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SYMPOSIUM REVIEW

Wnt pathway regulation of intestinal stem cells

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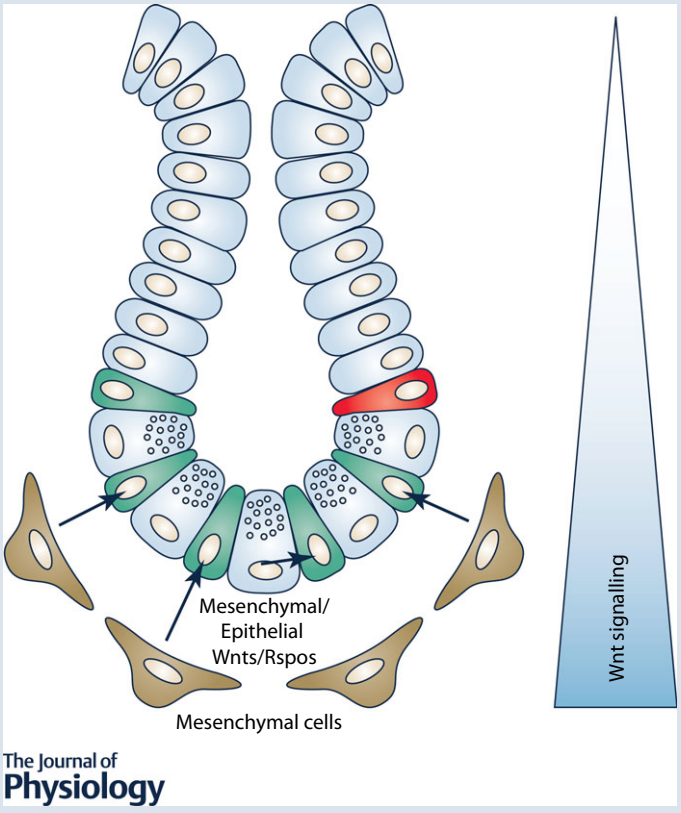

Abstract Wnt signalling is involved in multiple aspects of embryonic development and adult tissue homeostasis, notably via controlling cellular proliferation and differentiation. Wnt signalling is subject to stringent positive and negative regulation to promote proper development and homeostasis yet avoid aberrant growth. Such multi-layer regulation includes post-translational modification and processing of Wnt proteins themselves, R-spondin (Rspo)

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amplification of Wnt signalling, diverse receptor families, and intracellular and extracellular antagonists and destruction and transcription complexes. In the gastrointestinal tract, Wnt signalling is crucial for development and renewal of the intestinal epithelium. Intestinal stem cells (ISCs) undergo symmetric division and neutral drift dynamics to renew the intestinal epithelium. Sources of Wnts and Wnt amplifers such as R-spondins are beginning to be elucidated as well as their functional contribution to intestinal homeostasis. In this review we focus on regulation of ISCs and intestinal homeostasis by the Wnt/Rspo pathway, the potential cellular sources of Wnt signalling regulators and highlight potential future areas of study.

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Abstract figure legend Wnt signalling in intestinal homeostasis. The tightly regulated Wnt signalling pathway is active at the crypt base where Wnt and Rspo signals (blue) are coming from intestinal mesenchymal and epithelial compartments. It is crucial in intestinal homeostasis by acting on crypt base columnar intestinal stem cells (green) to promote self-renewal, yet has little effect on quiescent intestinal stem cells (red).

Abbreviations ISC, intestinal stem cell; LEF, lymphoid enhancer factor; LRP5/6, low density lipoprotein receptor-related protein 5/6; PCP, planar cell polarity; TCF, T-cell factor.

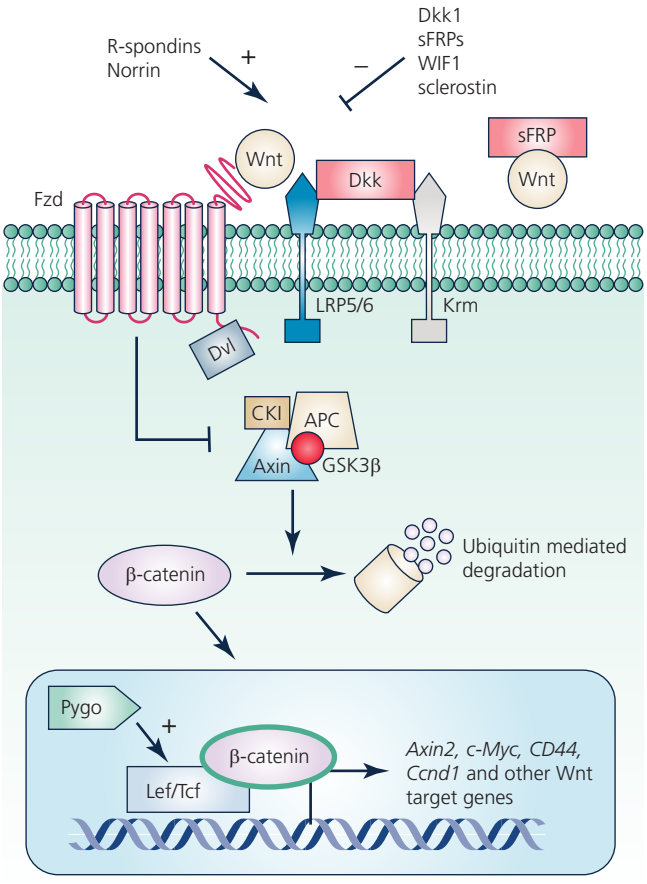
Wnt signalling: key players and pathways

The Wnt pathway is highly conserved across species and is broadly implicated in embryonic development, adult tissue homeostasis as well as disease pathophysiology (Clevers, 2006; van Amerongen & Nusse, 2009; Clevers & Nusse, 2012). Mammals possess genes for 19 Wnt ligands and 10 Frizzled (Fzd) receptors, which are seven-pass transmembrane receptors that mediate downstream Wnt signalling. Secretion of Wnt proteins relies on a number of processes including palmitoylation in the endoplasmic reticulum by the palmitoyltransferase Porcupine (Porcn). Lipidated Wnts can then bind to Wntless on the Golgi membrane where they are transferred to the plasma membrane for secretion (Kikuchi *et al.* 2011; Willert & Nusse, 2012). Wnts travel short distances to nearby cells to initiate downstream signalling that includes both canonical and non-canonical pathways. The canonical pathway is the best characterized and relies on β-catenin as its main effector protein. In canonical Wnt signalling, secreted Wnt proteins act in an autocrine or paracrine fashion by binding and forming a complex with two receptors: Fzd and low density lipoprotein receptor-related protein 5/6 (LRP5/6). This in turn activates signalling events including recruitment of proteins such as Dishevelled and conformational changes that collectively result in a block on β-catenin degradation by a destruction complex of proteins comprising Axin, adenomatous polyposis coli (APC), casein kinase I (CKI) and glycogen synthase kinase 3β (GSK3β). Stabilized β-catenin then translocates to the nucleus and associates with heterodimers of the lymphoid enhancer factor (LEF) and T-cell factor (TCF) family of transcription factors to transactivate expression of Wnt target genes (Fig. 1). In the absence of Wnt signalling, the destruction complex targets cytosolic β-catenin for proteasomal degradation (Niehrs, 2012). Non-canonical or β-catenin-independent pathways have been implicated in processes such as planar cell polarity (PCP) and calcium signalling. While the Wnt pathway is involved in these processes, the role of specific pathway components in these processes is not fully elucidated. PCP describes the orientation of a group of cells in a tissue along the plane and is important in gastrulation, neural tube closure and cell/tissue polarity (extensively reviewed in Simons & Mlodzik, 2008). In vertebrates, loss of Wnt by genetic deletion or secretion of Wnt inhibitors resulted in defects in PCP in the mouse inner ear (Heisenberg *et al.* 2000; Dabdoub *et al.* 2003; Qian *et al.* 2007). In addition to PCP, non-canonical Wnt signalling is implicated in calcium release mediated by Fzd (Slusarski *et al.* 1997; Sheldahl *et al.* 1999; Kuhl *et al.* 2000); however, it is unknown whether this effect is Wnt dependent or is mediated by interaction of Fzd with non-Wnt ligands eliciting downstream responses.

Multi-tiered regulation of Wnt signalling

Due to its central importance and involvement in myriad physiological pathways, Wnt signalling is appropriately subject to multi-tiered positive and negative regulation (Fig. 1). Wnt secretion itself is regulated post-translationally through palmitoylation by Porcn and Wntless as described above. Extracellular antagonists such as Dickkopf-1 (Dkk1), secreted Frizzled-related proteins (sFRPs), Wif1 and sclerostin inhibit Wnt signalling by

binding LRP5/6, Fzds or Wnts themselves thereby preventing pathway activation. Dkk proteins form ternary complexes with LRP and Kremen proteins, leading to LRP internalization, down-regulation and antagonism of canonical signalling (MacDonald *et al.* 2009). In contrast, sFRPs antagonize Wnt signalling by directly binding and neutralizing Wnt ligands and are therefore implicated in regulating both canonical and non-canonical Wnt pathways (Cruciat & Niehrs, 2013). At the intracellular level, Wnt signalling can be transcriptionally regulated by proteins that can bind or complex with β-catenin/TCF/LEF such as Pygopus and transducin-like enhancer of split proteins to activate or repress expression of Wnt target genes (Lien & Fuchs, 2014). Secreted Wnt agonists that enhance or promote Wnt signalling include Norrin and R-spondins (Rspos). Norrin binds to Fzd and activates Wnt signalling independent of Wnts (Xu *et al.* 2004; Cruciat & Niehrs, 2013). Rspos potently amplify Wnt signalling by binding and forming a complex with their receptors to increase the stability of Fzds at the plasma membrane, thereby potentiating Wnt signalling ability (de Lau *et al.* 2014) as we discuss in greater detail below.



The Journal of Physiology

Figure 1. Overview of the canonical Wnt pathway and points of regulation
Wnts bind to their receptors Fzd/Lrp5/6, recruit Dvl and block the destruction complex from degrading cytosolic β-catenin. Stabilized β-catenin translocates to the nucleus where it interacts with LEF/TCF to activate Wnt target genes. Wnt antagonists such as Dkk, sFRPs, WIF1 and sclerostin block Wnt signalling by binding and blocking Wnt ligands or their receptors. Norrin and R-spondins promote Wnt activity by binding or stabilizing membrane availability of Fzd/Lrp5/6. Pygo promotes Wnt signalling by activating transcription via interactions with the complex of proteins bound to the LEF/TCF transcription factors.

Renewal of the intestinal epithelium by intestinal stem cells

Stem cells are responsible for maintaining tissue function within diverse organs, such as the intestine. The intestinal epithelium is highly proliferative and is organized into invaginations ('crypts') and finger-like projections that protrude out into the lumen ('villi') that undergo complete renewal every 3–10 days driven by intestinal stem cells (ISCs). ISCs give rise to transit-amplifying (TA) cells that undergo differentiation as they exit the crypt and enter the villus compartment and are ultimately shed into the lumen. This rapidly proliferating and highly renewable nature renders the intestine a tissue of great interest to understanding stem cell biology. The past decade has seen an explosion of interest and advancement in our knowledge of ISC biology and regulation of ISC dynamics and behaviour. ISC maintenance is exquisitely controlled by a number of coordinated signals and pathways present in the niche in order to adapt to physiological demands as well as prevent aberrant growth.

Identification of ISC biomarkers through genetic lineage tracing has revealed two functionally distinct pools of ISCs, actively cycling and quiescent, that act in maintaining or restoring intestinal homeostasis. The two ISC pools have distinct but overlapping markers and crypt localization. Actively cycling crypt base columnar ISCs are located at the crypt base intercalated between Paneth cells. Quiescent or slowly cycling ISCs are found around the +4 region of the crypt above Paneth cells, which was identified by Potten and colleagues based on the ability of these cells to retain DNA label (label retaining cell) (Potten *et al.* 1974). *Lgr5*, encoding a seven-pass G protein-coupled receptor-like protein, was the first marker of actively cycling ISCs identified using genetic approaches to irreversibly mark and lineage trace $Lgr5^+$ cells and their multipotent progeny (Barker *et al.* 2007). *Olfm4* and *Ascl2* were also identified as actively cycling ISC markers that were enriched in $Lgr5^+$ ISCs and $Olfm4^+$ cells and have lineage tracing potential (van der Flier *et al.* 2009*a*,*b*; Schuijers *et al.* 2014). $Lgr5^+$ ISCs undergo symmetric division and niche competition with random clonal expansion and subsequent crypt

monoclonality, termed neutral drift dynamics (Lopez-Garcia *et al.* 2010; Snippert *et al.* 2010; Kozar *et al.* 2013). Lineage tracing of candidate quiescent ISC populations have yielded multiple biomarkers such as *Bmi1*, *Hopx*, *mTert* and *Lrig1*; however, these differ subtly in crypt position, indicating the potential co-existence of distinct quiescent ISCs (Sangiorgi & Capecchi, 2008; Montgomery *et al.* 2011; Takeda *et al.* 2011; Powell *et al.* 2012). Transcriptomic and proteomic profiling and multicoloured fluorescence *in situ* hybridization (FISH) have revealed that quiescent ISC markers are not exclusively present in these cells and can be broadly expressed along the crypt and co-expressed in $Lgr5^+$ ISCs (Itzkovitz *et al.* 2012; Munoz *et al.* 2012). Furthermore, distinct ISC populations are related by lineage, as toxin-mediated direct ablation of $Lgr5^+$ cells revealed that $Bmi1^+$ cells can replenish the ISC pool by giving rise to $Lgr5^+$ cells (Tian *et al.* 2011). Similarly, Epstein and colleagues demonstrated analogous bi-directional interconversion when they reported Hopx-expressing ISCs at the +4 position generate progeny expressing *Lgr5* and vice versa (Takeda *et al.* 2011).

Despite substantial expression overlap, the actively cycling and quiescent ISC populations are functionally distinct. Actively cycling $Lgr5^+$ ISCs rapidly lineage trace and are responsible for physiological intestinal renewal (Barker *et al.* 2007). Emerging evidence supports the notion that quiescent ISCs comprise a reserve stem cell population that is injury-inducible (Fig. 2). Both $mTert^+$ and $Bmi1^+$ ISCs exhibit relative baseline quiescence with rare homeostatic lineage tracing, which can be augmented upon intestinal injury (Montgomery *et al.* 2011; Yan *et al.* 2012). Van Landeghem and colleagues used the Sox9-EGFP transgenic mouse model, where distinct levels of the Sox9-EGFP transgene mark actively cycling *Lgr5* enriched ISCs ($Sox9\text{-}EGFP^{Low}$) and quiescent ISCs ($Sox9\text{-}EGFP^{High}$) within the same animal. In this system, $Sox9\text{-}EGFP^{High}$ cells, also enriched in mRNAs encoding enteroendocrine cell (EEC) hormones, re-enter the cell cycle following radiation-induced crypt ablation and generate actively cycling *Lgr5* enriched $Sox9\text{-}EGFP^{Low}$ ISCs providing additional evidence that this population containing reserve ISC are injury-inducible (Van Landeghem *et al.* 2012). Additionally, $Dll1^+$ secretory progenitors have been shown to be adopt ISC functional characteristics following radiation injury (van Es *et al.* 2012*b*). Buczacki *et al.* have further revealed that label-retaining cells (LRCs) are secretory progenitors co-expressing *Lgr5* and markers of Paneth cells and EECs under homeostatic conditions but can lineage trace following damage (Buczacki *et al.* 2013). Collectively, these studies support a model where the intestinal epithelium contains two categories of actively cycling versus quiescent ISC populations that coexist harmoniously and function in distinct situations to maintain intestinal homeostasis (Li & Clevers, 2010). Additionally, elegant genetic and crypt ablation studies have provided novel and exciting evidence that the concept of intestinal epithelial cell plasticity or dedifferentiation of progenitors potentially exists (van Es *et al.* 2012*b*; Van Landeghem *et al.* 2012; Buczacki *et al.* 2013); however, conclusive evidence implicating the ability of mature differentiated cells to adopt ISC function has yet to be shown.

Essential Wnt pathway regulation of intestinal epithelial self-renewal and maintenance

Wnt signalling is a critical component of the ISC niche. Clearly, Wnt pathway activation by *Apc* gene mutation and

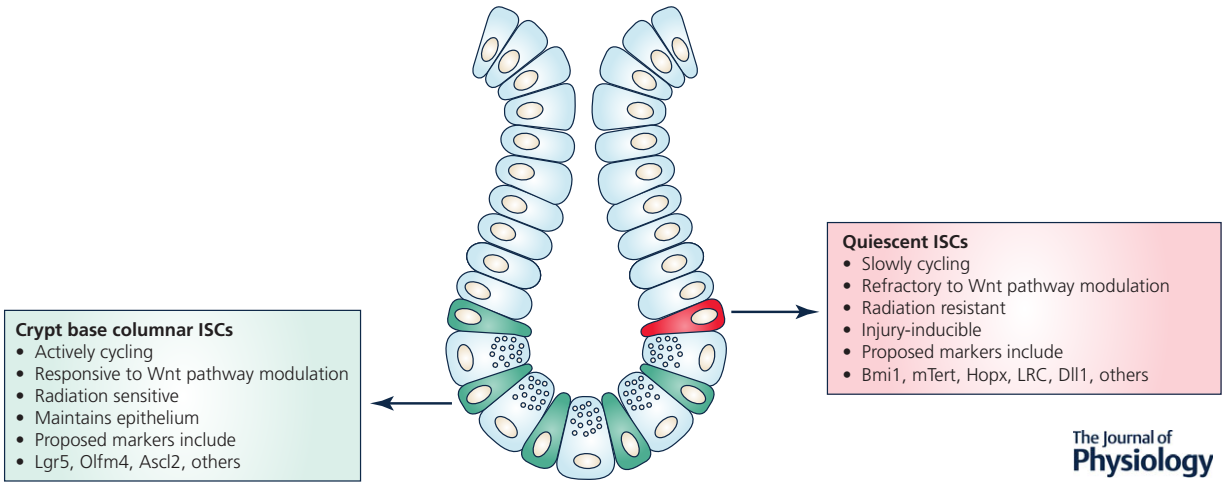


Figure 2. The intestinal epithelium contains two functionally distinct pools of intestinal stem cells (ISCs)
Crypt base columnar ISCs (green) and +4 quiescent ISCs (red) co-exist in the intestinal crypt and differ in their cycling kinetics, sensitivity to extracellular Wnt pathway manipulations and radiation.

constitutive activation of β-catenin, specifically in ISCs, is sufficient to induce intestinal epithelial hyperproliferation and polyposis (Barker *et al.* 2009; Krausova & Korinek, 2014; Powell *et al.* 2014). Conversely, loss-of-function gene ablation studies have been critical in identifying the vital role of canonical Wnt signalling in maintaining intestinal homeostasis, proliferation and ISC biology. Intestines of mice lacking the Wnt effector gene *Tcf4/Tcf7l2* were devoid of intestinal crypts and consisted of solely differentiated villus cells (Korinek *et al.* 1998). Subsequent studies using conditional genetic deletion of *Tcf4* and Wnt effector β-catenin (Ireland *et al.* 2004; Fevr *et al.* 2007; van Es *et al.* 2012*a*) or ectopic expression of the Wnt antagonist Dkk1 (Pinto *et al.* 2003; Kuhnert *et al.* 2004) resulted in loss of intestinal crypts and alterations in differentiated linages. Intestinal epithelial-specific Dkk1 transgenic expression elicits non-lethal loss of small intestine crypts, intestinal proliferation and secretory lineages (Pinto *et al.* 2003). An even more severe phenotype is observed upon adenovirus-mediated circulating Dkk1 expression, which results in global Wnt inhibition, rapid crypt and villus loss in small intestine, severe colitis and fully penetrant lethality (Kuhnert *et al.* 2004). Genetic deletion of Wnt target gene *Myc* has been shown to be important for postnatal intestinal crypt formation (Bettess *et al.* 2005) and regulating crypt size and proliferation during adulthood (Muncan *et al.* 2006). The effect on crypts and differentiated lineages in these loss-of-function studies reveals the critical role of the Wnt pathway in ISC function, differentiation and maintenance of epithelial architecture.

The intestinal effects of impaired Wnt ligand secretion have been evaluated by genetic *Porcn* deletion. Epithelial-specific *Porcn* loss did not affect intestinal homeostasis or intestinal regeneration following radiation injury indicating epithelial Wnt production is dispensable (Kabiri *et al.* 2014; San Roman *et al.* 2014); however, evidence for stromal Wnt contributions to intestinal homeostasis is less conclusive. Further, *Porcn* deletion from subepithelial myofibroblasts did not affect intestinal homeostasis (San Roman *et al.* 2014). On the other hand, pharmacological Porcn inhibition to mimic loss of epithelial and stromal Wnts resulted in decreased Lgr5$^+$ ISCs, loss of the Wnt-independent crypt base columnar ISC marker *Olfm4*, intestinal proliferation and impaired regenerative capacity (Kabiri *et al.* 2014). Taken together, these studies suggest that potential redundancy of Wnts between undefined compartments of intestinal stroma and/or epithelium may govern ISCs and intestinal homeostasis. Notably mouse knockouts of Wnt ligands have not thus far been associated with intestinal homeostatic phenotypes, although *Wnt5a* loss impairs colonic crypt regeneration following injury (Miyoshi *et al.* 2012). Recently, Foxl1$^+$ mesenchymal cells have been shown to be a crucial cell type providing both Wnt and Rspo within the intestinal niche, as they are enriched for Wnt2b, Wnt5a and Rspo3. Toxin-mediated loss of Foxl1$^+$ cells resulted in shortened intestinal length along with decreased crypt depth, villus height and intestinal epithelial proliferation. More strikingly, loss of Foxl1$^+$ cells ablated *Olfm4*$^+$ signal associated with loss of *Wnt2a*, *Wnt4* and *Wnt5a* mRNA (Aoki *et al.* 2016). This recent study implicates Foxl1$^+$ mesenchymal cells in Wnt/Rspo synthesis and intestinal homeostasis and provides potential future possibilities to elucidate the role of other mesenchymal cells in synthesis and secretion of other Wnts.

Identifying the sources of Wnt proteins is important to understanding their function on ISCs during intestinal homeostasis, regeneration or disease, particularly since the palmitoyl modification restricts long-range diffusion. *In situ* hybridization of Wnt ligands showed differential expression of various Wnts along the crypt–villus axis as well as in epithelial *versus* mesenchymal compartments (Gregorieff *et al.* 2005). *Wnt3* and *Wnt9b* expression was observed in Paneth cells while *Wnt6* was more diffusely expressed along the crypt epithelium. Expression of Wnts in the mesenchymal compartment included *Wnt2b* and *Wnt5a* (Gregorieff *et al.* 2005) from Foxl1$^+$ mesenchymal cells (Aoki *et al.* 2016). Future studies may allow functional and phenotypical correlation of Wnts with their localization in the ISC niche and their phenotypes, if any, on ISCs.

Role of R-spondins during development and intestinal homeostasis

R-spondin proteins (Rspo1–4) are secreted amplifiers of Wnt signalling that are highly conserved among vertebrates. Structurally, Rspo1–4 are unified by a common domain architecture comprising a C-terminal Thrombospondin type 1 repeat domain and two furin-like repeats, where the latter is essential for their function in enhancing Wnt signalling (de Lau *et al.* 2012). *In vitro* TOPflash studies in HEK293 cells demonstrated the Wnt signalling ability of Rspo proteins in its potent synergism with Wnt3a (Kazanskaya *et al.* 2004).

Single Rspo knockout mice have not revealed any adult intestinal phenotypes despite genetic data indicating key roles and functions of Rspo1–4 during embryogenesis and development. The functions of Rspo1–4 have been explored using genetic mutation studies and are reviewed in greater detail (de Lau *et al.* 2012). Briefly, Rspo1 is involved in development of the sex phenotype, differentiation of the skin and predisposition to skin carcinoma (Parma *et al.* 2006; Tomizuka *et al.* 2008). Loss of *Rspo2* has been shown to inhibit development of limbs, lungs, hair and ovarian follicles, associated with decreased Wnt activity, and mutants die shortly after birth due to limb defects (Nam *et al.* 2007; Bell *et al.* 2008; Cheng *et al.* 2013). Rspo3 is involved in angiogenesis and placental development. Targeted

disruption of *Rspo3* results in severe defects in placental vascularization and ultimately leads to death at E10 (Aoki *et al.* 2007; Kazanskaya *et al.* 2008). In C2C12 myoblasts, Rspo1 or -2 overexpression and Rspo2 or -3 knockdown were associated with increased and decreased myogenic differentiation, respectively, suggesting a role for Rspos in cellular differentiation (Han *et al.* 2011). Lastly Rspo4 is involved in fingernail development (Bergmann *et al.* 2006; Blaydon *et al.* 2006; Bruchle *et al.* 2008; Ishii *et al.* 2008). Overall, while genetic *Rspo* deletion phenocopies deletion of key Wnt signalling genes or that of reduced Wnt activity (Monkley *et al.* 1996; Vainio *et al.* 1999; Ishikawa *et al.* 2001), Rspo genetic deletion in regulation of ISCs or intestinal homeostasis has been elusive and has been potentially complicated by embryonic lethality (Aoki *et al.* 2007; Nam *et al.* 2007). Recently, Storm and colleagues treated mice with neutralizing antibodies raised against Rspo2 and Rspo3 and reported decreased *Lgr5* reporter gene expression suggesting a requirement for Rspo2/3 in $Lgr5^+$ ISC maintenance without any impairment of intestinal homeostasis or crypt architecture. However in response to radiation, anti-Rspo2/3 treated animals displayed impaired regeneration providing evidence for the role of both Rspo2 and Rspo3 in intestinal repair (Storm *et al.* 2016).

Conversely, Rspo has profound effects on intestinal homeostasis and ISC in gain-of-function contexts. *In vivo* experiments using ectopic Rspo1 administration leads to expansion of many intestinal parameters including small intestinal diameter, weight, crypt density, ISC number and proliferation (Kim *et al.* 2005; Yan *et al.* 2012). Rspo1 is a critical component in the growth and expansion of *in vitro* organoids ('miniguts') in three-dimensional Matrigel culture. In this system, a growth factor cocktail consisting of epidermal growth factor (EGF), Noggin and Rspo1 is used to mimic key signals present in the intestinal crypt to promote self-renewal, multipotency and organoid formation (Sato *et al.* 2009). Although potent, these gain-of-function effects *in vivo* and *in vitro* could reflect ectopic and/or non-physiological activation of ISC Wnt signalling. Thus, further genetic and/or pharmacological demonstrations of endogenous Rspo functions on ISCs and intestinal homeostasis are sorely needed.

The precise locations or sources of intestine-relevant Rspo proteins, within the gastrointestinal tract or at remote sites, are of considerable interest. Expression of *Rspo1–3* has been reported in the intestinal stromal fraction and minimally detected in the intestinal epithelium (Kabiri *et al.* 2014). *Rspo2* expression in myofibroblasts increased in response to *C. rodentium* infection, leading to aberrant proliferation and impaired differentiation of colonic epithelial cells, thus providing evidence that links Rspo proteins to pathophysiological states (Papapietro *et al.* 2013). The overlapping expression of Wnts and Rspos within different epithelial and stromal populations of the intestine may enable significant functional redundancy, perhaps to protect from ISC dysfunction and catastrophic loss (Kabiri *et al.* 2014).

R-spondins bind distinct receptors of the Lgr4–6 (seven-pass transmembrane) and Rnf43 and Znrf3 (Rnf43/Znrf3) (transmembrane E3 ubiquitin ligase) classes (Carmon *et al.* 2011; de Lau *et al.* 2011; Glinka *et al.* 2011; Xie *et al.* 2013). In the absence of Wnt signals, Rnf43/Znrf3 act as negative regulators of Wnt signalling by ubiquitination and subsequent endocytosis and lysosomal degradation of Fzd and Lrp (Hao *et al.* 2012; Koo *et al.* 2012), which is Dishevelled-dependent (Jiang *et al.* 2015). Rspo binding to Lgr4–6 and Rnf43/Znrf3, perhaps as a ternary complex, inhibits Rnf43/Znrf3-mediated degradation of Fzd/Lrp resulting in stabilization and accumulation of Fzd/Lrp receptors on the plasma membrane to enhance Wnt signalling (Fig. 3). Intestinal epithelium-specific *Lgr5* deletion surprisingly did not show an overt intestinal phenotype while *Lgr4* deletion decreased proliferation and induced crypt loss associated with decreased *Olfm4* mRNA. Furthermore, *Lgr4* null mice displayed impaired *ex vivo* organoid formation (Mustata *et al.* 2013). Combined *Lgr4/Lgr5* deletion further exacerbated the intestinal phenotype seen with loss of *Lgr4* alone (de Lau *et al.* 2011) and was similar to effects seen in mice ectopically expressing Dkk1 (Kuhnert *et al.* 2004). Conversely, conditional intestinal deletion of *Rnf43*/*Znrf3* in mice resulted in expansion of crypt size associated with hyperproliferation and expansion of $Olfm4^+$ cells (Koo *et al.* 2012). Despite evidence of alterations of ISC number by *Olfm4 in situ* hybridization, Rspo receptor knockout studies have not examined the subsequent fate of ISCs, as lineage-tracing studies have not been performed. Recently, Rnf43 has been shown to be present in the nucleus and interact with and prevent TCF4-mediated transcription, suggesting an additional mechanism by which Rnf43 negatively regulates Wnt signalling (Loregger *et al.* 2015) (Fig. 3).

Differential regulation of ISCs by the Wnt/Rspo signalling pathway

The profound intestinal defects observed in animals with disrupted Wnt signalling or Rspo1 overexpression are consistent with an important role for Wnt/Rspo signalling in ISC behaviour and function. The Wnt pathway is active as a gradient in the intestinal crypt, most prominently at the base and decreasing up the crypt–villus axis (Scoville *et al.* 2008). This expression gradient, coupled with the differential location of actively cycling *versus* quiescent ISCs within the crypt suggests differential responses to Wnt levels. A direct lineage tracing comparison of $Lgr5^+$ and $Bmi1^+$ ISCs in *Lgr5-eGFP-IRES-CreERT2* and *Bmi1-CreER;Rosa26-YFP* mice, respectively, indicates that modulation of Wnt signalling differentially affects

these two populations of ISCs. Rspo robustly expanded Lgr5$^+$ or Olfm4$^+$ cells with increased mitotic index while Bmi1$^+$ cells remained unaffected. Conversely, the Wnt antagonist Dkk1 potently depleted Lgr5$^+$ cells but not Bmi1$^+$ cells. Overall, the Lgr5$^+$ and Bmi1$^+$ ISC pools appear functionally distinct with differential response to Wnt pathway manipulations (Yan *et al.* 2012) (Fig. 2). Histological analyses and single molecule FISH revealed that conditional loss of *Tcf4* in intestinal epithelial cells resulted in loss of actively cycling ISC markers *Lgr5* and *Olfm4* with little effect on *Bmi1* transcript levels (van Es *et al.* 2012*a*). Additionally, intestinal epithelium-specific loss of *Rnf43*/*Znrf3* expanded the numbers of *Olfm4*$^+$ cells and led to adenoma formation (Koo *et al.* 2012). A combination of anti-Rspo2 and anti-Rspo3 monoclonal antibodies reduced crypt Lgr5 expression *in vivo* but this was not accompanied by crypt loss or lethality (Storm *et al.* 2016). Thus, Lgr5$^+$ ISCs in particular are responsive to modulation of Wnt signalling.

Future directions

There is a substantial body of evidence supporting roles of the Wnt/Rspo pathway in ISC behaviour and maintenance of the intestinal epithelium, but at the same time this raises numerous additional questions. While much of the known effects of Wnt and Rspo ligands on ISCs are inferred from gain-of-function or global inhibitor studies (Dkk1, Porcn antagonists), analogous loss-of-function data for specific individual Wnt or Rspo members have only begun to be explored (Storm *et al.* 2016), with open questions on regulation of ISC fate and differentiation. Indeed, genetic deletion of specific single Wnt, Fzd or Rspo family members have still not been associated with gross intestinal homeostatic phenotypes, perhaps complicated by mobilization of reserve stem cell populations, functional redundancy and/or need for conditional deletion approaches. The effects of Wnt5a deletion on colonic epithelial regeneration but not homeostasis (Miyoshi *et al.* 2012) suggests that additional complexity could exist whereby specific Wnt ligands might differentially regulate intestinal homeostasis *versus* repair or pathogenesis based on their anatomical location within the niche and their effect on ISCs. Similarly, this may also hold true for specific Rspo ligands (Storm *et al.* 2016). In this regard, it will be important to understand not only the spatial location of specific essential Wnt and Rspo ligands such as in Foxl1$^+$ mesenchymal cells, but also the circuitry governing their expression during homeostasis *versus* regeneration. It should be interesting to overlay Wnt/Rspo functional manipulation onto the elegant framework of Lgr5$^+$ ISC symmetric division and neutral drift dynamics, as well as interconversion between active and reserve stem cell pools. Lastly, the relative contribution of Wnt and Rspo ligands to physiological Wnt signalling and regulation of ISC fate is unknown, and could be substantially aided by development of highly specific gain-of-function methods for dissecting apart these two intersecting pathways. Regardless, continued exploration of Wnt or Rspo biology will illuminate how these fascinating ligand families regulate ISC fate during both intestinal homeostasis and disease.

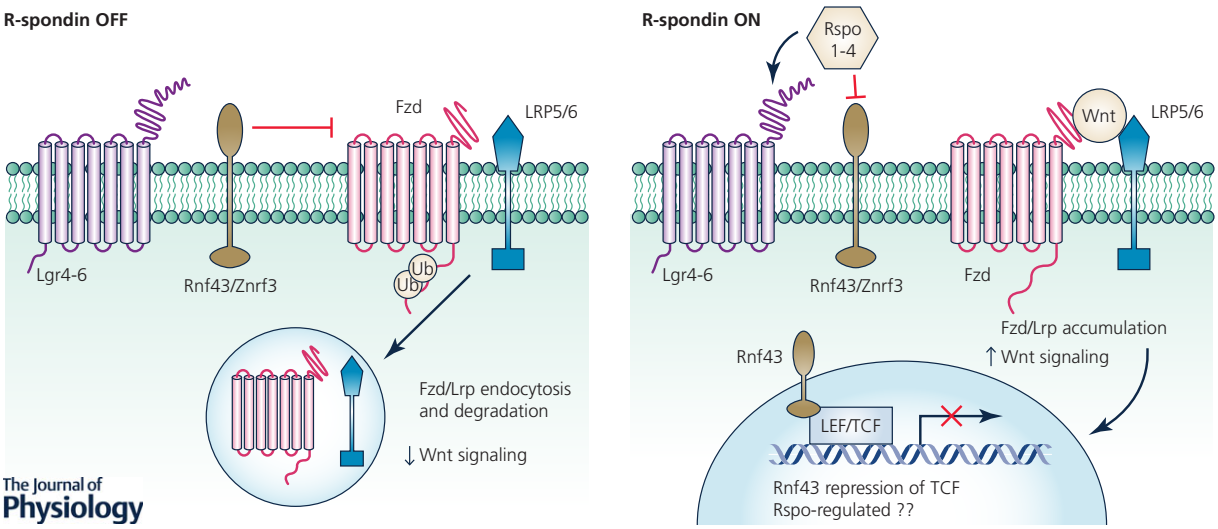


Figure 3. R-spondins potently amplify Wnt signalling
The transmembrane E3 ubiquitin ligases Rnf43/Znrf3 negatively regulate Wnt signalling by ubiquitinating the cytoplasmic tails of Fzds to promote their membrane clearance by endocytosis and degradation, ultimately down-regulating Wnt signalling. When R-spondins (Rspo1–4) are present, Rnf43/Znrf3 mediated degradation of Fzds is inhibited and Fzd/Lrp accumulation on the plasma membrane upregulates Wnt signalling. In an independent mechanism, Rnf43 present on the nuclear membrane interacts with and prevents LEF/TCF-mediated transcription.

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Additional information

Competing interests

The authors declare no competing interests, financial or otherwise, contributing to this work.

Author contributions

All authors have approved the final version of the manuscript and agree to be accountable for all aspects of the work. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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