B., and Miguel-Aliaga, I. (2013). The digestive tract of Drosophila melanogaster. Annu. Rev. Genet. 47, 377–404.

Leeuwhacke, M., Tan, S.H., Wong, A., Swathi, Y., Hajamohideen, A., Tan, L.T., Goh, J.J., Weng, E., Denl, S.L.J.J., Murakami, K., and Barker, N. (2017). Lgr5-expressing chief cells drive epithelial regeneration and cancer in the oxyntic stomach. Nat. Cell Biol. 19, 774–786.

Lim, K.H., Baines, A.T., Fiordalisi, J.J., Shiptins, M., Feig, L.A., Cox, A.D., Der, C.J., and Counter, C.M. (2005). Activation of RaA is critical for Ras-induced tumorigenesis of human cells. Cancer Cell 7, 533–545.

Lin, G., Xu, N., and Xi, R. (2008). Paracrine Wingless signaling controls self-renewal of Drosophila intestinal stem cells. Nature 455, 1119–1123.

Lopez-Garcia, C., Klein, A.M., Simons, B.D., and Winton, D.J. (2010). Intestinal stem cell replacement follows a pattern of neutral drift. Science 330, 822–825.

MacDonald, B.T., Tamai, K., and He, X. (2009). Wnt/beta-catenin signaling: components, mechanisms, and diseases. Dev. Cell 17, 9–26.

Madisen, L., Zwingman, T.A., Sunkin, S.M., Oh, S.W., Zarwila, H.A., Gu, H., Ng, L.L., Palminter, R.D., Hawrylcz, M.J., Jones, A.R., et al. (2010). A robust and high-throughput Cre reporting and characterization system for the whole mouse brain. Nat. Neurosci. 13, 133–140.

Martin, T.D., Samuel, J.C., Routh, E.D., Der, C.J., and Yeh, J.J. (2011). Activation and involvement of Ral GTPases in colorectal cancer. Cancer Res. 71, 206–215.

Metcalf, C., Kijjavin, N.M., Ybarra, R., and de Sauvage, F.J. (2014). Lgr5+ stem cells are indispensable for radiation-induced intestinal regeneration. Cell Stem Cell 14, 149–159.

Michelli, C.A., and Perrimon, N. (2006). Evidence that stem cells reside in the adult Drosophila midgut epithelium. Nature 439, 475–479.

Nalbasi, M., Carroll, L.R., and Cordero, J.B. (2015). Intestinal stem cell proliferation and epithelial homeostasis in the adult Drosophila midgut. Insect Biochem. Mol. Biol. 67, 9–14.

Neel, N.F., Martin, T.D., Stratford, J.K., Zand, T.P., Reiner, D.J., and Der, C.J. (2011). The RaGFP–RalEctor Signaling Network: The Road Less Traveled for Anti-Ras Drug Discovery. Genes Cancer 2, 275–287.

Neyen, C., Bretscher, A.J., Binggeli, O., and Lemaitre, B. (2014). Methods to study Drosophila immunity. Methods 68, 116–128.

Nol, R., Abbott, L.A., and Beilen, H.J. (2000). Senseless, a Zn finger transcription factor, is necessary and sufficient for sensory organ development in Drosophila. Cell 102, 349–362.

Ohlstein, B., and Spradling, A. (2006). The adult Drosophila posterior midgut is maintained by pluripotent stem cells. Nature 439, 470–474.

Olson, E.R., Pancratov, R., Chatterjee, S.S., Changkakoty, B., Pervaiz, S., and DasGupta, R. (2011). Yan, an ETS-domain transcription factor, negatively modulates the Wingless pathway in the Drosophila eye. EMBO Rep. 12, 1047–1054.

Pelchard, P., McCarthy, A., Leblanc-Dominguez, V., Yeo, M., Guichard, S., Stamp, G., and Marshall, C.J. (2012). Genetic deletion of RAL and RALb small GTPases reverses redundant functions in development and tumorigenesis. Curr. Biol. 22, 2065–2068.

Richiariya, S., Jayakumar, S., Abuzriki, K., Rosbash, M., and Hasan, G. (2017). A Drosophila transcripomics screen identifies Ral as a target of store-operated calcium entry in Drosophila neurons. Sci. Rep. 7, 42586.

Rodríguez-Viciana, P., and McCormick, F. (2005). RaGDS comes of age. Cancer Cell 7, 205–206.

Saha, S., Aranda, E., Hayakawa, Y., Bhanja, P., Atay, S., Brodin, N.P., Li, J., Asfaha, S., Liu, L., Tailor, Y., et al. (2016). Macrophage-derived extracellular vesicle-packaged WNTs rescue intestinal stem cells and enhance survival after radiation injury. Nat. Commun. 7, 13096.
Saito-Diaz, K., Benchabane, H., Tiwari, A., Tian, A., Li, B., Thompson, J.J., Hyde, A.S., Sawyer, L.M., Jodoin, J.N., Santos, E., et al. (2018). APC Inhibits Ligand-Independent Wnt Signaling by the Clathrin Endocytic Pathway. Dev. Cell 44, 566–581.

Sansom, O.J., Reed, K.R., Hayes, A.J., Ireland, H., Brinkmann, H., Newton, I.P., Battle, E., Simon-Assmann, P., Clevers, H., Nathke, I.S., et al. (2004). Loss of Apc in vivo immediately perturbs Wnt signaling, differentiation, and migration. Genes Dev. 18, 1385–1390.

Sato, T., Vries, R.G., Snippert, H.J., van de Wetering, M., Barker, N., Stange, D.E., van Es, J.H., Abo, A., Kujala, P., Peters, P.J., and Clevers, H. (2009). Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. Nature 459, 262–265.

Sato, T., van Es, J.H., Snippert, H.J., Stange, D.E., Vries, R.G., van den Born, M., Barker, N., Shroyer, N.F., van de Wetering, M., and Clevers, H. (2011). Paneth cells constitute the niche for Lgr5 stem cells in intestinal crypts. Nature 469, 415–418.

Schwarz-Romond, T., Fiedler, M., Shibata, N., Butler, P.J., Kikuchi, A., Higuchi, Y., and Bienz, M. (2007). The DIX domain of Dishevelled confers Wnt signaling by dynamic polymerization. Nat. Struct. Mol. Biol. 14, 484–492.

Shoshkes-Carmel, M., Wang, Y.J., Wangensteen, K.J., Toth, B., Kondo, A., Massasa, E.E., Itzkovitz, S., and Kaestner, K.H. (2018). Subepithelial telocytes are an important source of Wnts that supports intestinal crypts. Nature 557, 242–246.

Snippert, H.J., van der Flier, L.G., Sato, T., van Es, J.H., van den Born, M., Kroon-Veenboer, C., Barker, N., Klein, A.M., van Rheenen, J., Simons, B.D., and Clevers, H. (2010). Intestinal crypt homeostasis results from neutral competition between symmetrically dividing Lgr5 stem cells. Cell 143, 134–144.

Snippert, H.J., Schepers, A.G., van Es, J.H., Simons, B.D., and Clevers, H. (2014). Biased competition between Lgr5 intestinal stem cells driven by oncogenic mutation induces clonal expansion. EMBO Rep. 15, 62–69.

Strigini, M., and Cohen, S.M. (2000). Wingless gradient formation in the Drosophila wing. Curr. Biol. 10, 293–300.

Valenta, T., Degirmenci, B., Moor, A.E., Herr, P., Zimmerl, D., Moor, M.B., Hausmann, G., Cantu, C., Aguet, M., and Basler, K. (2016). Wnt Ligands Secreted by Subepithelial Mesenchymal Cells Are Essential for the Survival of Intestinal Stem Cells and Gut Homeostasis. Cell Rep. 15, 911–916.

Vermeulen, L., Morrissey, E., van der Heijden, M., Nicholson, A.M., Sottoriva, A., Buccazzi, S., Kemp, R., Tavare, S., and Winton, D.J. (2013). Defining stem cell dynamics in models of intestinal tumor initiation. Science 342, 995–998.

Wang, L., Zeng, X., Ryoo, H.D., and Jasper, H. (2014). Integration of UPRER and oxidative stress signaling in the control of intestinal stem cell proliferation. PLoS Genetics 10, e1004568.

Yamamoto, H., Komekado, H., and Kikuchi, A. (2006). Caveolin is necessary for Wnt-3a-dependent internalization of LRP6 and accumulation of β-catenin. Dev. Cell 11, 213–223.

Yan, C., Liu, D., Li, L., Wempe, M.F., Guin, S., Khanna, M., Meier, J., Hoffman, B., Owens, C., Wysoczynski, C.L., et al. (2014). Discovery and characterization of small molecules that target the GTPase Ral. Nature 515, 443–447.

Yan, K.S., Janda, C.Y., Chang, J., Zheng, G.X.Y., Larkin, K.A., Luca, V.C., Chia, L.A., Mah, A.T., Han, A., Terry, J.M., et al. (2017). Non-equivalence of Wnt and R-spondin ligands during Lgr5+ intestinal stem-cell self-renewal. Nature 545, 238–242.

Zeng, X., Chauhan, C., and Hou, S.X. (2010). Characterization of midgut stem cell- and enteroblast-specific Gal4 lines in Drosophila. Genesis 48, 607–611.

Zou, W.Y., Blutt, S.E., Zeng, X.L., Chen, M.S., Lo, Y.H., Castillo-Azofeifa, D., Klein, O.D., Shroyer, N.F., Donowitz, M., and Estes, M.K. (2018). Epithelial WNT Ligands Are Essential Drivers of Intestinal Stem Cell Activation. Cell Rep. 22, 1003–1015.
## STAR METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| mouse monoclonal anti Drosophila Armadillo | DSHB | Cat# N2 7A1 ARMADILLO; RRID:AB_528089 |
| mouse monoclonal anti Drosophila Prospero | DSHB | Cat# Prospero (MR1A); RRID:AB_528440 |
| chicken polyclonal anti GFP | AbCam | Cat# ab13970; RRID:AB_300798 |
| rabbit polyclonal anti Phospho-Histone H3 (Ser10) | Cell Signaling Technology | Cat# 9701; RRID:AB_331535 |
| mouse monoclonal anti Drosophila Wg | DSHB | Cat# 4d4; RRID:AB_528512 |
| rabbit polyclonal anti DsRed | Clontech Laboratories, Inc. | Cat# 632496; RRID:AB_10013483 |
| guinea pig polyclonal anti Drosophila Senseless | H. Bellen; (Nolo et al., 2000) | N/A |
| guinea pig polyclonal anti Drosophila Myc | G. Morata; (Herranz et al., 2008) | N/A |
| rabbit polyclonal anti β-Galactosidase | MP Biomedicals | Cat# 559761; RRID:AB_2687418 |
| goat anti-chicken-IgY(H+L)-Alexa Fluor 488 | Invitrogen | Cat# A-11039; RRID:AB_142924 |
| goat anti-mouse-IgG(H+L)-Alexa Fluor 488 | Molecular Probes | Cat# A-11029; RRID:AB_138404 |
| goat anti-guinea-pig-IgG(H+L)-Alexa Fluor 488 | Thermo Fisher Scientific | Cat# A-11073; RRID:AB_2534117 |
| goat anti-rabbit-IgG(H+L)-Alexa Fluor 488 | Thermo Fisher Scientific | Cat# A-11037; RRID:AB_2534095 |
| goat anti-mouse-IgG(H+L)-Alexa Fluor 594 | Molecular Probes | Cat# A-11032; RRID:AB_141672 |
| goat anti-guinea-pig-IgG(H+L)-Alexa Fluor 594 | Thermo Fisher Scientific | Cat# A-11076; RRID:AB_2534120 |
| goat anti-guinea-pig-IgG(H+L)-Alexa Fluor 647 | Thermo Fisher Scientific | Cat# A-21450; RRID:AB_2735091 |
| Mouse anti-BrdU | BD Biosciences | Cat# 51-75512X; RRID:AB_2314034 |
| Rat anti-CD44 | BD Bioscience | Cat# 550538, RRID:AB_393732 |
| Rabbit anti-Frizzled-5 | LSBio | Cat# LS-A4273, RRID:AB_591417 |
| Rabbit anti-Frizzled-6 | GeneTex | Cat.No: GTX64757 |
| Rabbit anti-Frizzled-7 | Abcam | Cat# ab64636; RRID:AB_1640522 |
| Rabbit anti- Frizzled-7 | LSBio | Cat# LS-C30350, RRID:AB_2263257 |
| Mouse anti- RALA | BD Biosciences | Cat# 610222, RRID:AB_397619 |
| Mouse anti- RALB | Millipore | Cat# 04-037, RRID:AB_612061 |
| Rabbit anti- RALBP1 | Abcam | Cat# ab33446, RRID:AB_945151 |
| Mouse anti- B-Actin | Sigma-Aldrich | Cat# A2228, RRID:AB_476697 |
| Rabbit anti- Histone H3 | Millipore | Cat# 06-599, RRID:AB_2115283 |
| Rabbit anti- RFP | Tebu-bio, UK | Cat# 600-401-379, RRID:AB_2209751 |
| Rabbit anti-Cleaved Caspase-3 | Cell Signaling | Cat# 9661, RRID:AB_2341188 |
| Rabbit anti- irp6 | Abcam | Cat# ab134146 |
| Mouse anti-β-catenin | BD Biosciences | Cat# 610153, RRID:AB_397554 |
| Rabbit anti-Notch1 | Cell Signaling | Cat# 2421, RRID:AB_2314204 |

**Bacterial and Virus Strains**

*Erwinia carotovora carotovora 15*
B. Lemaitre; (Basset et al., 2000)  
N/A

**Chemicals, Peptides, and Recombinant Proteins**

| High Capacity cDNA Reverse Transcription Kit | Applied Biosystems | Cat# 4368813 |
| MessageBOOSTER cDNA Synthesis Kit | Lucigen | Cat# MB060124 |
| PerfeCTa SYBR Green FastMix (Low ROX) | Quanta Bio | Cat# 95074-012 |
| VECTASHIELD Mounting Medium with DAPI | Vector Laboratories, Inc. | Cat# H-1200; RRID:AB_2336790 |

**Critical Commercial Assays**

| RNAeasy Mini Kit (50) | QIAGEN | Cat# 74104 |

**Experimental Models: Cell Lines**

| HEK293T cells | ATCC | RRID: CVCL_0063 |

(Continued on next page)
Continued

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Experimental Models: Organisms/Strains** | | |
| **Drosophila:** | | |
| ISC/EB driver: yw;esg-Gal4NP5130,UAS-GFP, UAS-GFPnLacZ/Cyo;tub-Gal80ts/Tm6B | S. Hayashi; (Goto and Hayashi, 1999) | N/A |
| EB driver: Su(H) GBE-gal4, UAS-CD8GFP/Cyo | S. Hou; (Zeng et al., 2010) | N/A |
| ISC driver: esg-gal4, UAS-2XEYFP; Su(H)GBE-gal80, tub-gal80ts/SMS-TM6 | S. Hou; (Wang et al., 2014) | N/A |
| ISC/EB Flipout system: w;esg-gal4,tub-gal80ts, UAS-GFP; UAS-flp, Act > CD2 > gal4, UAS-grp/TM6B | B. Edgar; (Jiang et al., 2009) | N/A |
| RalA reporter: pg85,FRT19A/FM7a;Sp/Cyo | C. Ghiglione; (Bourbon et al., 2002) | N/A |
| en-gal4 driver: y1 w*; P[w+mW.hs = en2.4-GAL4]16E | Bloomington Drosophila Stock Center (BDSC) | RRID:BDSC_30564 |
| RabBP1-RNAi: P[KK101635]VIE-260B | Vienna Drosophila Resource Center (VDR) | 105976; RRID:FlyBase_FBst0477802 |
| RabA-RNAi(1): P[KK108989]VIE-260B | VDRC | 105296; RRID:FlyBase_FBst0477124 |
| RabA-RNAi(2): y1 v1; P[y+1.7 v+1.8 = Thrp.JF03259]attP2 | BDSC | RRID:BDSC_29580 |
| RabA-RNAi(3): w1118; P[GD8562]v43622 | VDRC | 43622; RRID:FlyBase_FBst0465168 |
| UAS-Wg-RNAi: P[KK108857]VIE-260B | VDRC | 104579; RRID:FlyBase_FBst0476437 |
| UAS-GFP y1 w*; P[w+mC = UAS-mCD8::GFP.L]LL5, P[UAS-mCD8::GFP.L]2 | BDSC | RRID:BDSC_5137 |
| UAS-RalAwt(1): P[UAS-RalA]3 | G. Hasan; (Richhariya et al., 2017) | N/A |
| UAS-RalAwt(2): P[UAS-RalA]2 | G. Hasan; (Richhariya et al., 2017) | N/A |
| Control genotype w1118 | R. Cagan | N/A |
| Apc<sup>−/−</sup> w<sup>-</sup>;APC1q8,FRT82B/TM6B | Y. Ahmed; (Ahmed et al., 1998) | N/A |
| MARCM Control w<sup>-</sup>; FRT82B, con lacZ | R. Cagan | N/A |
| MARCM82B y,w,hsFLP,tub-gal4, UAS-GFP-myc; FRT82B,tub-gal80 CD2+ y/+Tm6B | N. Tapon | N/A |
| Fz3-RFP | Y. Ahmed; (Olson et al., 2011) | N/A |
| Delta<sup>-</sup>:GFP reporter y1 w*; Mt[PT-GFSTF.1] Df[In(3L)Gf(2)DSC.1/TM6C, Sb1 Tb1 | BDSC | RRID:BDSC_59819 |
| Su(H)/GBE-LacZ reporter w; Sp/CyO; cn ry | C. Micchelli; (Furriols and Bray, 2001) | N/A |
| Mouse lines: | | |
| Rala<sup>tm1.1Cjm</sup>/Rala<sup>tm1.1Cjm</sup> | PMID:23063435 | RRID:MGI:5505291 |
| Rala<sup>tm1.1Cjm</sup>/Rala<sup>tm1.1Cjm</sup> | PMID:23063435 | RRID:MGI:5505291 |
| Apc<sup>tm1Tno</sup>/Apc<sup>tm1Tno</sup> | PMID:20084116 | RRID:MGI:4429571 |
| VillCreER | (el Marjou et al., 2004) | N/A |
| Lgr5-EGFP-Cre<sup>ER</sup> | (Barker et al., 2007) | N/A |
| R26-LoxStopLox-ttdTomato | (Madisen et al., 2010), | N/A |
| RNAseq probes: | | |
| Mm-Axin2 2.5 LS | Advanced Cell Diagnostics, Hayward, CA | Product code: 400338 |
| Mm-Lgr5 2.5 LS Probe | Advanced Cell Diagnostics, Hayward, CA | Product code: 312178 |
| Mm-OLFM4 2.5 LS Probe | Advanced Cell Diagnostics, Hayward, CA | Product code: 311838 |
| Mm-Smoc2 2.5 LS Probe | Advanced Cell Diagnostics, Hayward, CA | Product code: 489688 |
| MM-PPIB 2.5 LS Probe Positive Control | Advanced Cell Diagnostics, Hayward, CA | Product code: 313918 |

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CONTACT FOR REAGENT AND RESOURCE SHARING

Requests for further information, reagents, and resources should be directed to and will be fulfilled by the Lead Contact, Owen J. Sansom (o.sansom@beatson.gla.ac.uk).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Experimental Animals
Species used: Drosophila melanogaster, Mus musculus

Drosophila breeding and maintenance
Flies were bred and maintained on standard food in humidity and temperature-controlled incubators in a 12h-12h light-dark cycle. Crosses for adult specific transgene manipulations were kept at 18°C. F1s of the desired genotype were collected following 2-3 days of adult eclosion. Animals with temperature sensitive transgenes were switched to 29°C and aged for the time needed to allow transgene activation. Apc full mutant animals were always maintained at 18°C. Otherwise, animals were aged at 25°C. Adult posterior midguts, and wing discs of late L3 larvae were analyzed. Standard food used: 10 g Agar, 15 g Sucrose, 30 g Glucose, 15 g Maize meal, 10 g wheat germ, 30 g treacle and 10 g Soya flour per liter of distilled water.

Sex
Only mated females were used for experiments on the Drosophila midgut.

Full Drosophila genotypes
Full fly genotypes as they appear in each Figure panel are listed in Table S1.

Mouse colonies
All experiments were performed according to UK Home Office regulations (Project License 70/8646), adhered to ARRIVE guidelines and were subject to review by the animal welfare and ethical review board of the University of Glasgow. Standard diet and water was given ad libitum, and under non-barrier conditions. The Mice strains used are indicated in the resource table. The Porcupine inhibitor WNT974(LGKT974) was administered in a concentration of 5mg/kg BID (oral) in a vehicle of 0.5% Tween-80/0.5% Methylcellulose. Tamoxifen (Sigma) IP was used to induce VilCreER and Lgr5CreER mice at the indicated concentrations. For regeneration

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Recombinant DNA     |        |            |
| M50 Super 8x TOPFlash | PMID:12699626 | RRID:Addgene_12456 |
| pSAR-MT-beta-gal    | PMID:8755583 | RRID:Addgene_16486 |
| SNAP-Fzd5           | (Koo et al., 2012) | N/A |
| SNAP-Fzd7           | Gift from Bienz lab | N/A |

Software and Algorithms

| Software and Algorithm | SOURCE | IDENTIFIER |
|------------------------|--------|------------|
| Fiji                   | NIH    | 1.51n; https://fiji.sc/ |
| ImageJ                 | https://imagej.net/Welcome | RRID:SCR_002074 |
| GraphPad Prism 6       | GraphPad | RRID:SCR_002798 |
| ZEN 2 lite             | Zeiss  | RRID:SCR_013672 |
| 7500 Real-Time PCR Software | Applied Biosystems | RRID:SCR_014596 |
| BatchQuantify          | This paper | https://github.com/emLtwc/2018-Cell-Stem-Cell |

Other

| Other | SOURCE | IDENTIFIER |
|-------|--------|------------|
| LSM780 microscope   | Zeiss  | N/A |
| BX51 microscope     | Olympus | N/A |
| 7500 Fast Real-Time PCR System | Applied Biosystems | N/A |
| AutostainerLink 48  | Dako   | N/A |
| Bond Rx autostainer | Leica  | N/A |
| FACSARIA FUSION      | BD Biosciences | N/A |
| Odyssey Clix        | LI-COR | N/A |
| Attune NxT flow cytometer | ThermoFisher Scientific | N/A |
experiments, mice were exposed to γ-irradiation from caesium-137 sources. This delivered γ-irradiation at 0.423 Gy min⁻¹. Mice were sampled 3 days following irradiation damage. The smallest sample size was used that would still give a significant difference in accordance with the 3Rs. No distinction between males and females has been made in all mice experiments. All mice were above 20 g of weight before eligible participate in any experiment. All mice experiments were performed on a C57BL/6 (n = 5 or more), except from clonal expansion experiments which perform on mixed background.

**METHOD DETAILS**

**Immunofluorescence of Drosophila tissues**
Tissues were dissected and fixed in 4% para-formaldehyde (Polysciences, Inc.) at room temperature for a minimum of 30 min. After fixation, tissues were washed 3 times in PBS + 0.2% Triton X-100 (PBST) for 20 min, followed by overnight incubation at 4°C with primary antibodies in PBST + 0.5% Bovine Serum Albumin (BSA) (PBT). Samples were then washed in PBT 3 × 20 minutes and incubated with secondary antibodies in PBT for 3h at room temperature, followed by washing and mounting.

Midguts stained for Wg were fixed in PEM-FA (0.1 M PIPES, 2mM EGTA, 1.0 mM MgSO4, 4% formaldehyde, pH 7.0). Tissues were washed in PBST and blocked in 5% normal goat serum (Sigma) in PBS for 2 hours at room temperature. Following the blocking step samples were subjected to the standard staining protocol described above. For detection of extracellular Wg, wing discs were dissected in PBS and moved to antibody for 1h at room temperature prior to fixation and subsequent processing following standard staining protocol. All samples were mounted onto glass slides (VWR) with 13mm x 0.12mm spacers (Electron Microscopy Science) and Vectashield mounting media containing DAPI (Vector Laboratories, Inc). Confocal images were obtained on a Zeiss LSM 780 and processed in the Zeiss ZEN software.

Antibody concentrations used: anti-Arm (1:10), anti-β-Gal (1:1000), anti-DsRed (1:500), anti-GFP (1:2000), anti-Myc (1:100), anti-Pros (1:20), anti-pH3S10 (1:100), anti-Senseless (1:2000), anti-Wg (1:10 or 1:3 for extracellular staining). Secondary antibodies were used as follows: anti-IgG-488 (1:200), anti-IgG-594 (1:100), IgG-647 (1:50).

**Drosophila midgut regeneration assay**
Regeneration assays was performed according to (Neyen et al., 2014). Oral infection was induced using *Erwinia carotovora subsp. carotovora* 15 (Ecc15) (Basset et al., 2000). Bacteria were grown overnight in LB medium in orbital shaker incubator at 30°C, 200 rpm. The bacterial culture was pelleted (Beckman Coulter JS-4.2 rotor, 10 min @3000rpm = 22547 k-factor) and adjusted to OD600 = 200 followed by mixing with a 5% sucrose solution 1:1. Flies used for regeneration experiments were starved for 2 hours prior to infection to synchronize feeding. Animals were moved into vials containing filter paper (Whatman) soaked into 5% sucrose solution (Mock) or the prepared bacterial solution. Flies were dissected 12-16 hours after infection.

**Quantification of pH3 positive cells in the Drosophila posterior midgut**
We used antibodies against phosphorylated Histone 3 to visualize ISC proliferation in the posterior midgut, which is defined as the region between the copper cell region and the hindgut. Number of midguts analyzed (N) for each experiment are indicated in the Figures.

**Drosophila MARCM and Flip-out clone analysis**
Recombinant clones were generated using the MARCM system (Lee and Luo, 1999) or the temperature-sensitive *esg flip-out (esg^F/O)* system (Jiang et al., 2009). MARCM adults of the desired genotype were subjected to three, 30-minute 37°C heat-shocks separated by 1h at room temperature. Animals were aged for ten or thirty days at 25°C for MARCM and 29°C for flip-out flies. Clonal size in MARCM or *esg^F/O* experiments was determined by counting the number of nuclei labeled by DAPI on an Olympus BX51 epi-fluorescent microscope. Clone size was not determined for 30-day old *esg^F/O* animals as no distinct clones cloud be identified.

**Drosophila RNA extraction and RT-qPCR**
Total RNA from a minimum of 15 midguts was extracted using QIAGEN RNAeasy kit, following manufacturer’s instructions. RNA was quantified using a NanoDrop 2000c Spectrophotometer.

cDNA was synthesized using the High-Capacity cDNA reverse transcription kit. Quanta SYBR green Master Mix (Low ROX, Fermentas) was used following manufacturer’s instructions. Data were obtained and analyzed using the Applied Biosystems 7500 software. Results represent biological triplicates ± SEM. Expression of target genes was measured and normalized to *rpl32*.

**Sorting of Drosophila ISCs and gene expression assessment**
For sorting of ISCs we dissected 100 posterior midguts (R4 + R5). Sample preparation, cell sorting, RNA extraction and RNA amplification were carried out as described by (Dutta et al., 2015) with a minor modification: MessageBOOSTER cDNA Synthesis Kit (Lucigen, Cat No. MB060124) was used to amplify RNA. To eliminate potential bias during RNA amplification, we included 500 pg of RNA from whole posterior midguts, which was subject to the same amplification procedure as the RNA obtained from sorted cells. Gene expression levels from sorted cells where first normalized against the *rpl32* levels in the same sample, and then presented as a ratio over gene expression in whole posterior midguts. Results represent biological triplicates ± SEM. Primers used for RT-qPCRs are detailed in Table S2.
**Drosophila staining quantifications**

Fz3-RFP and intestinal Wg was quantified as the average staining intensity within the GFP positive compartment; nuclear dMyc staining was quantified as the average staining intensity within the compartment positive for both DAPI and GFP, normalized by background intensity using a custom ImageJ macro, BatchQuantify. Extracellular Wg staining was analyzed in ZEN 2 (Zeiss).

**Immunohistochemistry and RNA in situ hybridization**

All IHC, special stains and in situ hybridisation staining was performed on 4um formalin fixed paraffin embedded sections that had previously been heated at 60°C for 2 hours.

The following antibodies were used on a Dako AutostainerLink 48, Brdu (BD Biosciences, UK), CD44 (Cell Signaling, UK), and RFP (Tebu-bio, UK). The tissue sections underwent manual dewaxing through xylene, graded alcohol and then washed in tap water before undergoing heat induced epitope retrieval (HIER). HIER was performed on a Dako PT module where the 4um sections were heated to 98°C for 25 minutes in appropriate retrieval buffer. Sections were placed in PT module buffer 1 (Thermo). After epitope retrieval sections were rinsed in Tris Buffered saline with Tween (Tbt) prior to being loaded onto the autostainer. The sections then underwent peroxidase blocking (Agilent, UK), washed in Tbt before application of primary antibody at a previously optimized dilution (Brdu 1/150; CD44 1/250; RFP 1/150) for 40 minutes. The sections were then washed in Tbt before application of appropriate EnVision (Agilent, UK) secondary antibody dependent on species of primary antibody for 30 minutes. Sections were rinsed in Tbt before applying Liquid DAB (Agilent, UK). The sections were then washed in water, counterstained with hematoxylin and coverslipped using DPX mountant (CellPath, UK).

Caspase 3 (Cell Signaling, UK) and Lysozyme (Agilent, UK) were stained on the Leica Bond Rx autostainer. Sections were loaded onto the autostainer and underwent dewaxing and epitope retrieval on board. Caspase 3 was retrieved using ER2 buffer (Leica, UK) for 30 minutes at 95°C and Lysozyme with Enzyme 1 (Leica, UK) for 5 minutes. The sections were then stained using an Intense R kit (Leica UK). Caspase 3 was diluted 1/500 and Lysozyme 1/300.

Rala, Notch-1, and β-catenin sections were stained manually. These sections underwent manual dewaxing through xylene, graded alcohol and then washed in tap water before undergoing antigen retrieval. Rala sections were boiled 30 min in citric acid buffer, Notch-1 sections 30 min in Protaqs IX buffer (BioCyc), and β-catenin sections 50 min in Tris-EDTA. Rala primary antibody (1:500) was incubated overnight at +4°C. Notch-1 antibody (1:50) at 1h RT, and β-Catenin 2h RT.

Sections were stained with Alcian Blue and a PAS stain. These sections underwent manual dewaxing through xylene, graded alcohol and then washed in tap water before undergoing appropriate standard staining protocol for each stain.

In situ-hybridization detection for Axin2, Ascl2, Lgr5, Olfm4, Ralb-E2, and Ppib (Advanced Cell Diagnostics, Hayward, CA) mRNA was performed using RNAscope 2.5 LS (Brown) detection kit (Advanced Cell Diagnostics, Hayward, CA) on a Leica Bond Rx autostainer strictly adhering to the manufacturer’s instructions.

ImageJ was used to quantify detected RNA on images taken at 40X. First the image was duplicated into two. The intestinal crypts were encircled manually in the original image, and added to the ROI manager. The Channels was then split into red, green, and blue. The blue channel was used to put a threshold to only mark the dots in each encircled crypt, and this setting was kept throughout the analysis for all images stained with a specific probe. The percentage of positive area within each crypt was then measured. For each sample at least 25 crypts have been measured, and the average represents one biological sample.

To false color the dots red, the image was first duplicated, and the channels split into red, green, and blue in one of them. The threshold was set in the blue image, and a selection created. This selection was added to the ROI manager, and used in the original image to mark the dots. Once marked, the dots were colored by the fill function. For presentation constraint have been increased to visualize the faint crypt structures obtain by counterstaining.

**Crypt and cell culture**

The proximal mouse small intestines were washed with PBS and open longitudinal, and cut to small pieces, that were washed in PBS repetantly until the PBS buffer is transparent and free from debris. Further the small pieces containing intestinal crypts were incubated in PBS containing 2mM EDTA during 30min in +4C. 10mL PBS is added and mechanically pipetted up and down to generate a first fraction containing intestinal crypts. This step was repeated and fraction 2-4 collected and filtered through a 70-um cell strainer. Isolated crypts then were mixed with 50 µL of Matrigel (BD Bioscence), plated in 24-well plates in Advanced DMEM/F12 with Noggin (100 ng mL^-1, Peprotech). Wild-type crypts were also supplemented with R-spondin (500 ng mL^-1; R&D Systems). Growth medium is replaced every other day.

For colony forming experiments, organoids were split into single cells by incubation in Tryple express (GIBCO) supplemented with Dnase (100U) for 30–45 minutes in a 37C water bath, and resuspended mechanically using a pipette every 15 minutes to disperse the cells. 5000 cells were seeded into 10ul Matrigel to allow colony formation over time.

Mycoplasma negative HEK293T cells were grown in DMEM medium supplemented with 10% FCS, 1% Glutamine, 1% Pest, and 1% HEPES. A Plasmids CRISPR-Cas9 containing gRNA was produced according to the manufacturer’s instructions (Invitrogen), and YFP positive clones were FACS sorted using the BD FACSARIA FUSION (BD Biosciences) 48h after transfection. All transfection experiments, including SNAP-tag Frizzled-5 and Frizzled-7 were performed using lipofectamine 2000 according to standard protocol (Invitrogen), gRNA oligonucleotides are detailed in Table S2.

For the TopFlash assay cells were transfeceted with SuperTopflash (Addgene), and B-gal (Addgene) plasmids in a ratio 10:1 using lipofectamine 2000 according to standard protocol. The Dual-Light Luciferase & β-Galactosidase Reporter Gene Assay System
was used as a dead/live markers. of Frizzled-7 on living cells were measured on the Attune NxT flow cytometer (ThermoFisher Scientific), and DAPI incorporation with Alexa Fluor fluorochrome (ThermoFisher Scientific) was incubated in blocking medium during 15 min RT. Cell surface levels using a 40-um cell strainer, washed twice in PBS, and blocked for 30 min in PBS/BSA in RT. Frizzled-7 antibody (1:500) labeled 37C water bath, and resuspended mechanically using a pipette every 15 minutes to disperse the cells. Single cells were isolated FACS sections were imaged using a Zeiss 710 confocal microscope.

m of cellular protein (30 μg) were separated on a 4%–12% gradient gel (Novex) and subsequently transferred to a PVDF membrane (Amersham). Total protein was visualized with Ponceau (Sigma). After blocking the membranes in TBS containing 5% BSA (Sigma), 0.02% Triton X-100 for 1 h, primary antibodies were added: After washing, the appropriate DyLight 800 or 580 fluorescency-conjugated secondary goat antibodies (Thermo Scientific, 1:10 000) were added in block solution for 1 h in room temperature on a rocking table. Antibody binding was detected using the Li-COR Odyssey CLx. Primary antibody (see resource table) incubations were carried out at 4° C overnight. All primary antibodies (resource table) were used (1:1000) for WB applications.

Myoplastma free HEK293T Cells were transfected with SNAP-tagged Frizzled constructs, and 24h later plated onto poly-d-lysine (Sigma-Aldrich) coated coverslips in 24-wells. After additional 24h incubation, cells were washed once with PBS, and incubated with diluted SNAP-Vista® Green 1:1000 (New England Biolabs inc.) in PBS for 5 minutes in room temperature. To follow internalization, standard DMEM medium containing 10% serum was added to cells and incubated according to indicated time points. Cells were fixed in 4% PFA and internalization was analyzed using a Zeiss 710 confocal microscope.

For internalization assay of endogenous protein cells were grown on poly-d-lysine (Sigma-Aldrich) coated 10 cm plates until confluent, and incubated in +4C during 1h in 0.13 mg/mL sulfo-NHS-SS- biotin (Sigma-Aldrich) in PBS. Cells were washed twice in cold PBS, and cell culture medium was added to plates, and then incubated for 2.5, 5, or 10 minutes in 37C in cell culture media as indicated. After incubation cells were washed 1h in 4C in 15/mg/mL MesNA(Sigma-Aldrich) diluted in PBS and supplemented with 10mM NaOH. To inactivate the MesNA 1mL of IAA (Sigma-Aldrich) in PBS was added to each sample and incubated 15min in 4C. After two PBS washes samples were lysed in RIPA buffer.

For detection ELISA plates (Thermo Scientific) were coated with primary antibody for 4h in RT, and blocked 1h in BSA-block. After two washes in TBST samples were added to the plates and incubated overnight in +4C. Next, Plates were washed twice and streptavidin-conjugated horseradish (1:1000) was added onto the plate. After two washes in TBST and two washes in PBS signal was detecting using SuperSignal Elisa Pico (ThermoFisher Science) on a Spark microplate reader (Tecan).

For mice samples approximately 1cm from the proximal small intestine was first isolated and stored in RINaLater. A small piece of the samples were further placed in vials containing CK14 ceramic beads and RLT buffer (QIAGEN RNAeasy kit) supplemented with β-mercaptoethanol. Samples were lysed using a Precellys machine. RNA was afterward extracted from lysed samples using the QIAGEN RNAeasy kit, following manufactuer’s instructions.

Further, cDNA was generated from mice RNA using Quantitect Reverse Transcription Kit (QIAGEN) in a reaction volume of 20 μl. qPCR reactions were prepared (15 μl), of 7.5 μl of 2 × DyNAmo HS master mix (Thermo Scientific), 0.5 μM reverse and forward primers, and 1.5 μl cDNA. The qPCR was performed according to manufactures protocol. Gapdh was used to normalize for differences in RNA input. Primers used for RT-qPCRs are detailed in Table S2.

Lgr5CreER tdTom mice were induced with 0.15mg tamoxifen (IP) as previously described (Snippert et al., 2014). The small intestines of mice were sampled at different time points and fixed with freshly prepared 4% paraformaldehyde (PFA) for 3 hours at room temperature. The small intestinal tissue was then incubated with DAPI (10ug/mL) in 0.1% PBS-T over night. Whole mount sections were imaged using a Zeiss 710 confocal microscope.

FACS Organoids were split into single cells by incubation in Tryple express ( GibCO) supplemented with Dnase (100U) for 30–45 minutes in a saline bath, and resuspended mechanically using a pipette every 15 minutes to disperse the cells. Single cells were isolated using a 40-um cell strainer, washed twice in PBS, and blocked for 30 min in PBS/BSA in RT. Frizzled-7 antibody (1:500) labeled with Alexa Fluor fluorochrome (ThermoFisher Scientific) was incubated in blocking medium during 15 min RT. Cell surface levels of Frizzled-7 on living cells were measured on the Attune NxT flow cytometer (ThermoFisher Scientific), and DAPI incorporation was used as a dead/live markers.
**Statistical analysis**
Graph Pad Prism 6 software was used for statistical analyses. Information on sample size, biological replicas, independent samples, independent experiments, and statistical tests used for each experiment are indicated in the figure legends.

**DATA AND SOFTWARE AVAILABILITY**

The custom ImageJ macro, BatchQuantify, used for *Drosophila* immunofluorescent staining quantification is available at [https://github.com/emLtwc/2018-Cell-Stem-Cell](https://github.com/emLtwc/2018-Cell-Stem-Cell).
Supplemental Information

RAL GTPases Drive Intestinal Stem Cell Function and Regeneration through Internalization of WNT Signalosomes

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**Figure S1. Related to Figure 1. RAL small GTPase signaling is dispensable for baseline tissue proliferation in the *Drosophila* midgut epithelium**

(A) Visualisation of *RalA* expression using a *RalA-gal4* enhancer trap line driving *UAS-gfp* (*RalA>GFP*; green) in the whole adult *Drosophila* gut. Expression is seen in enteric neuronal projections, circular strips of visceral muscle at the R2c-R3 region and small cells at the R4-R5 region. (Scale bar = 500 μm)

(B) Representative confocal images of 10 and 30 days old control and *RalA* knockdown (*RalA-RNAi(1)) escargot flipout* (ISC/EB Flipout>) clones (green). (Scale bar = 20 μm)

(C) Quantification of 10 days old control and *RalA* knockdown (*RalA-RNAi(1)) escargot flipout* (ISC/EB Flipout>) clone size (measured by cell number). 30-day clones were
not quantified as the tissue was uniformly labelled by GFP. Student’s t-test; N represents the number of clones quantified in 6 posterior midguts; Error bars indicate SD.

(D) Representative confocal images of 10 and 30 days old control and RalA knockdown (RalA-RNAi(1)) MARCM (MARCM82B>) clones (green). (Scale bar = 20μm)

(E) Quantification of 10 and 30 days old control and RalA knockdown (RalA-RNAi(1)) MARCM (MARCM82B>) clone size. Two-way ANOVA, followed by Sidak’s multiple comparisons test; N represents the number of clones quantified in >6 posterior midguts; Error bars indicate SD. ****p<0.0001.

(F) Representative confocal images of 30 days old control and RalA knockdown (RalA-RNAi(1)) MARCM (MARCM82B>) clones (green) stained for cell-type markers Armadillo (Arm; red/gray membrane staining) to label ISCs/EBs, and Prospero (Pros; red/gray nuclear staining) a marker of EEs. (Scale bar = 5μm)

(G) Quantification of 30 days old control and RalA knockdown (RalA-RNAi(1)) MARCM (MARCM82B>) clone cell-type distribution. Two-way ANOVA, followed by Sidak’s multiple comparisons test; N represents the number of clones quantified, the total number of cells were N=656 control and N=510 from >4 posterior midguts RalA-RNAi(1); Error bars indicate SEM.
Figure S2. Related to Figure 2. *Drosophila* RAL small GTPase regulates Wnt signaling in ISCs, upstream of the β-catenin destruction complex.
(A) Quantification of pH3+ cells in mock treated or regenerating (Ecc15) posterior midguts with two independent RalA knockdown constructs (RalA-RNAi(2) and RalA-RNAi(3)) within ISCs/EBs using the escargot-gal4, UAS-gfp driver. Two-way ANOVA, followed by Sidak’s multiple comparisons test; N represents the number of posterior midguts quantified; Error bars indicate SEM. ****p<0.0001.

(B) Representative confocal images of the endogenous Wnt pathway activity reporter dMyc (red/gray) in mock treated or regenerating (Ecc15) adult posterior midguts from control animals or animals subject to adult specific RalA knockdown (RalA-RNAi(1)) within stem/progenitor cells (ISCs/EBs; green) using the escargot-gal4, UAS-gfp driver (Scale bar = 20μm).

(C) Representative confocal images of the average nuclear staining intensity of the endogenous Wnt pathway activity reporter dMyc (red/gray) within the ISC/EB compartment in control animals or animals subject to adult specific wild-type RalA overexpression (RalAwt(1)) within stem/progenitor cells (ISCs/EBs) using the escargot-gal4, UAS-gfp driver (Scale bar = 20μm).

(D) Representative confocal images of 10 days old control and RalA knockdown (RalA-RNAi(1)) Apc mutant MARCM (APC/-/ MARCM82B>) clones (green). (Scale bar = 20μm)

(E) Quantification of 10 days old control and RalA knockdown (RalA-RNAi(1)) Apc mutant MARCM (APC/-/ MARCM82B>) clone size. Student’s t-test; N represents the number of clones quantified; Error bars indicate SD.

(F) Representative confocal images of wing discs from late L3 Drosophila larvae with posterior domain specific expression RFP only (w1118) or in combination with Wg-RNAi using the engrailed-gal4, UAS-RFP system, stained for the Wnt signaling target gene Senseless (Sens; green). (Scale bar = 50μm)

(G) Quantification of Senseless staining intensity perpendicular to the line Senseless expression in the control (RFP negative) versus the wild-type (w1118) compartment (RFP positive). Multiple t-tests, FDR=0.01; N=6; Error bars indicate SEM.

(H) Quantification of Senseless staining intensity perpendicular to the line Senseless expression in the control (RFP negative) versus the Wg knockdown (Wg-RNAi) compartment (RFP positive). Multiple t-tests, FDR=0.01; N=8; Error bars indicate SEM.

(I) Representative confocal images of wing discs from late L3 Drosophila larvae with posterior domain specific RFP only (w1118) or in combination with Wg-RNAi using
engrailed-gal4, UAS-RFP, stained for the secreted Wnt pathway ligand extracellular Wg (eWg; green). (Scale bar = 50μm)

(J) Quantification of extracellular Wg staining intensity perpendicular to the line Senseless expression in the control (RFP negative) versus the wild-type (w1118) compartment (RFP positive). Multiple t-tests, FDR=0.01; N=6; Error bars indicate SEM.

(K) Quantification of extracellular Wg staining intensity perpendicular to the line Senseless expression in the control (RFP negative) versus the Wg knockdown (Wg-RNAi) compartment (RFP positive). Multiple t-tests, FDR=0.01; N=6; Error bars indicate SEM.

(L) Quantification of pH3+ cells in mock treated or regenerating (Ecc15) posterior midguts expressing RalA-RNAi (RalA-RNAi(2)) within ISCs using the esg-gal4;Su(H)GBE-gal80 driver. Two-way ANOVA, followed by Sidak’s multiple comparisons test; N represents the number of posterior midguts quantified; Error bars indicate SEM.

(M) Quantification of pH3+ cells in mock treated or regenerating (Ecc15) posterior midguts expressing RalA-RNAi (RalA-RNAi(2)) within EBs using the Su(H)GBE-gal4, UAS-gfp driver. Two-way ANOVA, followed by Sidak’s multiple comparisons test; N represents the number of posterior midguts quantified; Error bars indicate SEM. ****p<0.0001.

(N) Representative confocal images of the endogenous Wnt pathway activity reporter dMyc (red/gray) in mock treated or regenerating (Ecc15) adult posterior midguts from control animals or animals subject to adult specific RalA knockdown (RalAwt(1)) within ISCs (green) using the esg-gal4;Su(H)GBE-gal80 driver. (Scale bar = 20μm)

(O) Quantification of the average nuclear dMyc staining intensity within the ISC compartment in control animals or animals subject to adult specific wild-type RalA overexpression (RalAwt(1)) normalized by the background staining. Student’s t-test; each dot represents a Z-stack confocal image from a posterior midgut; Error bars indicate SD. ****p<0.0001.

(P) Representative confocal images of the endogenous Wnt pathway activity reporter dMyc (red/gray) in mock treated or regenerating (Ecc15) adult posterior midguts from control animals or animals subject to adult specific RalA knockdown (RalA-
RNAi(1)) within EBs (green) using the Su(H)GBE-gal4, UAS-gfp driver (Scale bar = 20μm).

(Q) Quantification of the average nuclear dMyc staining intensity within the EB compartment in control animals or animals subject to adult specific wild-type RalA overexpression (RalAw7(1)) normalized by the background staining. Student’s t-test; each dot represents a Z-stack confocal image from a posterior midgut; Error bars indicate SD.
Figure S3. Related to Figure 3. Loss of either RALA or RALB suppresses Wnt signaling in the mammalian intestine

(A) Experimental design of Rala and Ralb knock out in mice gut

(B) IHC displaying Rala knock out in mice gut (scale bar = 50µm). WT (N=6), Rala$^{fl/fl}$ (N=4), Ralb$^{fl/fl}$ (N=4), and Apc$^{fl/fl}$; Ralb$^{fl/fl}$ (N=5); (Scale bar = 50µm).

(C) ISH staining displaying Ralb knock out in mice intestinal crypts. WT (N=6), Rala$^{fl/fl}$ (N=4), Ralb$^{fl/fl}$ (N=4), (Scale bar = 50µm).

(D) qPCR analysis of knock out efficiency of Rala and Ralb in mice gut WT (N=6), Rala$^{fl/fl}$ (N=4), and Ralb$^{fl/fl}$ (N=4). Statistical analysis was done with 1-way ANOVA
Tukey’s multiple comparisons test. Error bars represent standard derivation. **p<0.01

(E) H&E and BrdU images of small intestine of mice with impaired Ral small GTPase signaling. (scale bar = 50µm).

(F) Quantification of BrdU(50 Crypts per mouse) incorporation in small intestine of Rala and Ralb conditional knock out mice WT (N=7), Rala^fl/fl (N=4), Ralb^fl/fl (N=4). Statistical analysis was done with a non-parametric Mann-Whitney U test. Error bars represent standard derivation.

(G) Representative images of Lysosome, PAS, and Alcian Blue staining in Rala and Ralb knock out mice. WT (N=5), Rala^fl/fl (N=4), Ralb^fl/fl (N=4).

(H) Quantification of Lgr5+ cells in the small intestine of Rala and Ralb knock out mice, WT (N=5) Rala^fl/fl (N=5), and Ralb^fl/fl (N=5). Statistical analysis was done with a non-parametric Mann-Whitney U test. Error bars represent standard derivation. *p<0.05, **p<0.01

(I) Representative images of RFP staining in lgr5-CreER; tdTomato mice. Average of RFP positive crypts was calculated from a total of 50 crypts per mouse (N=4). (scale bar = 50µm).
Figure S4. Related to Figure 4. RAL small GTPase signaling mediates internalization, activation of Frizzled receptors and Wnt pathway activation in HEK293T Cells

(A) Clonogenicity measured as organoid formation from single cells after first passage in culture growth conditions, WT (N=4) Rala\textsuperscript{fl/fl} (N=4), and Ralb\textsuperscript{fl/fl} (N=4). Statistical analysis was done with a non-parametric Mann-Whitney U test. Error bars represent standard derivation.*p<0.05.

(B) Bright field, H&E, and Cd44 IHC of organoid with induced deletion of Ralb in vitro (Scale bar = 50µm). WT (N=4), Rala\textsuperscript{fl/fl} (N=4), Ralb\textsuperscript{fl/fl} (N=4)

(C) Representative IHC images of Cleaved Caspase-3 following Rala and Ralb deletion. (Scale bar = 50µm). WT (N=5), Rala\textsuperscript{fl/fl} (N=4), Ralb\textsuperscript{fl/fl} (N=4)

(D) Quantification of Cleaved Caspase-3 positive cells in small intestinal crypts following Rala and Ralb knock out, WT (N=5) Rala\textsuperscript{fl/fl} (N=4), and Ralb\textsuperscript{fl/fl} (N=4) Statistical analysis was done with a non-parametric Mann-Whitney U test. Error bars represent standard derivation.
Figure S5. Related to Figure 5. RAL small GTPase signaling mediates internalization, activation of Frizzled receptors and Wnt pathway activation in HEK293T Cells

(A) Representative western blot images on RALBP1, RALA, and RALB protein levels following Crispr mediated knock out. β-actin was used as a loading control.

(B) Maximal projection of confocal images depicting internalization events of SNAP-FZD5 and SNAP-FZD7 over time. (Scale bar = 20µm).

(C) Western blot analysis on samples from cell surface Sulfo-NHS-SS-Biotin labelled protein samples after streptavidin pulldown. β-actin was used as a loading control.
Figure S6. Related to Figure 6. RAL Small GTPases are functionally important for ISCs

(A) Representative images of *Ralb-E2* expression on ISH, and RFP on IHC in serial sections of mouse intestine 30 days after low dose tamoxifen induction using the *Lgr5-CreER* promoter. (Scale bar = 50μm)
Figure S7. Related to Figure 7. Complete ablation of RAL GTPase causes crypt death and can be rescued by APC Loss

(A) Rala and Ralb expression analysis by qPCR of combined Rala and Ralb knock out in the small intestine of mice and in combination or not with Apc deletion, WT (N=6), Rala\(^{fl/fl}\); Ralb\(^{fl/fl}\) (N=4), and Apc\(^{fl/fl}\); Rala\(^{fl/fl}\); Ralb\(^{fl/fl}\) (N=5). Statistical analysis was done with 1-way ANOVA Tukey’s multiple comparisons test. Error bars represent standard derivation. **p<0.01, ***p<0.001

(B) Representative images of Caspase 3, PAS, Alcian Blue and Lysosyme staining in Rala and Ralb knock out mice.

Quantification of Cleaved Caspase-3 positive cells in small intestinal crypts following Rala and Ralb knock out, WT (N=5), and Rala\(^{fl/fl}\); Ralb\(^{fl/fl}\) (N=4). Statistical analysis
was done with a non-parametric Mann-Whitney U test. Error bars represent standard derivation.