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Review

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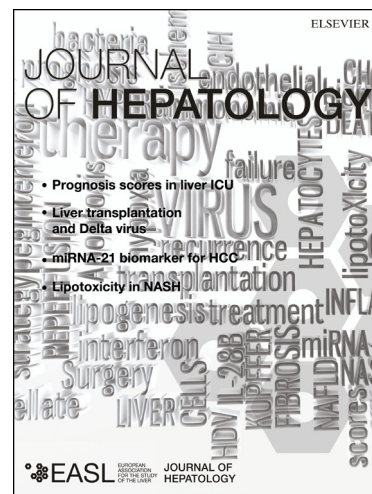
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**Wnt signaling and hepatocarcinogenesis: molecular targets for the
development of innovative anticancer drugs**

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HCC, Hepatocellular carcinoma; FZD, Frizzled; CSC, cancer stem cells; PCP, Planar Cell Polarity; TCF/LEF, T-cell factor/lymphoid enhancer factor; TLE-1, Transducin Like Enhancer-1; APC, Adenomatous Polyposis Coli protein; GSK3 β , Glycogen Synthase Kinase 3 β ; CK1, Casein Kinase 1; β -TRCP, beta-transducin repeat containing protein; SCF, Skp1/cullin F-box complex; Dvl/Dsh, disheveled; Pygo, pygopus; CBP, CREB-Binding Protein; PKC, Protein Kinase C; NFAT, Nuclear Factor of Activated T-cell; NLK, NemoLike Kinase; Wif1, Wnt inhibitory protein-1; sFRP, secreted FZD-related proteins; Dkk, Dickkopf; Krm, Kremen; Rspo, R-spondin; Gpc3, Glypican-3; DIFs, Differentiation-Inducing Factors.

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Abstract: (236 words)

Hepatocellular carcinoma (HCC) is one of the most common causes of cancer death worldwide. HCC can be cured by radical therapies if early diagnosis is done while the tumor has remained of small size. Unfortunately, diagnosis is commonly late while the tumor has grown and spread. Thus, palliative approaches are usually applied such as transarterial intrahepatic chemoembolization and sorafenib, an anti-angiogenic agent and MAP kinase inhibitor. This latter is the only targeted therapy that has shown significant, although moderate, efficiency in some individuals with advanced HCC. This highlights the need to develop other targeted therapies, and to this goal, to identify more and more pathways as potential targets. The Wnt pathway is a key component of a physiological process involved in embryonic development and tissue homeostasis. Activation of this pathway occurs when a Wnt ligand binds to a Frizzled (FZD) receptor at the cell membrane. Two different Wnt signaling cascades have been identified, called non-canonical and canonical pathways, the latter involving the β -catenin protein. Deregulation of the Wnt pathway is an early event in hepatocarcinogenesis and has been associated with an aggressive HCC phenotype, since it is implicated both in cell survival, proliferation, migration and invasion. Thus, component proteins identified in this pathway are potential candidates for pharmacological intervention. This review focuses on the characteristics and functions of the molecular targets of the Wnt signaling cascade and how they may be manipulated to achieve anti-tumor effects.

Introduction

HCC represents a major public health problem with a high impact on society. HCC is the sixth most common tumor worldwide in terms of incidence (about one million *per year*). Projections are that this incidence will substantially increase during the next decades due to persistent infection with the hepatitis C virus as well as the emergence of non-alcoholic steato-hepatitis as a major health problem. HCC portends a poor prognosis since ranking third in terms of “cause of death” by cancer, and often presents as a major complication of cirrhosis related to chronic hepatitis B and C infections, or nonvirus-related [1-3]. The dismal prognosis is generally related to a late diagnosis after HCC cells have infiltrated the liver parenchyma as well as spread through the portal venous system and/or form distant metastases. However, if HCC is diagnosed early (< 20% of patients), these smaller tumors may be cured by surgical resection, liver transplantation or radiofrequency ablation. In more advanced tumors (> 80% of patients at diagnosis), only palliative approaches can be applied. In this regard, transarterial intra-hepatic chemoembolization has been shown to be somewhat effective for increasing overall survival of individuals with tumors that have spread only into the liver parenchyma without extrahepatic metastasis (median overall survival is increased from 15 to 20 months compared to the best supportive care). In HCC with extra-hepatic spread, only sorafenib, an anti-angiogenic and MAP kinase inhibitor, has been shown to increase overall survival of patients (from 8 to 11 months) [4]. All other systemic approaches such as cytotoxic chemotherapy have not been shown to be effective; thus, to date, no targeted therapy except sorafenib, has been proven to prolong life in patients with HCC. However, there are ongoing or ended clinical trials with agents that target FGF, VEGF, PDGF, EGF, IGF, mTOR and TGF β signaling pathways but none yet has been shown to have a significant impact on patient survival [5].

Recently, cancer stem cells (CSC) have been hypothesized to play a key role in tumor maintenance as well as relapse after surgical resection. There is accumulating information that supports a role for CSC in hepatocarcinogenesis to maintain the tumor size and to initiate tumor recurrence following therapy [6]. The pool of CSC is maintained by self-renewal capabilities that are largely driven by reactivation of embryonic signaling programs mediated by Wnt, Notch, Bmi and Hedgehog pathways, similar to what has been previously demonstrated during breast carcinogenesis [7]. Preclinical studies further underline the potential value of inhibiting activation of these signaling programs in some tumor types [8-11].

In this review, we describe the features of a therapeutic target, *i.e.* the Wnt pathway, for potential therapy of HCC. We will discuss experimental and preclinical studies regarding the use of Wnt inhibitors as a therapeutic approach for HCC.

The Wnt-mediated signaling

The first member of the Wnt family of ligands was identified from the *int-1* gene found in a mammary adenocarcinoma, located at the integration site of the Mouse Mammary Tumor Virus (MMTV); subsequently, it was demonstrated to have oncogenic properties [12]. More important, *int-1* homolog genes have been found in human tumors as well [13]. In addition, a highly conserved *int-1* homolog was also discovered in drosophila and designated *Wingless* “Wg” [14]. The combination of *int-1* and *Wingless* led to the common Wnt1 terminology and recently has been used to designate the Wnt family of ligands [15].

Wnt proteins are secreted extracellular auto-paracrine glycoproteins that interact with Frizzled receptors (FZD), a seven transmembrane domain protein, resembling the G-protein-coupled receptor (GPCR) family. Vinson and colleagues revealed that FZD contains an extracellular cysteine-rich domain (CRD) which is the putative binding site for the Wnt

ligands. These investigators demonstrated the functional role of the *frizzled* locus to coordinate development of the cytoskeleton in drosophila epidermal cells [16]. Subsequently, Wnt/FZD-mediated signaling has been extensively studied, and although it has been widely implicated in cellular homeostasis, these ligand/receptor interactions have now been appreciated as key factors during the oncogenesis process and therefore, could serve as new therapeutic targets.

Thus, Wnt proteins represent members of a highly conserved family that is involved in several processes including embryonic development, cell fate determination, proliferation, polarity, migration and stem cell maintenance. In addition, Wnt/beta-catenin signaling has been found to play key roles in metabolic zonation of adult liver, regeneration [17]. In adult organisms, deregulation of Wnt signaling may lead to tumor development [18, 19]. The Wnt-mediated pathway is activated through the binding of one Wnt ligand to a FZD receptor. Ten different FZD receptors and 19 Wnt ligands have been identified in humans. The binding of Wnt to a FZD receptor can trigger activation of at least three different pathways. The first is the Wnt/ β -catenin cascade, also called the Wnt-canonical pathway; the remaining two are the Planar Cell Polarity (PCP) and the Wnt/Calcium pathways, respectively. The two latter are β -catenin independent and represent examples of the non-canonical cascades. In this regard, a multitude of combinations between the 19 Wnt ligands and the 10 FZD receptors such as co-receptors and other molecules are theoretically possible. Classically, Wnt1/2/3/3a/8a/8b/10a/10b and FZD1/5/7/9 are classified as the canonical elements, whereas Wnt4/5a/5b/6/7a/7b/11 and FZD2/3/4/6 are designated as non-canonical components. The remaining Wnt2b/9a/9b/16 and FZD8/10 proteins remain unclassified [19, 20]. However, it remains elusive how selectivity between Wnt/FZD as well as specificity of downstream signaling are achieved. Some Wnt/FZD elements can share dual canonical and non-canonical functions. For instance it has been shown that in absence of Ror2 co-receptors, Wnt5a can

activate β -catenin signaling with FZD4 and Lrp5 [21]. FZD3 has been described to act likely through canonical pathway in mice neurogenesis [21]. Zhang et al. demonstrated that in *Xenopus* foregut, FZD7 can activate low level of β -catenin and non-canonical JNK signaling in which both pathways contributed to foregut fate and proliferation while JNK pathway regulated cell morphology [22]. It is of interest that canonical and non-canonical pathways can not only be driven by specific Wnt/FZD combinations, but also by the cell type, differentiation status, localization and composition of the microenvironment [23].

The canonical Wnt/FZD pathway

The β -catenin protein, encoded by the *CTNNB1* gene, is a key component in Wnt-canonical pathway signaling. β -catenin has a central region which presents armadillo domain repeats important for the binding of partners, such as Axin1 and Adenomatous Polyposis Coli protein (APC) as well as transcription factors [24]. The C- and N-terminal regions are important. C-terminus of β -catenin serves as a binding factor for a multitude of complexes promoting β -catenin-mediated transcription, whereas phosphorylation of the N-terminus promotes degradation of β -catenin. Indeed, β -catenin may be present in several cellular compartments, such as the inner plasma membrane having a role in cell-cell junctions, the cytoplasm and the nucleus where it forms an active complex containing TCF/LEF transcription factors (T-cell factor/lymphoid enhancer factor) [25]. In the absence of nuclear β -catenin, TCF/LEF interact with the transcriptional co-repressor Transducin Like Enhancer-1 (TLE-1) (*Drosophila* homolog Groucho), thus preventing β -catenin target gene expression [26]. Following translocation into the nucleus, β -catenin binds TCF/LEF and replaces the TLE-1 repressor to form a transcriptional complex that activates the expression of its target genes (**Fig. 1**).

In absence of the canonical Wnt signaling, cytosolic β -catenin is targeted for degradation by a complex composed of a scaffold of proteins named Axin1, APC, and two

serine/threonine kinases: the Glycogen Synthase Kinase 3 β (GSK3 β) and the Casein Kinase 1 (CK1) [27] (**Fig. 1A**). Axin1 and APC act together as scaffolding proteins through binding of β -catenin, and enhance its N-terminal phosphorylation by GSK3 β and CK1. The first phosphorylation event is generated by CK1 at Ser45 which allows the GSK3 β -mediated sequential phosphorylation of Thr41, Ser37 and Ser33 [28, 29]. Ser37 and Ser33 phosphorylations provide a binding site for the E3 ubiquitin ligase β -TRCP (β -transducin repeat containing protein), leading to β -catenin ubiquitination in a β -TRCP/Skp1/cullin F-box complex (SCF) dependent manner followed by proteasomal degradation [30, 31].

Activation of the canonical Wnt signaling cascade leads to disruption of the β -catenin degradation complex, resulting in β -catenin accumulation in the cytoplasm followed by translocation into the nucleus where it serves as a transcription factor to activate downstream target genes (**Fig. 1B**). In brief, this process is as follows: Wnt ligand binds to the extracellular domain of a FZD receptor and Lrp5/6 co-receptors. This ternary complex (Wnt/FZD/Lrp) recruits the scaffolding phosphoprotein dishevelled (Dvl/Dsh) at the plasma membrane which in turn traps the Axin-bound-GSK3 β complex, thus preventing proteasomal degradation of cytosolic β -catenin. When stabilized, β -catenin is able to translocate into the nucleus, where it binds to TCF/LEF transcription factors and then forms a transcriptionally active complex with pygopus (Pygo), CBP (CREB-Binding Protein) and Bcl9 proteins [32]. In mammals, four *TCF* genes have been described, which adds further complexity to the mechanism(s) of activation of the Wnt canonical cascade [33]. Of notice is the β -catenin pool localized at plasma membrane that plays a key role in cell-cell junctions. To this aim, a complex including either p120 catenin/ γ -catenin(plakoglobin)/ α -catenin or p120 catenin/ β -catenin/ α -catenin [25] binds the cytoplasmic carboxyl terminus domain of E-cadherin adhesion molecule, in order to join cadherins to the actin cytoskeleton. More precisely, p120 catenin bind the juxtamembrane and then β -catenin or γ -catenin binds to the cytoplasmic

domain of E-cadherin. The remaining α -catenin served as a link between actin and β/γ -catenin which lead to the stabilization of cell adhesion [34]. The possible consequences of inhibiting β -catenin at adherent junctions has to be discuss in regard of their role in epithelio-mesenchymal transition (EMT). Disruption of E-cadherin-mediated adherent junctions is a major event in EMT [35] and because of the interplay between cadherin-mediated cell adhesion and canonical/ β -catenin signaling [36], targeting β -catenin could also promote the disruption of these junctions leading to enhance EMT. However Wickline et al. have shown that in hepatocyte-specific β -catenin-conditional null mice, γ -catenin is upregulated and associated with E-cadherin and actin to maintain adherent junctions. In addition, no nuclear γ -catenin was detected in liver of KO mice, leading to the conclusion that despite armadillo domains on γ -catenin, there is no compensation at nuclear level. Nevertheless, authors warn us about preventing concurrent γ -catenin suppression that may increase tumor cell invasion [37]. More recent study confirmed these results in in vitro experiments with HCC cell lines and identified the mechanism of γ -catenin stabilization as serine/threonine phosphorylation induced by protein kinase A [38]. In regard to this recent data, targeting β -catenin in HCC therapies may not disturb cell junctions since the design of Wnt inhibitors for therapeutic intervention specifically design soluble active β -catenin as preferential target.

The non-canonical Wnt/FZD pathways

In contrast to the canonical Wnt pathway, non-canonical signaling does not depend on β -catenin and requires Ror2/Ryk co-receptors instead of Lrp5/6 (**Fig. 2**). In the Wnt/PCP pathway, Wnt/FZD interaction promotes the recruitment of Dvl/Dsh, which in turn binds to the small GTPase protein called Rac, leading to both the induction of ROCK (Rho-associated protein kinase) pathway and the activation of the MAP kinase cascade and subsequently to the activation of AP1-mediated target gene expression [39, 40]. In the Wnt/Calcium pathway, the

complex formation between FZD, Dvl/Dsh and G proteins results in PLC (Phospho Lipase C) activation which cleaves PIP2 (Phosphatidyl Inositol 4,5 biphosphate) into DAG (DiAcylGlycerol) and IP3 (Inositol 1,4,5-triphosphate). This process results in the activation of PKC (Protein Kinase C) through DAG while IP3 promotes calcium release from the endoplasmic reticulum. Increased intracellular concentration of calcium enhances phosphorylation and activation of PKCs. This also triggers the activation of Ca²⁺-calmodulin-dependent Calcineurin and CAMKII (Ca²⁺-calmodulin dependent kinase II), leading to NFAT (Nuclear Factor of Activated T-cell) and NLK (Nemo Like Kinase) translocation, respectively. NLK acts as a β -catenin pathway inhibitor through phosphorylation and degradation of TCF/LEF transcription factors [41].

Antagonists and agonists of Wnt/FZD-mediated signaling

Several secreted proteins are known to negatively or positively regulate the Wnt/FZD complex. Four classes of antagonistic molecules have been described. Wnt inhibitory protein-1 (Wif1) and secreted FZD-related proteins (sFRP1, 2, 3, 4, 5) bind and sequester the soluble Wnt ligands, thus inhibiting their interaction and binding to FZD receptors [42-45]. The Dickkopf family is composed of four members (Dkk1, 2, 3, 4) that can interact with both Lrp5/6 and Krm1,2 (Kremen1,2) co-receptors [46]. The ternary complex Lrp-Dkk-Krm prevents β -catenin stabilization by promoting Lrp5/6 endocytosis [47]. Wise and Sost proteins form the other class of secreted antagonists. They bind Lrp5/6 and thus disrupt the Wnt-induced FZD-Lrp5/6 interaction [48, 49].

Three agonistic molecules have recently been identified; the R-spondins (Rspo1, 2, 3, 4), Norrin and Glypican-3 (Gpc3). The Gpc3 is a heparan sulfate proteoglycan bound to the cell membrane through a glycosyl-phosphatidylinositol anchor. Gpc3 increases autocrine/paracrine canonical Wnt signaling by binding to Wnt ligands, thus facilitating the interaction between Wnt ligands and FZD receptors [50]. Mechanisms by which Rspo and

Norrin activate the canonical Wnt pathway have not been clarified. Rspo1 is able to bind to both Lrps and FZDs but it has also been proposed that Rspo prevents Lrp6 internalization through binding to Krm instead of Dkk [51-53].

Wnt signaling deregulation in human hepatocarcinogenesis

Similar to other tumor tissue types, the canonical Wnt/FZD signaling is a critical contributor to HCC pathogenesis. Indeed, 40-70% of HCCs harbor nuclear accumulation of the β -catenin protein, one of the hallmarks of the Wnt/ β -catenin pathway activation [54-56]. Activating mutations of the β -catenin gene (*CTNNB1*) occur in 8-30% of tumors, while loss-of-function/mutations in *APC* and *Axin* genes occur in 1-3% and 8-15%, respectively and are mutually exclusive to *CTNNB1* mutations [54, 57-62]. Some observations suggest that the *CTNNB1* mutation could be a late event during hepatocarcinogenesis. However, accumulation of β -catenin was detected in the early stage of HCC development, suggesting that other mechanisms could contribute to β -catenin stabilization (**Table 1**) [60, 63]. Strikingly, extrinsic activation of Wnt/ β -catenin pathway and *CTNNB1* mutation do not lead to the same molecular expression pattern, supporting different roles for wild-type and mutated β -catenin. The Wnt/ β -catenin activated HCC sub-class with a *CTNNB1* mutation is characterized by upregulation of liver-specific Wnt-targets, low grade and well-differentiated tumors, with chromosome stability and a favorable prognosis. The Wnt/ β -catenin activated HCC sub-class without *CTNNB1* mutation is characterized by dysregulation of classical Wnt targets, high chromosomal instability, aggressive phenotype and is preferentially associated with chronic HBV infection [54, 63, 64].

Modulation of Wnt ligands or FZD receptor expression could account for Wnt/ β -catenin pathway activation without any other mutations in *CTNNB1*, *APC*, or *Axin* genes. Indeed, upregulation of activators, such as ligands (Wnt1/3/4/5a/10b) or receptors/co-receptors

(FZD3/6/7, Lrp6), and downregulation of inhibitors (sFRP1/4/5, Wif1, Dkk3, Dkk4) have been reported both in HCC tumors and surrounding precancerous liver tissues, which emphasizes that their over and/or under-expression may be early molecular events during hepatocarcinogenesis [65-73].

Although β -catenin activation is crucial for liver development and regeneration, it is not sufficient *per se* for initiation of hepatocarcinogenesis. Indeed, animal models overexpressing an active β -catenin protein do not spontaneously form HCC [74-76]. However, β -catenin activation may cooperate with other oncogenic pathways such as Insulin/IGF-1/IRS-1/MAPK, H-RAS, MET, AKT and chemicals to induce HCC formation in mice [75, 77-79]. It's described that beta-catenin mutation is a late event in hepatocarcinogenesis since present in some HCC tumors whereas absent in preneoplastic lesions, thus prompting us to speculate that only nonmutated beta-catenin could play a role in very early steps of hepatocarcinogenesis such as initiation and promotion. However, mutated forms of beta-catenin are used in experimental models to assess the role of activated beta-catenin in hepatocarcinogenesis. In these experimental mouse models, it's well shown that mutated beta-catenin is insufficient alone and *per se* for initiation of HCC but only enhance tumor promotion either in a context of chromosomal instability and increase of susceptibility to DEN-induced HCC formation [78, 80], or in a context of *Lkb1*^{+/-} mice that spontaneously develop multiple hepatic nodular foci (NdFc) followed by HCC [81], or in a context of H-Ras transgenic mice where mutated beta-catenin appears as a strong carcinogenic co-factor collaborating with the mutated Ras oncogene [82]. In contrast and apparently paradoxically, invalidation of beta-catenin in hepatic beta-catenin conditional knockout mice has been found as enhancing DEN-induced tumorigenesis [83]. Of interest is an another model of HCC developing in mice under exposure to phenobarbital (PB, potent tumor promoter in mouse liver) and DEN as tumor initiator. A tumor initiation–promotion study was conducted in mice

with conditional hepatocyte-specific knockout (KO) of Ctnnb1 and in Ctnnb1 wild-type controls. As expected, DEN+PB strongly enhanced liver tumor formation in Ctnnb1 wild-type mice. Amazingly, the prevalence of tumors in Ctnnb1 KO mice was 7-fold higher than in wild-type mice, suggesting an enhancing effect of the gene KO on liver tumor development [84]. *Thus there is a paradox where absence of wild-type beta-catenin or presence of the mutated form, both lead to enhanced DEN-induced hepatocarcinogenesis. The issue is that the discussion is speculative since the mechanism of increased HCC in conditional beta-catenin KO is unknown. In the design of Wnt inhibitors for therapeutic intervention, these agents do target the Wnt pathway through the soluble beta-catenin cascade, but do not impact on invalidation of the beta-catenin pool involved in the membrane catenin/cadherin complexes involved in cell homeostasis. The beta-catenin therapeutic targeting may need to be personalized, based on the unexpected findings of enhanced tumorigenesis after chemical exposure in hepatocyte-specific beta-catenin conditional knockout mice.*

Although the role of Wnt/ β -catenin pathway is debated with respect to the initiation of hepatocarcinogenesis, it is definitively implicated in determining HCC aggressiveness, due to its promotion of increased cell proliferation, migration and invasion. This finding has been further substantiated by ectopic expression of Wnt3 and FZD7, Lrp6 or down-regulation of sFRP1, Dkk1 and Dkk4 in HCC cell lines [66, 69, 73, 85, 86]. Moreover, recent studies have revealed that the Wnt/ β -catenin pathway is also involved in the self-renewal and expansion of HCC initiating cells (*i.e.* the so-called liver CSC) which also influences tumor aggressiveness and resistance to chemo- radio-therapeutic agents [87, 88]. Furthermore, Wnt/FZD-mediated signaling could influence tumor microenvironment that supports tumor survival, growth, and size. Recent investigations emphasize the role of sFRP1 in the induction of senescence of tumor-associated fibroblasts after chemotherapeutic treatment [89]-[90, 91].

It is noteworthy that the canonical and non-canonical Wnt/FZD pathways may have complementary roles in the pathogenesis of HCC. Indeed, β -catenin activation appears to be involved in the tumor initiation phase of hepatic oncogenesis, whereas subsequent activation of non-canonical pathways associated with inactivation of β -catenin may enhance tumor promotion and progression [88]. However, non-canonical pathways can also exhibit opposite effects on tumor behavior, since specific Wnt/FZD combinations are able to function as tumor suppressors [92]. Although little is known about the role of Wnt/PKC pathway in HCC, it has been demonstrated that inhibition of PKC β activity reduces motility and invasion properties of HCC cells [93]. Finally, activation of the Wnt/JNK pathway during HCC progression would presumably support tumor growth, since enhanced JNK activity appears to be involved in HCC cell proliferation both *in vitro* and *in vivo* [94].

Identification of molecular targets for therapeutic interventions

There is some evidence to link the Wnt pathway activation to tumor cell properties characteristic of the malignant phenotype, such as enhanced cell proliferation, migration and invasion, which raises the possibility to target members of this signaling cascade as an attractive therapeutic approach for treatment of HCC [95, 96] (**Fig. 3**).

Targeting extracellular molecules of the Wnt pathway

Antibody-based therapies directed against the overexpressed Wnt ligands and FZD proteins could provide a therapeutic approach. For instance, preclinical experiments have shown that an anti-Wnt1 monoclonal antibody inhibits the Wnt signaling pathway resulting in enhanced apoptosis and inhibiting cell proliferation, both *in vitro* and *in vivo* in a xenograft model of HCC [67]. These findings have been experimentally validated for several other types of tumors, such as sarcomas, colon, breast, non-small-cell lung cancer and head-neck squamous cell carcinomas [97-100]. Interestingly, as demonstrated with a colon cancer cell

line, this anti-Wnt antibody was able to induce apoptosis even in the presence of downstream mutations in *APC* or *CTNNB1* genes and appeared to be synergistic with docetaxel chemotherapy with respect to therapeutic response [97]. Although not tested in HCC tumors thus far, anti-Wnt2 antibodies may be useful to inhibit the Wnt/ β -catenin cascade. Such antibodies induce apoptosis and inhibit tumor growth *in vivo* in several tumor types, including melanoma, mesothelioma and non-small-cell lung cancer [101-103]. Since non-canonical pathways seem to be implied in tumor progression, the inhibition of Wnt-related ligand could be considered for therapy. For instance, WNT5A, which seems to be involved in non canonical pathway in HCC [88], could be antagonized by the use of anti-WNT5a antibodies. Indeed, in gastric cancer cells where WNT5A activates non canonical pathway, its inhibition reduces migration and invasion activities *in vitro* and *in vivo* [104]. **Nevertheless, since the noncanonical pathway could antagonize the canonical one, it might be deleterious to inhibit the former.** Anti-FZD7 antibodies that induce apoptosis and decrease cell proliferation both *in vitro* and *in vivo* of FZD7 positive Wilms' tumor cells are also available [105]. More recently, a multispecific antibody that targets both FZD 1, 2, 5, 7 and 8 and mainly affects the canonical signaling pathway, has been developed. It triggers a therapeutic reduction of breast, colon, lung and pancreas tumor growth and synergizes with other chemotherapeutic agents as well [106]. Strikingly, this antibody remains effective even in tumor cells with *APC* or *CTNNB1* gene mutations. In addition, FZD co-receptors could also be attractive targets for monoclonal antibody therapy since, in a retinal pigment epithelial cell line, anti-Lrp6 antibody has been shown to inhibit Wnt signaling [107].

Another therapeutic strategy would be to trap the endogenous Wnt ligands with the exogenous soluble form of FZD receptors. This approach was reported for FZD7 by Tanaka and colleagues in esophagus carcinoma cells and confirmed later in HCC cells [86, 108]. More recently, Wei and coworkers have developed the same approach using a FZD7

extracellular domain peptide (sFZD7) that can bind and sequester the soluble Wnt3 ligand. This peptide decreased the viability of HCC cell lines with high specificity, since normal hepatocytes were not sensitive to sFZD7. Moreover, sFZD7 cooperates with doxorubicin to reduce HCC cell proliferation *in vitro* and in a xenograft murine model as well. Interestingly, it has been shown to be highly efficient and independent of the β -catenin mutational status [109]. Inhibition of Wnt secretion by the small molecules, IWP2 and Wnt-C59 may also prevent autocrine Wnt signaling activation, as observed in colon cancer cell lines. These small molecules are also able to inhibit the progression of mammary tumors in Wnt1 transgenic mice [110, 111]. Addition of Wnt antagonist, such as sFRP1 or Wif1, has shown encouraging therapeutic results in HCC cell lines by blocking the Wnt/ β -catenin signaling. These soluble molecules induce apoptosis, reduce angiogenesis and cell proliferation both *in vitro* and *in vivo* and are not influenced by the *CTNNB1* mutation status [112]. Other Wnt antagonists such as sFRP2 and sFRP5 should also be considered, since they show similar treatment effects in colon cancer as sFRP1 exhibits in HCC [113]. Interestingly, Dkk1 and sFRP1 addition cooperates with anti-FZD7 antibodies to increase apoptosis in Wilm's tumor demonstrating the importance of combinatorial therapies [105]. Therapeutic small molecules such as niclosamide and silibinin, display anti-tumor activity *in vitro* and *in vivo* by suppressing Lrp6 expression, leading to inhibition of Wnt/ β -catenin signaling in human prostate and breast tumor cells, as well as by promoting induction of apoptosis [114, 115].

Targeting the Wnt-mediated pathway in the cytosol

The straight-in approach to inhibit Wnt/ β -catenin pathway is to directly target β -catenin by small interfering-RNA or antisense based therapy, which can reduce cell proliferation and survival of HCC cell line, providing a proof of principle for this approach [116-118]. However, its potential use as a therapeutic tool remains unlikely since β -catenin protein is

essential for cell junction. Thus, targeting the soluble active pool of β -catenin seems more appropriate.

The interaction between the cytosolic tail of FZD and its adaptor Dvl protein is of importance in mediating Wnt signaling. A proof-of-principle has clearly been established in HCC cells, by using small interfering peptides capable of entering the tumor cells and disrupting the interaction between a specific motif on the FZD7 cytosolic tail and the PDZ domain of Dvl [11]. Similar results have been obtained in melanoma and non-small-cell lung cancer cells with small molecules using this same strategy [119].

Targeting the β -catenin destruction complex (APC, Axin, CK1 and GSK3 β) as a therapeutic target has not been assessed in HCC so far. However, using other tumor model systems, such a strategy has demonstrated some potential. Since Axin1 overexpression induces apoptosis in HCC harboring *APC*, *Axin1* or *CTNNB1* mutations, stabilization of Axin1 would be an attractive approach to trigger β -catenin degradation [120]. This may be achieved by using inhibitors of the Axin1 or/and 2 degradation, such as the small peptides IWR2, JW55 or XAV939 that inhibit the Wnt/ β -catenin pathway, leading to a decreased proliferation of colon and breast cancer cell lines. Nevertheless, recent findings support the idea that this decrease may be restricted to low nutrient conditions, and emphasizes that stabilization of Axin needs to be combined with other therapeutic approaches [110, 121-123]. Preventing β -catenin stabilization through GSK3 β activation would also be possible due to the discovery of differentiation-inducing factors (DIFs), which are natural metabolites expressed by *Dictyostelium discoideum*. Although the mechanisms of action of DIFs activity remain poorly understood, it is well known that DIFs induce β -catenin degradation and subsequently reduce cyclin D1 expression and function [124]. CK1 α , another component of the destruction complex, may be stabilized by pyrvinium that inhibits both Wnt signaling and cell proliferation, even in the presence of *APC* or *CTNNB1* mutations, as observed in colon cancer

cell lines [125]. Another therapeutic approach would be to enhance β -catenin proteasomal degradation. In HCC, colon and prostate cancer cell lines, the small molecule antagonist CGK062 has been shown to exert such an effect, *via* the induction of β -catenin phosphorylation in the N-terminal domain which promotes its degradation [126]. Two chemicals agents, hexachlorophene and isoreserpine, upregulate Siah-1, an ubiquitin ligase that induces β -catenin degradation, independent of its phosphorylation status, thereby inhibiting Wnt signaling and subsequently has been shown to reduce colon cancer cell proliferation [127].

Targeting the Wnt pathway in nucleus

Finally, an alternative way to block Wnt-mediated signaling is to target the nuclear β -catenin *per se* and/or the co-factors responsible for transcription of downstream Wnt-responsive genes. To accomplish this aim, several small molecules have been identified. The FH535 agent prevents both Wnt- and PPAR- (Peroxisome Proliferator-Activated Receptors) mediated signaling by suppressing the recruitment of β -catenin coactivators to target gene promoters and has been shown to have activity in HCC, colon and lung tumor cell lines [128]. The PKF115-584, PKF118-310 and CGP049090 are inhibitors of TCF/ β -catenin binding to DNA target sequences. They induce apoptosis *in vitro* and *in vivo*, as well as cell cycle arrest at the G1/S phase and suppress tumor growth *in vivo* independently of the mutated status of *CTNNB1* [129]. Furthermore, inhibition of β -catenin/CBP interaction by ICG-001 both selectively induces apoptosis in transformed, but not in normal colonic cells and reduces growth of colon carcinoma cells *in vitro* as well as *in vivo* [130]. A second generation ICG-001 (PRI-724) is also available and in phase-I clinical trial (<http://clinicaltrials.gov/show/NCT01302405>). Other β -catenin binding proteins such as TBP, Bcl9, Pygo also represent attractive approaches for inactivating Wnt signaling. Finally,

interferon can inhibit β -catenin signaling through upregulation of RanBP3 that is a nuclear export factor, serving as extruding β -catenin outside the nucleus [131].

Conclusions and perspectives

Developmental regulated signaling pathways such as Notch, Hedgehog and Wnt, have become important targets for new cancer drug development. While Notch and Hedgehog inhibitors are already in clinical trials, the Wnt inhibitors are still under preclinical assessment and only a few compounds have started to reach the phase-I clinical trials, since only recently has this pathway been recognized as playing a key role in tumor development. However, many studies have established proof-of-principle that specific targeting of molecules in this pathway can partially or fully switch off canonical as well as non-canonical Wnt signaling and lead to substantial anti-tumor activity. Thus, biotechnology and pharmaceutical organizations are currently developing Wnt signaling inhibitors. These inhibitors can target upstream or downstream proteins in this pathway. Targeting the Wnt cascade upstream of APC is controversial because downstream activating mutations in APC would, in theory, still drive tumor development. To cover the broadest number of activating mutations that occur in tumors, it seems that the ideal antagonist would be one that exerts its anti-tumor effect in the nucleus. Nevertheless, several experiments show that upstream targeting can also be very effective. Of importance is the potential toxicity of Wnt inhibitors on normal cells. Indeed, the Wnt pathway is critical for tissue and liver regeneration and for the ability of stem cells to self-renewal. Wnt pathway inhibitors could therefore have substantial and long-term side effects including anemia, immune suppression, as well as damage to the gastrointestinal tract. It is unknown as to what may occur in an adult mammal when this pathway is shut down or reduced in normal activity. Despite these known and unknown pitfalls, drug development is

moving steadily forward to generate and characterize Wnt pathway inhibitors both *in vitro* and *in vivo*. Indeed, agents that inhibit Wnt/ β -catenin signaling as a means to produce anti-tumor effects are currently being assessed in clinical trials.

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Figure legends

Figure 1: Canonical Wnt/FZD signaling pathway. **A.** In the absence of Wnt signaling, soluble β -catenin is phosphorylated by a degradation complex consisting of the kinases GSK3 β and CK1 α and the scaffolding proteins APC and Axin1. Phosphorylated β -catenin is targeted for proteasomal degradation after ubiquitination by the SCF protein complex. In the nucleus and in absence of β -catenin, TCF/LEF transcription factor activity is repressed by TLE-1; **B.** Activation of the canonical Wnt/FZD signaling leads to phosphorylation of Dvl/Dsh, which in turn recruits Axin1 and GSK3 β adjacent to the plasma membrane, thus preventing the formation of the degradation complex. As a result, β -catenin accumulates in the cytoplasm and translocates into the nucleus, where it promotes the expression of target genes *via* interaction with TCF/LEF transcription factors and other proteins such as CBP, Bcl9 and Pygo.

Figure 2: Non-canonical Wnt/FZD signaling pathways. Interaction of Wnt, FZD and ROR2/RYK co-receptors leads to either (1) JNK activation, (2) PKCs activation, (3) NFAT transactivation, or (4) inhibition of β -catenin activity through binding of NLK to TCF/LEF.

Figure 3: Potential Wnt-component targets for therapeutic intervention on tumor development and growth. Inactivation of Wnt signaling pathway could be achieved by: (1) targeting extracellular signaling molecules with monoclonal antibodies, soluble factors or small molecules; (2) preventing the FZD/Dvl interaction; (3) stabilizing the destruction complex or (4) increasing β -catenin proteasomal degradation and (5) preventing the interaction between β -catenin and its co-factors for transactivity in the nucleus. The relationship between therapeutic molecules and their protein targets are indicated by a color

code. Molecules in bold have been tested in HCC model, those in italics in other models of tumor growth.

Key points

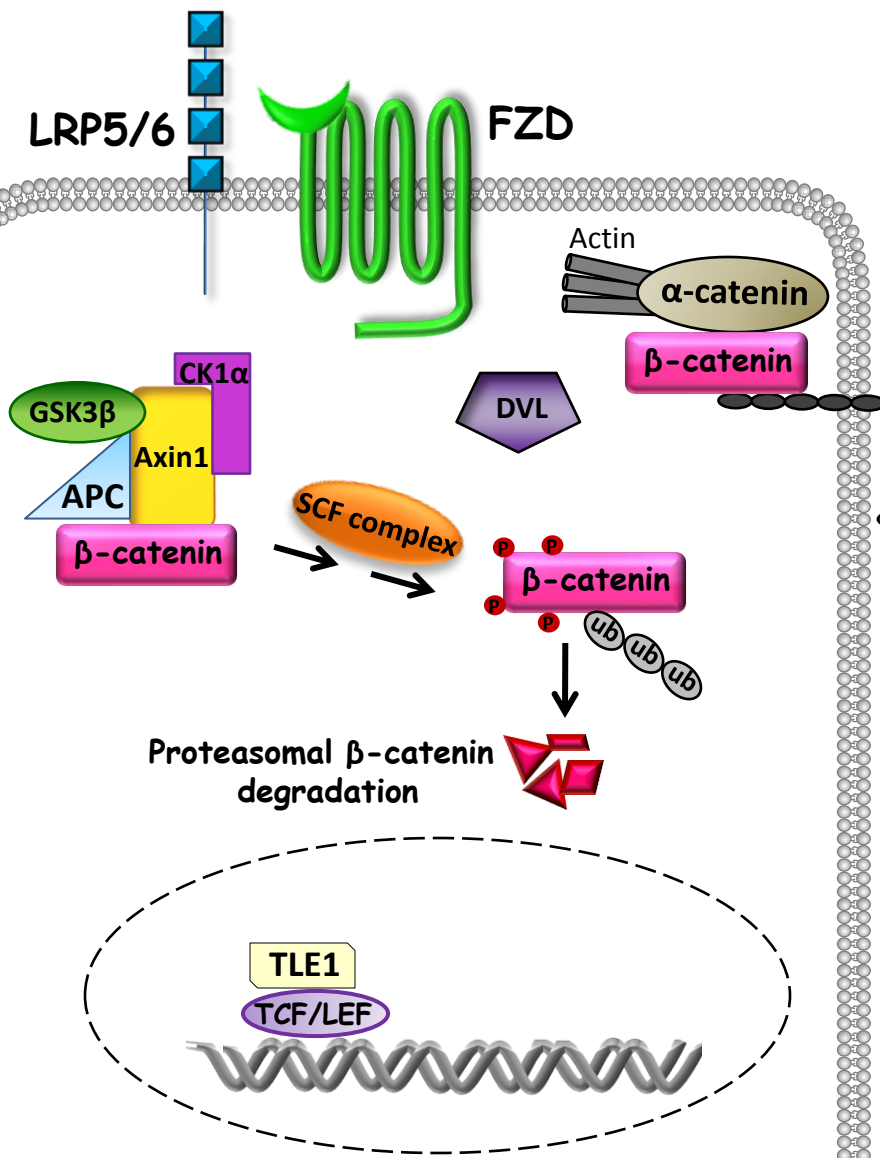
- The increasing incidence of HCC and the frequent ineligibility of patients for curative options due to a widespread dissemination of cancerous cells at diagnosis, highlights the urgent need of effective systemic therapies. Cytotoxic chemotherapy has never shown significant efficiency so far, and targeted therapies are under evaluation.
- Thus far, only sorafenib (anti-angiogenic agent and MAPK inhibitor) has brought significant benefit on survival but the outcome of patients still remains poor.
- Innovative targeted therapies onto oncogenic addiction loops involved in HCC tumor aggressiveness and maintenance, is an attractive strategy. Among them, the Wnt pathway might play a central role *via* transactivity of β -catenin.
- Pharmacological inhibitors of the Wnt pathway are under investigation, and identification of the key targets in the complex network of the Wnt components remains a challenge. Among them, Wnt ligands, Frizzled receptors and β -catenin appear as the most evident targets.
- The development of Wnt inhibitors has not reached clinical trials thus far, but preclinical studies have given promising data.

Table 1. Most prevalent potential mechanisms involved in activation of beta-catenin found so far in HCCs.

Mechanism	Prevalence in HCC tissues
CTNNB1 mutation	8-30%
AXIN mutation	8-15%
APC mutation	1-3%
FZD7 overexpression	23-59%
WNT3 overexpression	35-42%
sFRP1 repression	44-60%
sFRP5 repression	21-35%

Figure 1

A: Absence of canonical signaling



B: Activation of canonical signaling

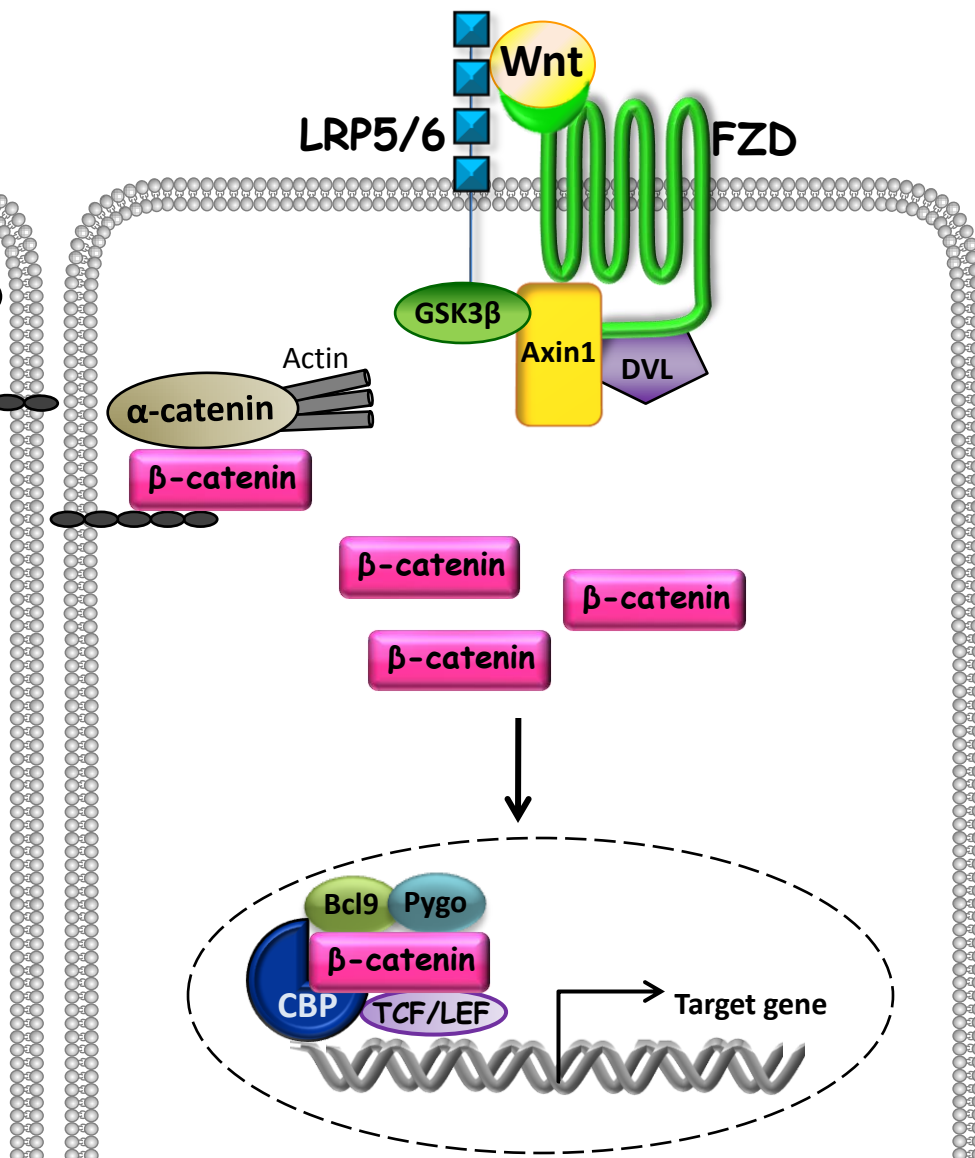


Figure 2

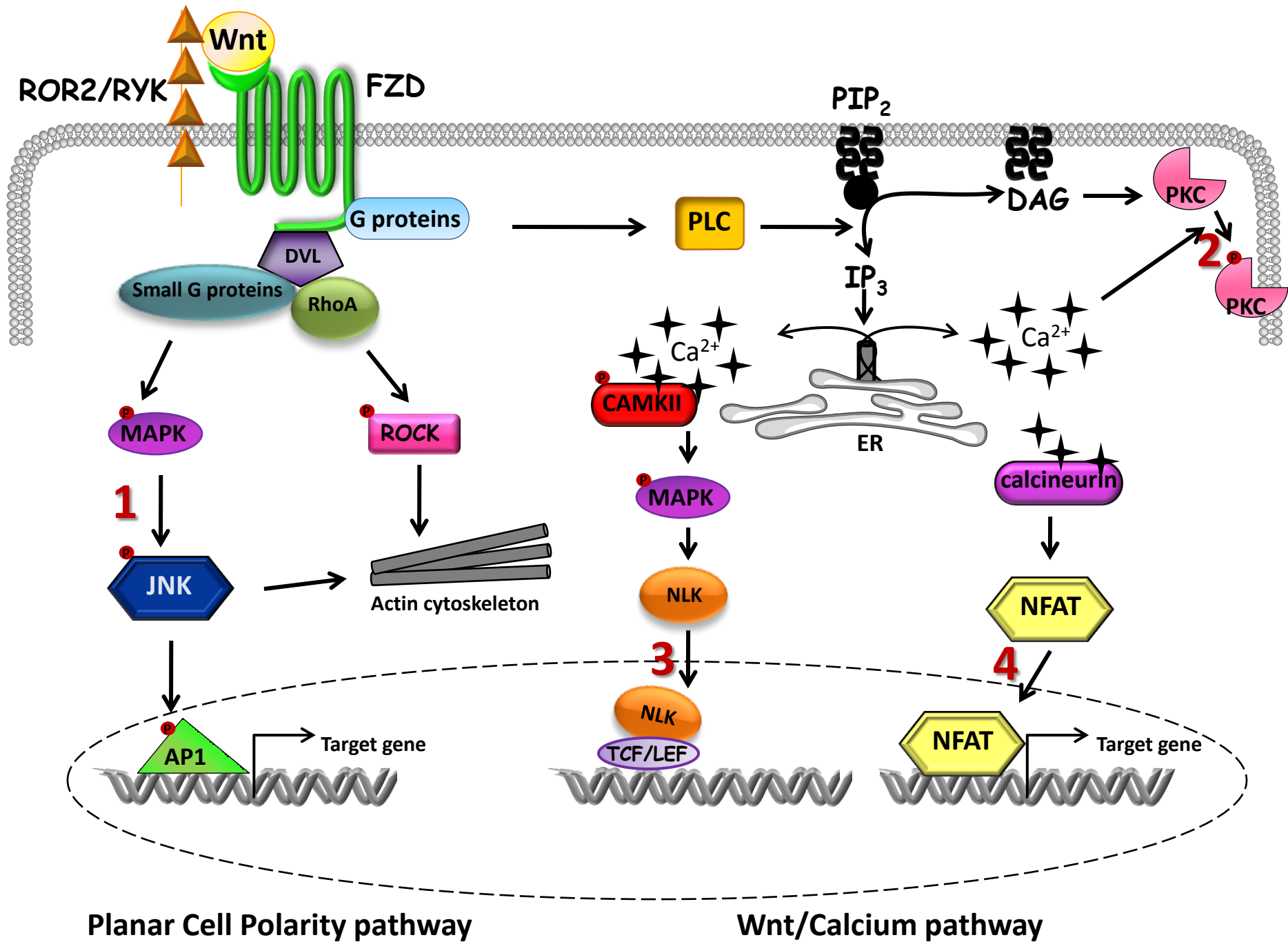
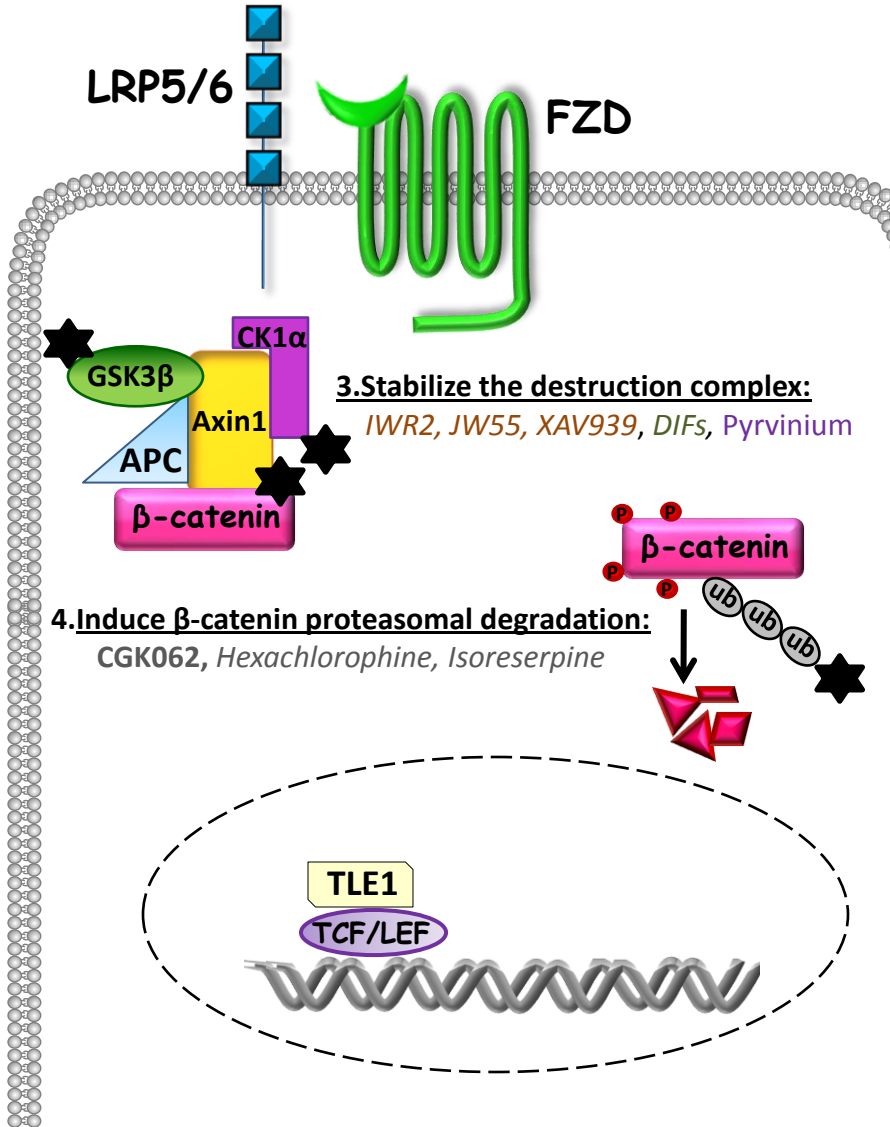


Figure 3

A: Absence of canonical signaling



B: Activation of canonical signaling

1. Block extracellular signaling:

- Monoclonal antibodies : *Wnt1, Wnt2, FZD7, FZD1/2/5/7/8, LRP6*
- Soluble inhibitors: *sFZD7, WIF1, sFRP1, sFRP2, sFRP5*
- Small molecules : *IWP2, Wnt-C59, Niclosamide, Silibinin*

