# Wnt signaling in epithelial stem cells and cancer

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## Abstract

Wnt/ß-catenin signaling is integral to the homeostasis and regeneration of many epithelial tissues due to its critical role in adult stem cell regulation. It is also implicated in many epithelial cancers, with mutations in core pathway components frequently present in patient tumors. In this chapter, we discuss the roles of Wnt/ß-catenin signaling and Wnt-regulated stem cells in homeostatic, regenerative and cancer contexts of the intestines, stomach, skin and liver. We also examine the sources of Wnt ligands that form part of the stem cell niche. Despite the diversity in characteristics of various tissue stem cells, the role(s) of Wnt/ß-catenin signaling is generally coherent in maintaining stem cell fate and/or promoting proliferation. It is also likely to play similar roles in cancer stem cells, making the pathway a salient therapeutic target for cancer. While promising progress is being made in the field, deeper understanding of the functions and signaling mechanisms of the pathway in specific epithelial tissues will expedite efforts to modulate Wnt/ß-catenin signaling in cancer treatment and tissue regeneration.

**Keywords**: Wnt/ß-catenin signaling, epithelial stem cells, epithelial cancer, intestine, stomach, skin, liver

## Introduction

Epithelia comprise the largest tissue type in our body. They serve as barriers against external agents, absorptive surfaces for water and nutrients, and/or glands secreting important hormones or enzymes. Most epithelia line surfaces of our body that come into contact with the external environment (e.g. skin, cornea), or tissue cavities within our body that could be exposed to external agents (e.g. gastrointestinal tract, lung, urogenital tissues). As a result, it is necessary for the tissues to turnover frequently to replace lost or damaged cells so as to maintain the normal function and integrity of the epithelial tissues. Small populations of multipotent adult epithelial stem cells are critical for effecting this relentless tissue renewal throughout life.

### *Stem cells*

Adult stem cells fuel tissue renewal during both homeostasis and regeneration. They constitute specialized classes of cells, each representing a specific lineage of their resident organs, that give rise to rapidly proliferating progenitors responsible for producing the various differentiated cells that carry out the requisite physiological tissue functions. Stem cells are the only population that can persist long-term, thereby sustaining the supply of differentiated cells over the organism's lifetime. The two hallmarks of adult stem cells are (1) infinite self-renewal capability as a population and (2) an ability to generate all differentiated lineages of an organ.

Stem cells can be broadly classified as homeostatic and facultative. Homeostatic stem cells are responsible for the day-to-day maintenance of the tissue, while facultative stem cells are not typically active during homeostasis but are capable of performing stem cell functions when called to duty following injury. These two types

of stem cells are not mutually exclusive and can co-exist as distinct populations in the same tissue.

## *Cancer*

Cancer, a disease of unrelenting cell proliferation and compromised normal tissue function, is a frequent and deadly disease of the modern world. Epithelial cancers, a.k.a. carcinomas, is the most common type of cancer, as highlighted by the fact that the 10 cancers with highest incidence worldwide in 2012 are all carcinomas, regardless of geography[1].

Cancers arise from a series of molecular changes that endow the tumors with eight distinct survival advantages, now famously referred to as "Hallmarks of Cancer"[2]. The cells in which these changes initially accumulate are the cell-of-origin (a.k.a. tumor initiating cell) of the cancer. In many carcinomas, the cell-of-origin is often thought to be a stem or progenitor cell that has been transformed through the acquisition of multiple genetic mutations, equipping it with a survival and proliferative advantage[3]. Once established, the progression of the tumor then becomes fueled by cancer stem cells (CSCs), which may or may not be the same as the cancer cell-of-origin.

## *Wnt/ß-catenin signaling*

Wnt/ß-catenin signaling is instrumental in directing tissue development, homeostasis and regeneration, particularly in the stem cell context. Unsurprisingly, it is also commonly subjugated by tumor cells to drive cancer. There are two other known Wnt

pathways, commonly referred to as the planar cell polarity pathway and Wnt/$Ca^{2+}$ pathway. Here, we will be focusing on Wnt/ß-catenin pathway, which has been established as a principal signaling pathway in stem cell and cancer biology.

The detailed mechanics of Wnt/ß-catenin signaling are extensively described elsewhere[4-6]. To summarize (Figure 1), in the absence of Wnt ligands, a destruction complex which includes Adenomatous polyposis coli (Apc), Glycogen synthase kinase-3 (Gsk-3ß) and Axin, binds to cytoplasmic ß-catenin and marks it for proteosomal degradation, thereby preventing Wnt/ß-catenin signaling activity in the nucleus. At the same time, the T-cell factor (Tcf)/Lymphoid-enhancing factor (Lef)/Groucho nuclear complex actively represses transcription of Wnt/ß-catenin target genes. When Wnt ligands bind to Frizzled (Fzd) and LDL-receptor related protein 5/6 (Lrp5/6) receptors in the Wnt-responding cells, the receptors sequester Axin, causing the destruction complex to disassemble, thereby allowing cytosolic ß-catenin to accumulate. ß-catenin subsequently enters the nucleus, and converts the Tcf/Lef complex from a repressor to a transcriptional activator[7-10], inducing expression of target genes like Axin2[11] and Leucine-rich repeat containing G protein-coupled receptor 5 (Lgr5, a.k.a. Gpr49)[12] (Figure 1). Nuclear localization of ß-catenin is commonly used as an indicator of high Wnt/ß-catenin signaling activity. Interestingly, while Axin2 is a weak negative feedback regulator of Wnt/ß-catenin signaling, Lgr5 is a potentiator of Wnt signaling. When Lgr5 (or closely related family members Lgr4 and Lgr6) binds to its secreted ligand R-spondin (there are four R-spondins in humans and mice), they form a complex with the membrane-bound E3 ligases Zinc and ring-finger 43 (Znrf3) and Ring-finger protein 43 (Rnf43), thereby preventing them from ubiquitinating Fzd receptors which would target them for

degradation[13-15]. Hence, when Lgr4/5/6 is expressed in the presence of R-spondins, Fzd receptors accumulate on the cell membrane to enhance Wnt/ß-catenin signaling.

*[Insert Figure 1 here]*

Figure 1: Wnt/ß-catenin signaling. In the absence of Wnt ligand, the Frizzled (Fzd) receptor is targeted for degradation by Rnf43 and Znrf3. In addition, cytosolic ß-catenin is also marked for degradation by the destruction complex comprising of Axin, Gsk-3ß and Apc. In the absence of ß-catenin, the Tcf/Lef/Groucho complex in the nucleus acts as a transcriptional repressor and the Wnt/ß-catenin targets are not expressed. When Wnt ligand binds to the Fzd/Lrp receptor complex, they sequester *Axin* and cause the destruction complex to disintegrate leaving ß-catenin molecules to accumulate in the cytosol and translocate into the nucleus. In the nucleus, ß-catenin associates with Tcf/Lef complex at promoters/enhancers of Wnt/ß-catenin target genes such as Axin2 and Lgr5 to effect their transcription. When Lgr5 is expressed at the membrane, binding of R-spondin ligand sequesters Rnf43/Znrf3, thereby inhibiting Fzd degradation and potentiating Wnt/ß-catenin signaling activity.

*In vivo* lineage tracing models employing Cre-lox technology have identified *Lgr5* and/or *Axin2* as markers of adult stem cell populations in a wide range of tissues. These lineage tracing models comprise two essential components – a CreERT2 cassette whose expression is controlled by an endogenous Lgr5 or Axin2 promoter, and a conditional reporter allele such as Rosa26-LacZ[16], mTmG[17], Confetti[18] or Ai6/9/14[19]. The CreERT2 cassette encodes for the Cre recombinase fused to the ligand-binding domain of estrogen receptor, which sequesters the Cre recombinase

at the cell membrane in the absence of tamoxifen (an estrogen analog)[20]. When tamoxifen binds to the ER domain, the Cre recombinase is released from the membrane and translocates to the nucleus where it catalyzes the recombination of a genomic region flanked by loxP sites. Specifically, in inducible reporter alleles, the Cre recombinase removes the loxP-flanked transcriptional stop cassette that precedes the reporter gene, thereby facilitating its constitutive expression driven by the ubiquitous Rosa26 promoter. Consequently, cells that expressed Axin2 or Lgr5 when the tamoxifen was administered are labeled with an indelible, heritable genetic mark that is subsequently passed on to all their progeny in a living tissue. Long-term labelling of all differentiated cell lineages in a given tissue identifies the Cre-expressing Lgr5/Axin2 cells as self-renewing, multipotent stem cells.

*Sources of Wnt ligands are essential stem cell niche components*

The stem cell niche is the microenvironment that supports the stem cells and regulates their behavior during tissue homeostasis and regeneration. As Wnt/ß-catenin signaling is one of the critical pathways for stem cell maintenance, the Wnt ligand-producing cells define a core component of the niche. Since Wnt ligands are palmitoylated, they have limited diffusion range in the extracellular matrix and therefore typically act as short-range signals[21]. Wnt ligands can theoretically be produced by any of the cell types present in the niche, such as fibroblasts, hematopoietic cells, endothelial cells or other epithelial cells, which would consequently form a paracrine signaling axis. Alternatively, autocrine signaling is possible when Wnt-responding stem cells supply the Wnt ligands for their own use.

Regardless of the precise mode of signaling, the production of mature Wnt ligands and their transport to the membrane for secretion requires two proteins – Porcupine (Porcn) and Wntless (Wls, a.k.a. Gpr177, Evenness interrupted/Eva, MIG-14) (Figure 2). Porcn catalyzes the palmitoylation which is required for the function of all Wnt ligands[22-24], as the modification likely facilitates binding to the Fzd receptors[25,26]. Once palmitoylated, the Wnts are insoluble in cytosol and require transport within exocytic vesicles for secretion, where Wls acts as a chaperone for the docking of the Wnt ligands to the vesicular membrane[27-29]. Therefore, inactivating Wls or Porcn in Wnt-producing niche cells using pharmacological inhibitors or via genetic mouse models effectively disrupts the supply of functional Wnts to the stem cells, attenuating Wnt/ß-catenin signaling *in vivo*. This has proven to be a useful approach for functionally examining the role of Wnt-producing niches in stem cell-driven homeostasis, regeneration and cancer. Interestingly, small molecules that inhibit Porcn activity have already shown promising results for treating Wnt-driven cancers[30-35]. Some of these are currently in cancer clinical trials, indicating that targeting Wnt-producing cells in cancer is potentially a viable therapeutic strategy.

*[Insert Figure 2 here]*

Figure 2: Post-translational modification of Wnt ligands. In the Wnt-producing cells (left), nascent Wnt proteins are palmitoylated by the enzyme Porcupine (Porcn). The lipid modification is required for their secretion and function. Thereafter, the lipidated Wnt molecule associates with a multispan transmembrane protein Wntless (Wls) in a secretory vesicle, and is secreted from the cell via exocytosis.

The study of Wnt/ß-catenin signaling in stem cells has proven useful in two major aspects. First, we learn about the underlying mechanism of stem cell and tissue homeostasis *in vivo*, as well as the more ominous pathway alterations that lead to tumorigenesis and carcinogenesis. Second, the list of Wnt/ß-catenin target genes has yielded useful adult stem cell markers by studying the central role of this pathway in effecting stem cell maintenance *in vivo*. Using these markers, the stem cells can be identified and isolated for a range of applications, including *ex vivo* expansion, manipulation using DNA editing technologies and expression profiling. These advances have facilitated major advances for the stem cell field and regenerative medicine.

In this chapter, we will discuss the role of Wnt/ß-catenin signaling in several prototypical epithelial stem cell systems, focusing on its roles in adult stem cells and cancer, with a particular emphasis on adult stem cells marked by Wnt target genes such as Axin2 and Lgr5.

## 1) Gastrointestinal stem cells and cancer

The gastrointestinal tract is made up of a single layer of columnar epithelial cells, and exhibits the highest turnover in the body – the gastric and intestinal epithelia renew every 7-10 and 3-14 days respectively[36,37]. The gastrointestinal system, which spans the mouth, esophagus, stomach and intestines, is primarily responsible for the digestion and absorption of nutrients and water from our food intake. In this section, we will examine stem cells of the intestine and stomach.

## *Intestine*

The intestinal epithelium is undoubtedly where the function and mechanism of Wnt/ß-catenin signaling has been characterized most comprehensively, and is a classical prototype for the study of adult stem cells (reviewed in Clevers[38]). The small intestine and colon exhibit turnover rates of 3-5 days and 10-14 days respectively, which are the highest among all adult tissues[36]. Effecting this rapid turnover during homeostasis are intestinal stem cells (ISCs) governed by Wnt/ß-catenin signaling, which can also be the causative force in intestinal cancer when dysregulated.

### Wnt/ß-catenin signaling in intestinal crypts are crucial during homeostasis

The functional unit of the small intestinal mucosa comprises the crypt and villus. Stem and progenitor cells located in the crypts of Lieberkühn generate differentiated progeny – absorptive enterocytes, enteroendocrine cells, goblet cells, tuft cells or Paneth cells. Except for Paneth cells which remain at the base of the crypts, all the other differentiated cells migrate upwards along the villi towards the lumen (Figure 3). While it has long been known that the cellular source of the intestinal epithelium resided in the crypts, the exact identity of the ISCs and their genetic markers were not defined until the late 2000s (reviewed in Barker et al[39]). Nonetheless, the importance of Wnt/ß-catenin signaling for maintenance of the intestinal epithelium was revealed through the use of the Villin-CreERT2 driver, which facilitated genetic studies in the entire intestinal epithelium spanning the crypt/villus axis[40,41]. Wnt/ß-catenin signaling activity in the crypt exists as a gradient, with robust levels at the crypt base tapering off towards the crypt-villus junction (Figure 3). Indeed, nuclear

beta-catenin, a hallmark of active Wnt signaling, is confined to the crypt base columnar (CBC) cells and Paneth cells populating the crypt base[42].

[Insert Figure 3 here]

Figure 3: Small intestinal stem cell. (A) Villi of the small intestine contain differentiated cells, which originate from the stem and transit amplifying (TA) cells in the crypts. Wnt/ß-catenin signaling, which is strongest at the base of the crypts, maintain Lgr5-expressing intestinal stem cells (ISCs), the crypt base columnar (CBC) cells. The CBC cells are interdigitated between Paneth cells, which are their progeny and a component of their niche. (B) Paneth cells secrete Wnts, which are immediately received by Fzd receptor on the adjacent ISCs, leading to expression of target genes such as Lgr5, Ascl2, and EphrinB3 which are widely used as ISC markers. In particular, Lgr5 expression potentiates Wnt/ß-catenin signaling as explained in Figure 1, reinforcing the robust Wnt/ß-catenin signaling status at the crypt base. Stromal myofibroblasts surrounding the crypt epithelial cells are also constituents of the ISC niche as they secrete Wnts to complement those from the Paneth cells.

Abrogating Wnt/ß-catenin signaling in the intestinal epithelium severely perturbs homeostasis and survival. Administering the Wnt antagonist Dkk1 or knocking out critical effectors such as ß-catenin or Tcf4 (*Tcf7l2*) impairs progenitor/transit amplifying cell proliferation in the crypts and blocks differentiation towards secretory lineages, resulting in severely compromised villi comprising exclusively absorptive enterocytes and increased mortality[43-47]. Similarly, Tcf4 null mutant mice lack proliferative cells and enteroendocrine cells in intestinal crypts at birth[48]. In contrast,

knocking out Tcf1 and Tcf3 in the intestinal epithelium had no phenotype[47], identifying Tcf4 as the workhorse of the Tcf family during Wnt-driven intestinal homeostasis.

Conversely, hyperactivating Wnt/ß-catenin signaling increases transit amplifying cell proliferation, accompanied by ß-catenin stabilization leading to crypt expansion, and impaired differentiation towards the enterocyte lineage[49-53]. Such hyperactivation is typically achieved through forced expression of a ß-catenin mutant that is immune to degradation or injection of the agonist protein R-spondin1. Interestingly, R-spondin1 administration protected ISCs against various adverse challenges to the intestines, like chemoradiation[52,54] and graft versus host disease (from allogeneic bone marrow transplantation)[55], implying that amplifying Wnt/ß-catenin signaling could increase robustness of the ISCs, and hence the health of the intestinal tissue as a whole.

## ISCs express Wnt/ß-catenin target genes

Blocking dysregulated Wnt/ß-catenin signaling in colorectal cancer cell lines via inducible expression of dominant negative TCF4 (dnTCF4) revealed a list of potential direct Wnt/ß-catenin target genes[56]. Following validation of these target genes in normal murine intestinal tissue and human intestinal tumor samples, the target genes were shortlisted and classified into three categories – (i) genes associated with ISCs (e.g. Lgr5, Ascl2, Troy), (ii) genes associated with proliferating stem and transit amplifying progenitors (e.g. Axin2, c-Myc, CD44, Cyclin D1), and (iii) genes expressed in differentiated Paneth cells (e.g. MMP7, EhpB3)[12,57]. The ISC marker genes were subsequently confirmed by an independent study that profiled transcriptomes and proteomes of $Lgr5^{+}$ ISCs[58]. From there, Lgr5, EphB3 and

Achaete-scute complex homolog 2 (Ascl2, discussed on Page 17) have emerged as the most prominent and widely used ISC markers.

Lgr5 is expressed by rapidly cycling CBC cells in the intestines, the candidate intestinal stem cells proposed by Cheng and Leblond in 1974[59], as well as colonic crypt base cells. *In vivo* lineage tracing using the Lgr5-EGFP-ires-CreERT2 knockin mouse showed that these Lgr5+ cells in small intestine and colon can continuously give rise to all differentiated lineages over a mouse's lifespan, and generate long-term organoids *in vitro*[57,60]. Hence, $Lgr5^+$ cells are the *bona fide* adult intestinal stem cells during homeostasis. The use of Lgr5 as a marker of homeostatic and facultative adult stem cell marker was subsequently extended to a plethora of tissues, some of which will be reviewed in later sections.

Ephrin signaling governs cell positioning along the crypt-villus axis by imposing an upward migratory force from the crypt base in order to maintain discrete compartments within the intestinal architecture. Wnt/ß-catenin signaling inversely regulates the expression of the EphB receptors and their ephrinB ligands[42,56]. The B-subfamily of Ephrins comprises the receptors EphB2 and EphB3, and their ligands ephrinB-1 and ephrinB-2. EphB3 expression is enriched in $Lgr5^+$ CBCs, whilst EphB2 is expressed throughout the proliferative compartment of the crypt base, with the exception of terminally differentiated Paneth cells[42]. The ephrinB-1 and ephrinB-2 ligands are highly expressed at the crypt-villus junction and in the lower third of the villus. Deletion of the EphB2 and EphB3 receptors leads to mislocalization of the Paneth cells along the crypt-villus axis, and erroneous intermingling of cells in the crypts but is not lethal. Furthermore, $EPHB2^{Hi}$ human colonic stem cells can be

isolated using antibodies against EPHB2, and propagated *in vitro* long term while retaining their multi-lineage potential[61], presenting opportunities for downstream research, diagnostic and therapeutic applications.

## Use of ISC marker Lgr5 reveals ISC dynamics and properties

Following the identification of Lgr5 as an ISC marker, questions about ISC dynamics and properties have been widely studied. For instance, are these ISCs required during homeostasis and regeneration? How does this population reconcile with the other purported ISC population at the $^{+}4$ position? What are the population dynamics of the $Lgr5^{+}$ ISCs? What are the constituents of the ISC niche? The resulting collection of studies stemming from the discovery of $Lgr5^{Hi}$ ISCs demonstrate the utility of a robust marker in understanding stem cell biology.

*In vivo* ablation of endogenous $Lgr5^{+}$ stem cells using Lgr5-DTR-EGFP mice has been used to study the role of $Lg5^{+}$ stem cells during homeostasis and regeneration in adult tissues. In this mouse model, $Lgr5^{+}$ ISCs selectively express Diphtheria toxin receptor (DTR) which renders them susceptible to Diphtheria toxin[62]. Surprisingly, intestinal $Lgr5^{+}$ CBCs were found to be dispensable during homeostasis[62]. Also, damage induced by a near lethal dose of irradiation (12Gy) in wildtype intestines permitted regeneration despite transient loss of $Lgr5^{+}$ ISCs[53]. These indicate the presence of an alternative stem cell population, and/or plasticity within the crypt cells to rapidly replace the lost $Lgr5^{+}$ ISCs.

The alternative stem cell hypothesis was supported by the ability of a purported Lgr5-independent, $Bmi1^{+}$ population to regenerate all intestinal epithelial cells

including the Lgr5$^+$ ISCs[62]. Bmi1[63], along with Hopx[64], Lrig1[65] and Tert[66], are proposed markers of a slower-cycling ISC population at the +4 position (the fourth cell from the crypt base). Potten and colleagues had originally proposed crypt cells at the +4 position as ISCs due to their radiosensitivity and label retention property[67,68]. Contrary to more recent studies, Potten and colleagues showed that +4 cells are actively cycling but demonstrate label retention because of asymmetric DNA strand segregation during division[69].

It is ambiguous whether the +4 markers indeed label an Lgr5-independent ISC population. Expression of the +4 markers are not confined to the +4 position, but found throughout the crypts[58,70]. In fact, their expression patterns overlap with Lgr5's by transcriptome analysis and multiplex in situ hybridization[58,70]. Additionally, single cell qRT-PCR of label-retaining cells (LRCs, occupying positions +3 to +6) ascertained their overlap with Lgr5$^+$ ISCs[71]. Thus, it is possible that tracing using these +4 markers was initiated from Lgr5$^+$ ISCs co-expressing these markers. In fact, the relatively short timeline of the emergence of crypt-to-villus tracing units for Bmi1[63] and Lrig1[65] is similar to that of Lgr5$^+$ ISCs and contrasts with the behavior expected from a quiescent nature (short term tracing dynamics were not shown for Hopx[64] and Tert[66]). Therefore, it is unclear whether these +4 markers truly label a stem cell population distinct from the Lgr5$^+$ ISCs.

The rapid replacement of Lgr5$^+$ ISCs upon ablation could also be explained by inherent plasticity of cell populations residing within the crypts. Tetteh et al found that differentiated enterocytes can be induced to dedifferentiate upon loss of Lgr5$^+$ cells to restore the ISC pool *in vivo*[72]. Furthermore, Hopx$^+$ cells can give rise to Lgr5$^+$

CBCs and vice versa[64]. Using an inducible H2B-YFP mouse, Buczacki et al[71] labeled a population of LRCs that overlaps with the Dll1$^+$ cells identified by van Es et al[73]. During homeostasis, both populations contain secretory precursors and do not generate multi-lineage clones. However, epithelial injury induced by a sublethal dose of irradiation (6Gy) activates the stem potential of LRCs and Dll1$^+$ precursors to re-populate the small intestine with all known differentiated lineages. Since Dll1$^+$ cells originate from Lgr5$^+$ ISCs, plasticity built within the crypts serves to maintain intestinal survival during times of assault[73]. This suggests that ISC identity is determined by its location in the crypt, i.e. the ISC niche. Strikingly, although wildtype intestines can recover from a near lethal dose of irradiation (10-14Gy), intestinal regeneration is severely impeded if Lgr5$^+$ cells were ablated prior to irradiation[74]. This suggests that following sub-lethal damage events, endogenous Lgr5$^+$ ISCs are still necessary for complete and efficient regeneration of the intestine.

The identification of Lgr5$^+$ ISCs also facilitated a detailed investigation of their endogenous population dynamics using the reporter Confetti, which is a stochastic multicolor reporter for clonal output analysis[18]. Lgr5$^+$ ISCs initially formed multi-colored crypt bases which gave way to single colored crypts, indicating that crypts drift towards clonality over time[18]. Mathematical modeling of the clonal dynamics indicate that the ISCs divide symmetrically, and engage in neutral competition in a stochastic manner, a behavior subsequently ascertained in human colon samples[18,75]. Such clonal dynamics indicate that Lgr5$^+$ ISCs are functionally equivalent, in agreement with results from single cell transcriptome analysis of Lgr5$^{Hi}$ cells[76]. *In vivo* live imaging of Lgr5$^+$ cells and their progeny reveal a more complex picture – while the Lgr5$^+$ cells may be intrinsically equivalent, their fates are

determined by their positions in the crypt. Specifically, Lgr5$^{+}$ ISCs positioned at the bottom of the crypt (positions 0 to +2) are more likely to generate progeny that will remain as stem cells at the base while those along the side of the crypts (positions +3 and +4) are biased towards leaving the ISC niche and differentiating[77]. However, the positions the cells occupy are not fixed due to constant transfer between these two groups. Therefore, Lgr5$^{+}$ cells are functionally equivalent to one another. This underscores that stemness and longevity of the ISC are determined by the position it occupies, rather than its intrinsic properties.

It has been postulated that niche space is a major defining factor for ISC population size as ISCs compete for niche space to maintain their stemness. The *Lgr5*$^{+}$ ISCs are nested between Paneth cells, descendants of the ISCs, forming a regular geometric pattern such that every ISC contacts a Paneth cell[18,57,78] (Figure 3). Paneth cells secrete Wnt3, 6 and 9b, acting as a source of Wnt ligands for the adjacent ISCs[78-80]. ISCs directly sequester Wnt ligands from adjacent Paneth cells via direct binding to Fzd receptors rather than relying primarily on intercellular diffusion of Wnt ligands[81]. Since Fzd expression is negatively regulated by Rnf43/Znrf3[14], this suggests that availability of Fzd receptor would be the rate limiting factor for Wnt/ß-catenin signaling activity. The main Fzd receptor in the ISCs is Fzd7 as it is the most highly expressed Fzd receptor and knocking it out in ISCs causes ISC loss and impairs regeneration[82]. In the colon where Paneth cells are lacking, Reg4$^{+}$ deep crypt secretory cells serve as the stem cell niche and are required for colon stem cell survival[83].

However, ablating Paneth cells[84,85] or removing epithelial sources of Wnt via Villin-Cre-mediated deletion of Porcn[86] did not impair stem cell-driven epithelial homeostasis or injury in the intestine, indicating a non-epithelial contribution to the ISC niche *in vivo*. While Porcn-deficient intestinal crypts could not establish themselves in culture, co-culturing them with stromal myofibroblasts supported organoid growth in the absence of exogenous R-spondin1[86]. These myofibroblasts are marked by Gli1 expression, and highly express Wnt2b, R-spondin2 and R-spondin3, enabling them to support the growth of Porcn or Wls-knockout organoids *in vitro*[12,58,62,70,86-89] (Figure 3). However, concomitantly deleting Porcn from epithelial cells and myofibroblasts did not yield a phenotype[88]. In contrast, systemic deletion of Wls using Rosa26-CreERT2 caused depletion of crypts and ISCs[87], and systemic inhibition of Porcn using a pharmacological small molecule C59 during homeostasis and post-injury caused crypt loss or atrophy together with villi blunting[86]. Thus, additional source(s) of Wnts contributing to the ISC niche remain to be elucidated. Possible candidates include endothelial cells, lymphocytes, and nerve cells. Furthermore, it would be interesting to determine if the constituents of the ISC niche are functionally equivalent, or if various niche constituents function in specific contexts (e.g. homeostasis or injury).

## Wnt/ß-catenin signaling regulates multiple aspects of ISC behavior

During homeostasis, Wnt/ß-catenin plays multiple roles to ensure a healthy population of ISCs for efficient tissue renewal throughout the organism's lifetime. The major functions of Wnt/ß-catenin signaling can be summarized as (i) maintaining stemness, (ii) inducing proliferation of stem cells, and (iii) preserving genomic integrity.

First, high levels of Wnt/ß-catenin signaling reinforce the stem cell state through induction of its target gene Ascl2[12,90], which has been proposed as the master regulator of ISC identity. This sets up a positive feedback loop as Ascl2, Tcf4 and ß-catenin act synergistically to effect transcription of the Wnt program[90,91]. Forced expression of Ascl2 resulted in increased proliferation, increased c-Myc expression and expansion of crypt structures and the ISC compartment as marked by $Lgr5^+$ and $Olfm4^+$ (a commonly used ISC marker)[92]. However, Ascl2 is not an oncogene as its overexpression in a $Apc^{Min}$ cancer-predisposed background (described on page 22) does not accelerate tumorigenesis[93]. Conversely, deletion of Ascl2 reduced ISC population size, although there was no notable phenotype due to 'escaper' wildtype crypts[92]. Forced expression of Ascl2 rescues Tcf4 knockout organoids from lethality, underscoring Ascl2's role as an effector to induce expression of vital stem cell-associated genes to promote stemness. The positive feedback loop between Wnt/ß-catenin and Ascl2 expression ensures that Ascl2-mediated regulation of ISC identity is confined to the crypt base, where Wnt/ß-catenin signaling is strongest.

The Ascl2/Tcf/Lef complex competes with the Smad/Hdac complex activated by Bone morphogenetic protein (BMP) signaling for occupancy of promoters of ISC marker genes like Lgr5 and Ascl2[94]. BMP signaling represses Wnt/ß-catenin signaling in the crypts and consequently blocks ISC renewal, as evidenced by the fact that BMP receptor 1a (Bmpra1) null mutant crypts exhibit expansion of the $Lgr5^+/Olfm4^+$ stem compartment and epithelial hyperproliferation that manifested as polyps[94,95].Therefore, BMP signaling counterbalances Wnt/ß-catenin-derived forces

promoting stemness and proliferation in the crypt, thereby maintaining normal tissue architecture.

As mentioned, the high levels of Wnt/ß-catenin signaling at the crypt base are critical for ISC maintenance. Wnt/ß-catenin signaling renders ISCs responsive to R-spondin/Lgr5 signaling by providing Lgr5 receptors on the cell surface, enabling them to respond to available R-spondin ligands, which in turn further augments Wnt/ß-catenin signaling[96]. Furthermore, concomitant deletion of Lgr4 and Lgr5 caused loss of crypts and ISCs, eventually leading to mortality[15]. Hence, potentiation of Wnt/ß-catenin signaling by R-spondin/Lgr signaling axis is indispensable for ISC self-renewal and ensures Wnt/ß-catenin signaling is the highest at the crypt base.

Second, Wnt/ß-catenin signaling induces proliferation of ISCs and the transit amplifying compartment through its mitogenic effects exerted via downstream target genes CyclinD1 and c-Myc. c-Myc is a transcriptional repressor of cell cycle inhibitors p21 and p27[56,97,98], while CyclinD1 promotes G1 entry and progression by binding to Cyclin-dependent kinases 3/4/6[56,63,65,99-102]. Dysregulation of upstream regulators of CyclinD1 and c-Myc such as Apc results in their overexpression, leading eventually to cancer formation[56]. In contrast, c-Myc-deficient crypts are smaller and proliferate more slowly, indicating that Wnt/ß-catenin signaling is involved in controlling ISC proliferation[103]. CyclinD1, Cyclin E1 and c-Myc are G1/S regulators, which can be phosphorylated by GSK-3ß for degradation[101,102,104]. Since Wnt/ß-catenin signaling inhibits GSK-3ß activity, activating the pathway also blocks proteosomal degradation of these G1/S regulators, resulting in their accumulation. Collectively, Wnt pathway activity delivers higher expression and increased stability

of the G1/S regulators to promote cell cycle progression in the crypt. While Wnt/ß-catenin signaling has also been shown to play a role in mitosis, it has yet to be demonstrated in the intestine[100].

Third, Wnt/ß-catenin signaling is involved in telomere maintenance to preserve integrity of the genetic information, which is especially important given that ISCs cycle every 24 hours[57,105]. In the intestine, ß-catenin and Tert regulate each other's expression reciprocally. ß-catenin binds to the Tert promoter in $Lgr5^{+}$ ISCs from normal mouse small intestine[106]. In turn, Tert interacts with Tcf-binding elements of promoters of Wnt/ß-catenin target genes including Axin2, CyclinD1 and c-Myc in whole intestinal lysates, thus serving as a transcriptional effector of the Wnt/ß-catenin signaling pathway and establishing a positive feedback loop[107]. Although the ISCs or crypts were not specifically isolated for the latter study, the $Lgr5^{Hi}$ compartment was found to have the highest Tert expression, telomerase activity and telomere length[108]. Therefore, through interaction with Wnt/ß-catenin signaling, Tert is likely acting on the cycling ISCs to protect genome integrity and preserve normal function of the intestines (reviewed in Günes and Rudolph[109]).

## ISCs can be isolated and maintained *in vitro* as organoids in the presence of Wnt and R-spondin

The discovery of Lgr5 as an adult intestinal stem cell marker and the availability of the Lgr5-EGFP-ires-CreERT2 reporter mouse model facilitated the development of a new primary culture technique – organoid culture[60]. $Lgr5^{Hi}$ cells from the reporter mouse intestine could be distinguished from the $Lgr5^{Low}$ and $Lgr5^{Neg}$ counterparts by FACS, and isolated for *in vitro* culture. The cells are grown in Matrigel, and

supplemented with media containing growth factors such as Wnt3a, R-spondin1, Estrogen Growth Factor (EGF), Fibroblast Growth Factor 10 (Fgf10), Noggin and Y27632 (ROCK inhibitor). The fact that Wnt3a and R-spondin1 are obligate components of the medium to maintain ISCs in organoids underscores the importance of R-spondin/Lgr-potentiated Wnt/ß-catenin signaling in the intestines.

Single $Lgr5^{Hi}$ cells can give rise to self-renewing three-dimensional budding structures that contained crypt-villus regions along with a central lumen akin to the native tissue[60]. The buds of the organoids contain the $Lgr5^{+}$ stem cells intermingled with Paneth cells, while the regions between the buds contain the differentiated lineages such as enterocytes, enteroendocrine cells and goblet cells. These differentiated cells would be continually extruded into the lumen of the organoids, which necessitated passaging of the organoids by breaking them up and re-seeding them.

The organoids can be propagated long term without the chromosomal abnormalities commonly observed in immortalized tissue culture cell lines, making them a near-physiological *in vivo* system for studying stem cells without stromal contribution. Furthermore, normal and cancerous intestinal tissue from humans can be maintained as organoids in culture, with the addition of nicotinamide, A83-01 and SB202190[110,111]. Due to the technique's tractability and utility in studying stem cell populations in a reductionist setting, organoid culture has since been extended to a wide array of tissues from both mouse and humans for assaying the stem potential of putative stem cell populations, with the core growth factors retained across the

systems, particularly those required for maintaining potentiated Wnt/ß-catenin signaling (reviewed in Fatehullah et al[112]).

## ISCs are a cell-of-origin of intestinal cancer driven by aberrant Wnt/ß-catenin signaling

Wnt/ß-catenin signaling was one of the earliest pathways to be associated with cancer, in particular with colorectal cancer (CRC) where aberrant Wnt/ß-catenin signaling is frequently the initiating event. Additional mutations in Kras, Tgf-ß, PI3-Kinase and p53 signaling pathways promoted colorectal tumorigenesis and carcinogenesis. From this, the notion that CRC progression followed a sequential accumulation of known genetic mutations was conceived and extended to other cancers[113]. The large proportion of human CRCs that harbor mutations in Wnt/ß-catenin pathway underscores the requirement for hyperactive Wnt/ß-catenin signaling, which is likely acts on the colorectal CSCs.

The tumor suppressor gene APC is the most commonly mutated component of the Wnt/ß-catenin signaling in CRC, accounting for up to 85% of sporadic CRCs[114]. These mutations can be found in sporadic CRC tumors and in the germlines of patients with the hereditary cancer syndrome Familial Adenomatous Polyposis (FAP)[115,116]. These mutations tend to truncate the gene to yield null, hypomorphic or dominant negative alleles of APC, which result in a defective ß-catenin destruction complex. As ß-catenin molecules accumulate and translocate to the nucleus, Wnt/ß-catenin signaling becomes hyperactivated[117,118]. FAP patients inherit one mutated APC allele, and subsequent loss of heterozygosity at this gene locus inevitably leads to a significant load of adenomatous polyps in the colon by mid-teens, some of which

eventually progress to malignant adenocarcinoma. In most sporadic CRCs, both APC alleles are mutated[119]. Such mutations in APC, either inherited or acquired, tend to be one of the earliest events in CRC tumorigenesis[120]. The APC Multiple intestinal neoplasia (Min) allele was the first to be modeled in mice[121]. However, the $APC^{Min}$ mice developed lesions predominantly in the small intestines, in contrast to human FAP in which the polyps are found in the colon. Mouse models bearing other hypomorphic versions of Apc also present intestinal lesions with varying severity which corresponds to the degree of hypomorphism of the Apc mutant allele [122-127].

The oncogenic impact of uncurtailed Wnt/ß-catenin signaling was unequivocally demonstrated in humans and mouse models with dysregulated Wnt/ß-catenin signaling. Upon systemic deletion of Apc in adult mice, the intestinal architecture was grossly altered with overproliferation of crypt-like progenitors, causing mortality within five days of induction[49]. Similarly, loss of AXIN2, a Wnt target gene and negative feedback inhibitor of Wnt/ß-catenin signaling, is also commonly found in human CRCs[128]. Besides loss of pathway inhibitors like Apc and Axin2, CRC can be initiated via mutations in exon 3 of the ß-catenin gene (Ctnnb1) which prevent ß-catenin degradation even in the absence of Wnt ligands. The resulting accumulation of ß-catenin promotes constitutive formation of Tcf4/ß-catenin complex and hyperactivation of the Wnt/ß-catenin target gene program[56]. Impressively, re-expressing Apc in CRCs promotes tumor regression and restores homeostatic crypt behaviour despite the presence of oncogenic Kras and absence of p53, indicating that regulation of Wnt/ß-catenin signaling is central to CRC therapy[129].

Mutations in the R-spondin/Lgr signaling axis are also found in CRC. Recurrent gene fusions with R-spondin 2 (RSPO2) and R-spondin 3 (RSPO3) respectively accounted for 3% and 8% of the CRCs sampled in the Seshagiri et al study[130]. In particular, RSPO3 was frequently found to be fused in frame with the Protein tyrosine phosphatase, receptor type K (PTPRK) gene. Tumors with RSPO2 and RSPO3 fusions express higher RSPO and LGR4, LGR5 and LGR6 than tumors without the RSPO fusions. Moreover, these RSPO2 and RSPO3 fusions are sufficient for activating and amplifying Wnt/ß-catenin signaling *in vitro*, indicating escalated levels of Wnt/ß-catenin signaling in these tumors. Interestingly, targeting patient-derived xenograft samples that harbor RSPO3 gene fusions with anti-RSPO3 antibody led to stasis or regression, indicating that RSPO3 is a key driver of these colorectal tumor growths[131]. While AXIN2, MYC and CyclinD1 were only slightly downregulated with anti-RSPO3 treatment, LGR5 and ASCL2, both markers of ISCs, were among the top 5 most downregulated genes. Cellular differentiation markers were also significantly downregulated, indicating that the anti-RSPO3 treatment significantly reduces Wnt/ß-catenin signaling levels and diminishes stem-like properties in the tumor. Similarly, loss-of-function mutations in RNF43 have been found in 18% of human CRCs[132], but ZNRF3 mutations in cancer are less common[13]. Simultaneous knockout of Rnf43 and Znrf3, which increases availability of Frizzled receptors at the cell surface, induces hyperproliferation and intestinal metaplasia[133]. As expected, these Wnt ligand-dependent adenomas are responsive to C59 treatment, which inhibits Porcn activity[133].

Colorectal tumors capitalize on the homeostatic functions of Wnt/ß-catenin signaling to advance tumorigenesis. Inhibition of Wnt/ß-catenin signaling in CRC cell lines by

expressing the dnTCF4 led to cell cycle arrest and induced differentiation[56], outcomes similar to *in vivo* inhibition of ß-catenin in xenografts of human CRC tumors[134]. Approximately 70% of human CRCs were found to over-express MYC[135], a prominent oncogene and a Wnt/ß-catenin target gene which prevents cell cycle arrest[56,97,98]. Deletion of c-Myc in *Apc*-null background prevented tumorigenesis despite the presence of high nuclear ß-catenin[136]. Furthermore, loss of a single allele of c-Myc is sufficient to slow down tumorigenesis, although the cancer still progressed[137]. Hence, c-Myc is a key effector of hyperactive Wnt/ß-catenin signaling that drives hyperproliferation, apoptosis and aberrant cell migration in colorectal tumorigenesis.

Additionally, Wnt/ß-catenin signaling could protect genomic integrity of colorectal tumors. Similar to observations during homeostasis, ß-catenin binds to the Tert promoter in the hyperproliferative cells of the Villin-CreERT2;ß-catenin$^{(_{\Delta}ex3)fl/+}$ intestinal epithelium, which will form adenomas[106]. In the intestinal adenomatous lesions of these mice, it is possible that the resulting Wnt-driven increase in Tert expression endows the tumor, including its resident CSC population, with the ability to preserve its telomeres as an essential part of its long-term strategy to ensure survival and growth[138,139].

Wnt/ß-catenin signaling in CRC can also be enhanced by oncogenic Kras signaling, with its oncogenic form Kras$^{G12V}$ found in 50% of CRCs[114,140]. Oncogenic effects of Apc inactivation (Apc$^{1638N}$) are exacerbated by oncogenic Kras$^{G12V}$ which phosphorylates E-cadherin-bound ß-catenin, releasing it from the adherens junction into the cytoplasm[141]. The resulting accumulation of nuclear ß-catenin drives

expression of Wnt target genes, leading to malignant intestinal adenomas and carcinomas in mice. In CRC cell lines *in vitro*, oncogenic Ras has also been found to increase Wnt/ß-catenin signaling by phosphorylating LRP6 through the Ras-activated MEK pathway. Phosphorylation of LRP6 then activates ß-catenin/TCF4 transcriptional activity[142]. Therefore, oncogenic Kras signaling synergizes with Wnt/ß-catenin signaling to drive the tumorigenesis program.

Given the emerging plasticity of the intestinal epithelium, are all intestinal cells poised to serve as CRC cell-of-origin upon hyperactivation of Wnt/ß-catenin signaling? Or are stem cells the prime tumor-initiating candidates for accumulating the oncogenic mutations due to their longevity? In support of the latter hypothesis, knocking out *APC* in murine Lgr5$^{+}$ ISCs rapidly generated a lethal burden of intestinal adenomas with high nuclear ß-catenin, Ki67 and c-Myc within 36 days[143]. Similarly, a single allele of stabilized mutant ß-catenin (ß-catenin$^{\Delta ex3}$) in Lgr5$^{+}$ ISCs is sufficient to induce small intestinal adenomas[144]. These tumor-initiating effects can be enhanced by haploinsufficiency for E-cadherin as fewer E-cadherin molecules are present in the membrane to act as sinks for ß-catenin[144]. In contrast, differentiated cells cannot form tumors as mutation of Apc alone in the differentiated compartment of the intestine is insufficient for tumorigenesis. However, differentiated cells can be reprogrammed to a more stem cell-like state by certain oncogenic events or changes to the local environment such as colitis-induced inflammation[145], increased NFKB signaling[146], or reduced BMP signaling[147]. As a result, these differentiated cells acquire stem cell markers and subsequently drive tumor formation.

Existing CRC tumors are thought to rely on resident CSCs to fuel tumor growth. In the heterogeneous colorectal tumor, cells with highest Wnt/ß-catenin signaling activity mark CSCs as they have the highest clonogenic and tumorigenic capabilities. $Wnt^{Hi}$ human colorectal tumor cells and $LGR5^{Hi}$ cells from organoids derived from primary human colorectal tumors generate tumors in xenografts efficiently[148,149]. Their self-renewal ability was also demonstrated in serially-transplanted xenografts[148] and in *in vitro* organoids respectively[149]. Transcriptome profiling of single cells from human colorectal tumors confirmed upregulation of Wnt signaling pathway components in the $LGR5^{+}$ stem/progenitor subpopulations[150]. Additionally, $Lgr5^{+}$ cells in murine colorectal adenomas actively contribute to adenoma growth *in vivo*[151]. While $Lgr5^{+}$ CSCs are necessary for tumor growth and metastasis, they are dispensable for tumor maintenance[152]. Hence, colorectal CSCs that are responsible for fueling tumor growth are characterized by high Wnt/ß-catenin signaling and Lgr5 expression.

Similar to the spatial arrangement in the homeostatic ISC niche, tumor-resident $Lgr5^{+}$ CSCs are intermingled with Paneth-like cells, reminiscent of the arrangement in the normal crypt[151], suggesting that Paneth cells also form an essential component of the CSC niche. Also, while it is unclear if tumor myofibroblasts secreted Wnt ligands, they appear to support the CSCs via secretion of Hepatocyte growth factor (Hgf), which could increase ß-catenin activity through c-Met signaling[148]. Co-transplantation of $Wnt^{Low}$ tumor cells lacking intrinsic tumorigenic potential with tumor myofibroblasts re-programmed the $Wnt^{Low}$ tumor cells to serve as tumor initiating cells tumor following transplantation[148], highlighting the instructive nature of the microenvironment in helping cells acquire stem cell-like characteristics.

Currently, clinical studies of several promising therapeutic strategies are underway. CRCs with RSPO translocations can be targeted by the anti-RSPO3 antibody mentioned earlier (designated OMP131R10) which is currently in Phase 1 clinical trial (trial identifier NCT02482441), and Porcn inhibitors that deprive the tumors of functional Wnt ligands. Existing small molecule PORCN inhibitors C59, ETC-159 and LGK974 (or WNT974) have shown efficacy in reducing proliferation and stem cell marker expression of patient-derived xenografts of colorectal tumors[30,32,35]. Murine intestinal tumors harboring null mutations in both Rnf43 and Znrf3 are also responsive to C59[133]. Encouragingly, these small molecules appear to be effective across multiple cancers. ETC-159 is currently in Phase 1 clinical trial (trial identifier NCT02521844), while LGK974 is currently in Phase 2 (trial identifier NCT02649530). However, both RSPO3 and PORCN-targeted therapeutic approaches will only work on CRCs that rely on paracrine Wnt for growth, in stark contrast to *Apc*-deficient adenomas that are Wnt ligand-independent. One promising therapeutic agent for this class of CRC tumors could be RPI-724, a small molecule that disrupts the binding of ß-catenin and its activator, thereby inhibiting Wnt/ß-catenin signaling-driven transcription[153]. It was shown to be effective in human CRC cell lines, $Apc^{Min}$ mice and xenografts[153], and is currently in Phase 2 clinical trial in combination with bevacizumab in patients with metastatic CRC (trial identifier NCT02413853). Furthermore, additional therapeutic strategies would be required to prevent relapse of the cancer as ablating $Wnt^{Hi}$ CSCs in murine colorectal adenomas does not obliterate the tumors[152], potentially due to reprogramming of other tumor cells by the oncogenic niche. Hence, further investigation of the Wnt-promoting tumor

microenvironment will help to develop a therapeutic strategy to complement current tumor-targeted treatments.

*Stomach*

The stomach is the main site where ingested food is digested into chime, through the actions of the suite of enzymes and acid produced by specialized epithelial cells in the stomach lining. The chime is then released into the intestines where the nutrients can be absorbed. The mouse stomach comprises two distinct parts: the proximal non-glandular part, and the distal glandular part. The glandular part is further subdivided into corpus (proximal) and pylorus (distal) (Figure 4). In contrast, the human stomach lacks the non-glandular part, and has counterparts to the murine corpus and pylorus.

In contrast to the intestines, Wnt/ß-catenin signaling-related studies on the homeostatic corpus and pylorus thus far have mostly been limited to Lgr5 (and Troy in the corpus), with a lack of mechanistic studies. Hence, this section will mainly discuss the current findings about the corpus and pyloric stem cells in relation to Lgr5, and Wnt/ß-catenin signaling in gastric cancer.

[Insert Figure 4 here]

Figure 4: Gastric stem cell in the corpus and pylorus. (A) Schematic of the murine stomach. (B) Facultative stem cells of the corpus. The chief cells situated at the base of the corpus glands express Lgr5 and do not contribute to regular homeostasis of the corpus, as observed by paucity of RFP+ traced units after 6 months. However,

when oxyntic atrophy (loss of parietal cells) is induced by a high dose of tamoxifen, whole RFP+ glands comprising all differentiated lineages are observed. The initiation of oxyntic atrophy is marked by reduced Sostdc1 which indicates elevated Wnt/ß-catenin signaling. (B) Homeostatic stem cells of the pylorus. $Lgr5^{+}$ pyloric stem cells are found at the base of the glands, with the proliferative cells in the isthmus region. Only a small subset of $Lgr5^{-+}$ cells are proliferating. When lineage tracing is induced in $Lgr5^{+}$cells, whole RFP+ pyloric glands are observed after 10 days.

## Facultative stem cells of the corpus are Wnt-dependent

The corpus/body of the stomach secretes acid via parietal cells, and the epithelium is protected from the acid and other assaults by a mucous layer produced by the surface mucinous cells. In murine and human corpus, Lgr5 is expressed in basal chief cells, which normally function as sources of zymogens to aid digestion[154]. Troy, another Wnt/ß-catenin target gene, is also expressed in both chief and parietal cells in the corpus[155], marking a broader population than $Lgr5^{+}$ chief cells. Both $Lgr5^{+}$ and $Troy^{+}$ cells remain quiescent and contribute minimally to homeostasis[154,155]. Nonetheless, these cells are able to generate organoids *in vitro* with an obligate requirement for Wnt and R-spondin1, an indication of their Wnt-dependent stem potential[154,155].

However, $Lgr5^{+}$ and $Troy^{+}$ cells can be activated by ablation of parietal cells or proliferating cells respectively, and subsequently produce all differentiated lineages of the gastric epithelium over time[154,155]. Interestingly, during early regeneration following parietal cell loss, Wnt signaling is transiently hyperactivated via downregulation of the extracellular Wnt inhibitor Sostdc1. Soon after, $Lgr5^{+}$ chief

cells start to proliferate and display stem cell properties[154]. It is tempting to hypothesize that during homeostasis, Sostdc1 dampens Wnt/ß-catenin signaling to a basal level to ensure quiescence of the $Lgr5^+$ cells while retaining their stem potential. If this is true, this would mean that differential levels of Wnt/ß-catenin signaling dictate distinct outcomes, indicating an intricately calibrated mechanism.

Since the $Lgr5^+$ and $Troy^+$ cells are not daily stem cells, the day-to-day maintenance of the corpus epithelium must be sustained by another population, possibly the proliferating cells of the isthmus. While Lgr5 is not expressed by the isthmus cells, it has been shown that Wnt5a is expressed in the stromal cells adjacent to the isthmus epithelial cells[156], suggesting the involvement of Wnt5a-mediated signaling in the isthmus region. However, Wnt5a is likely to be acting through a non-ß-catenin-mediated pathway given its known association with the non-canonical Wnt pathways[157] and the absence of Wnt/ß-catenin target genes in the isthmus region. Other proposed markers of homeostatic corpus stem cells include Mist1[156], Sox2[158] and enhancer of Runx1[159]. However, as these markers do not label isthmus cells exclusively, whether isthmus cells are the stem cells of the homeostatic corpus remains inconclusive.

## Wnt-responding stem cells maintain pyloric epithelium during homeostasis

The pylorus secretes the hormone gastrin, among many other hormones and enzymes, which stimulates the parietals cells in the corpus to produce acid for digestion. The pylorus comprises gastric glands which harbor around eight $Lgr5^+$ $Axin2^+$ stem cells at each gland base and $Axin2^+$ $Lgr5^-$ proliferating progenitors in the

lower isthmus[160,161]. Axin2's expression pattern is reminiscent of Fzd7's, which is the most highly expressed Frizzled in the pylorus[162].

The Lgr5$^{+}$ stem cells divide symmetrically[160], undergo neutral drift[160], and fuel the turnover of the pyloric epithelium every 7-10 days [37]. Single Lgr5$^{Hi}$ cells generate long-term organoid cultures in the presence of Wnt and R-spondin1[37], indicating the importance of Wnt/ß-catenin signaling in pyloric stem cell maintenance. The requirement for recombinant R-spondin1 in culture media can be substituted with co-culturing pyloric organoids with stromal myofibroblasts[161]. *In vivo*, R-spondin3 secreted by stromal myofibroblasts promotes proliferation of Axin2$^{+}$ cells, and is required for normal expression of Axin2 and Lgr5[161]. Hence, Wnt-responding stem and progenitor cells of the pylorus are supported by a myofibroblast niche.

## Wnt/ß-catenin signaling and inflammation due to *Helicobacter pylori* infection drives gastric cancer originating from Lgr5$^{+}$ stem cells

The current working model of gastric carcinogenesis by Correa[163] begins with *Helicobacter pylori* (*H. pylori*)-induced inflammation which leads to chronic gastritis, followed by atrophy, intestinal metaplasia, dysplasia and eventually neoplasia. While environmental factors such as diet and smoking are known to correlate with progression in the Correa model, the underlying molecular mechanisms remain poorly understood.

In humans, *H. pylori* infection presents the highest risk factor for gastric cancer. Long-term infection of *H. pylori* leads to chronic gastric inflammation, and approximately 3% of *H. pylori*-infected individuals will later develop gastric cancer[164].

When the lower halves of pyloric glands are colonized by *H. pylori*, there is increased proliferation of $Lgr5^+$ and $Axin2^+$ stem and progenitor cells, driving hyperplasia and chronic inflammation[165]. The virulence factor responsible for driving $Lgr5^+$ stem cell proliferation was determined to be the contact-dependent virulence factor CagA, indicating the need for direct contact between *H. pylori* and the pyloric epithelium[165]. Stromal myofibroblasts, the homeostatic niche of pyloric stem cells, secrete more R-spondin3 upon *H. pylori* infection, thereby inducing higher proliferation in $Axin2^+$ cells[161], pointing to a direct effect of *H. pylori* infection on Wnt/ß-catenin signaling activity.

The Wnt/ß-catenin pathway is often hyperactivated in both intestinal and diffuse types of gastric cancer, as evidenced by the presence of nuclear ß-catenin[166]. This likely occurs as a result from mutations in Wnt pathway components such as APC, CTNNB1 and RNF43, which are commonly found in human gastric adenocarcinomas[167]. Human gastric cancer samples have elevated WLS expression compared to those from matched normal control gastric tissue[168]. This robust WLS expression was correlated with HER2 expression, increased proliferation and shorter progression-free survival rates, suggesting that Wnt/ß-catenin signaling may cooperate with HER2 in promoting tumor progression. Another factor that interacts with the Wnt/ß-catenin signaling machinery is the transcription factor Runx3. However, the actual role of Runx3 is unclear as it has been shown to play both activating[169] and repressing[170,171] roles in the modulation of Tcf4/ß-catenin complex activity.

However, hyperactivation of Wnt/ß-catenin alone only generates pre-neoplastic lesions in mice[166], indicating that additional 'hits' are required for gastric carcinogenesis. *Helicobacter felis*, a close relative of *H. pylori*, induces cyclooxygenase-2 (Cox-2) and microsomal prostaglandin E synthase-1 (mPGES-1) expression in the stomach[172]. These enzymes synthesize prostaglandins which are associated with inflammation. Cox-2 and mPGES-1 overexpression synergizes with Wnt1 overexpression to generate dysplastic gastric tumors in the Gan mouse model[166], highlighting the critical role of inflammation in driving the progression of gastric tumors displaying hyperactivated Wnt/ß-catenin signaling.

Wnt-responding gastric stem cells can act as the cell-of-origin of gastric cancer. In the murine corpus, the $Lgr5^{+}$ chief cells can serve as the origin of pseudopyloric metaplasia (SPEM) after targeted mutations of $Kras^{G12D}$ and APC/PTEN are exclusively activated in them[154]. Importantly, human corpus tumors express elevated levels of LGR5 and AXIN2, underscoring hyperactive Wnt/ß-catenin signaling as a feature of corpus tumors in humans. Similarly, the $Lgr5^{+}$ pyloric stem cells are able to initiate tumors in mice following targeted conditional deletion of the Apc tumor suppressor gene, resulting in polyps with hyperactive Wnt/ß-catenin signaling[37]. However, the broad action of the Lgr5-CreERT2 driver in multiple Lgr5+ stem cell compartments resulted in rapid lethality due to the resulting tumor burden in the intestines, precluding any progression of the pyloric tumors towards more advanced cancer with time. Subsequently, $Lgr5^{+}$ pyloric stem cells were formally shown to serve as a cell-of-origin of invasive pyloric tumors when concomitant targeted deletion of Smad4 and Pten tumor suppressor genes in the stem cells generated

pyloric adenocarcinomas after two months[173]. However, due to technical limitations, a metastatic model of gastric adenocarcinoma is still unavailable.

Many questions regarding stem cells in the homeostatic stomach and gastric cancer remain unsolved. In the corpus, what are the homeostatic stem cells? Are different levels of Wnt/ß-catenin signaling required to effect distinct homeostatic and regenerative outcomes? In the pylorus, how are the $Axin2^{+}Lgr5^{+}$ and $Axin2^{+}Lgr5^{-}$ populations maintained as distinct populations? Do they also experience differential Wnt regulation as well? Do Wnt/ß-catenin signaling and the prostaglandin synthesis pathway interact with each other or do they act independently on the tumor initiating population? The stomach presents an interesting opportunity to examine crosstalk between long-term inflammation and Wnt-driven cancer that can yield insight applicable to other tissues and cancers.

### 2) Skin stem cells and cancer

The skin is the largest organ of the body and acts as the first line of defense against pathogens and physical environmental assaults. Multiple ‘mini-organs’ are found in the skin, such as hair follicles, interfollicular epidermis (IFE) and appendages such as sweat and sebaceous glands. Each of these ‘mini-organs’ is maintained by a dedicated population of adult epidermal stem cells. We will examine Wnt/ß-catenin signaling in hair follicular stem cells (HFSCs), IFE stem cells and cutaneous cancers in greater detail.

*Hair follicles*

Hair follicles are embedded in the IFE and undergo three phases of hair cycle throughout life – anagen (growth), catagen (destruction) and telogen (rest). During anagen, HFSCs travel from the bulge along the outer root sheath to the matrix, where they proliferate to generate new cells that migrate upwards to form the growing hair follicle (Figure 5A). In catagen, HFSCs migrate along the retracting follicle to return to the bulge where they remain during telogen until the next wave of anagen arrives. The repeated cycles of HFSC activation and quiescence make the hair follicle an attractive model for studying stem cell regeneration.

[Insert Figure 5 here]

Figure 5: Skin stem cell in the hair follicle and inter follicular epidermis (IFE). (A) Hair follicular stem cell (HFSC). During the telogen (resting) phase, the outer bulge stem cells express Axin2 and Lgr5. The outer bulge HFSCs secrete their own Wnts to drive autocrine Wnt/ß-catenin signaling. The outer bulge HFSCs prevent the adjacent inner bulge cells from being receptive to Wnt pathway activation by secreting diffusible Dkk protein. During anagen, these HFSCs migrate from the outer bulge along the outer root sheath to enter the matrix, where they proliferate and differentiate to generate the hair shaft. (B) Interfollicular epidermal (IFE) stem cell. The IFE stem cell at the base express Axin2, and secrete their own Wnts to set up autocrine Wnt/ß-catenin signaling. The IFE stem cells also secrete Dkk proteins, which diffuse to the suprabasal layers and inhibit their response to the Wnt ligands in the environment, thereby restricting Wnt/ß-catenin signaling to the basal stem cells.

## Wnt/ß-catenin signaling induces anagen in cooperation with other signaling pathways

High levels of Wnt/ß-catenin signaling in late telogen phase have long been known to promote anagen entry, in part promoted by increased levels of R-spondins1-4 in this stage[174]. Constitutive Wnt/ß-catenin signaling in basal keratinocytes (which include HFSCs) from development caused *de novo* formation of hair follicles[175,176], and increased Wnt/ß-catenin signaling during telogen led to precocious anagen entry along with thicker and longer hair follicles[177]. Conversely, inhibiting Wnt/ß-catenin signaling from birth prevents hair follicle formation[178], and abrogating Wnt/ß-catenin signaling during anagen halts proliferation in the matrix (where differentiated cells of the hair shaft are produced)[179,180]. Similarly, ectopic Dkk1 expression or injection causes the hair follicles to enter catagen prematurely by inducing expression of pro-apoptotic protein Bax, making Dkk1 a candidate for the molecular mediator of male pattern baldness[180,181]. Surprisingly, sustained proliferation due to constitutive Wnt/ß-catenin signaling could also lead to premature hair loss due to stem cell exhaustion, as seen in the murine model in which Wnt1 is overexpressed in basal keratinocytes[182]. Therefore, Wnt/ß-catenin signaling during telogen induces hair follicles to enter anagen, and high levels of Wnt/ß-catenin signaling promote hair follicle growth.

The telogen-to-anagen transition is actually a combination of two distinct processes – the specification of the hair follicular fate in HFSCs, followed by the proliferation of the committed cells to generate the new hair follicle. As modulating either process would result in the same phenotype *in vivo*, it has been challenging to determine the exact stage that Wnt/ß-catenin signaling acts on. Through a combinatorial *in vivo*/*in*

*vitro* approach to uncouple the two processes, Lien et al. found that ß-catenin-knockout HFSCs retain proliferative capacity, but are unable to specify follicular fate upon grafting[183]. Additionally, plucking ß-catenin knockout follicles resulted in sebocyte differentiation instead of new follicle formation[183], reminiscent of the *in vivo* ß-catenin knockout phenotype[179]. Hence, Wnt/ß-catenin signaling is essential for specifying hair follicle fate and preventing sebocyte fate during the telogen-to-anagen transition.

Several Wnt ligands are expressed in the hair follicle during this transition - The bulge expresses Wnts 1, 4, 7b, whilst Wnts 6, 7b, 10a, 10b and R-spondins1-4 are upregulated at the junction of the secondary germ to dermal papilla[174,184,185] (Figure 5A). The Wnts and R-spondins signal to the proliferative matrix where Wnt/ß-catenin signaling is active. Most notably, genetic studies have revealed the functional relevance of Wnt10a and Wnt10b in the transitioning hair follicle. In agreement with the other studies augmenting Wnt/ß-catenin signaling, ectopically expressed Wnt10b increases proliferation at the derma papilla, matrix and hair shaft, resulting in larger hair follicles[186]. However, the Wnt10b null mouse does not have any hair follicle phenotype[187], possibly due to redundancy with other Wnt ligands such as Wnt10a. Wnt10a is associated with Odonto-onycho-dermal dysplasia, a rare autosomal recessive disease presenting palmoplantar hyperkeratosis (thickened skin on palms and soles) among a host of other symptoms[188]. Importantly, since Wnt10a and Wnt10b expression is not affected in the Shh null mutant[184], these Wnts likely regulate the expression of Sonic Hedgehog (Shh), which is a Wnt/ß-catenin target gene that initiates anagen. In turn, Shh, together with Notch signaling, regulates Wnt5a expression in the dermal papilla[184,189]. Without Wnt5a, cysts are generated in

place of hair follicles as its target gene *FoxN1*, a critical regulator of keratinocyte differentiation, is not expressed[189,190].

On the other hand, BMP signaling negatively regulates Wnt/ß-catenin signaling during telogen-to-anagen transition. BMPs, namely Bmp2 and Bmp4, are expressed by dermal neighbors comprising fibroblasts, adipocytes and dermal papillae. These dermis-derived BMP ligands promote BMP signaling in the HFSCs, thereby inhibiting Wnt/ß-catenin signaling and anagen entry[191-195]. However, before anagen initiates, the dermal cells will reduce BMP expression and the dermal papillae express the BMP antagonist Noggin. Knocking out Noggin prevents the activation of ß-catenin/Lef1-mediated transcription and anagen entry[196]. Hence, it is imperative to inhibit BMP signaling so that the HFSCs are able to respond to Wnt/ß-catenin signaling, which then initiates the transition to anagen. Once Wnt/ß-catenin signaling kicks off, a suite of ß-catenin/Lef1-mediated Wnt target genes such as *Shh*, *Jagged1* (Notch ligand), *Dlx3* (homeobox transcription factor), *FoxN1* and keratins are expressed, all of which are required for the proliferation and differentiation of the hair follicular cells during anagen[175,176,197-200].

## Active Wnt/ß-catenin signaling in HFSC during telogen maintains stemness

For a long time, Wnt/ß-catenin was thought to be inactive in the quiescent HFSCs during telogen as there is no proliferation in the bulge during this phase and Wnt/ß-catenin signaling has primarily been thought of as a mitogen. However, *in vivo* lineage tracing approaches employing Axin2 and Lgr5 Cre drivers have formally proven that telogen HFSCs respond to Wnt/ß-catenin signaling. Axin2 and Lgr5 are both expressed in the secondary hair germ during telogen and in the matrix and

outer root sheath during anagen. Lineage tracing with Axin2CreERT2 and Lgr5-EGFP-ires-CreERT2 show that Axin2$^+$ and Lgr5$^+$ cells are able to give rise to the differentiated lineages in the entire hair follicle over many hair cycles[185,201]. Furthermore, Lgr5-EGFP$^+$ cells can generate new hair follicles when transplanted onto the backs of nude mice, indicative of their stem and transplantation potential[201].

HFSCs produce their own Wnts to drive autocrine Wnt/ß-catenin signaling for maintenance of their stem cell identity, while restricting Wnt/ß-catenin signaling response in adjacent daughter cells by secreting Dkk3. During telogen, Wnts 1, 4, 7b are the most highly expressed Wnts ligands, which are maintained throughout the transition to anagen[185]. The epithelial origin of Wnts was confirmed when Wls was knocked out in basal keratinocytes and outer root sheath cells (including HFSCs), causing a termination of hair growth[180]. Inactivating ß-catenin specifically in Axin2$^+$ HFSCs during telogen in adult mice largely abrogates hair growth and reduces proliferation[180,185], similar to the phenotype observed with pan-basal keratinocyte deletion of ß-catenin during telogen[179]. Thus, Wnt-responding HFSCs require ß-catenin-mediated Wnt signaling for proliferation and proper hair follicle growth. However, it remains unclear whether Wnt/ß-catenin signaling maintains follicular fate during telogen as observed during the transition to anagen[183], and if it serves additional functions.

In contrast, another study proposed that during telogen, Wnt/ß-catenin target gene expression is repressed by the Tcf3/Tcf4/Groucho complex, thereby rendering Wnt/ß-catenin signaling inactive[183]. In the presence of Wnt ligands and nuclear ß-catenin, Tcf1/Lef1 activator complex replaces Tcf3/Tcf4/Groucho repressor complex

at the promoters of Wnt target genes, thereby effecting their transcription. As Tcf1 and Lef1 are Wnt/ß-catenin target genes, Wnt/ß-catenin signaling is engaged in a positive feedback loop that further enhances its activity. This study proposes that Wnt/ß-catenin signaling is only active upon telogen-anagen transition, when ß-catenin alleviates transcriptional repression to facilitate activation of follicular fate genes.

Despite the apparent contrast, the findings from the abovementioned studies could be reconciled by examining the relative levels of Wnt/ß-catenin signaling activity. It is conceivable that Wnt/ß-catenin signaling is present in the HFSCs from telogen through anagen to specify follicular fate and maintain stemness. However, similar to the corpus, it is present at a basal level during telogen, which can only be detected by sensitive reporters such as the Axin2 and Lgr5-Cre drivers[185,201]. Once the transition into anagen is initiated, positive feedback loops drive Wnt/ß-catenin signaling activity to reach the threshold needed to enter anagen and actively drive follicular fate to induce hair follicle growth. Hence, more studies are required to assess the fine tuning of Wnt/ß-catenin signaling in HFSC maintenance and activation.

### *Interfollicular epidermis*

The IFE refers to the epidermal layer without the hair follicles and other appendages. It is organized as multiple stratified squamous layers, with the innermost basal cells in contact with the basement membrane, separated from the dermis, and progresses upwards towards the increasingly keratinized layers of epidermal cells that forms the outermost layer of the skin.

## Wnt/ß-catenin signaling is required for IFE homeostasis

Several early studies ablating ß-catenin and Lef1 expression in the skin produced hair follicle phenotypes, but not IFE phenotypes[179,200,202], leading to the view that Wnt/ß-catenin signaling is not active in the IFE.

However, some evidence indicate that the IFE is indeed regulated by Wnt/ß-catenin signaling. Hyperactivating Wnt/ß-catenin signaling often results in thickened IFE. For instance, individuals harboring gain-of-function mutation in RSPO1 have palmoplantar hyperkeratosis (thickening of the epidermis on the palms and soles) and predisposition to squamous cell carcinoma of the skin[203]. Overexpression of stabilized ß-catenin mutant or Wnt1 in keratinocytes also results in a thickened IFE[175,176,182]. Additionally, primary human keratinocyte cultures harbouring a gain-of-function ß-catenin mutant also has higher colony formation efficiency[204].

In contrast, abrogating Wnt/ß-catenin signaling leads to thinner IFE. Knocking out Porcn (and consequently Wnt signaling) in epidermal cells resulted in thinner skin displaying extensive hair-deficient areas and focal dermal hyperplasia[205], indicating that epidermal cells are an important source of Wnts for skin and hair follicle morphogenesis. Notably, the murine mutants, like human patients with focal dermal hyperplasia, have thinner, more fragile skin and fewer hair follicles[205,206]. Knocking out Wls in basal keratinocytes reduces Wnt/ß-catenin signaling levels and also leads to skin thinning with associated diminished proliferation. Importantly, this phenotype can be rescued by transgenic expression of Bmp4, placing BMP signaling downstream of Wnt/ß-catenin signaling in the IFE[207]. Other Wnt signaling ablation

models, including the Tcf3/Tcf4 double knockout mouse model, also show thinner IFE and compromised self-renewal ability[179,208]. Surprisingly, constitutive loss of ß-catenin in keratinocytes during development leads to epidermal thickening accompanied by loss of hair follicles as developmental multipotent stem cells are directed towards the IFE fate than follicular fate[179], underscoring the complex and context-dependent roles of Wnt/ß-catenin signaling in the skin.

Wnt/ß-catenin signaling acts on IFE stem cells, which reside in the basal keratinocyte layer and express Axin2[209] (Figure 5B). When Wnt/ß-catenin signaling was inhibited in adult mice by conditional deletion of ß-catenin in Axin2$^{+}$ cells, the IFE was thinner and had fewer proliferating cells[209]. This suggests that Wnt/ß-catenin signaling maintains the basal stem cells by regulating their proliferation to support a healthy IFE, similar to conclusions from earlier studies with keratinocyte-specific drivers.

Clonal analysis with multicolor lineage tracing in the mouse epidermis from the palm, tail and ear using Axin2CreERT2 and other generic inducible Cre drivers showed that like ISCs, the basal stem cells are functionally equivalent. They maintain IFE homeostasis as a population by giving rise to equal numbers of stem and daughter cells during divisions, through a combination of symmetric and asymmetric divisions[209-211]. While it is unclear if Wnt/ß-catenin signaling is involved in the decision of division symmetry, a precedent has been set in the mouse embryonic stem cells, where a localized Wnt source is able to direct asymmetric division such that the daughter stem cell proximal to the Wnt source retains stem cell markers while the distal daughter cell tends to differentiate[212].

Interestingly, like the HFSCs, the Axin2$^+$ basal stem cells act as their own niche by producing their own Wnts – namely Wnt4 and Wnt10a[209]. To limit Wnt/ß-catenin signaling to the stem cells of the basal layer, the basal cells also secrete Dkk3, which diffuses to the suprabasal daughter cells to prevent them from responding to the Wnt ligands.

## Multipotent stem cells of the IFE, HF and SG in adulthood?

Lgr6 was initially reported to be selectively expressed in cells of the isthmus region, above the bulge where Lgr5$^+$ bulge stem cells reside. Lineage tracing analyses showed that these Lgr6$^+$ cells give rise to sebaceous glands, IFE and hair follicles, constituting a long-term stem cell population that contributes to multiple mini organs of the skin during homeostasis and regeneration over the organism's lifetime[213]. However, more recent studies observed additional Lgr6$^+$ populations in the IFE and sebaceous glands[214,215]. These distinct populations of Lgr6$^+$ cells were able to give rise to tracing units in their own respective compartments, exhibiting population asymmetry as previously shown in the skin and other organs[214]. Hence, Lgr6 marks three distinct Wnt-responding adult stem cell populations in the skin.

## Cutaneous cancers

The mot frequent cancer in the United States is skin/cutaneous cancer[216]. Of all skin cancers, basal cell carcinoma (BCC) is the most common form of cutaneous cancer. Hedgehog-driven BCCs display active Wnt/ß-catenin signaling and human BCCs exhibit nuclear ß-catenin localization[217-219]. Strikingly, ectopic expression of Dkk1 in early superficial BCCs blocked tumor formation[217], and deletion of ß-catenin in

Hedgehog-responding initiating cells prevented tumor formation[219]. Promoters of APC and SFRP5 are significantly more frequently methylated in BCCs, which is predicted to increase Wnt/ß-catenin signaling activity[220]. Another factor associated with BCC development is reduced Vitamin D receptor levels in the presence of high Wnt/ß-catenin signaling activity[221]. If Vitamin D receptor levels are high, trichofolliculomas (tumors of the sebaceous glands) will develop instead. This echoes the dichotomous cell fates seen in adult murine genetic studies in which Wnt/ß-catenin signaling is inhibited by the dominant negative mutant of Lef1[222], one of the commonly mutated genes in human sebaceous tumors[223]. Hence, Wnt/ß-catenin signaling is required for BCC initiation and progression. The pathway is most likely acting on HFSCs and IFE stem cells, both of which have shown to be potential candidates for the cell-of-origin of BCCs[219,224-226].

Squamous cell carcinoma (SCC) is the second most common form of cutaneous cancer. Murine models of cutaneous SCC can be induced chemically by dimethylbenz(a)anthracene/12-O-tetradecanoylphorbol-13-acetate (DMBA/TPA). The majority of these tumors demonstrate cytoplasmic and nuclear accumulation of ß-catenin, and express Wnt/ß-catenin target genes like c-Myc and c-Jun, indicating active Wnt/ß-catenin signaling in SCC[227]. Cytoplasmic and nuclear accumulation of ß-catenin is similarly observed in the tumor-propagating $CD34^+$ population and human SCC tumors[228]. Strikingly, these tumors regress upon keratinocyte-specific deletion of ß-catenin or ectopic expression of Tcf4, indicating that ectopic expression of Tcf4 reinforces its repressive role and constitutive Wnt/ß-catenin signaling is required for sustained tumor growth via the survival of CSCs. Likewise, promoters of SFRP1, SFRP2, SFRP3, SFRP4 and SFRP5, a class of Wnt inhibitors, are more

significantly methylated in SCC tumors than normal epidermis[229]. In K14-ΔNLef-1 mice in which Wnt/ß-catenin signaling in basal keratinocytes is inhibited, DMBA/TPA treatment readily generated sebaceous tumors which contain differentiated sebocytes[202], demonstrating the dichotomy between hair follicle and sebaceous fates. HFSCs and IFE progenitors have been proposed as the cell of origin for cutaneous SCC (reviewed in Blanpain[3]), both of which are Wnt-responding during homeostasis[185,201,209].

Hyperactive Wnt/ß-catenin signaling has been implicated in a subset of hair follicle tumors which are densely packed with cysts containing hair follicles, referred to as pilomatricomas (also known as trichomatricomas). Pilomatricomas are benign tumours derived from the hair follicular matrix that often display mutations in the ß-catenin gene and/or ß-catenin expression concentrated in basophilic cells within the tumor[230]. In mice, hyperactivating the Wnt/ß-catenin pathway led to epitheloid cysts that progressed to pilomatricomas. The resulting pilomatricomas were addicted to continuous ß-catenin expression, highlighting the requirement for constant hyperactive Wnt/ß-catenin signaling in these tumors[175]. In human pilomatricomas, the basaloid cells show strong nuclear ß-catenin staining[231], reaffirming active Wnt/ß-catenin signaling in the tumors as observed in the mouse model.

Wnt/ß-catenin signaling undoubtedly plays an instructive role in lineage determination and adult stem cell maintenance in the skin. The specific role of Wnt/ß-catenin signaling is clearly dictated by the particular mini-organ and state of development. During early development, while high levels of Wnt/ß-catenin signaling specify a follicular fate, lower levels direct towards sebaceous and IFE fates.

Interestingly, upon establishment of the lineages, the respective adult stem cells require Wnt/ß-catenin signaling to maintain homeostasis. However, the exact levels of Wnt/ß-catenin signaling required to maintain the stem cells are unclear. Specifically, how do the Wnt/ß-catenin signaling activities in the anagen and telogen hair follicles compare to that of the IFE? Is Wnt/ß-catenin signaling present at a basal level during mid-telogen before transitioning to much higher levels to induce anagen? If so, what are the underlying mechanics regulating this rheostat? Could potentiation of Wnt/ß-catenin by the R-spondin/Lgr signaling axis be involved in this fine-tuning? If a basal level is required for skin-related lineages, is there a common determinant factor among the Wnt-regulated skin lineages? Addressing these questions would further our understanding of how Wnt/ß-catenin signaling achieves such precise regulation of tissue homeostasis and how it is exploited by cutaneous cancers.

### 3) Liver stem cells and cancer

The liver is the largest internal organ and carries out a variety of functions including drug metabolism, chemical detoxification and regulation of glycogen storage. It is organized into hepatic lobules, delineated by the central vein (which brings blood out of the liver), and the portal triad consisting of hepatic artery, hepatic portal vein (both of which transport blood into the liver), and intrahepatic bile duct (Figure 6). Blood flows from the hepatic artery and hepatic portal vein to the central vein through sinusoids, which are lined by endothelial cells. While the hepatic epithelial cells surrounding the portal triad and central vein are broadly labeled as hepatocytes, it is clear that the hepatocyte population is heterogeneous and the liver can be divided into multiple zones based on the distinct functions, localizations and markers of the

hepatocytes – (i) pericentral hepatocytes (those surrounding the central vein), (ii) periportal hepatocytes (those surrounding the portal triad) and (iii) mid-lobular hepatocytes residing between the first two zones[232].

[Insert Figure 6 here]

Figure 6: Liver stem cell. (A) Functional unit of the liver. The portal triad, which consists of portal vein (PV), hepatic artery (HA) and biliary duct (BD), is a region devoid of Wnt/ß-catenin singling, while the region surrounding the central vein (CV) is a site of active Wnt/ß-catenin signaling. Lgr4 is expressed in hepatocytes in all three regions. (B) The pericentral hepatocytes immediately surrounding the CV express Lgr5 and Axin2, and are purported to be homeostatic hepatocyte stem cells of the liver. The sinusoid endothelial cells lining the CV express Wnt2 and Wnt9b, forming the niche for the pericentral hepatocytes.

## Wnt/ß-catenin signaling regulates liver zonation and hepatocyte renewal during homeostasis and regeneration

R-spondin/Lgr-potentiated Wnt/ß-catenin signaling is required for liver zonation. Pericentral hepatocytes around the central vein express Wnt/ß-catenin target genes such as Axin2, Lgr5, glutamine synthetase (GS), transporter-1 of glutamate (Glt1), leukocyte cell-derived chemotaxin-2 (Lect2), ornithine aminotransferase (Oat), cytochrome proteins like Cyp2e1 and Cyp1a2, whereas Wnt/ß-catenin signaling is suppressed in the periportal regions[233-237].

Modulating Wnt/ß-catenin signaling disrupts metabolic zonation in the liver[236-240]. Hyperactivation of Wnt/ß-catenin signaling by knocking out hepatocyte-specific Apc

rapidly induces GS expression in the entire liver, extending to periportal regions where periportal markers are lost, leading to a fatal metabolic disorder[237]. In contrast, disrupting Wnt/ß-catenin signaling via adenoviral Dkk1 administration or knocking out core signaling components such as ß-catenin, Lrp5/Lrp6, or Lgr4/Lgr5 abrogates expression of pericentral markers while periportal markers are ectopically expressed in pericentral regions[236-240]. These studies underscore the differential regulation by Wnt/ß-catenin signaling in the two regions. Mice injected with adenoviral Dkk1 died after a week due to intestinal phenotype[46] but mice with liver-specific knockout of ß-catenin or Lrp5/6 were viable despite reduced liver mass with lower proliferation levels[238-240]. The lack of strong phenotype cannot be explained by gamma-catenin (plakoglobin), which can act redundantly with ß-catenin, as gamma-catenin was not found in the nucleus, suggesting it is unlikely to have a signal transduction role[241]. Therefore, while Wnt/ß-catenin signaling is important in maintaining liver zonation and normal proliferation during homeostasis, it is not essential for survival.

The adult liver has a remarkable regenerative capacity, and can repair itself fully even after two-thirds of the liver has been resected (partial hepatectomy). This is accomplished in part via activation of Wnt/ß-catenin signaling, which is thought to regulate the S phase entry of hepatocytes. Hepatocyte-specific knockout of ß-catenin or Lrp5/6 delays liver regeneration due to later S phase entry and reduced proliferation after partial hepatectomy, though the liver mass recovers eventually[238,240,242]. This suggests redundancy in the functions of ß-catenin or Wnt signaling with other molecules and pathways in the liver during regeneration. Similarly, affecting the R-spondin/Lgr axis by deleting Lgr4 and Lgr5 in the hepatocytes also delays their S phase entry upon partial hepatectomy[236].

Conversely, S phase entry and proliferation rates are accelerated upon partial hepatectomy when Wnt/ß-catenin signaling level is increased through expressing stabilized ß-catenin mutant in hepatocytes, hydrodynamic delivery of *Wnt1* DNA, systemic injection of R-spondin 1 and knocking out both Znrf3 and Rnf43 in hepatocytes[236,243]. Hence, Wnt/ß-catenin signaling is required for efficient liver regeneration.

The cellular source of hepatocytes during homeostasis has been a long-standing question in the field. Recently, pericentral hepatocytes and parenchymal hepatocytes have been both proposed as the origin of new hepatocytes in the adult liver. The Wnt-responding pericentral hepatocytes express Axin2[235] and Lgr5[236]. The Axin2$^+$ pericentral hepatocytes proliferate more quickly than the rest of the hepatocytes[235,244], and give rise to progeny that migrate to the periportal regions over time during homeostasis[235], suggesting that they are capable of acting as hepatocyte progenitors. However, a separate study using Lgr5-CreERT2 knock in lines generated by Kinzel et al[245] found that hepatocytes expressing Lgr5 did not produce significant progeny[236]. Instead, Lgr4$^+$ hepatocytes present throughout the liver without zonal restriction produce new hepatocytes during homeostasis[236].

Using an independent Lgr5 reporter line (Lgr5-LacZ), Lgr5 expression is not detected during homeostasis, but is induced in small cells near biliary ducts upon injury by carbon tetrachloride administration, which causes acute liver fibrosis[246]. Lineage tracing with a third independent Lgr5-CreERT2 mouse model demonstrated that these cells give rise to biliary and hepatocyte daughter cells, indicating bipotency[246]. These characteristics are reminiscent of the ‘oval cells’, a bipotent population that

only appears upon hepatic injury[247]. Single Lgr5$^{+}$ cells induced upon injury can generate organoids, which require R-spondin1 in the media for survival, once again underscoring the importance of Wnt/ß-catenin activity potentiation by R-spondin/Lgr axis[246,248].

Given the discrepancies amongest the various *in vivo* studies, it is important to ensure that the mouse models faithfully recapitulate endogenous expression of the genes of interest, and the physiological behaviors of the cells they mark. For instance, the absence of Lgr5-LacZ expression in liver during homeostasis in the Huch et al study[246] is likely to be technical issue with reporter sensitivity since Lgr5 mRNA is present in pericentral hepatocytes during homeostasis[235,236]. Therefore, complementary approaches are necessary in order to establish the true identity of the homeostatic hepatocyte progenitor.

Two niche sources of Wnt signals have been proposed. Sinusoidal endothelial cells secrete Wnt2 and Wnt9b during homeostasis, which are required for normal Axin2 and GS expression[235]. In addition, Wnt2 from sinusoidal endothelial cells is also necessary for complete liver regeneration[249]. The other proposed niche is the hepatic macrophage, Kupffer cell. Knocking out Wls in Kupffer cells delays liver regeneration after partial hepatectomy, likely due to later S phase entry as demonstrated by reduced Cyclin D1 levels[238]. In contrast, Wls-deficient hepatocytes do not display any phenotype during homeostasis and regeneration[238]. Hence, hepatocytes require paracrine sources of Wnts for homeostasis and regeneration.

## Aberrant Wnt/ß-catenin signaling is a crucial pathway in hepatocellular carcinoma

Hepatocellular carcinoma (HCC) is the fifth most common cancer and the third most lethal cancer worldwide[250]. HCC is the uncontrolled growth of hepatocytes, fueled by several aberrant signaling pathways including Wnt/ß-catenin signaling. In fact, mutations in Wnt pathway components leading to hyperactivation of the pathway are found in 66% of HCCs[251], and expression levels of Wnt/ß-catenin pathway components correlate significantly with disease progression[252].

Unsupervised clustering of patient HCC samples identified various subclasses of HCC, some of which are associated with hyperactivated Wnt/ß-catenin signaling[253,254]. In another study, Wnt/ß-catenin signaling was found to be one of the two major signaling networks, with alterations in Wnt/ß-catenin signaling pathway accounting for 65% of the HCC cases[255]. Furthermore, Wnt/ß-catenin signaling is highly active in the invasive HCC characterized by positive EpCAM and alpha-fetoprotein expression. Therefore, Wnt/ß-catenin signaling plays a crucial role in HCC. Active Wnt/ß-catenin signaling is also associated with HCC progression as hepatocellular adenomas with activating mutations in CTNNB1 are more likely to develop into malignant HCC[256-259]. Additionally, nuclear ß-catenin localization is correlated with unfavorable prognosis, disease progression, and expression of the proliferation marker KI67[257-259], particularly in later stages of HCC[260].

Multiple Wnt/ß-catenin signaling components are commonly mutated in HCC. Mutations in exon 3 of CTNNB1, which prevents degradation of ß-catenin, is the Wnt-related mutation most commonly associated with HCC. CTNNB1 exon 3 mutations are found in up to 44% of human HCCs across various studies[255,261-263],

and correlate with overexpression of known Wnt/ß-catenin target genes, including Lgr5, GS and Glt[263,264]. In addition, inactivating mutations in Wnt/ß-catenin negative regulators AXIN1, AXIN2 and APC were repeatedly observed in HCCs[255,264-266]. Other alterations found to activate Wnt/ß-catenin signaling in HCC include overexpression of the FZD7 receptor[267-269], increased expression of the ligand WNT3[267], inactivation of the negative regulator GSK-3ß[270], and epigenetic silencing of the inhibitors sFRPS[271,272]. Of the FZD receptors involved, only FZD7 has been shown to induce nuclear/cytoplasmic accumulation of ß-catenin in HCC[267,269,273]. Amplifications in WNT9A, RSPO2 and FZD6 leading to their overexpression were also found in 21.6-25.0% of the HCC tumors analyzed[255].

While hyperactivated Wnt/ß-catenin signaling is prevalent in HCC cases, multiple studies have shown that it is insufficient for inducing HCC on its own. Hepatotoxin-induced HCC originates from mature hepatocytes, not biliary progenitors[274,275], indicating that the genetic and epigenetic changes promoting tumorigenesis occur in hepatocytes. Murine hepatocytes expressing a stabilized form of mutant ß-catenin do not develop HCC spontaneously, though Wnt/ß-catenin signaling is active as evidenced by nuclear ß-catenin localization[233,243,276,277]. Therefore, to generate mouse models of HCC, additional genetic and/or epigenetic changes are required in a genetic background with hyperactivated Wnt/ß-catenin signaling. Additional genetic changes include other tumorigenic factors like the Ha-Ras oncogene[278,279] or loss of tumor suppressors like Liver kinase B1 (Lkb1)[280]. These changes can also be induced chemically in a Wnt-hyperactivated background by the carcinogen diethylnitrosamine[243] or phenobarbital, which activates CAR (Constitutive Androstane Receptor, NR1I3), a xenobiotic nuclear receptor[281]. Nonetheless, one

study showed that it is possible to induce HCC by modulating Wnt/ß-catenin signaling on its own. Apc in the liver was inactivated via a low, non-lethal dose of adenoviral-Cre, which led to liver tumorigenesis over a long latency period of 8 months, with associated high levels of Wnt/ß-catenin signaling[282]. However, it is unclear if Wnt-independent effects of APC inactivation or viral infection could account for the additional 'hit'. Overall, other genetic or epigenetic factors are required in combination with a sensitized Wnt/ß-catenin background for liver tumorigenesis.

In HCC, Wnt/ß-catenin signaling is important in the maintenance of CSCs marked by CD133[283], EpCAM[284], OV6[285] and Lgr5[286]. In fact, EpCAM is a Wnt/ß-catenin target gene in HCC[284]. Interestingly, tumor-associated EpCAM is able to perform signaling functions by cleaving its intracellular domain that binds to ß-catenin and Lef1 to induce transcription of genes with Lef1 binding sites in the promoter, as well as the hepatic CSC gene signature[287,288].

The $EpCAM^+$ CSCs from patients with advanced cirrhosis and HCC express high amounts of WLS, MYC, CyclinD1 and WNT3. Spheroids formed from these $EpCAM^+$ cells are sensitive to IWP-2, a Porcupine inhibitor, indicating that the CSC-like cells in advanced cirrhosis and CSCs in HCC engage in autocrine Wnt signaling[289], contrasting the paracrine mode of signaling observed during homeostasis[235,238,249]. However, this therapeutic approach is unlikely to be useful in HCCs harboring mutations in downstream components such as ß-catenin, APC and AXIN1, which render Wnt/ß-catenin signaling in the tumor independent of Wnt ligands. Wnt/ß-catenin signaling in HCC can also be potentially targeted by FH535, a small

molecule, which inhibits ß-catenin activity (both wildtype and mutant) and has shown efficacy in HCC cell lines and isolated hepatic CSCs marked by CD133, CD44 and CD24 expression[290].

While it is known that Wnt/ß-catenin signaling plays a role in HCC and regulates the CSCs, several outstanding questions remain. It is clear that HCC originates from hepatocytes. However, does HCC arise from a specific subpopulation of hepatocytes, such as the pericentral hepatocytes that have high Wnt/ß-catenin signaling activity during homeostasis[235], or the $Lgr4^+$ parenchymal hepatocytes[236]? Strikingly, while hyperactivation of Wnt/ß-catenin signaling appears to be sufficient for tumorigenesis in other tissues such as the intestines, it is dependent on additional factors to induce the onset of HCC. What are the other crucial functions required for HCC tumorigenesis that are not provided by Wnt/ß-catenin signaling? Finally, the precise identity of the liver progenitors during homeostasis remains to be elucidated, as well as their roles in the various models of liver injury and regeneration. Studies of Wnt/ß-catenin signaling in the liver will undoubtedly gain further momentum in the future, by harnessing the plethora of tools for dissecting the pathway, which will contribute towards translation impact.

## Concluding remarks

Wnt/ß-catenin signaling is instrumental in orchestrating organismal development, and mutations in the pathway often lead to congenital disorders. Post-development, the importance of the pathway in ensuring proper adult homeostasis and regeneration is also evidenced by the increasing number of adult stem cell populations found to require Wnt/ß-catenin signalling for maintenance. Through a

combination of genetic experiments to modulate the Wnt pathway and lineage tracing, we have achieved a deeper understanding of adult tissue stem cells, with the intestines and skin being prime examples. However, we have only begun to scratch the surface for most other epithelial tissues. While numerous adult stem cells have been found to be Wnt-responding (Table 1), further mechanistic studies are required for a deeper understanding of how Wnt/ß-catenin signaling regulates stem cell behavior.

In cancer, Wnt/ß-catenin signaling is also intricately involved in tumor initiation, progression and metastasis. Most of these cancers are associated with hyperactivated Wnt/ß-catenin signaling, through genetic mutations or epigenetic misregulation of the pathway components. Such changes associated with Wnt/ß-catenin signaling are believed to play a role in the earliest stages of oncogenic transformation. These changes can be broadly classified into two categories - those that are Wnt ligand-dependent, and those that are Wnt ligand-independent. Genes implicated in the former category would include the Wnt ligands, R-spondins, soluble inhibitors and other pathway components needed for post-translational modifications and exocytosis of the Wnt ligands (e.g. Porcn and Wls). For the Wnt ligand-dependent cancers, a new class of drugs targeting Porcn are garnering great interest in their early clinical trials for treating various epithelial cancers[291]. Combining Porcn-targeted treatment with other emerging treatments including checkpoint inhibitors like PD-1 and immunotherapies such as CAR-T is likely to boost therapeutic efficacy. For instance, the Porcn inhibitor LGK974 has been combined with PD-1 in an ongoing clinical trial for multiple epithelial cancers (NCT01351103).

However, for cancers harboring genetic and epigenetic changes downstream of Wnt/Fzd binding, including mutations in APC and CTNNB1, Wnt/ß-catenin signaling is independent of Wnt ligands. Such cancers would therefore be refractory to Porcn modulators, instead requiring drugs that target the signal transduction machinery in the Wnt-responding cell. A comparison of patents related to Wnt modulators and Wnt-related clinical trials showed that although modulators of ß-catenin formed the largest class of patents, the proportion of clinical trials targeting ß-catenin was small[292]. This small group of clinical trials (NCT02195440, NCT02413853, NCT01606579) mostly involved one molecule, PRI-724, which blocks the binding of ß-catenin and its co-activator, thereby inhibiting Wnt/ß-catenin signaling transcription[153]. Given the large proportion of Wnt ligand-independent cancers, it is imperative to develop therapies that can target the Wnt/ß-catenin transcription machinery.

A longstanding challenge in drugging the Wnt pathway lies in minimizing off target effects, as many homeostatic adult stem cells are Wnt-dependent. Many of these adult stem cells have been identified or proposed as the cell-of-origin of cancers after accumulating a suite of genome and epigenetic alterations. CSCs, which are responsible for propagating the tumor, are also often dependent on Wnt/ß-catenin signaling. CSCs often bear characteristics of adult stem cells, such as self-renewal ability, expression of stem markers like Lgr5, CD44 and CD133 and elevated Wnt/ß-catenin signaling activity. The tumor microenvironment also plays a critical role in maintaining CSCs. Therefore, modulating Wnt/ß-catenin signaling in CSCs and their microenvironment could prove to be crucial for developing effective therapeutic strategies.

## Table 1: Wnt-responding adult tissue stem cells

| Epithelial tissue | Wnt-responding Adult stem cell type | Homeostatic/ facultative | Wnt marker | Capability to form organoids or be transplanted? | Cancer cell-of-origin? | Aberrant Wnt/ß-catenin pathway involved in cancer? (selected references) |
|---|---|---|---|---|---|---|
| Small intestine and colon | Crypt columnar base cells | Homeostatic | Lgr5[57] | Organoid[293] | Yes[143] | Yes[114] |
| Gastric corpus | Chief cells | Facultative | Lgr5[154] | Organoid[154] | Yes[154] | Yes[167] |
| Gastric corpus | Chief cells (and parietal cells) | Facultative | Troy[155] | Organoid[155] | NT | - |
| Gastric pylorus | Basal cells | Homeostatic | Lgr5[37] | Organoid[37] | Yes[37,294] | Yes[167] |
| Interfollicular epidermis | Basal cells | Homeostatic | Axin2[180,209] | NT | NT | Yes[220,229] |
| Hair follicle | Outer bulge cells | Homeostatic | Axin2[185], Lgr5[201] | Transplantation[201] | NT | Yes[220,229] |
| Liver | Pericentral hepatocytes | Homeostatic | Axin2[235] | NT | NT | Yes[295] |
| Liver | Parenchymal hepatocytes | Homeostatic | Lgr4[236] | NT | NT | Yes[295] |
| Liver | Biliary duct cells | Facultative | Lgr5[246] | Organoid[246] | NT | - |

| | | | | | | |
|---|---|---|---|---|---|---|
| Mammary gland | Basal cells | Homeostatic | Axin2[296], Lgr5[297,298], Procr [299] | Transplantation [298-300] | NT | Yes[301] |
| Ovary | Hilum ovarian surface epithelial cell | Homeostatic | Lgr5[302] | NT | NT | |
| Inner ear cochlea | Tympanic border cells | Homeostatic | Axin2[303] | NT | NT | - |
| Cornea | Limbal epithelial stem cells | Homeostatic | Lgr5, suggested but not proven[304] | NT | NT | - |
| Tongue (posterior) | Base of circumvallate papillae | Homeostatic | Lgr5[305-307] | Organoid[306] | NT | - |
| Nail | Nail matrix cells | Homeostatic | Lgr6[308] | NT | NT | - |
| Kidney | Nephron segment-specific stem cell | Homeostatic | Axin2[309], Lgr5[310] | NT | NT | |
| Pancreas | Pancreatic duct cells | Facultative | Lgr5[311] | Organoid[311] | NT | |
| Prostate | Luminal cells | Homeostatic | Lgr5[312] | Transplantation[312] | NT | |

NT – Not tested

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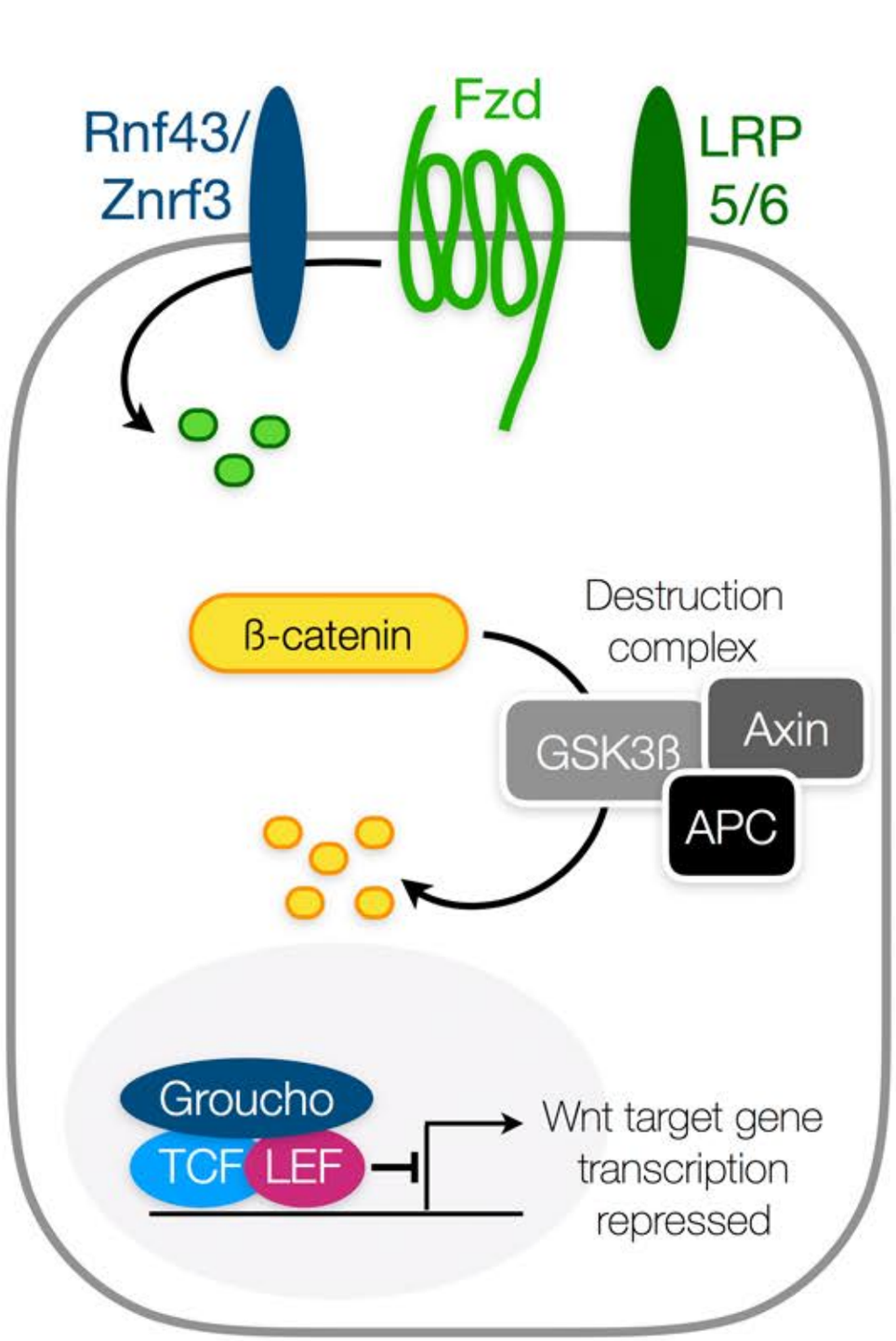
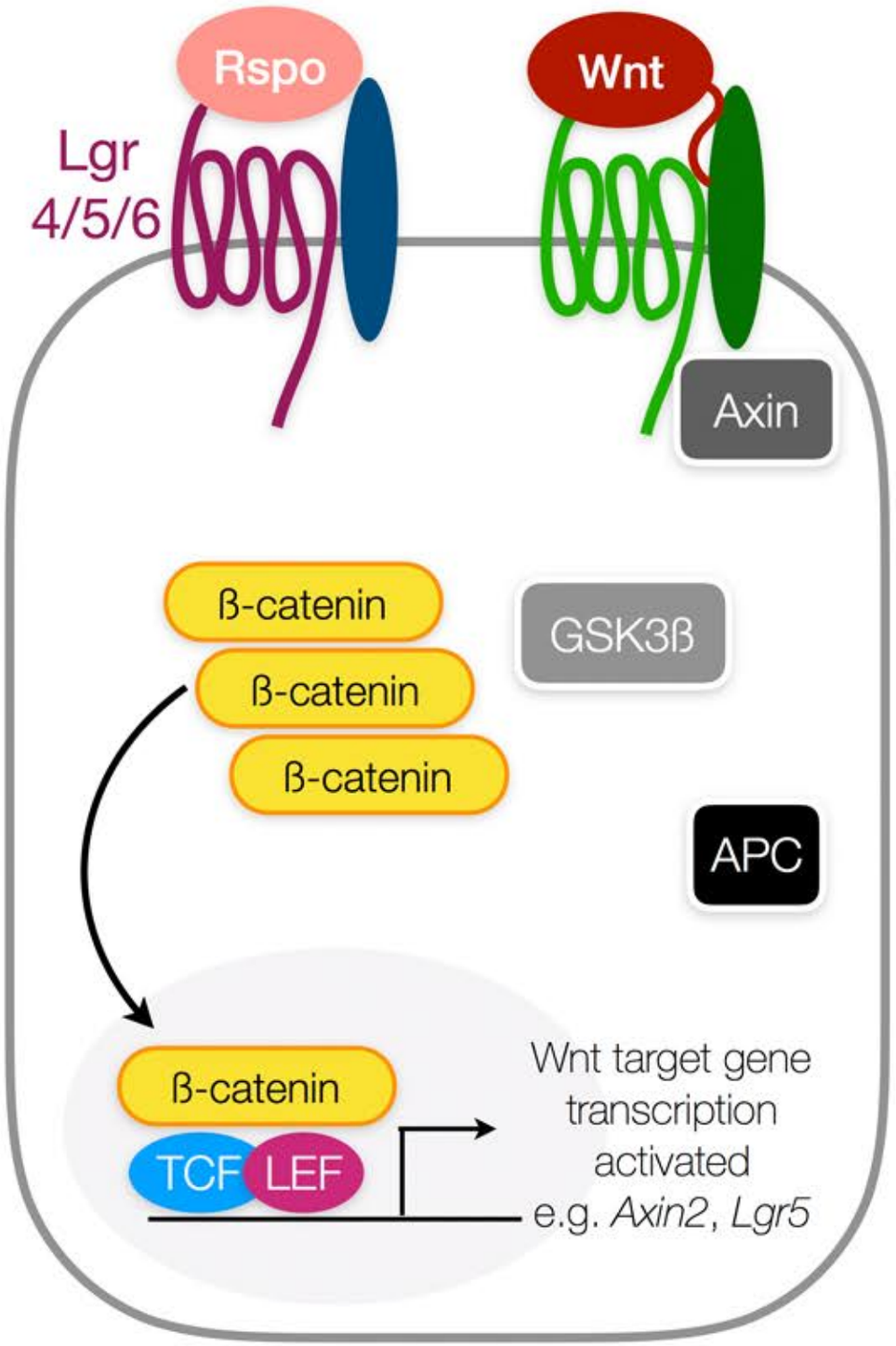
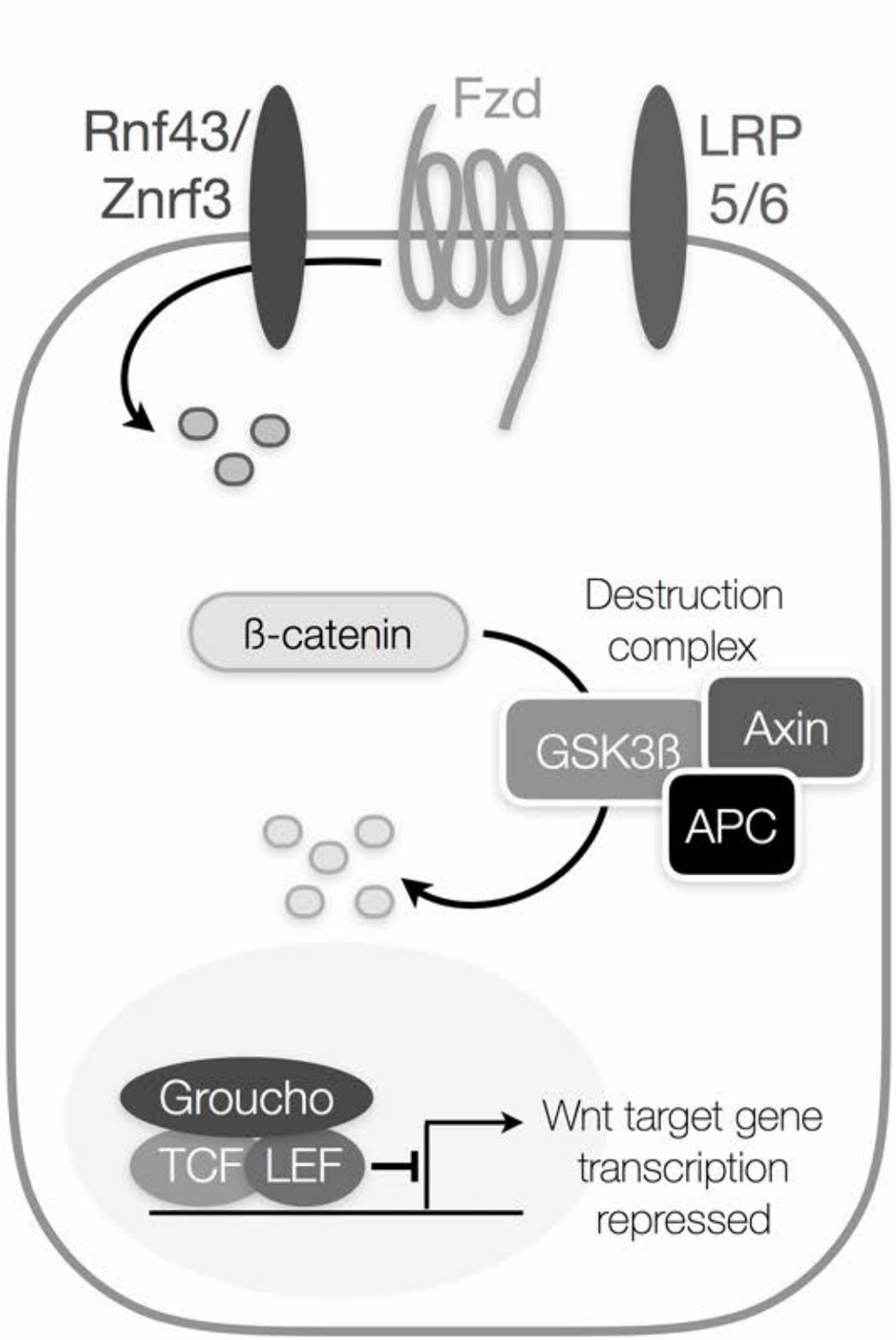



**Without Wnt**
OFF state




**With Wnt and Rspo**
ON state

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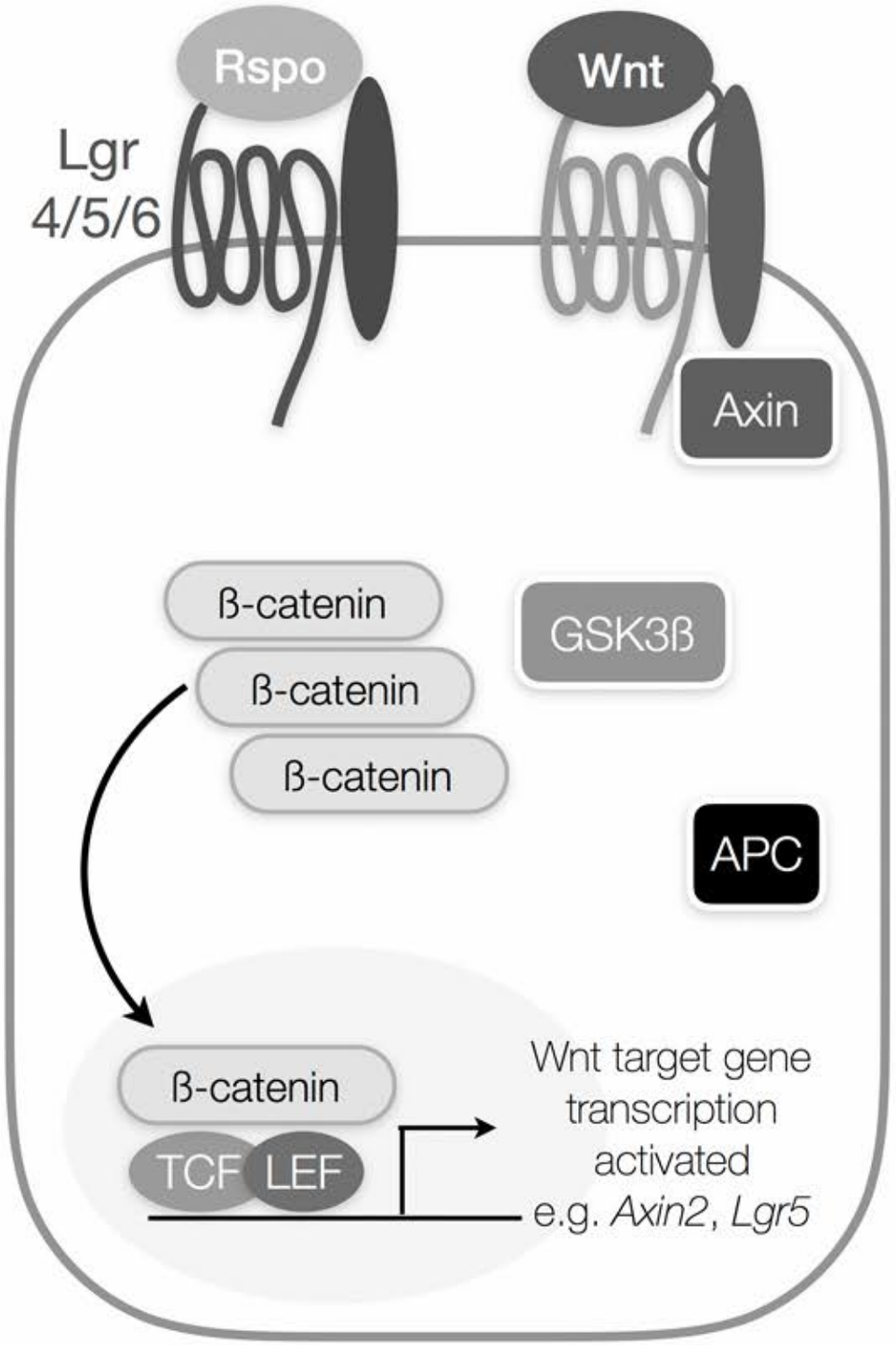
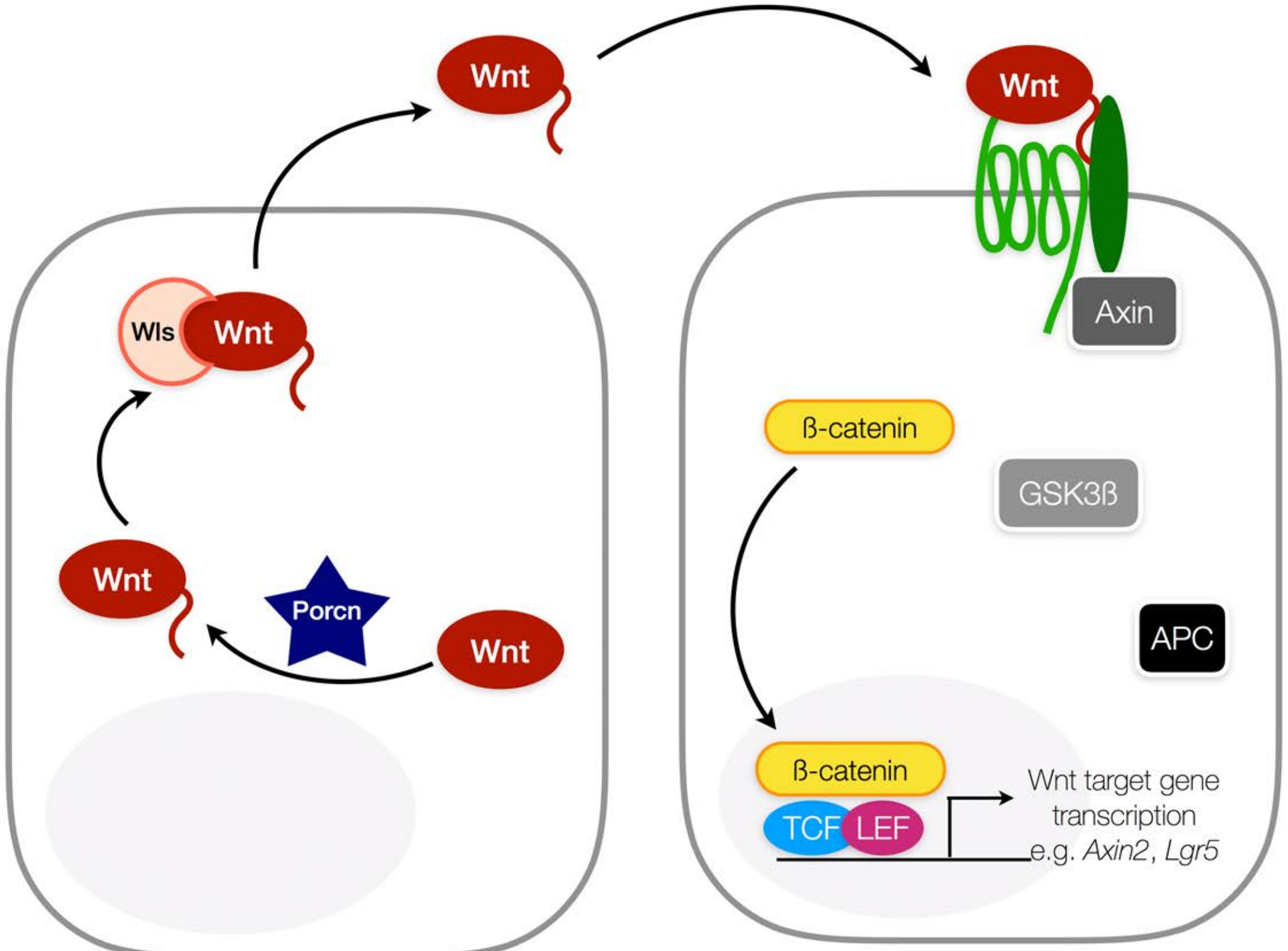
OFF state

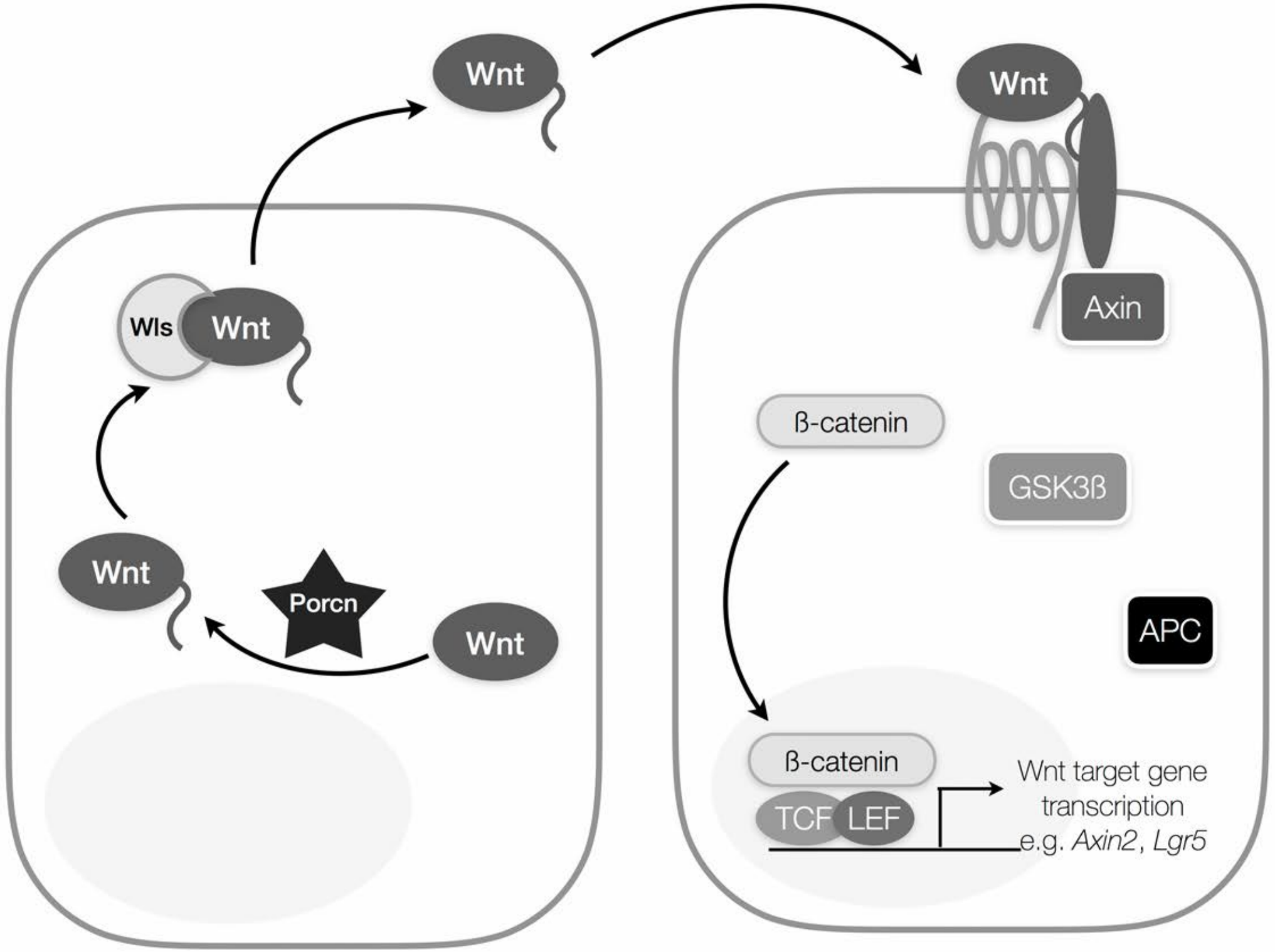



**With Wnt and Rspo**
ON state



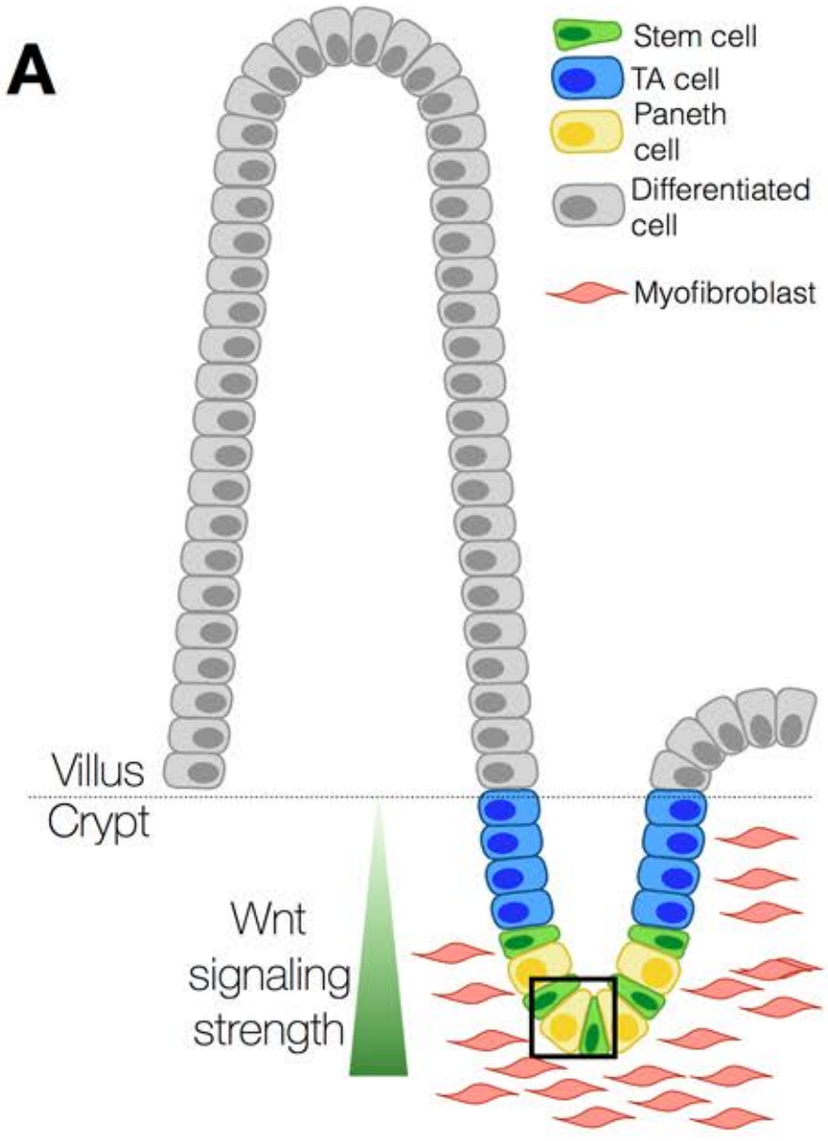

**Wnt-secreting cell**
(e.g. Niche)

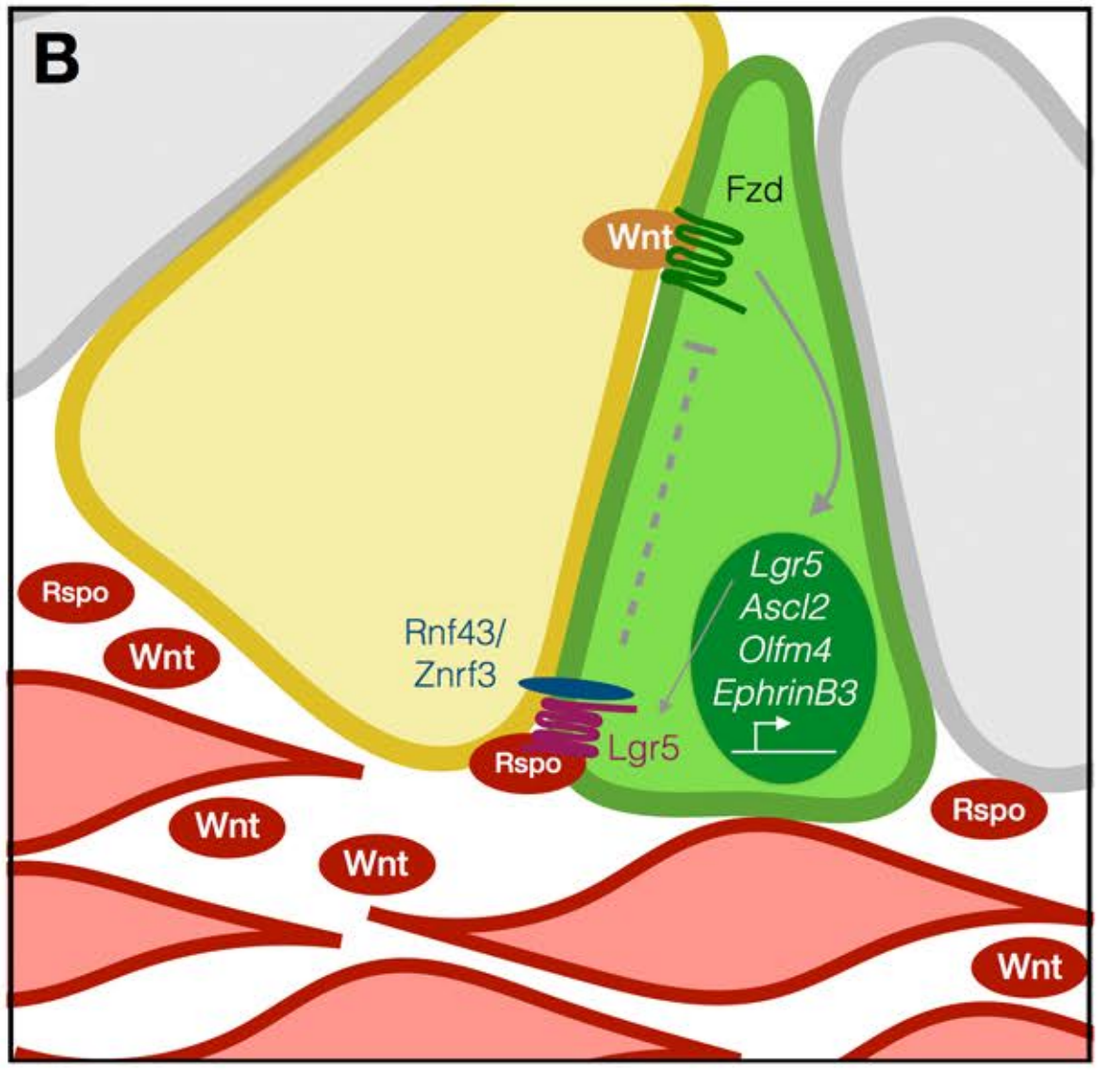
**Wnt-responding cell**
(e.g. Stem cell)

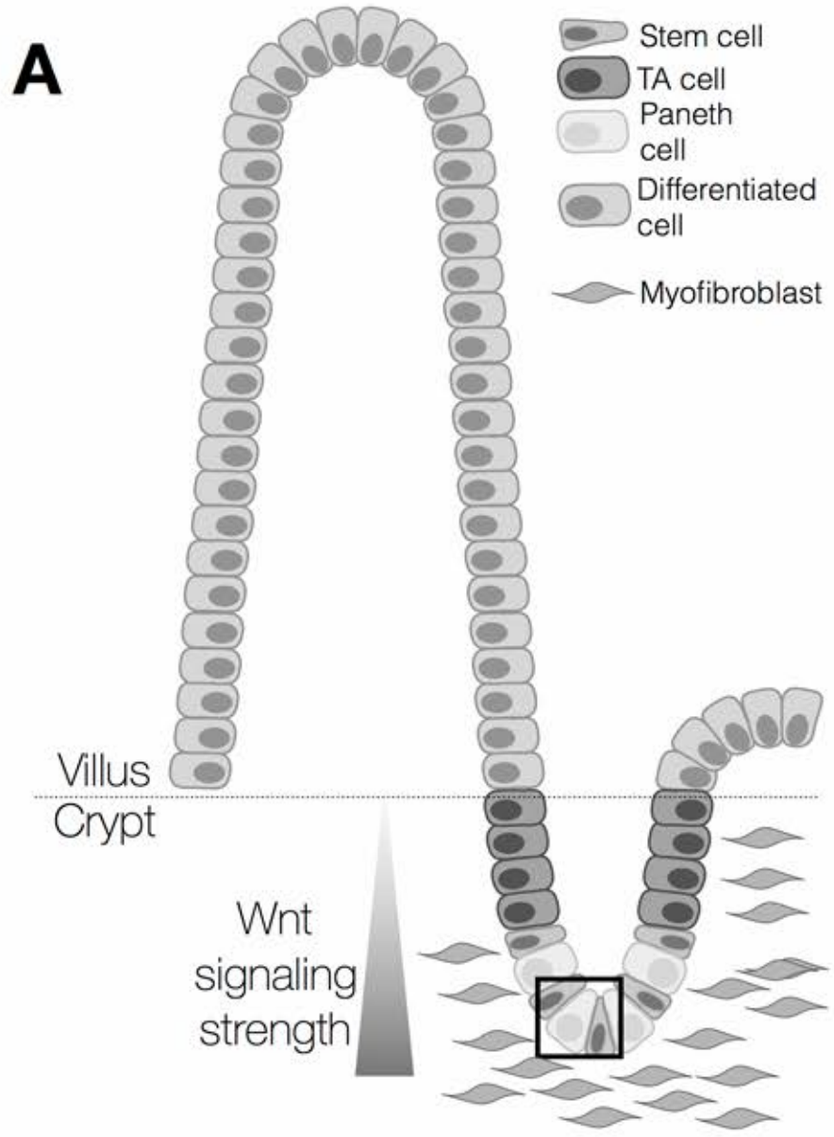


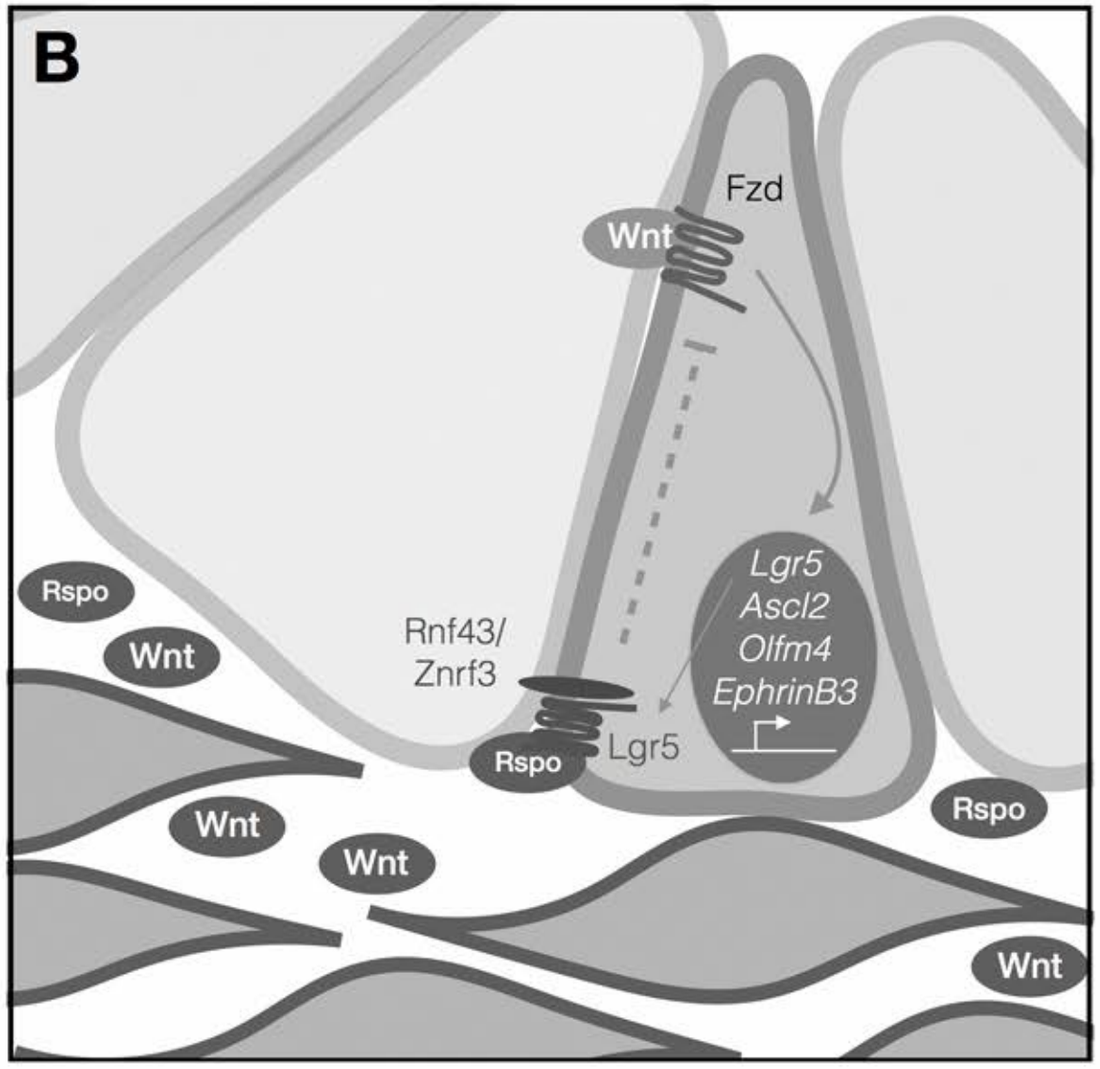

**Wnt-secreting cell**
(e.g. Niche)

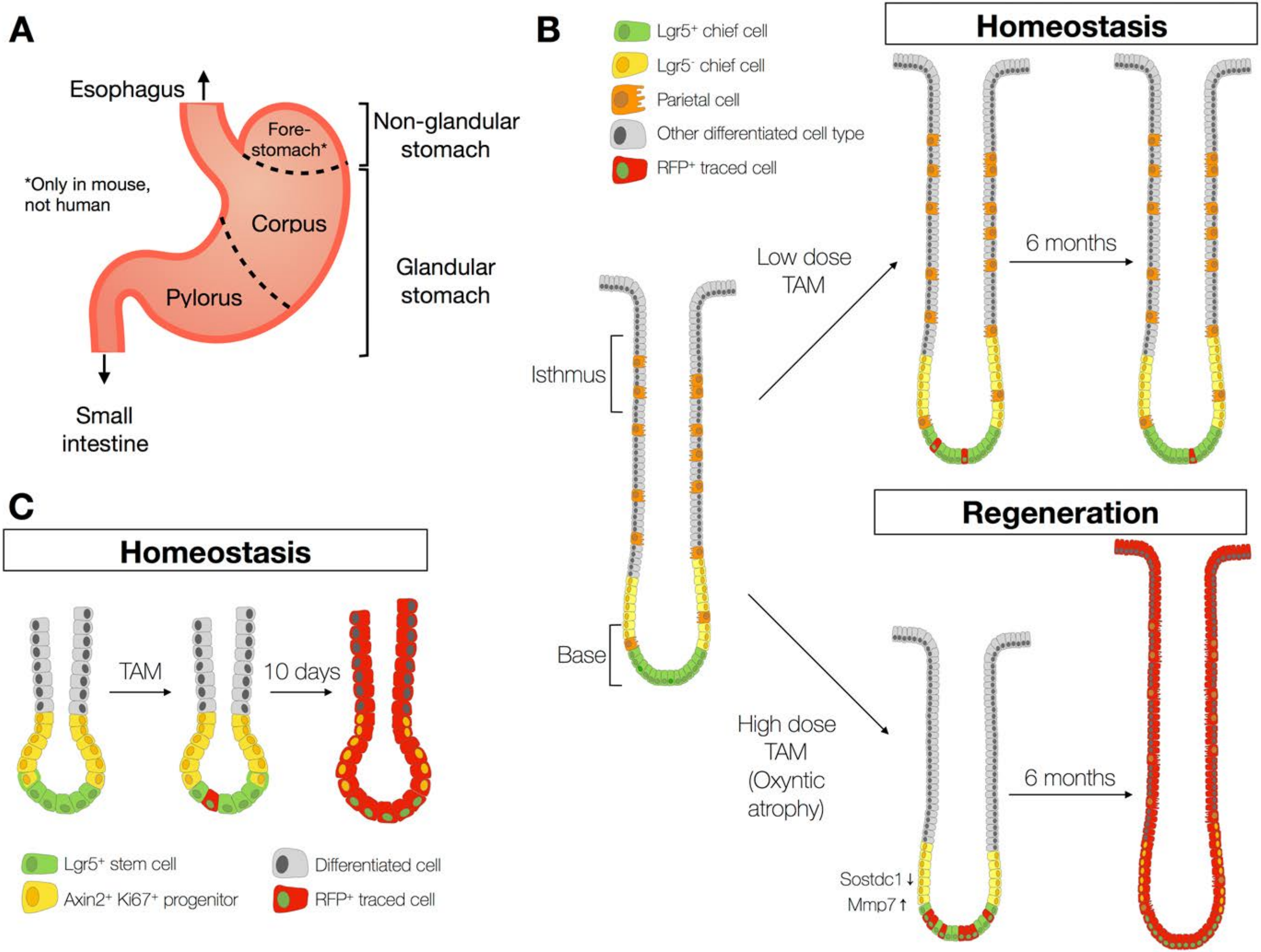
**Wnt-responding cell**
(e.g. Stem cell)

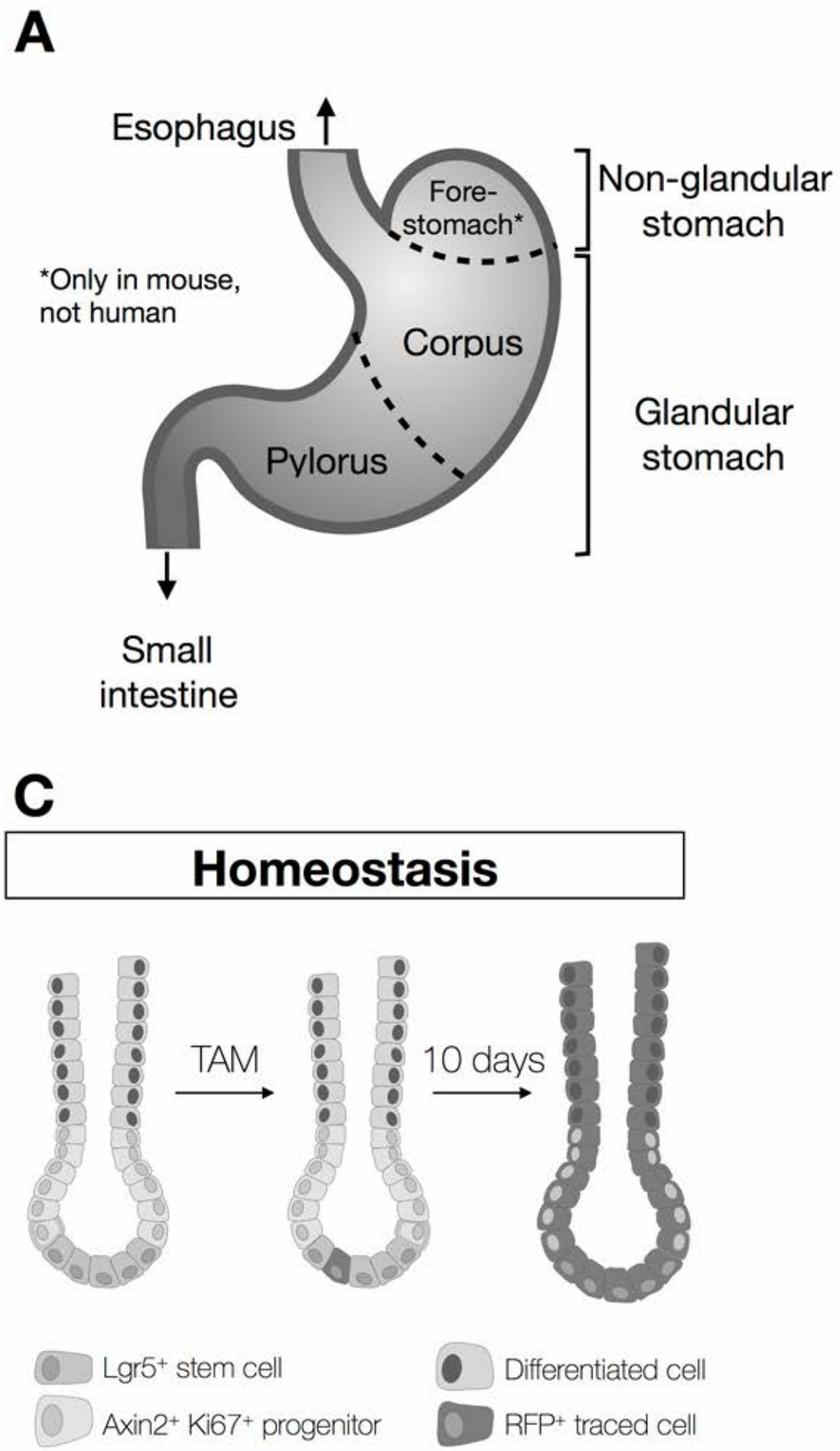

A
Stem cell
TA cell
Paneth cell
Differentiated cell
Myofibroblast
Villus
Crypt
Wnt signaling strength


B
Fzd
Wnt
Rspo
Wnt
Rnf43/
Znrf3
Lgr5
Ascl2
Olfm4
EphrinB3
Rspo
Lgr5
Rspo
Wnt
Wnt
Wnt

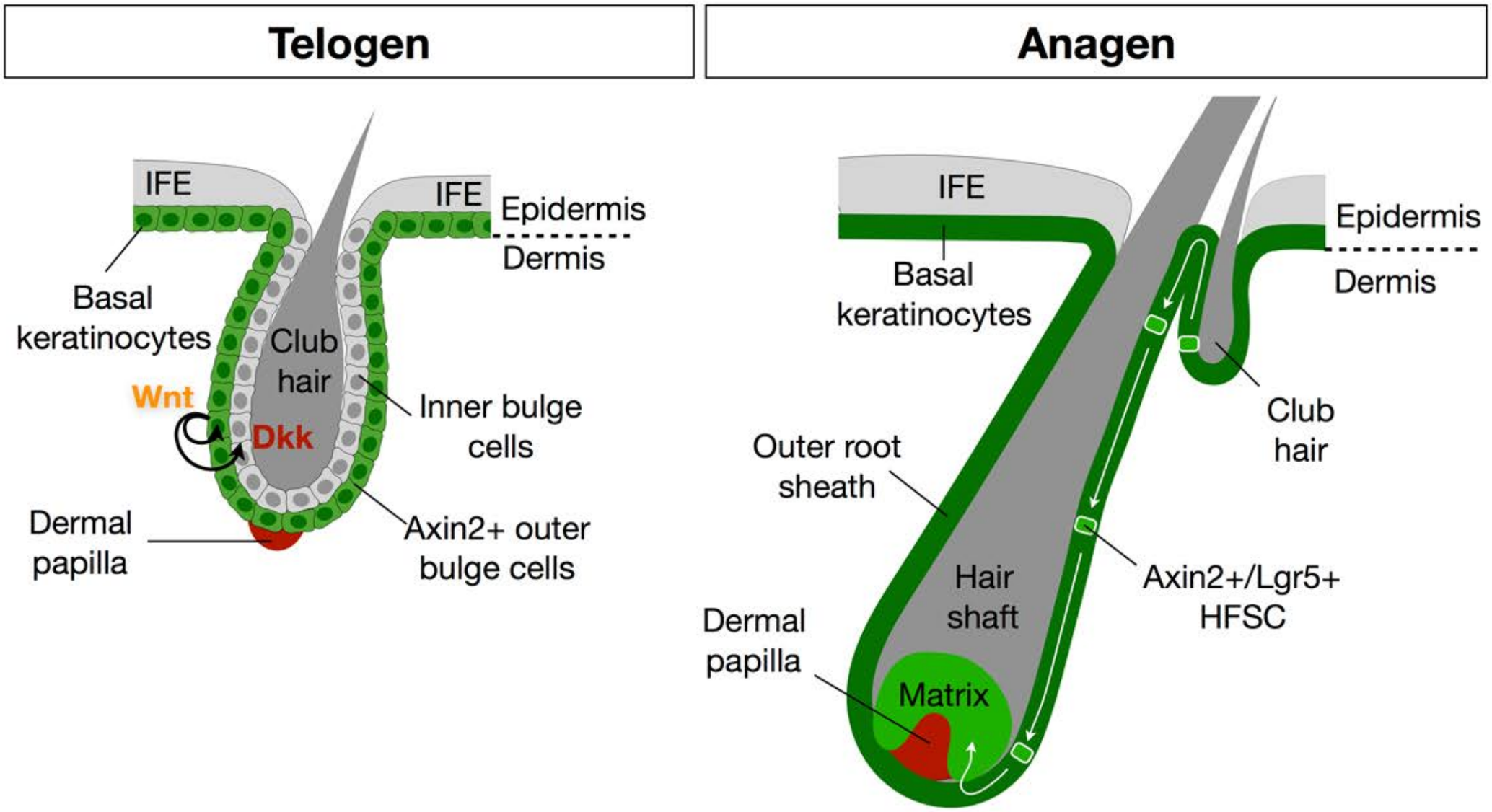

A
Stem cell
TA cell
Paneth cell
Differentiated cell
Myofibroblast
Villus
Crypt
Wnt signaling strength


B
Fzd
Wnt
Rspo
Wnt
Rnf43/
Znrf3
Lgr5
Ascl2
Olfm4
EphrinB3
Rspo
Lgr5
Rspo
Wnt
Wnt
Wnt

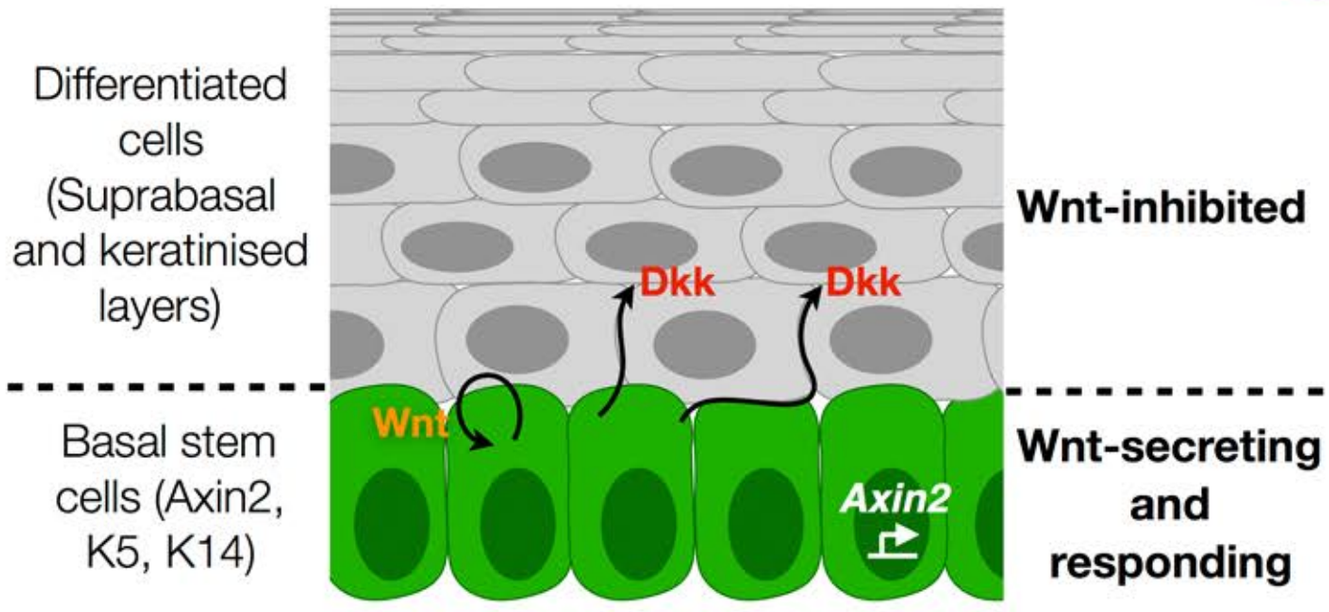

A
Esophagus
Fore-
stomach*
Non-glandular
stomach
*Only in mouse,
not human
Corpus
Glandular
stomach
Pylorus
Small
intestine
B
Lgr5+ chief cell
Lgr5- chief cell
Parietal cell
Other differentiated cell type
RFP+ traced cell
Homeostasis
Low dose
TAM
6 months
Isthmus
Base
Regeneration
High dose
TAM
(Oxyntic
atrophy)
6 months
Sostdc1↓
Mmp7↑
C
Homeostasis
TAM
10 days
Lgr5+ stem cell
Axin2+ Ki67+ progenitor
Differentiated cell
RFP+ traced cell


A
Esophagus
Fore-
stomach*
Non-glandular
stomach
*Only in mouse,
not human
Corpus
Glandular
stomach
Pylorus
Small
intestine
B
Lgr5+ chief cell
Lgr5- chief cell
Parietal cell
Other differentiated cell type
RFP+ traced cell
Homeostasis
Low dose
TAM
6 months
Isthmus
Base
Regeneration
High dose
TAM
(Oxyntic
atrophy)
6 months
Sostdc1↓
Mmp7↑
C
Homeostasis
TAM
10 days
Lgr5+ stem cell
Axin2+ Ki67+ progenitor
Differentiated cell
RFP+ traced cell

A

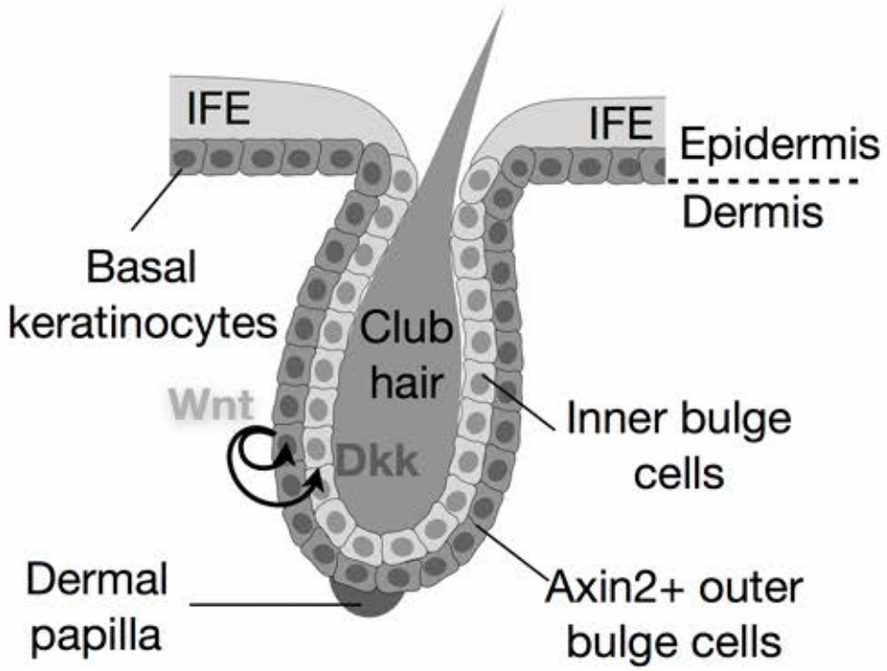

Telogen
Anagen
IFE
IFE
Epidermis
Dermis
Basal keratinocytes
Club hair
Wnt
Dkk
Inner bulge cells
Dermal papilla
Axin2+ outer bulge cells
IFE
Basal keratinocytes
Epidermis
Dermis
Club hair
Outer root sheath
Hair shaft
Axin2+/Lgr5+ HFSC
Dermal papilla
Matrix

B

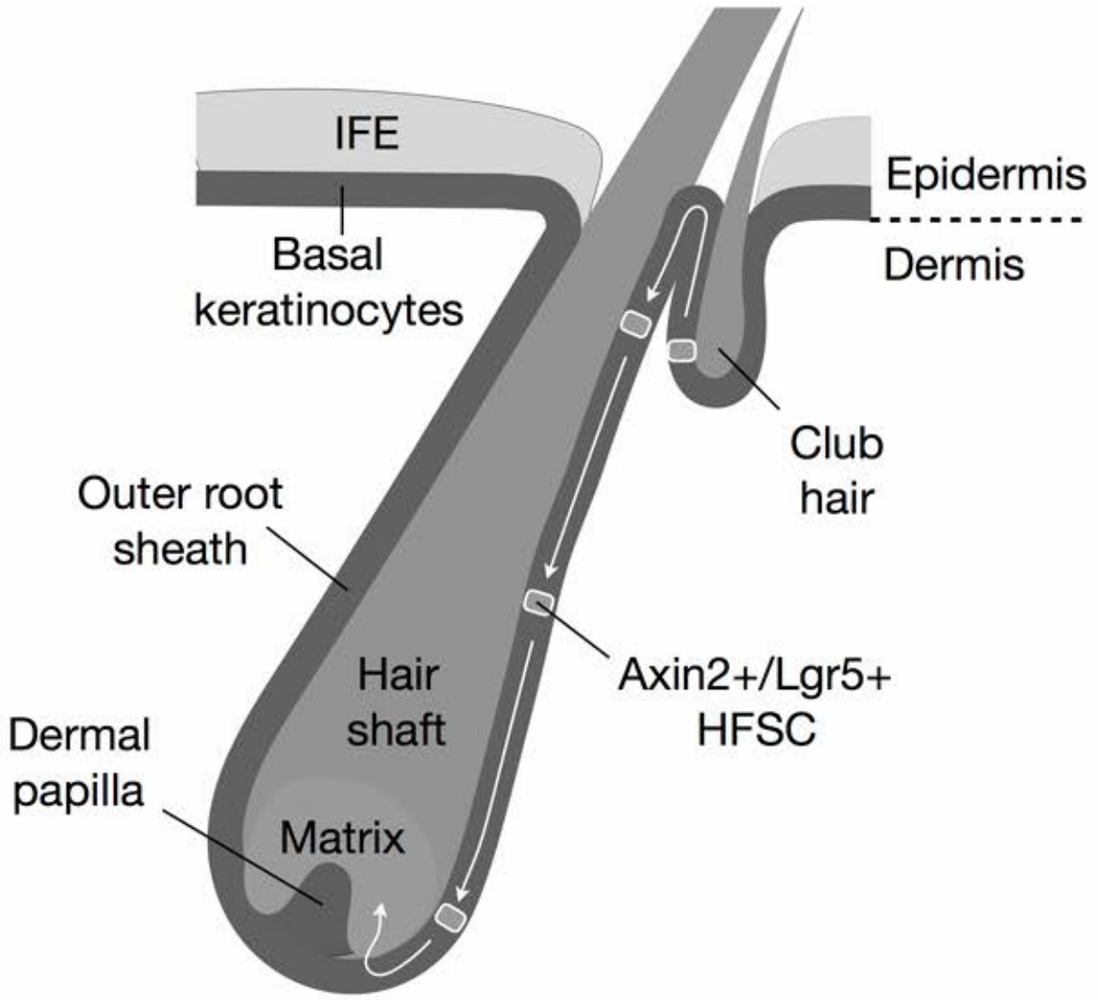

Differentiated cells (Suprabasal and keratinised layers)
Wnt-inhibited
Dkk
Dkk
Wnt
Axin2
Basal stem cells (Axin2, K5, K14)
Wnt-secreting and responding

**A**

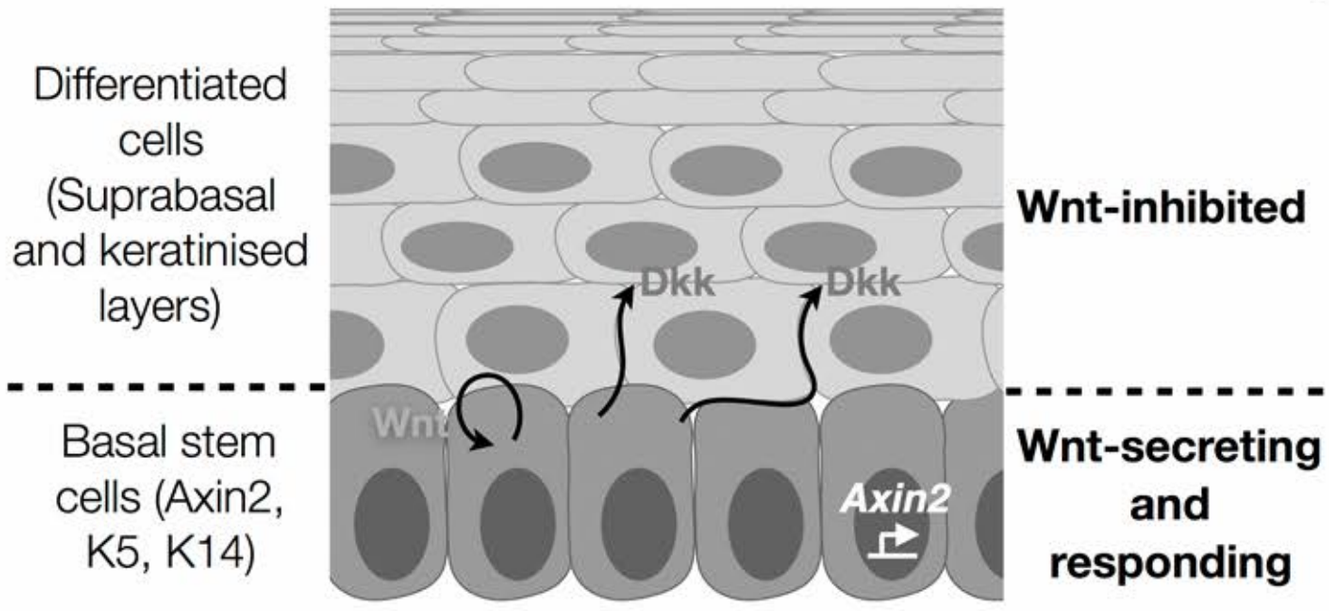
## Telogen

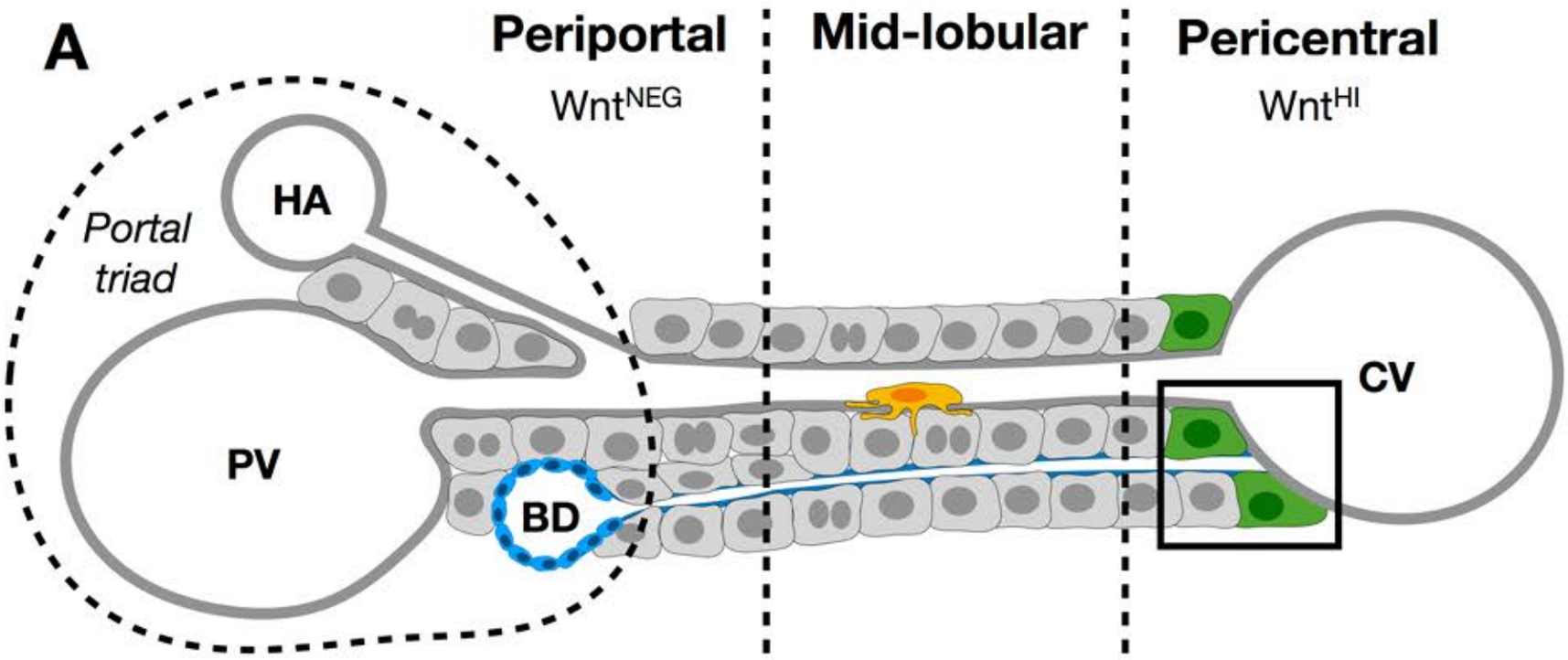


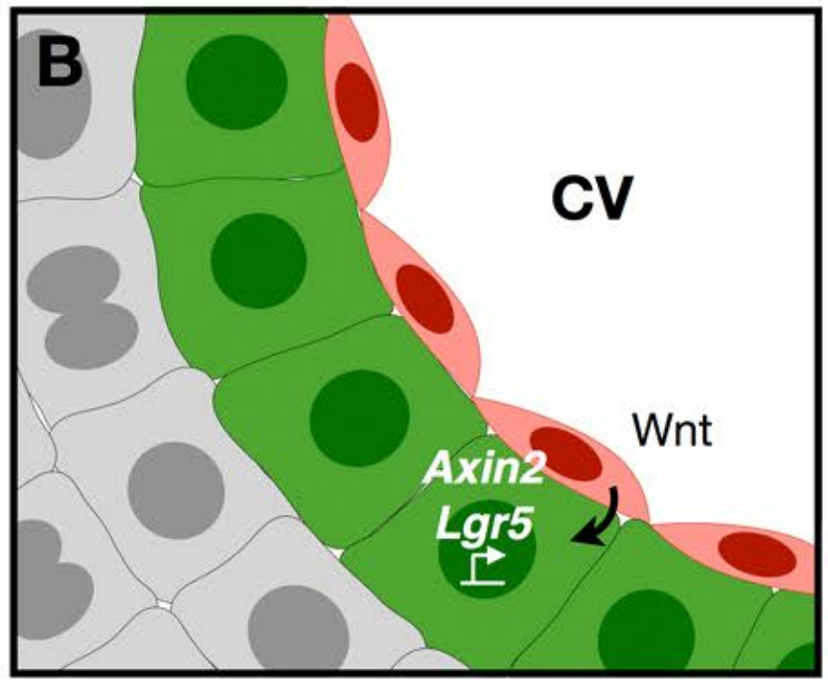

## Anagen




**B**





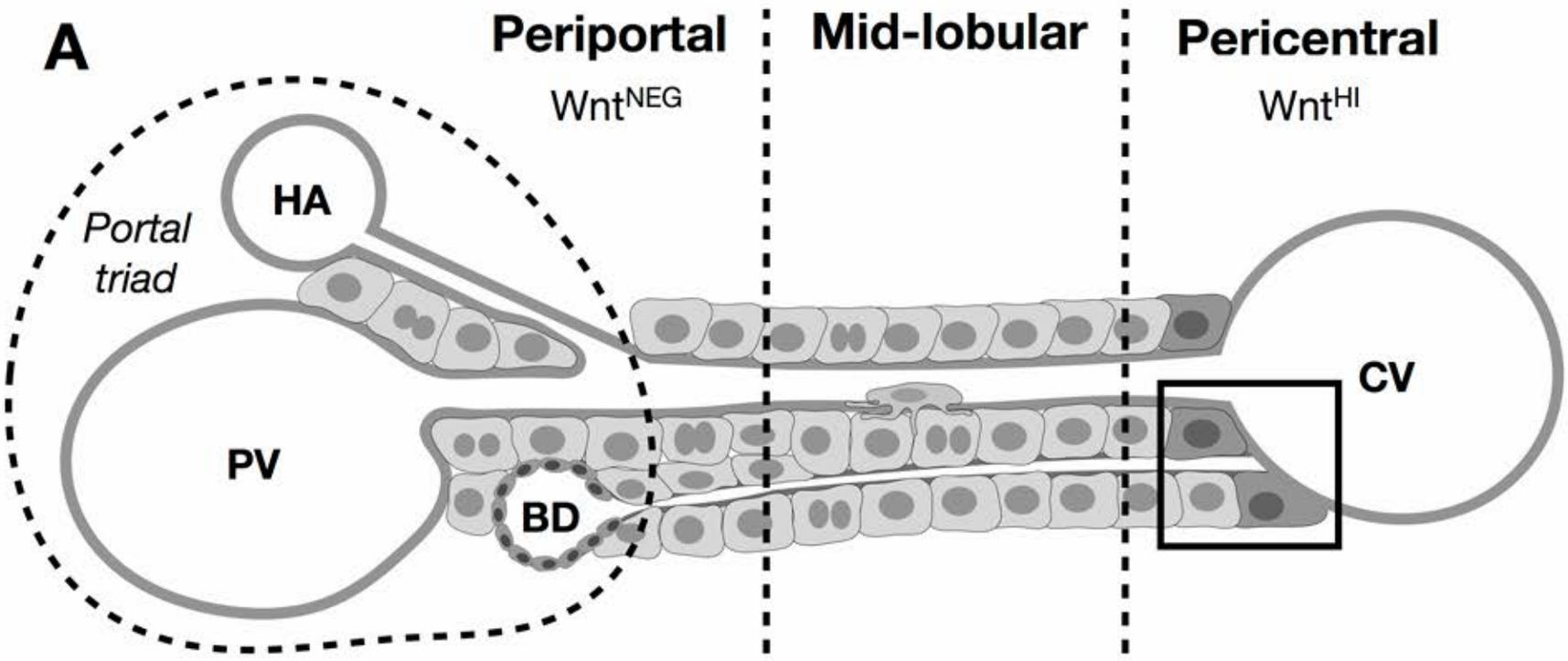

Axin2⁺ Lgr5⁺ hepatocyte

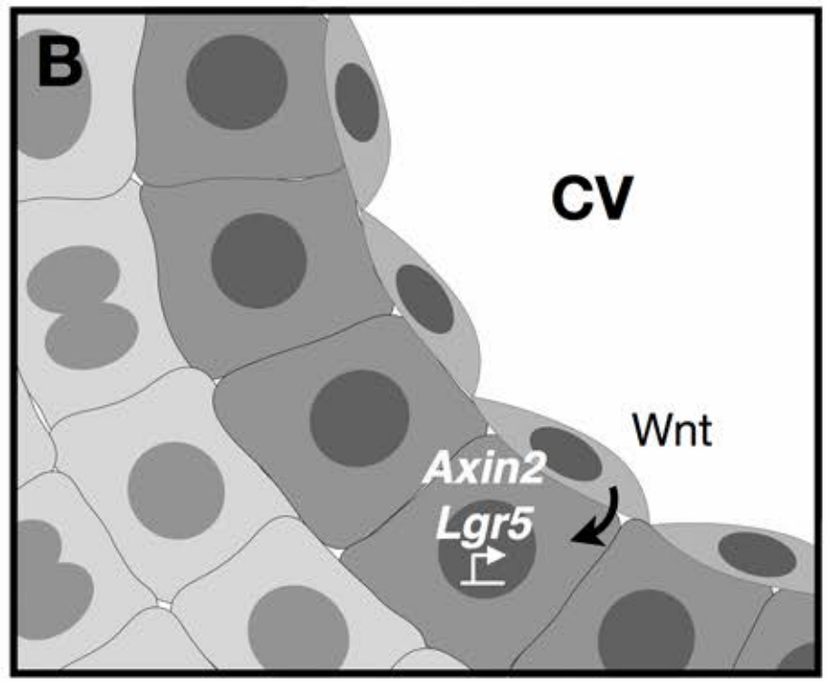
Hepatocyte

Biliary duct cell

Sinusoidal endothelial cell

Kupffer cell




Axin2⁺ Lgr5⁺ hepatocyte

Hepatocyte

Biliary duct cell

Sinusoidal endothelial cell

Kupffer cell