Stem cells are defined by their intrinsic capacity to self-renew and differentiate. Cancer stem cells retain both these features but have lost homeostatic mechanisms which maintain normal cell numbers. The canonical Wnt/\(\beta\)-catenin signaling pathway plays a central role in modulating the delicate balance between stemness and differentiation in several adult stem cell niches such as the hair follicles in the skin, the mammary gland, and the intestinal crypt. Accordingly, constitutive Wnt signaling activation, resulting from mutations in genes encoding its downstream components, underlies tumorigenesis in these tissues. In the majority of sporadic colorectal cancer cases, the rate-limiting event is either loss of APC function or oncogenic \(\beta\)-catenin mutations. However, although the presence of these initiating mutations would predict nuclear \(\beta\)-catenin accumulation throughout the tumor mass, heterogeneous intracellular distributions of this key Wnt signaling molecule are observed within primary tumors and their metastases. In particular, tumor cells located at the invasive front and those migrating into the adjacent stromal tissues show nuclear \(\beta\)-catenin staining. Hence, different levels of Wnt signaling activity reflect tumor heterogeneity and are likely to account for distinct cellular activities such as proliferation and epithelial-mesenchymal transitions, which prompt tumor growth and malignant behavior, respectively. Several intrinsic (cell-autonomous and/or autocrine) and extrinsic (paracrine, derived from the tumor microenvironment) factors may explain this heterogeneity of Wnt/\(\beta\)-catenin signaling activity within the tumor mass.

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**Introduction: Wnt signaling in epithelial homeostasis and cancer**

Epithelial cancers represent unique models to study the molecular and cellular mechanisms that underlie tumor onset, progression towards malignancy, and metastasis in distant organs. Moreover, the elucidation of these mechanisms has greatly contributed to the understanding of their essential role in preserving homeostasis in normal epithelial linings in mammals. Colorectal cancer (CRC) possibly embodies the most illustrative example, mainly because of two features. First, it arises and progresses through the adenoma-to-carcinoma sequence, a well-defined series of histological stages, each characterized by distinct mutations in well-known oncogenes and tumor suppressor genes. Second, in the vast majority of sporadic CRC cases in human, the rate-limiting and initiating event is represented by the constitutive activation of the Wnt/\(\beta\)-catenin signaling pathway [1].

The canonical Wnt signal transduction pathway is schematically illustrated in Figure 1a. In the absence of Wnt signaling, intracellular levels of \(\beta\)-catenin are regulated by a multiprotein complex encompassing kinases such as GSK3\(b\) (glycogen synthase kinase-3\(b\)) and CK1 (casein kinase 1), and the scaffolding proteins APC (adenomatous polyposis coli), Axin1 and Axin2 (conductin). This ‘destruction complex’ binds and phosphorylates \(\beta\)-catenin at serine and threonine residues, thus targeting it for ubiquitination and proteolytic degradation. In the presence of Wnt ligands, co-activation of the Frizzled and LRP (low-density lipoprotein receptor-related proteins) receptors leads to inhibition of the destruction complex and the consequent stabilization of \(\beta\)-catenin. Intracellular \(\beta\)-catenin accumulation eventually results in its nuclear translocation. In the nucleus, \(\beta\)-catenin binds to members of the TCF/LEF family of transcription factors, thus modulating expression of a broad range of target genes. In the majority of sporadic colorectal cancers and familial adenomatous polyposis (FAP), a hereditary predisposition to the formation of hundreds of benign adenomas in the colon–rectum, inactivating mutations in the APC gene underlie tumor formation [1] (Figure 1b, left panel). Loss of APC function leads to intracellular \(\beta\)-catenin stabilization and its constitutive signaling to the nucleus [2]. Notably, in colorectal cancers without APC mutations, \(\beta\)-catenin is found to carry oncogenic mutations that render it resistant to proteolytic degradation [3] (Figure 1b, right panel). In both cases, constitutive transcriptional deregulation of the broad spectrum of known Wnt target genes occurs (http://www.stanford.edu/~rnusse/). This altered gene expression pattern affects the finely tuned equilibrium between stemness, proliferation and differentiation in intestinal epithelial cells along the crypt-villus axis, leading to the disruption of tissue architecture and the formation of...
benign growths [4]. In addition to its central role in intestinal homeostasis and cancer, canonical Wnt signaling also regulates stemness, proliferation and differentiation in other adult stem cell niches, including the skin and hair follicle [5*], the mammary gland [6] and hematopoietic tissues [7]. Accordingly, constitutive activation of Wnt/β-catenin signaling triggers tumorigenesis in the skin [8], breast [9] and bone marrow [10].

**The β-catenin paradox**

As depicted in Figure 1, the Wnt signaling model predicts that each tumor cell within a colorectal cancer initiated by mutations in *APC* or β-catenin should exhibit intracellular and/or nuclear β-catenin accumulation. However, immuno-histochemical analysis of colorectal cancers reveals a very heterogeneous intracellular distribution of β-catenin: whereas well-differentiated parenchymal cells located in the tumor center retain membranous expression comparable to normal colon epithelium, nuclear β-catenin expression is predominantly observed in tumor cells localized at the invasion front and scattered in the adjacent stromal compartment [11] (Figure 2). Notably, tumor cells with nuclear β-catenin accumulation appear to undergo cell-cycle arrest [12] and epithelial–mesenchymal transitions (EMTs), as shown by the progressive loss of E-cadherin expression [13] and acquisition of mesenchymal markers such as fibronectin [14]. These alleged ‘migrating cancer stem cells’ (MCSCs) may retain the capacity to self-renew and differentiate, allowing them to transiently trans-differentiate and to invade adjacent tissues more efficiently (by EMT), and eventually to form metastases in distant organs by reciprocal mesenchymal–to-epithelial transitions (METs) (Figure 2) [15].

The non-random distribution of tumor cells with intracellular β-catenin accumulation within primary masses and distant metastases suggests a scenario where differential Wnt signaling activation regulates cancer stemness in a dosage-dependent fashion [16]: the initiating *APC* or β-catenin mutation is necessary but insufficient for full-blown Wnt activation and only tumor cells located at the invasive front are exposed to growth factors and cytokines able to further enhance β-catenin nuclear translocation. In addition, somatic mutations in other tumor suppressors and oncogenes may act synergistically in promoting Wnt signaling. Overall, both intrinsic (tumor cell-autonomous) and extrinsic (secreted by the tumor microenvironment) factors are likely to play rate-limiting roles in cancer stemness, local invasion and metastasis by differentially modulating Wnt/β-catenin and other signaling pathways such as Notch, TGFβ and Sonic hedgehog (Shh) [17]. In this review, we discuss the most relevant examples of these intrinsic and extrinsic Wnt signaling promoting factors and their effect on tumor growth and malignant behavior.

**Intrinsic Wnt/β-catenin-promoting factors**

Along the adenoma-to-carcinoma sequence, loss of *APC* function is usually followed by oncogenic activation of the *KRAS* oncogene. Also, the combination of *KRAS* oncogenic mutations and nuclear β-catenin accumulation at the invasive front is predictive of poor prognosis among CRC patients [18]. Recently, a study by Janssen *et al.* has shown that *APC* and *KRAS* mutations are synergistic in promoting β-catenin nuclear translocation, thus enhancing canonical Wnt signal transduction [19**]. Mice carrying compound *Apc* and *KRAS* mutations are characterized by a striking increase in intestinal tumor multiplicity and progression, when compared with *Apc*-only mutant animals. Reporter assays and immuno-fluorescence analysis indicated that cells with both oncogenic *KRAS* and loss-of-function *Apc* mutations are characterized by enhanced Wnt signaling activation, which is likely to underlie the observed increase in tumor onset and malignant transformation. Accordingly, the number of cells with nuclear β-catenin within intestinal tumors from compound *Apc/KRAS* animals was increased in comparison to those from *Apc*-only mutant mice [19**]. Activated *KRAS* is known to trigger tyrosine phosphorylation of β-catenin, leading to its release from E-cadherin at the adherens junction and increased Wnt signaling to the nucleus [20]. Similarly, somatic mutations in other genes in the RAS pathway, such as *BRAF* [21], or in members of the tyrosine kinase and phosphatome [22,23] frequently found in sporadic colorectal cancers are likely to enhance Wnt signaling through β-catenin tyrosine phosphorylation.

An alternative autocrine mechanism for increased Wnt signaling activation has been shown by the study by Bafico *et al.* [24**]. Colorectal carcinoma cells (HCT116) harboring β-catenin mutations were found to express canonical Wnt ligands including Wnt-3a. Notably, HCT116 engineered by homologous recombination to express only the wild type β-catenin allele did retain high levels of intracellular β-catenin. Expression of the Wnt antagonist *sFRP-1* (secreted frizzled-related protein) — which encodes a protein able to bind Wnt, dimerize with the Frizzled receptor and thereby form nonfunctional complexes — effectively down-regulated intracellular β-catenin levels in cells carrying the wild type allele but failed to do so in the β-catenin mutant HCT116. Likewise, *sFRP-1* expression significantly reduced the *in vivo* tumorigenicity of β-catenin wild type HCT116 cells but had no effect on the parental cells. These results clearly demonstrate the existence of an autocrine feedback loop for Wnt/β-catenin signaling in colorectal cancer, regardless of the presence of mutations in downstream components of the pathway. In the same study, breast and ovarian cancer cell lines were found to share a similar Wnt autocrine loop, although mutations in downstream components were not seen [24**]. Notably, *sFRP-1* expression is frequently down-regulated in breast cancer cases [25], and nuclear β-catenin accumulation is significantly correlated with poor prognosis [26].
Extrinsic Wnt/β-catenin-promoting factors

In addition to the above cell-autonomous mechanisms, the non-random distribution of tumor cells with nuclear β-catenin at the invasive front of colorectal cancers is likely to be at least partly explained by interactions with the tumor microenvironment. The invasive front of epithelial tumors represents a micro-ecosystem where myofibroblasts interact with parenchymal cells by producing extracellular matrix (ECM) and by secreting cytokines and growth factors that locally promote cell proliferation and invasion [27]. In fact, expression of canonical and non-canonical Wnt ligands has been shown by in situ RNA hybridization in both mesenchymal and epithelial cells of the small intestine and colon [28].

Nevertheless, the paracrine modulation of Wnt signaling in epithelial cells by myofibroblasts and other stromal cells is likely to be coordinated by a more complex network of promoting and inhibiting secreted factors. The mesenchymal forkhead transcription factors Foxf1 and Foxf2 have been shown to limit paracrine Wnt signaling and promote extracellular matrix production in the gut [29**]. In Foxf1 and Foxf2 mouse mutants, mesenchymal expression of Wnt5a is increased, possibly via Bmp4 down-regulation. The latter is accompanied by β-catenin nuclear accumulation in epithelial cells along the entire villus axis. Notably, inhibition of Sonic and Indian Hedgehog (Hh) signaling by cyclopamine reduces Foxf1 and Foxf2 expression, indicating cross-talk between intestinal fibroblasts and epithelial cells involving Hh, Bmp and Wnt signaling [29**]. Foxf1, another forkhead gene expressed in intestinal fibroblasts, also controls β-catenin intracellular accumulation in epithelial cells. Foxf2 mutant mice show increased expression of extracellular proteoglycans, which act as co-receptors for Wnt signaling activation and markedly expand the proliferative zone [30,31]. Likewise, loss of Foxf2 function in an Apc<sup>−/−</sup> genetic background strikingly increases gastric and colonic tumor multiplicity [32**]. In these Apc<sup>−/−</sup>/Foxf1<sup>−/−</sup> mice, the increase in the number of intestinal transient amplifying cells is thought to result from an accelerated rate of loss of heterozygosity (LOH) at the wild type Apc locus. Although nuclear β-catenin levels were increased in Foxf1<sup>−/−</sup> mice, they did not differ between the normal colon of Apc<sup>−/−</sup>/Foxf1<sup>−/−</sup> and Apc<sup>−/−</sup>/Foxf1<sup>+/+</sup> mice [32**]. Therefore, it is plausible to think that the primary cause of the observed increase in intestinal tumor numbers is a more subtle expansion of (cancer) stem cells and their progenitors caused by enhanced Wnt/β-catenin signaling activation.

In addition to Wnt ligands, other growth factors secreted by stromal cells in the tumor microenvironment are likely to enhance Wnt/β-catenin signaling to the nucleus. In colorectal cancer, hepatocyte growth factor (HGF) is found in the tumor microenvironment, where it binds its receptor (HGF-R, encoded by the MET proto-oncogene) [33], possibly promoting tumor invasion in a paracrine fashion. A recent study by Rasola and collaborators [34] has shown that HGF and β-catenin physically interact in a complex. Upon HGF stimulation of CRC cells, MET is dissociated from β-catenin followed by β-catenin’s tyrosine phosphorylation, thus enhancing Wnt signaling to the nucleus in a fashion similar to that observed for KRAS oncogenic activation [19**,20]. Moreover, HGF upregulates β-catenin expression via the phosphatidylinositol 3-kinase (PI3K) pathway. As well as causing increased expression and intracellular accumulation of β-catenin, HGF stimulation leads to increased cell scattering and motility, and apoptosis inhibition. Hence, the cross-talk between HGF secreted from the tumor microenvironment and Wnt/β-catenin signaling in colorectal cancer cells promotes tumor growth and invasion [34**].

Like HGF, other growth factors can activate EMT in epithelial cells by enhancing Wnt signaling. Platelet-derived growth factor (PDGF) has been shown to be expressed in several mesenchymal and epithelial cell types and to exert its effects on target cells by activating two tyrosine kinase receptors, namely PDGF-Rα and PDGF-Rβ [35]. Notably, a recent study by Yang and collaborators has revealed a novel Wnt-independent pathway that enhances β-catenin signaling to the nucleus [36**]. PDGF stimulation of HT-29, a colorectal cancer cell line harboring loss-of-function APC mutations, prompts the tyrosine phosphorylation of p68, a RNA helicase, by c-Abl kinase. Tyrosine-phosphorylated p68 binds β-catenin and inhibits its Ser/Thr-phosphorylation by GSK3β. The p68/β-catenin interaction is necessary for PDGF-induced nuclear accumulation of β-catenin and EMT stimulation of HT-29 cells [36**]. Given that epidermal growth factor (EGF) and transforming growth-factor-β (TGF-β) can also lead to p68 phosphorylation through receptor tyrosine kinases, the p68-driven enhancement of Wnt/β-catenin signaling may represent a common intracellular response to paracrine stimulation of EMT in tumor epithelial cells [36**,37].

The tumor-promoting role of inflammation is widely recognized and is consistent with the association between the increase of inflammatory cells in colorectal tumors and...
Heterogeneous patterns of β-catenin intracellular distribution in primary and metastatic colorectal cancer (CRC). (a) β-catenin immunohistochemical (IHC) analysis (performed as described in [13]) of a typical primary colorectal adenocarcinoma (i). While cells located in the inner tumor show membranous staining, the invasive front (ii) reveals clustering of cells with nuclear β-catenin accumulation (iv). A sentinel lymph node biopsy (iii) shows a similar degree of heterogeneity in β-catenin intracellular distribution with membranous and cytoplasmic staining in well-differentiated cells (v), and nuclear accumulation in parenchymal cells located along the outer rim of the lesion and scattered in the surrounding tissue (vi). (b) β-catenin IHC analysis of a typical liver colorectal metastasis (i and ii). The staining recapitulates the pattern of intracellular β-catenin distribution of the primary lesion with nuclear accumulation in cells at the outer rim of the main metastasis (iii) and scattered among the surrounding liver and inflammatory cells (iv).
their progression to malignancy. Progressive up-regulation of Wnt2 and Wnt5α expression was observed by in situ RNA hybridization in macrophages during the progression from colorectal adenoma to carcinoma [38]. This observation indicates the existence of yet another mechanism of paracrine Wnt activation by macrophages, and possibly other inflammatory cells. Non-steroidal anti-inflammatory drugs (NSAIDs) have been shown to effectively reduce the number and size of intestinal polyps in FAP patients carrying germline APC mutations and in Apc-mutant mice by inhibiting cyclooxygenase 2 (COX-2), one of the main enzymes involved in prostaglandin biosynthesis [39]. The preventive and even curative effects of NSAIDs in colorectal cancer point to a role for prostaglandin E2 (PGE2), the main metabolite of COX-2, in colorectal cancer progression. In a recent study, Castellone et al. elucidated an Axin1/β-catenin signaling mechanism triggered by PGE2 that enhances colon cancer cell growth [40**]. PGE2, through its heterotrimeric guanine nucleotide-binding protein (G protein)-coupled receptor EP2, activates PI3K and the protein kinase Akt by free G protein βγ subunits and the direct association of a G protein subunit with Axin1. This results in the inactivation and release of GSK3β from the destruction complex and the consequent intracellular accumulation of β-catenin [40**]. Together with the increased expression of Wnt ligands by tumor-infiltrating macrophages [38], these findings provide a molecular basis for the activation of paracrine Wnt signaling by inflammatory cells in colorectal cancer.

**Downstream effectors of Wnt/β-catenin signaling depend on signal strength**

Enhanced canonical Wnt signaling in colorectal cancer cells located at the invasive front has been postulated to trigger EMTs, thus promoting local invasion [15]. As discussed above, both Wnt autocrine and paracrine mechanisms have been elucidated that might explain the observed intra-tumor heterogeneity in intracellular and nuclear β-catenin accumulation. However, the downstream effects resulting from different levels of Wnt/β-catenin signaling and in particular those affecting cell proliferation, EMT and local invasion are as yet poorly understood.

That different strengths of Wnt/β-catenin signaling differentially affect cell fate in adult stem cell niches by activating specific genetic programs has been elegantly shown in the skin and hair follicle [41]. Here, a stem cell population capable of giving rise to all the differentiated lineages of the epidermis, sebaceous gland and hair follicle is localized within the bulge, a permanent structure in the outer root sheath of the hair follicle. These stem cells are quiescent and can be visualized by their long-term capacity to retain labeling in pulse-chase experiments with BrdU. Lowry and coworkers showed that low-level Wnt/β-catenin signaling triggers the proliferation and mobilization of bulge stem cells, causing new hair follicle growth without affecting the morphology and size of the niche [57]. Accordingly, genetic ablation of β-catenin results in loss of label-retaining capacity and stem cell marker expression in cells located in the bulge. These changes are accompanied by specific transcriptional profiles encompassing genes known to regulate the equilibrium between bulge stem cell quiescence and proliferation, and hair growth [57]. Higher levels of canonical Wnt signaling in the stem cell progeny activate different transcriptional programs directing proliferation of keratinocytes, hair morphogenesis and differentiation. This differential activation of Wnt/β-catenin signaling along the hair follicle is likely to be caused by the differential expression of a complex network of secreted Wnt antagonists (sFRP1, Dickkopf3), and transcription-promoting (Lef1) and -inhibiting (Tcf3) factors [41].

Apart from the direct transcriptional responses to different Wnt/β-catenin signaling strengths, secondary mechanisms acting at the protein level may also underlie tumor invasion and malignant behavior upon enhanced Wnt activation. BCL9-2 (also known as BCL9L or B9L) is a BCL9 homologue originally identified because of its ability to bind tyrosine phosphorylated β-catenin in a yeast two-hybrid screen [42]. BCL9-2 has been shown to represent one of the main factors in the regulation of the switch between the distinct functions of β-catenin in cell adhesion and Wnt signaling. Upon tyrosine phosphorylation of β-catenin (by kinases like MET [34]) or as a result of oncogenic KRAS activation) [19**,20], the resulting BCL9-2/β-catenin complex cannot interact with α-catenin, thus interfering with the adhesive functions of the E-cadherin–catenin complex [43**]. However, the binding of BCL9-2 to β-catenin does not affect its interaction with TCF/LEF transcription factors and localizes it to the nucleus, where it increases transcription of Wnt target genes [43**]. Moreover, BCL9-2 up-regulation in colorectal cancer cells is required for enhanced Wnt signaling and activation of the invasive phenotype [44**]. Accordingly, BCL9-2 down-regulation by siRNA reverses this process by inhibiting cell migration. Finally, the observation of increased BCL9-2 expression in low-grade colorectal tumors in comparison to normal mucosa is indicative of the important role of this gene in enhancing Wnt signaling activation in the progression of adenomas towards more dysplastic and advanced stages [44*,45].

An Axin2-dependent pathway leading to the stabilization of the transcription factor Snail1, a key EMT-inducing gene, has recently been elucidated in breast cancer cells [46*]. Axin2 is a transcriptional downstream target of canonical Wnt signaling and an integral member of the destruction complex that earmarks β-catenin for proteolytic degradation. Moreover, it can also act as a chaperone of GSK3β, regulating its subcellular distribution between
the nucleus and the cytoplasm. By doing so, Axin2 controls GSK3β-mediated phosphorylation of multiple target proteins, including c-Myc, Cyclins and Snail1 [47]. Upon Wnt/β-catenin activation, Axin2 is upregulated and redirects GSK3β compartmentalization to the cytoplasm, leaving Snail1 in its unphosphorylated and transcriptionally active form. Snail1 stabilization subsequently represses E-cadherin expression and induces EMT and tissue invasion [46]. Hence, Axin2, and possibly also Axin1, can act both as tumor suppressor genes, by serving a scaffolding function within the destruction complex, and as oncogenes, by promoting EMT when upregulated. As axin mutations have been frequently reported among CRC cases [48], similar mechanisms for modulating EMT and local invasion are also likely to be active in cancers of the gastrointestinal tract.

Conclusions
The heterogeneous intracellular localization of β-catenin within colorectal tumors and the non-random distribution at the invasive front of parenchymal cells with nuclear accumulation of this key factor in canonical Wnt signaling can be explained by several intrinsic (cell-autonomous, autocrine) and extrinsic (paracrine) factors acting locally in response to the tumor microenvironment. As observed in several adult stem cell niches, such as the intestinal crypt, the hair follicle and the mammary gland, Wnt/β-catenin signaling regulates self-renewal and differentiation in a dosage- and context-dependent fashion. Likewise, the interaction between epithelial tumor cells and the different components of the surrounding microenvironment — including tumor-infiltrating inflammatory cells, myofibroblasts and other stromal cells — can locally affect the intracellular levels of canonical Wnt signaling and differentially trigger stemness, cell proliferation, EMT and invasive behavior. In a proportion of breast and colon cancer cases [49], similar local effects are achieved by somatic mutations in genes such as KRAS and BRAF, and in members of the kinome and phosphatome. A combination of autocrine and paracrine factors differentially modulates Wnt signaling activation and locally promotes migration and invasion. The prospective isolation of these Wnt-activated migrating cancer stem cells and the characterization of their transcriptional, epigenetic and protein profiles will allow the elucidation of the genetic programs activated in this clinically relevant sub-population of tumor cells and will open the way for novel and tailor-made diagnostic and therapeutic approaches.

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References and recommended reading
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- of special interest
- of outstanding interest

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This study demonstrates that canonical Wnt signaling prompts tumor cell de-differentiation and tissue invasion through an Axin2-dependent pathway that stabilizes Snail1, a key regulator of normal and neoplastic EMT programs. Axin2 regulates EMT by controlling intracellular distribution of GSK3β, the kinase responsible for controlling Snail1 protein turnover and activity.

