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KEYNOTE (GREEN)

# Unlocking the Wnt pathway: Therapeutic potential of selective targeting $FZD_7$ in cancer

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**The Wnt signaling is of paramount pathophysiological importance. Despite showing promising anticancer activities in pre-clinical studies, current Wnt pathway inhibitors face complications in clinical trials resulting from on-target toxicity. Hence, the targeting of pathway component(s) that are essential for cancer but dispensable for normal physiology is key to the development of a safe Wnt signaling inhibitor. Frizzled$_7$ ($FZD_7$) is a Wnt pathway receptor that is redundant in healthy tissues but crucial in various cancers. $FZD_7$ modulates diverse aspects of carcinogenesis, including cancer growth, metastasis, maintenance of cancer stem cells, and chemoresistance. In this review, we describe state-of-the-art knowledge of the functions of $FZD_7$ in carcinogenesis and adult tissue homeostasis. Next, we overview the development of small molecules and biomolecules that target $FZD_7$. Finally, we discuss challenges and possibilities in developing $FZD_7$-selective antagonists.**

## Introduction

Cancer remains a major health burden worldwide, with 19 million new cases and nearly 10 million deaths in 2020 according to the WHO global cancer observatory (https://gco.iarc.fr/).[1] Since the first anticancer therapy in the 1940s, research and development for oncology indications has been the most productive area of pharmaceutical industry. Between 2015 and 2020 alone, 69 new anticancer drugs were approved by the FDA, accounting for 29% of new approvals.[2]

From its discovery nearly 40 years ago,[3] the Wnt signaling pathway has been shown to contribute to carcinogenesis because the genes that encode pathway components are either mutated or undergo epigenetic dysregulation of their expression levels in cancer. Historically, the Wnt pathway was associated with β-catenin-dependent signal transduction, but currently, the pathway has been expanded to include several β-catenin-independent branches and broadly to encompass the entire spectrum of signaling events initiated by the Wnt and FZD proteins. The major role of aberrant Wnt signaling in cancer resulted in the first generation of drug candidates that inhibit the pathway. However, although the Wnt pathway is a validated target in cancer, it also plays critical roles in adult physiology by regulating tissue renewal, stem cell proliferation, cell migration, cell differentiation, and other functions.[4–5] More than 100 protein components of the Wnt pathway have been identified, the majority of which function throughout all tissues, whereas a small subset is more tissue specific (Fig. 1). As a result, the first generation of Wnt pathway inhibitors are hampered by on-target side effects, which result from their non-selective inhibition of the Wnt pathway.[6] Therefore, the next generation of Wnt pathway inhibitors should target Wnt pathway components that are involved in tumors while sparing physiological processes.

A unique feature of the Wnt pathway is the component redundancy at multiple levels of the pathway, especially at the

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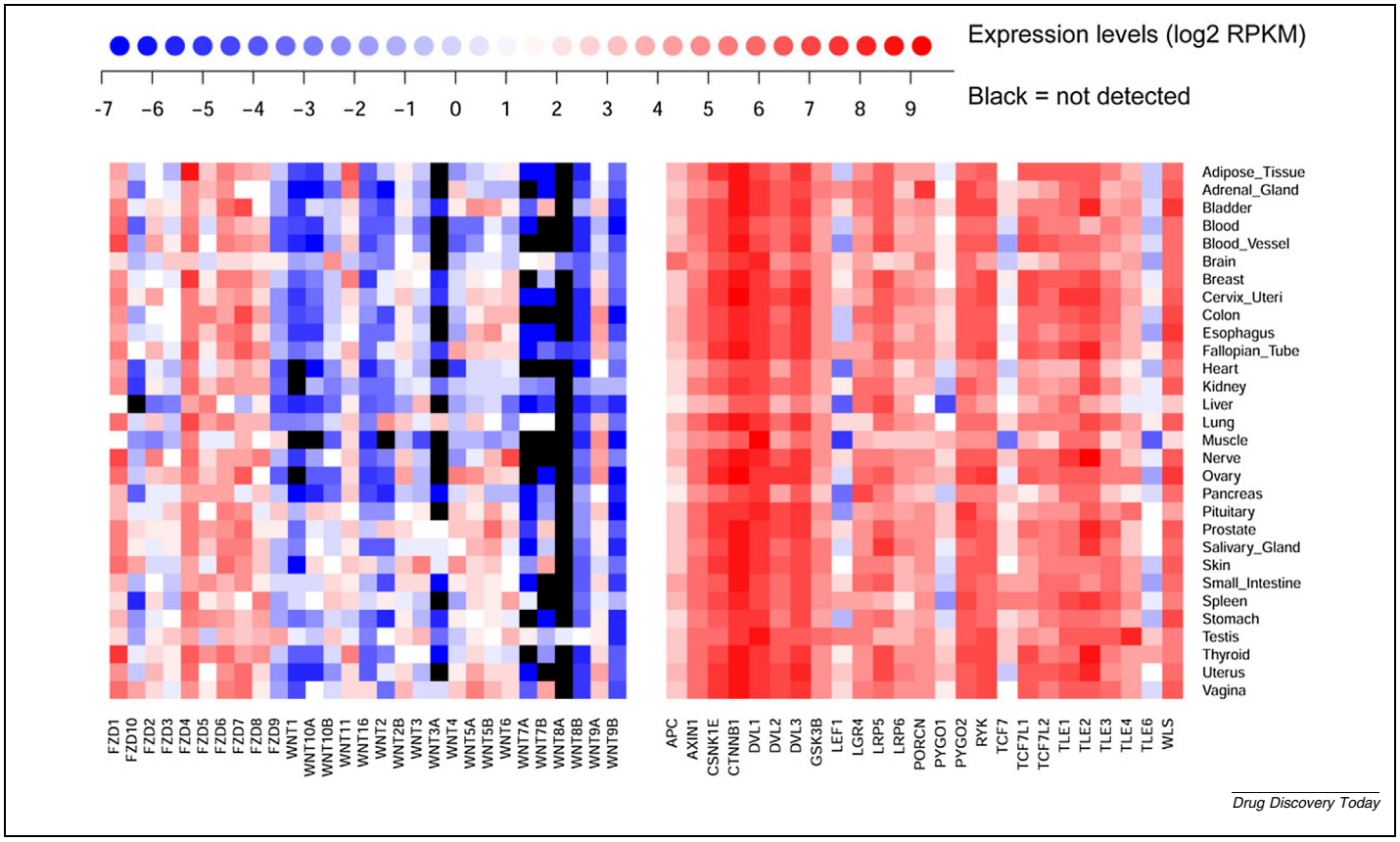


**FIGURE 1**

**Expression levels (RNA-seq v2, GTex project) of the Wnt and FZD genes (left panel) and the genes encoding downstream components of the pathway (i.e. Axin, β-catenin, and T-cell factor (TCF) homologs; right panel) in various healthy tissues.** The almost uniformly 'read' appearance of the right panel highlights the fact that the downstream Wnt signaling components are expressed ubiquitously across the tissues. It is expected, therefore, that the pharmacological targeting of any such pathway component in cancer will inevitably produce adverse on-target effects in healthy tissues. By contrast, the 'salt-and-pepper' pattern of the left panel highlights the differential expression of Wnts and FZDs across tissues, revealing these upper-level pathway components as potential drug targets with fewer anticipated adverse side effects. RPKM, reads per kilobase of transcript per million reads mapped.

plasma membrane and the nuclear levels, which could be exploited to provide selective inhibition of the Wnt pathway in the disease context.[6] At the plasma membrane, Frizzled (FZD) family proteins serve as the principal receptors for Wnt. Ten FZDs are encoded in mammalian genomes, and among these, $FZD_7$ has been highlighted in recent years because of its particular contributions to tumor development.[7–8] $FZD_7$ plays an essential role in carcinogenesis by regulating tumor proliferation and metastasis, maintenance of cancer stem cells, and chemoresistance.[7–8] In this review, we update the current knowledge of the involvement of $FZD_7$ in various cancers, as well as in healthy tissue homeostasis. This knowledge serves as the rationale for the development of $FZD_7$-selective antagonists as anticancer therapeutics. We elaborate on the current state of development of $FZD_7$ antagonists, both small molecules and biomolecules.

## FZDs: The key to selective inhibition of the Wnt pathway?

The first Wnt protein was identified in the 1980s as a putative mammary oncogene in mice.[3] In humans, 19 distinct Wnts that contain a conserved pattern of 23–24 cysteine residues have been characterized.[9] Upon expression but prior to secretion, Wnts are posttranslationally acylated by the Porcupine enzyme, a modification that is essential for Wnt functions.[9] A Wnt binds to a FZD on the plasma membrane, additionally engaging a coreceptor, such as low-density lipoprotein receptor-related protein 5/6 (LRP5/6), a Tyr kinase-like orphan receptor (ROR1 and ROR2), a receptor Tyr kinase (RYK), or a syndecan.[10]

There are ten FZDs in humans ($FZD_{1–10}$), which have sequences of 500–700 amino acids that show 20–40% identity.[11] FZDs can be categorized into four subfamilies on the basis of their sequence: $FZD_{1/2/7}$, $FZD_{4/9/10}$, $FZD_{5/8}$, and $FZD_{3/6}$. There are three domains within FZD proteins: i) a highly conserved extracellular cysteine-rich domain (CRD); ii) the transmembrane domain (TMD); and iii) the intracellular C-domain. The CRD is the Wnt-binding domain, comprising 120–125 amino acids with 10 conserved cysteines.[12] Several studies have proven that the FZD protein family is part of G-protein coupled receptor (GPCR) class F, as is the Smoothened (SMO) 7-TM protein.[13]

Resolving the structure of Wnt and FZD has proven challenging because of difficulties in isolating the active proteins. Nevertheless, the Wnt–FZD interactions have been elucidated



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progressively over the past several years. The first resolved structure of an interaction was that of *Xenopus* Wnt-8 (XWnt-8) and the CRD of human $FZD_8$, which displayed a topology resembling a 'grasping-hand'.[14] There are two binding sites in the interaction between XWnt-8 and $FZD_8$-CRD: (i) a palmitoleic acid lipid group on Ser187 at the tip of the 'thumb' of XWnt-8 that projects into a deep groove in the $FZD_8$-CRD; and (ii) the conserved tip of the index finger' of XWnt-8 that forms hydrophobic amino acid contacts with a depression on the opposite side of the $FZD_8$-CRD.[14] Another study has recently found that the acyl group of Wnt facilitates the formation of dimers of the CRD of $FZD_7$,[15] although such dimerization remains to be proven for the full-length receptors. Moreover, FZDs might respond to Wnt binding in a polar manner. For example, $FZD_6$ forms homodimers via its transmembrane (TM)4 and TM5 domains, and Wnt stimulation induces $FZD_6$ dimer dissociation followed by re-association.[16]

The interaction between Wnt, FZD, and co-receptors activates several downstream Wnt pathways. The best-studied branch of Wnt signaling is the β-catenin-dependent pathway, which culminates at β-catenin-induced gene expression. In the absence of Wnt, the newly synthesized β-catenin is continuously phosphorylated by the so-called destruction complex, a multiprotein complex consisting of Axin, adenomatous polyposis coli (APC), glycogen synthase kinase 3 beta (GSK3β), and casein kinase 1 alpha (CK1α).[17] Phosphorylation of β-catenin leads to its ubiquitination and proteasomal degradation, and sustains the low level of cytoplasmic β-catenin. Wnt binding to FZD and LRP5/6 initiates β-catenin-dependent signaling by disintegrating the destruction complex, leading to cytoplasmic accumulation of β-catenin. β-catenin then translocates into the nucleus where it interacts with transcription factors of the T-cell factor/lymphoid enhancer factor (TCF/LEF) family to induce the transcription of target genes, such as *c-Myc* and *cyclin D1*. Several Wnts are known to bind to $FZD_7$ to activate the β-catenin-dependent pathway, including Wnt-2b, Wnt-3, Wnt-3a, Wnt-7a, Wnt-7b, Wnt-8b, and Wnt-9b (Fig. 2).[8,18–19]

Wnt–FZD might also activate β-catenin-independent pathways, such as the planar cell polarity (PCP) pathway. The PCP pathway drives cell polarization by controlling cytoskeleton rearrangements.[20] Within the PCP pathway, Ryk, receptor tyrosine kinase-like orphan receptor-1 and -2 (ROR1/2), or syndecan, instead of LRP5/6, serve as the Wnt co-receptor.[10] Downstream of the Wnt–FZD–co-receptor complex, several components transduce the PCP signaling, including small GTPases of the Rho subfamily (Rho, Rac, and Cdc42), the Rho-associated kinase (ROCK), and the JNK-type mitogen-activated protein kinase (MAPK).[21] Multiple Wnts have been shown to activate the PCP pathway through binding to $FZD_7$, including Wnt-5a, Wnt-5b, Wnt-7a, Wnt-8b, Wnt-10b and Wnt-11 (Fig. 2).[8,22–23]

Even though the majority of Wnt pathway signaling branches involve co-receptors, some downstream pathways can be activated exclusively through the interaction of Wnt and FZD. An example is the activation of heterotrimeric G protein and its downstream effectors. Using co-immunoprecipitation and various bioluminescence resonance energy transfer (BRET) assay systems, $FZD_7$ has been shown to interact with Gαs, an isoform of Gα subunit of heterotrimeric G proteins.[24–27] Multiple Wnts activate the $FZD_7$–Gαs signaling cascade, including Wnt-5a and Wnt-7a.[24–25] In the skeletal muscle, the Wnt-7a–$FZD_7$–Gαs axis activates the Akt–mTOR anabolic growth pathway and is essential for the repair of skeletal muscles.[24]

As discussed above, 11 out of 19 Wnts are known to interact with $FZD_7$. However, these Wnts also interact with several different FZD homologs, creating complex and functionally redundant Wnt–FZD interactions. Furthermore, the co-receptors are also not specific for any FZD. As a consequence, at the initiation of the Wnt-pathway, a single FZD homolog might serve as the key target for the selective inhibition of a specific Wnt subpathway.

## The role of FZDs in normal tissue homeostasis

FZD homologs are expressed in different tissues and organs throughout the mammalian body, especially in the nervous, cardiovascular, bone, and gastrointestinal systems. Here, we focus on elaborating the function of FZDs in maintaining physiological conditions postnatally. The essential role of FZDs in organism development has been reviewed elsewhere.[28]

$FZD_1$ and $FZD_3$ are the main FZD proteins expressed in the nervous system. $FZD_1$ is enriched in the central nervous system and exhibits neuroprotective roles.[29–31] Loss of $FZD_3$ from the spinal cord leads to a defect in the transmission of sensory information between limbs and the brain.[32]

$FZD_4$ is essential in the vasculature and cardiovascular systems. A $FZD_4$ mutation has been linked to familial exudative vitreoretinopathy, a retinal vasculature disease.[33] A $FZD_4$ knockout led to the abnormal development of retinal vasculature in mice.[34] By using antibodies to downregulate $FZD_4$ in adult mice, Paes *et al.*[35] demonstrated that $FZD_4$ is required to maintain the integrity of the blood–retina barrier. $FZD_4$ deletion also impairs the formation of small arteries and capillaries in peripheral organs.[36]

Bone fractures are one of the major side effects caused by the first generation of Wnt pathway inhibitors. Multiple FZDs regulate bone homeostasis: $FZD_1$, $FZD_4$, $FZD_8$, and $FZD_9$.[37–41] In general, bone homeostasis is regulated by two processes: bone formation by osteoblasts and bone resorption by osteoclasts. $FZD_1$ plays an important role in osteoblast differentiation and mineralization.[37–38] Loss of $FZD_8$ increased osteoclastogenesis without affecting bone formation.[39] By contrast, *FZD9* loss impaired bone formation without any effect on bone resorption.[40] However, both *FZD8* and *FZD9* loss resulted in osteopenia and risk of osteoporosis.[39–40] A recent study reported that $FZD_4$ is expressed in osteoblasts and is essential for normal bone acquisition.[41] *FZD4* loss is compensated by upregulation of $FZD_8$, and these two FZDs might function redundantly in osteoblasts.[41]

Gastrointestinal (GI) homeostasis is regulated by Wnt signaling, mainly through $FZD_5$ and $FZD_7$.[19,42–43] $FZD_5$ is expressed in the Paneth cells of intestinal crypts, which are highly specialized epithelial cells that secrete antimicrobial peptides and immunomodulating proteins to regulate the intestinal flora.[42] $FZD_5$ is essential for the maturation of Paneth cells and directs the positioning of these cells within the intestinal crypt tissue network.[42] $FZD_7$ is expressed in both stomach and intestine.[19,43] In the intestine, $FZD_7$ is enriched in the leucine-rich repeat-



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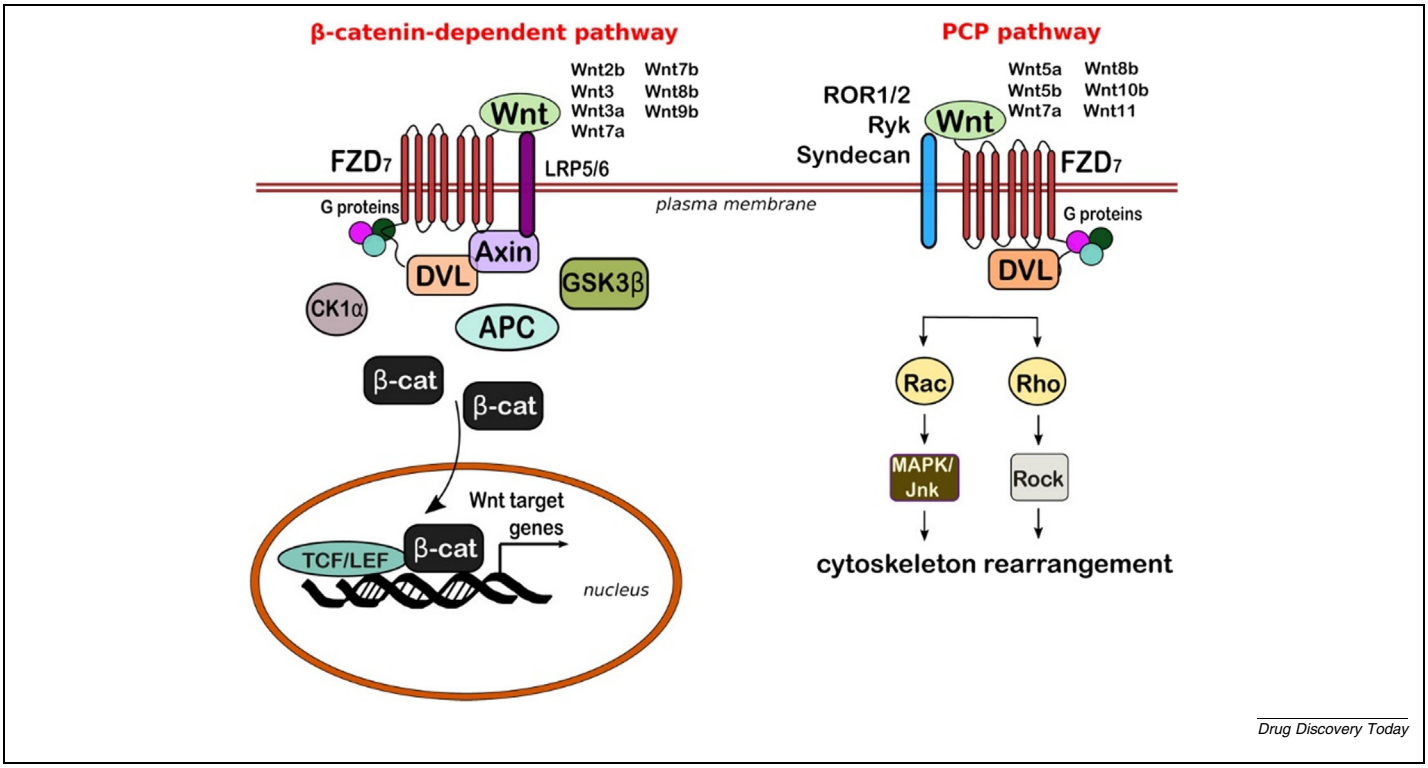


**FIGURE 2**

**$FZD_7$ activates at least two branches of the Wnt pathway, the β-catenin-dependent Wnt pathway and the planar cell polarity (PCP) pathway.** In the β-catenin-dependent pathway, $FZD_7$ and the co-receptor LRP5/6 can bind to several Wnt proteins to activate downstream signaling, thereby inducing the β-catenin-dependent gene expression. In the PCP pathway, $FZD_7$ together with one of several other co-receptors can bind to several Wnts to activate downstream signaling, culminating in cytoskeleton rearrangements. APC, adenomatous polyposis coli; β-cat, β-catenin; CK1α, casein kinase 1 α; DVL, Dishevelled; $FZD_7$, Frizzled$_7$; GSK3β, Glycogen synthase kinase 3 β; Jnk, c-Jun N-terminal kinases; LRP5/6, Low-density lipoprotein receptor-related protein 5/6; MAPK, mitogen-activated protein kinase; Rock, Rho-associated kinase; ROR1/2, receptor tyrosine kinase-like orphan receptor-1 and -2; TCF/LEF, T-cell factor/lymphoid enhancer factor.

containing G-protein coupled receptor $5^+$ ($Lgr5^+$) crypt stem cells.[19] Conditional deletion of $FZD_7$ from $Lgr5^+$ crypt stem cells is deleterious to these cells and triggers the repopulation of the intestinal epithelium with non-recombined $FZD_7$-proficient stem cells. These data, taken together with the failure of $FZD_7^{-/-}$ intestinal organoids to regenerate upon passage, indicate the critical importance of $FZD_7$ in maintaining intestinal homeostasis. An injury challenge to the intestine of $FZD_7$-knockout mice showed that tissue regeneration in these mice is impaired compared to that in wild-type mice.[19] The intestine of wild-type mice regenerates within 70 hours of the induction of injury, whereas it takes 120 hours for the intestine of $FZD_7$-knockout mice to regenerate. Altogether, these results indicate that $FZD_7$ is important for the homeostasis and robust regeneration of intestinal epithelium.[19] In the stomach epithelium, $FZD_7$ is expressed at drastically higher levels in the antrum compared to the corpus.[43] Conditional deletion of $FZD_7$ in the antral gastric epithelium resulted in a phenomenon similar to that observed in the intestine: conditional $FZD_7$ deletion from the gastric epithelium was deleterious but triggered rapid repopulation of epithelium with $FZD_7$-proficient cells.[43] However, in the gastric antral epithelium (in contrast to the intestinal epithelium), $FZD_7$ is not required for the activity of $Lgr5^+$ stem cells.[44–45] Hence, $FZD_7$ is needed to maintain at least one population of stem cells in the gastric antrum, but this population has yet to be identified.

Given the importance of different FZD proteins in maintaining GI homeostasis, the effect of the specific inhibition of a single receptor has been evaluated in GI organoid models. Nile *et al.*[46] engineered a $FZD_{1/2/7}$-selective peptide (dFz7-21) and evaluated it activity in organoid cultures established from adult mouse intestinal epithelium. The peptide was found to disrupt the functional intestinal stem cells within the organoids. Another study developed genetically engineered antibody-mimetic proteins, named designed repeat protein binders (DRPBs), that targeted different FZD subtypes and tested these DRPBs on intestinal organoids as well as *in vivo*.[47] DRPB_Fz4 (which binds to $FZD_4$) and DRPB_Fz7 (which binds to $FZD_{1/2/7}$) did not affect the intestinal organoids, whereas DRPB_Fz8 (which binds to $FZD_{5/8}$) inhibited intestinal organoid growth in the 0.1–1 nM range.[47] *In vivo*, adenoviruses expressing DRPB_Fz8 induced rapid loss of duodenal crypts and villi, resulting in lethality within 7 days.[47] On the other hand, DRPB_Fz4 and DRPB_Fz7 were not detrimental to the intestine *in vivo*.[47] Another recent study used engineered Wnt surrogates consisting of the FZD and LRP binding domains, which can selectively bind to and activate different FZD subtypes.[48] By using only the FZD-binding arms, the surrogates were turned into FZD-subtype-selective antagonists.[48] When these subtype-specific pharmacological tools were used, either $FZD_{5/8}$ or $FZD_{1/2/7}$ agonists were able to support the regeneration of intestinal organoids follow-



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ing pan-Wnt inhibition using a porcupine inhibitor; only inhibition of both FZD subtypes resulted in organoid loss.[48]

Altogether, these studies show that specific FZD homologs function in the maintenance of different organs, mostly in a redundant manner. This conclusion is of paramount importance for the concept of FZD-selective anticancer therapy.

## Prospects for developing $FZD_7$ antagonists as cancer therapeutics

In various cancers, the Wnt pathway regulates nearly all aspects of tumorigenesis. The branches of the Wnt pathway demonstrate a certain degree of specialization in driving particular events: the β-catenin-dependent Wnt pathway is more frequently involved in cancer initiation, progression, dormancy, and stem cell maintenance, whereas Wnt–PCP signaling has often been shown to contribute to cancer cell migration and invasiveness, hence promoting tumor metastasis.[49–50] In this review, we elaborate the vital role of $FZD_7$ in various cancers. $FZD_7$ can activate both the β-catenin-dependent and the β-catenin-independent pathway, and thus can influence cancer proliferation, differentiation, chemoresistance, and metastasis. Importantly, $FZD_7$ is upregulated and performs a crucial function in various cancers, making the inhibition of $FZD_7$ a promising strategy to fight cancer, with multiple potential oncology indications. A number of reports described below have directly addressed the role of $FZD_7$ in cancers, and their main findings are summarized in the Table 1.[8]

### *Triple negative breast cancer*

Breast cancer (BC) is the most frequent cancer in women and was known to be responsible for 684,996 deaths worldwide in 2020.[1] Between 15% and 20% of BC cases are triple-negative BC (TNBC), which behaves more aggressively than other types of BC and results in a poorer short-term prognosis. TNBC has the worst outcome with a five-year overall survival rate of 78.5%, compared to 90% for other BC types.[51] The lack of effective targeted treatments and the high risk of relapse following surgery contribute to the increased mortality from TNBC. To date, no general biomarkers have been identified for TNBC, probably due to the heterogenicity of TNBC subtypes. Although novel therapies have been approved in the past three years, cytotoxic chemotherapy remains the only available systemic treatment.[51] In recent years, the Wnt pathway has arisen as a prospective target in TNBC.[52]

$FZD_7$ is the only member of the FZD family that is significantly overexpressed in TNBC tissues.[53] $FZD_7$ plays an important role in TNBC tumor transformation, promoting the proliferation and invasion of TNBC cell lines *in vitro* and *in vivo*.[53–55] Multiple studies have concluded that $FZD_7$ signals through the β-catenin-dependent Wnt pathway in TNBC.[53,56] However, a recent study pointed out that $FZD_7$ might also transmit the β-catenin-independent Wnt pathway, as bioinformatic and co-immunoprecipitation analyses have revealed that Wnt-5a and Wnt-5b (the Wnts known to initiate mainly β-catenin-independent signaling) bound $FZD_7$ in MDA-MB-231 and Hs578T TNBC cell lines.[23] Consequently, $FZD_7$ regulates several intracellular oncogenic molecules, including phosphorylated Stat3, Smad3, and Yes-associated protein 1, driving tumorigenesis, metastasis, and stemness in TNBC.[23] Further, downregulation of $FZD_7$ inhibited both TNBC tumor progression and metastasis *in vitro* and *in vivo*.[23,53]

$FZD_7$ promoted the activity of mammary stem cells.[56] An isoform of transformation-related protein 63 (ΔNp63) regulated $FZD_7$ expression, and ΔNp63 overexpression induced luminal cells to enter a stem-like state.[56] Bioinformatic studies have identified a positive correlation between ΔNp63 and $FZD_7$ in TNBC tissues.[56] A study *in vivo* using a patient-derived-xenograft (PDX) model revealed that the ΔNp63–$FZD_7$–Wnt signaling axis regulates the tumorsphere-forming ability, highlighting the importance of $FZD_7$ in tumor-initiating cells.[56]

The TNBC cell line IOWA-1 T shares many features of cancer stem cells[57] and aggressively forms a tumor upon xenotransplantation into immune-compromised mice.[58] Our analysis of the $FZD_{1–10}$ expression profile in these TNBC stem cells highlights $FZD_7$ as the primary overexpressed receptor, with its expression exceeding that of other FZD members by one or two orders of magnitude (Fig. 3).

Collectively, these data pinpoint $FZD_7$ as the main Wnt receptor in TNBC, especially in the cancer stem cells that are known to mediate tumor relapse and chemoresistance.[59]

### *Colorectal cancer*

Colorectal cancer (CRC) is the second most common cancer worldwide, with 935,173 known deaths in 2020.[1] Although there are multiple targeted treatments, there is no universal treatment regimen for CRC. The emergence of drug resistance has been unavoidable and most of the targeted therapies are associated with adverse effects. The registered worldwide incidence of CRC is expected to increase to 2.5 million cases in 2035 as the result of improved diagnostic screening in developing countries, as well as lifestyle and environmental factors.[60]

Within the Wnt pathway, mutations of *APC* and *CTNNB1* (the gene encoding β-catenin) are the major tumorigenesis drivers in CRC.[61] Interestingly, a study reported that $FZD_7$ is involved in the activation of the β-catenin-dependent Wnt pathway in colon cancer cells, despite the presence of the *APC* or *CTNNB1* mutations downstream in the pathway, at least *in vitro*.[62] $FZD_7$ knockdown suppressed CRC proliferation and metastasis.[62–63] Besides its role in the β-catenin-dependent Wnt pathway, $FZD_7$ might also transmit signals via β-catenin-independent pathways in CRC. $FZD_7$ knockdown decreased c-Jun, p-JNK, and p-c-Jun protein levels and RhoA activation, which are indicators of the Wnt–PCP pathway.[63] Another study has showed that R-spondin 2 (RSPO2) suppresses CRC metastasis by antagonizing the Wnt-5a–$FZD_7$ β-catenin-independent pathway.[64]

The role of $FZD_7$ in CRC metastasis is peculiar because, within the β-catenin-dependent pathway, $FZD_7$ promotes tumor growth by invoking more epithelial (rather than mesenchymal) characteristics, therefore reducing the potential of cells to disperse.[65] This phenomenon causes CRC cells to remain cohesive, and thus advances local tumor growth. Using an *in vitro* model of CRC morphogenesis that spontaneously undergoes cyclic transitions between two-dimensional monolayer (migratory, mesenchymal) and three-dimensional sphere (carcinoid, epithelial) states, Vincan *et al.*[66] have reported that $FZD_7$ regulates either the CRC migratory or epithelialization events, depending on the context.



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**TABLE 1**

**Roles of $FZD_7$ in various types of cancer based on studies assessing its direct involvement in tumorigenesis.**

| Cancer type | Role of $FZD_7$ in tumorigenesis | Branch of Wnt pathway activated | Reference (s) |
|---|---|---|---|
| Triple negative breast cancer | • Cell invasion, motility and clonogenicity *in vitro*<br>• Tumor growth *in vivo* | β-catenin-dependent | 53–55 |
| | • Tumorsphere formation *in vivo* | β-catenin-dependent | 56 |
| | • Mesenchymal phenotype<br>• Breast cancer cell stemness | β-catenin-independent | 23 |
| Colorectal cancer | • Tumor proliferation and invasion | β-catenin-dependent and -independent (JNK/c-jun, RhoA, and PKC/ERK pathways) | 62–64 |
| | • Tumor growth<br>• Mesenchymal-epithelial transition of metastatic cells | β-catenin-dependent and -independent pathways | 65–66 |
| Gastric cancer | • Tumor proliferation in Helicobacter *pylori* infection-induced cells<br>• Cancer cell growth, migration, invasion and stem-cell-like properties | β-catenin-dependent pathway | 44,71,73–74 |
| | • Cancer stemness and chemoresistance toward cisplatin | Not determined | 70 |
| Hepatocellular carcinoma | • Cell proliferation and motility | β-catenin-dependent | 77–79,81 |
| | • Cell invasion and anchorage-independent growth in non-transformed hepatic cells | β-catenin-dependent | 80 |
| | • Chemoresistance toward 5-fluorouracil | β-catenin-dependent | 82 |
| Ovarian cancer | • Cell proliferation, cell cycle progression, and cell–cell adhesion | β-catenin-independent (Wnt–PCP pathway) | 84 |
| | • Cancer stemness and chemoresistance toward cisplatin | β-catenin-dependent | 85 |
| Melanoma | • Cell growth and viability in both naive and BRAF inhibitor-resistant melanoma cells | β-catenin-independent (PI3K–AKT pathway) | 87 |
| | • Melanoma tumor initiation and metastasis *in vivo* | β-catenin-independent (Wnt11–FZD7–DAAM1–RhoA–ROCK1/2) | 88 |
| | • Metastasis formation of melanoma cell lines | β-catenin-independent (JNK pathway) | 89 |
| Pancreatic cancer | • Cancer stemness and chemoresistance toward gemcitabine | β-catenin-dependent | 18,92 |
| Kidney cancer | • Clonogenicity and proliferation of Wilms' tumor cells | β-catenin-dependent | 93 |
| | • Renal cancer cell proliferation | β-catenin-dependent | 94 |
| Cervical cancer | • Cervical cancer cell migration and invasion | β-catenin-independent (JNK/c-jun pathway) | 95 |
| Glioma | • Glioma cell proliferation *in vitro* and *in vivo* | β-catenin-dependent | 96 |
| Esophageal cancer | • Esophageal cancer cell growth, migration and invasion<br>• Chemoresistance toward cisplatin | β-catenin-dependent | 98 |
| Leukemia | • Chronic myeloid leukemia (CML) proliferation<br>• Chemoresistance toward imatinib | β-catenin-dependent | 99 |

$FZD_7$ knockdown using RNAi impaired cell migration. The same study also revealed that $FZD_7$ is essential for the mesenchymal–epithelial transition (EMT) of metastatic cells, which is an important step in initiating tumor growth at the metastasis sites.

Despite these interesting findings, *in vivo* treatment with vantictumab (an antibody targeting $FZD_{1/2/5/7/8}$) suppressed colorectal tumor growth only in the tumor subset that had wild-type APC and β-catenin, but was inactive against the majority of colorectal tumors bearing mutations in *APC* and CTNNB1.[67] Hence, the downstream mutations, which are a characteristic feature of CRC, appear to counteract FZD-targeting and thus future $FZD_7$-selective inhibitors efficiently. The potential use of $FZD_7$-selective inhibitors in this type of cancer will therefore have to be studied in the context of specific tumor subtypes and potentially in combination with other Wnt-targeting agents, with the possibility of using these agents at lower doses than are used in monotherapy.

### *Gastric cancer*

Gastric cancer is the fifth most diagnosed cancer worldwide, with 768,793 deaths reported in 2020.[1] As the result of late diagnosis and lack of targeted treatments for gastric cancer, patient prognosis is poor, with a five-year survival rate of just 23–36%; advanced gastric cancer has a median survival of less than one year.[68–69]

The role of $FZD_7$ in gastric cancer has been appreciated in recent years. $FZD_7$ has been observed to be upregulated in gastric cancer tissues when compared to adjacent non-cancerous gastric tissues.[70–71] $FZD_7$ expression in gastric cancer tissues also correlates with poor patient survival.[71–72] *Helicobacter pylori* infection decreased the expression of microRNA-27b (miR-27b), which negatively regulates $FZD_7$ expression.[73] A decrease in miR-27b resulted in $FZD_7$ upregulation, promoting the proliferation of gastric cancer cells in *H. pylori* infection-induced cells via the β-catenin-dependent Wnt pathway.[73] Another known regulator of $FZD_7$ expression in gastric cancer is YTH domain family member 1 (YTHDF1), a protein that regulates $FZD_7$ mRNA stability and translation via N-methyladenosine (m6A) modification.[74]

$FZD_7$ contributes to the growth and metastasis of gastric cancers.[71,74] $FZD_7$ drives cancer growth in both APC-wild type and APC-mutant gastric adenoma through the β-catenin-dependent Wnt pathway.[44,71,74] Gastric cancer stem cells (CSCs) play a critical role in chemoresistance and the recurrence of



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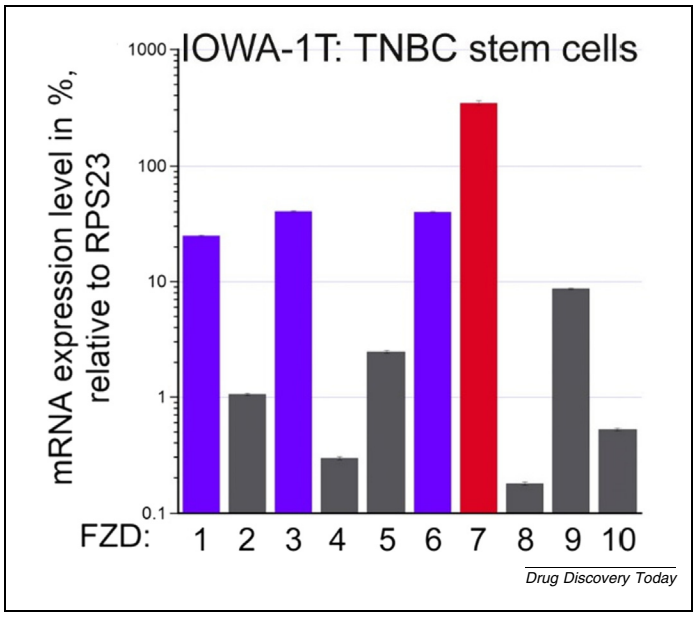


**FIGURE 3**

**qPCR analysis of $FZD_{1-10}$ mRNA expression in the triple-negative breast cancer (TNBC) stem cell line IOWA-1 T.** Levels of expression are presented as a percentage of those of the ribosomal protein S23 (RPS23) and are the means from a triplicated experiment. Color-coding highlights the FZD homologs with the strongest expression levels.

gastric cancer. $FZD_7$ knockdown decreases the expression of stemness markers and the ability of gastric cancer cells to form spheroids, suggesting that $FZD_7$ is essential for gastric CSCs.[70] Furthermore, both $FZD_7$ knockdown and treatment with vantictumab were able to overcome the chemoresistance of gastric cancer cells toward cisplatin.[44,70]

### *Hepatocellular carcinoma*

Hepatocellular carcinoma (HCC) represents 90% of cases of liver cancer, and is the sixth most common cancer and the third most prevalent cause of cancer death with 830,180 known deaths in 2020.[1] Despite improvement in the early detection of HCC that allows liver transplantation, surgery, or radiation therapy, HCC is often diagnosed at late stages. The prognosis for HCC remains poor, with a median overall survival of 11 months for advanced HCC resulting from the lack of an effective biomarker and the low rate of response to the available targeted therapies.[75–76] Upregulation of the β-catenin-dependent Wnt pathway contributes to both immune evasion and resistance to immunotherapy in HCC.[75]

$FZD_7$ is overexpressed in HCC tumors compared to peritumoral areas, and this overexpression is associated with the intracellular accumulation of β-catenin.[77–78] In addition to $FZD_7$, Wnt-3a is also upregulated in HCC tumors compared to noncancerous liver tissues.[79] The Wnt-3a–$FZD_7$ interaction activates the β-catenin-dependent Wnt pathway in HCC cell lines.[79–80] Furthermore, overexpression of Wnt-3a and $FZD_7$ activates the Wnt pathway and promotes proliferation in non-transformed hepatic cells.[80]

Wnt-3a–$FZD_7$ signaling also promotes cell migration, cell invasion, and anchorage-independent growth, which might be associated with the EMT.[80] Dominant-negative mutant constructs that encoded a C-terminally truncated $FZD_7$ protein reduced HCC cell motility, confirming the role of $FZD_7$ in the migration of HCC cells.[78] In addition, $FZD_7$ knockdown promoted apoptosis in HepG2 and Huh-7 HCC cell lines.[81]

$FZD_7$ is also involved in HCC chemoresistance. The expression of $FZD_7$ and multidrug resistance protein 1 (MDR1) are higher in 5-fluorouracil (5-FU)-resistant HCC cells than in the parental cells.[82] $FZD_7$ siRNA decreased the expression of MDR1 in 5-FU-resistant HCC cells and sensitized these cells to chemotherapy by inducing apoptosis.[82]

### *Ovarian cancer*

Ovarian cancer was linked to 207,252 deaths in 2020.[1] Avastin (an antibody against vascular endothelial growth factor (VEGF)) and poly adenosine diphosphate-ribose polymerase (PARP) inhibitors are the standard of care, have significantly reduced cancer progression and are used as maintenance therapy further to surgery and chemotherapy.[83]

Ovarian cancer is classified, on the basis of gene expression patterns, into five subtypes: epithelial-A (Epi-A), Epi-B, Mes, Stem-A, and Stem-B. $FZD_7$ expression has been found to be enriched in Mes and Stem-A molecular subtypes.[84] *In vitro*, $FZD_7$ expression is enriched in SKOV3 cell spheroids and in PA1 ovarian teratocarcinoma cells, a cell line that harbors pluripotency and stem cell characteristics.[84] A recent study confirmed the involvement of $FZD_7$ in ovarian CSCs. It found that $FZD_7$ is upregulated in platinum-tolerant (Pt-T) ovarian cancer cells, which exhibit stemness properties.[85] Furthermore, $FZD_7$ knockdown decreased the expression of stemness-associated transcription factors.[85] Altogether, these results suggest that $FZD_7$ plays an essential role in driving stem cell properties in ovarian cancer.

$FZD_7$ knockdown suppressed the proliferation of ovarian cancer cells, and also inhibited spheroid formation.[84–85] *In vivo*, knockdown of $FZD_7$ in OVCAR5 cells delayed tumor initiation and decreased tumor size.[85] Interestingly, $FZD_7$ knockdown inhibited cell proliferation by modulating cell cycle progression, but it showed no effect on apoptosis.[84] $FZD_7$ also regulates rearrangements of the actin cytoskeleton, as well as cell migration and motility, via phospho-myosin light chain (pMLC) and Rho GTPases, which are downstream effectors of the Wnt–PCP pathway.[84]

Prolonged exposure to platinum chemotherapeutic agents upregulates $FZD_7$ expression, at both mRNA and protein levels.[85] $FZD_7$ knockdown sensitized ovarian cancer cells to platinum. $FZD_7$ also marks a cell population that is enriched in glutathione metabolism-related genes that are highly susceptible to ferroptosis, a type of programmed cell death that is dependent on iron and characterized by the accumulation of lipid peroxides.[85]

### *Melanoma*

On the basis of the molecular characteristics of advanced melanoma (staging from III to IV according to the American Joint Committee in Cancer), the standard of care treatments for melanoma are anti-PD-1 immunotherapy or a combination of anti-PD-1 with BRAF and MEK inhibition. These therapies have reduced the risk of recurrence to 30–50% but are associated with treatment-related toxicity.[86]





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In melanoma cells, it has been shown that $FZD_7$ signals mainly through β-catenin-independent Wnt pathways, including the DAAM1–RhoA–ROCK1/2 axis, the JNK pathway, and a crosstalk with AKT.[87–89] Chronic treatment of melanoma cells with the BRAF inhibitor PLX4720 elevated Wnt-5a expression.[87] In addition, Wnt-5a activated AKT signaling in melanoma cells via the receptors $FZD_7$ and RYK, leading to increased growth and chemoresistance.[87] Knockdown of Wnt-5a, $FZD_7$, and RYK inhibited tumor growth and sensitized melanoma cells to BRAF inhibitor.[87]

Another study reported that $FZD_7$ is essential for the initiation of melanoma cell tumors *in vivo*.[89] $FZD_7$ is required for the proliferation of melanoma cells during metastatic growth; nevertheless, the effects of $FZD_7$ on cell proliferation are not intrinsic to the cancer cells but depend on the microenvironment *in vivo*.[89] The role of $FZD_7$ in the formation of metastatic melanoma is independent of BRAF mutation status or sensitivity to BRAF inhibitors.

Further, $FZD_7$ and Wnt-5a promote melanoma metastasis via crosstalk with the JNK pathway.[89] A recent study highlighted that $FZD_7$ supports melanoma spheroid (melanosphere) formation and amoeboid melanoma cell invasion.[88] Mechanistically, the Wnt-11–$FZD_7$–DAAM1 axis activates Rho-ROCK1/2–Myosin II and regulates the tumor-initiating potential, local invasion, and distant metastasis formation of melanoma.[88]

### *Pancreatic cancer*

Pancreatic cancer is a deadly disease that caused 466,003 known deaths in 2020, with a very low five-year survival rate of 9%. This poor prognosis results from late-stage diagnosis and a lack of efficacious treatments.[1,90] One of the areas of research into possible new treatments targets CSCs. Among the potential targets, Wnt/β-catenin is thought to play a role in the development of pancreatic CSCs that are responsible for tumor initiation, progression, and metastasis, as well as chemotherapy resistance.[90]

A recent study documented the role of $FZD_7$ in chemoresistance in pancreatic cancer. ATP-binding cassette superfamily G member 2 (ABCG2) is one of the xenobiotic transporters involved in multi-drug resistance to chemotherapeutic agents.[91] ABCG2 is overexpressed in human pancreatic cancer tissues when compared to adjacent tissues, and this overexpression correlates with a lower probability of survival.[92] An *in vitro* study has demonstrated that Wnt-5a upregulates ABCG2 expression through $FZD_7$ in Capan-2 pancreatic cancer cells. The overexpression of Wnt-5a and $FZD_7$ drives gemcitabine resistance in Capan-2 cells.[92] In addition to Wnt-5a, Wnt-7b has also been validated as a binding partner for $FZD_7$, which also regulated the expression of ABCG2, in pancreatic cancer.[18] $FZD_7$ knockdown sensitized cancer cells to gemcitabine, highlighting the role of $FZD_7$ in chemoresistance in pancreatic cancer.[18,92] Furthermore, $FZD_7$ knockdown decreased the stemness phenotypes of pancreatic CSCs.[18]

### *Other types of cancer*

The role of $FZD_7$ has been documented in other types of cancer, such as kidney cancer, cervical cancer, glioma, esophageal cancer, and leukemia.

The role of $FZD_7$ in kidney cancer was first appreciated in Wilms' tumors, a type of kidney cancer that mainly affects young children. A subset of Wilms' tumors express $FZD_7$ ($FZD_7^+$ cells), but it was difficult to isolate this subset of cells.[93] An anti-$FZD_7$ antibody that was intended to isolate Wilms' tumor cells turned out to induce apoptosis in these cells.[93] This indicated the essential role of $FZD_7$ in the survival of at least a subset of Wilms' tumor cells. Further exploration demonstrated that $FZD_7^+$ cells in Wilms' tumors are highly clonogenic and proliferative when compared with $FZD_7^-$ cells.[93] $FZD_7$ is upregulated in another type of kidney cancer, clear cell renal cell carcinoma (ccRCC), as compared to peritumor tissues.[94] Wnt-3a activated $FZD_7$ in RCC cells and promoted the proliferation of these cells.[94]

In cervical cancer, $FZD_7$ is involved in cancer metastasis. $FZD_7$ knockdown inhibited the expression and activities of matrix-metalloproteinase 2 (MMP2) and MMP9, proteins that are necessary to break down the extracellular matrix during cancer metastasis.[95] In addition, $FZD_7$ knockdown increased the expression of epithelial markers and decreased mesenchymal markers in HeLa and SiHa cells, suggesting that $FZD_7$ plays an important role in the EMT.[95] Phenotypically, $FZD_7$ knockdown suppressed the migration and invasion capacities of HeLa and SiHa cervical cancer cells. The effect of $FZD_7$ on cervical cancer motility seems to be mediated by a β-catenin-independent Wnt pathway, especially by JNK/c-jun signaling.[95]

In glioma, $FZD_7$ overexpression correlates positively with advanced tumor stages,[96] but negatively with the median survival of glioma patients.[97] $FZD_7$ promotes glioma cell proliferation *in vitro* and *in vivo*. In a recent study, the transcriptional co-activator TAZ was found to be a target gene of the β-catenin-dependent Wnt pathway.[96] $FZD_7$ promotes the proliferation of glioma cells though upregulation of TAZ.[96]

Esophageal cancer is also regulated by $FZD_7$. $FZD_7$ affects the growth, migration, and invasion of esophageal cancer cells.[98] Furthermore, the overexpression of $FZD_7$ upregulates MDR1 expression, leading to chemoresistance in esophageal cancer; $FZD_7$ knockdown sensitized esophageal cancer cells to cisplatin.[98]

$FZD_7$ has also been found to regulate cancer cell proliferation and chemoresistance in chronic myeloid leukemia (CML).[99] $FZD_7$ inhibition sensitized CML cells to imatinib, the first-line therapy for CML.[99]

## $FZD_7$ antagonists in current development

The identification of specific $FZD_7$ inhibitors has proven to be challenging. The Wnt–FZD pathway is initiated by Wnt proteins, promiscuous ligands that each can engage multiple FZDs. In addition, Wnt binds to the CRD, a highly conserved extracellular region of FZD proteins. Therefore, the lack of an *in vitro* assay that would distinguish any one individual FZD from the other nine family members has hampered classical high-throughput screening (HTS) approaches for the discovery of selective FZD antagonists. As regards structure-based approaches, molecular understanding of the FZD protein family has improved in the past decade, but to-date, the whole receptor has not been crystalized. The available structures for $FZD_7$ are limited to the CRD in its apo form (PDB ID: 5 T44), in complex with a fatty acid (PDB



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ID: 5URV) or in complex with the peptide dFz7-21 (PDB ID: 5WBS).[100] Despite these challenges, several approaches have led to the identification of $FZD_7$ inhibitors. Here, we elaborate on some agents that target $FZD_7$, including small molecules and biomolecules such as peptides, proteins, and antibodies (Tables 2 and 3). Below, we focus on exploring molecules that physically bind $FZD_7$ and antagonize the downstream Wnt pathway, without restricting our review to the context of cancer.

### *Small molecules*

To-date, no $FZD_7$-selective small-molecule inhibitors have been identified through HTS. Instead, structure-based drug discovery has been the driving force in the identification of compounds that interact with the $FZD_7$ (Table 2).

The GPCR class F family is composed of SMO and FZDs. SMO has a high sequence similarity to the FZD proteins: the TMD of $FZD_7$ and SMO share 28% identical and 47% homologous residues. Zhang *et al.*[101] developed a homology model for $FZD_7$ TMD based on the crystal structure of SMO interacting with a SMO antagonist LY2440680 (Taladegib). This homology model then was used for the structure-based virtual screening of 500,000 diverse small molecules and then 5000 analogs of the top-scored compounds. This *in silico* screening resulted in the identification of six hits that inhibited Wnt/β-catenin signaling *in vitro* in Wnt-3a-expressing HEK293 cells. SRI37892, the best hit ($IC_{50}$: 0.66 μM), inhibited the proliferation and suppressed the colony formation of several TNBC cell lines, with an $IC_{50}$ value corresponding that seen for Wnt pathway inhibition.[101] Treatment with SRI37892 also decreased LRP6 phosphorylation, a molecular event close to the Wnt–FZD interaction. Although the authors did not assess the selectivity of SRI37892 for FZDs, these findings suggest possible on-target activity of SRI37892 on FZDs. Further analysis of the putative binding site in the $FZD_7$ TMD in the model identified that the common phenyl benzimidazole unit of these inhibitors binds a hydrophobic pocket via multiple Pi–Pi interactions, while the other end of the compounds occupies a second hydrophobic pocket.[101] The sequence similarity of the binding pocket within the FZD family suggests that SRI37892 and its analogs might also act on multiple FZDs.[101]

With no available structure solved for Wnt-3, Wnt-3a, or full-length $FZD_7$, Pinto *et al.*[102] generated 100 homology models using the pdb4f0a template (41% sequence identity) for Wnt-3, Wnt-3a and three crystals of the $FZD_7$ CRD domain. Ranking of the homology models and further assessment of their quality resulted in the selection of the best homology model for each protein.[102] Pinto *et al.*[102] searched the ZINC database for commercial analogs of palmitoleic acid (PAM) because of the role of this compound in activating the Wnt pathway and identified 29 compounds with 99% similarity. Docking into the homology model identified four fatty acids (illustrated by ZINC05972969 in Table 2) that had a calculated binding energy similar or better than that of PAM.[102] *In vitro* studies are now required to assess and validate the effects of these ligands on the Wnt pathway and to evaluate their selectivity among FZDs.

### *Biomolecules*

Biomolecules that mimic or antagonize $FZD_7$ have been used to study the role of $FZD_7$ in the Wnt pathway. Besides being used as a molecular tool, some of those molecules exhibit anticancer properties (Table 3).

### *Peptides*

Several peptides that target $FZD_7$ have been developed to antagonize the Wnt pathway. From a phage peptide library, Nile *et al.*[46] identified peptides that bound specifically to an Fc-tagged $hFZD_7$ CRD but not to an Fc-tagged $hFZD_8$ CRD. Five peptides were synthesized and the most potent peptide, Fz7-21, both inhibited the Wnt-3a-induced Wnt-β–catenin pathway in HEK293 ($IC_{50}$: 100 nM) and blocked the Wnt-3a-mediated stabilization of β-catenin in L-cells ($IC_{50}$: 50 nM).[46] The Cys10 residue of the peptide appeared to be key for the interaction as its replacement with a serine or with an unnatural stereoisomer reduced by 30-fold or completely abolished the inhibitory activity.[46] Fluorescence size-exclusion chromatography (FSEC) using a fluorescein-labeled version of Fz7-21 confirmed the peptide's subtype selectivity for the $FZD_{1/2/7}$ isoforms. The authors were able to solve the structure of a construct consisting of the N-terminus of Fz7-21 fused to the C-terminus of the $hFZD_7$ CRD (PDB ID: 5WBS).[46] This crystal structure showed that the peptide dimerizes through a disulfide bond made by Cys10, interacts with residues at a new site proximal to the lipid-binding groove of the $hFZD_7$ CRD, and traps the $FZD_7$ dimer in an open and inactive state.[46] This result was validated by a shotgun alanine scan that confirmed the key

**TABLE 2**

**Small molecules targeting $FZD_7$ identified by computational docking.**

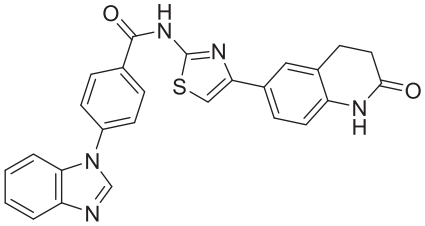
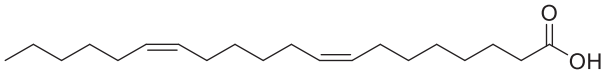
| Molecule name | Structure | Putative binding site | Biological activities | Reference |
|---|---|---|---|---|
| SRI37892 |  | $FZD_7$ transmembrane domain (TMD) | Inhibited cell proliferation and β-catenin-dependent Wnt pathway in triple-negative breast cancer (TNBC) cells *in vitro* | [101] |
| ZINC05972969 |  | $FZD_7$ cysteine-rich domain (CRD) | Not determined | [102] |



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**TABLE 3**

**Biomolecules targeting $FZD_7$.**

| Molecule name | Binding site | Biological activities | Reference (s) |
|---|---|---|---|
| **Peptide** | | | |
| Fz7-21 | $FZD_7$ cysteine-rich domain (CRD) | • Inhibited Wnt3a-induced Wnt/β-catenin pathway in HEK293 cells<br>• Disrupted the formation of the Wnt3a–FZD7–LRP6 ternary complex<br>• Inhibited *Clostridium difficile* interaction with FZD7 | 46,103 |
| **Engineered protein and antibodies** | | | |
| DRPB_Fz7 | $FZD_{1/2/7}$ CRD | • Bound to $FZD_{1/2/7}$ (subtype specific)<br>• DRPB_Fz7 moderately downregulated the expression of Wnt target genes in the liver *in vivo* | 47 |
| Antibody anti-human $FZD_7$ | $FZD_7$ CRD | • Depleted stem cell properties in $FZD_7$-sensitive Wilms' tumor<br>• Reduced the survival of $FZD_7$-sensitive Wilms' tumor cells | 93,105 |
| Antibody anti-human $FZD_7$ | $FZD_7$ CRD | • Blocked the β-catenin-dependent Wnt pathway in various cancer cell lines<br>• Inhibited cell migration in triple-negative breast cancer (TNBC) and ovarian cancer cells<br>• Suppressed glioblastoma cell clonogenicity | 106 |
| $FZD_7$ scFv | $FZD_7$ CRD | • Inhibited cell growth and increased cell apoptosis in TNBC cells<br>• Decreased the expression of Wnt target genes | 107–108 |
| $FZD_7$-NS | $FZD_7$ CRD | • Inhibited the Wnt/β-catenin-dependent pathway<br>• Decreased TNBC cell viability and migration *in vitro* | 109 |
| $FZD_7$-Fab | $FZD_7$ | • Bound selectively to $FZD_7$<br>• Inhibited the Wnt-3a induced β-catenin-dependent Wnt pathway | 110 |
| F2 scFv | $FZD_{1/2/7}$ CRD | • Bound to $FZD_{1/2/7}$ (subtype specific)<br>• Combination with F3 ($FZD_{5/8}$ antagonist) was detrimental to intestinal organoid | 48 |
| Vantictumab (OMP-18R5) | $FZD_{1/2/5/7/8}$ CRD | • Bound to $FZD_{1/2/5/7/8}$<br>• Inhibited the β-catenin-dependent Wnt pathway<br>• Inhibited the growth of several types of tumors<br>• Synergist with several standard-of-care chemotherapeutics | 67,112 |

interactions, by the 40-fold potency improvement of a dimeric form dFz7-21, and by the concentration-dependent induced dimerization of $hFZD_7$ CRD. Interestingly, the dFz7-21 peptide does not compete with Wnt-3a for binding to $FZD_7$ but disrupts the formation of the Wnt-3a–$FZD_7$–LRP6 ternary complex.[46]

Further peptide optimization, driven by inhibition of the *Clostridium difficile* toxin B interaction with $FZD_7$, resulted in a significant potency improvement.[103] The new peptide inhibited the Wnt-3a-induced β-catenin-dependent pathway with an $IC_{50}$ in the low-digit nanomolar range.[103] The unique mode of action of these peptides offers a viable approach for the treatment of multiple diseases associated with $FZD_7$, including cancer. Eventually, the subtype selectivity of these peptides will help us to further understand the role of $FZD_{1/2/7}$ in physiological processes and diseases.

### *Engineered proteins and antibodies*

Ankyrin-repeat proteins are genetically engineered antibody-mimetic proteins that typically exhibit highly specific and high-affinity binding to target proteins. Dang *et al.*[47] used ankyrin-repeat proteins, which they called designed repeat protein binders (DRPBs), to develop FZD subtype-specific inhibitors. They utilized computational design to target a defined and large surface region in the CRD of each FZD subtype, then generated subtype-specific variants by exploiting contacts at subtype-specific positions within this broadly conserved buried interface.[47] First, they developed DRPB_Fz8, which binds $FZD_5$ and $FZD_8$. The crystal structure of the DRPB_Fz8 –$FZD_8$ CRD complex reveals a binding mode identical to the 'grasping-hand' model, which prevents the binding of Wnts to the FZD lipid-binding groove. Dang *et al.*[47] then redesigned DRPB_Fz8 for two other FZD subtypes, $FZD_4$ (DRPB_Fz4) and $FZD_7$ (DRPB_Fz7 binding to the CRD of $FZD_{1/2/7}$), as well as for DRPB binding to both $FZD_7$ and $FZD_8$ subtypes (DRPB_Fz7/8).[47] Some of the key residues that define FZD subtype specificity were solved from the crystal structure. One example is the Ala111Asp mutation from DRPB_Fz8 to DRPB_Fz7/8, which enables the formation of a hydrogen bond and a salt bridge with a corresponding Lys of $FZD_7$, which is a Glu in the $FZD_8$ subtype.[47] However, epitopes of CRD outside the lipid binding groove can also serve to inhibit FZD. A set of small molecule antagonists bind the $FZD_8$ CRD at residues Leu97, Met149, Asp150 (corresponding to Ile117, Val161 and Gly162 of $FZD_7$) at micromolar concentrations, corresponding to similar $IC_{50}$ Wnt pathway inhibition in the cellular assay[104] and thus providing a proof of principle for this mechanism of receptor targeting.

Antibodies against $FZD_7$ comprise a large portion of the biological molecules that target $FZD_7$ in cancer. Initially used as a tool to isolate $FZD_7^{\pm}$ Wilms' tumors (WTs), a commercial human anti-$FZD_7$ antibody (Anti-$FZD_7$ Ab) induced extensive cell death of $FZD_7^{\pm}$ WT.[93,105] Anti-$FZD_7$ Ab treatment depleted the stem cell properties of $FZD_7$-sensitive WT.[93] Furthermore, Anti-$FZD_7$ Ab reduced the proliferation and survival of $FZD_7$-sensitive WT cells grafted to chick embryos.[93] Another anti-human $FZD_7$-specific antibody was developed to block the β-catenin-dependent Wnt pathway in various cancer cell lines, including TNBC and ovarian cancer cells.[106] This antibody also inhibited the migration of TNBC cells and ovarian cancer cells.[106]



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Other antibodies that have been developed against FZDs include single-chain fragment variable (scFv) antibodies against the extracellular domain of $FZD_7$.[107–108] These scFv antibodies bind specifically to $FZD_7$. Anticancer assays demonstrated that the scFv antibodies inhibited the growth of BC cells and increased their apoptosis.[107–108] Several studies have explored the use of nanoparticles to improve the target affinity of the $FZD_7$ antibody. Riley *et al.*[109] utilized nanoshells composed of silica cores and thick gold shells, then coated these nanoshells with the $FZD_7$ antibody to form a $FZD_7$ antibody–nanoshell complex ($FZD_7$–NS). $FZD_7$–NS selectively bound TNBC cells overexpressing $FZD_7$ and antagonized the β-catenin-dependent Wnt pathway, with higher efficacy than that of free $FZD_7$ antibodies.[109]

$FZD_7$-specific fragment antigen binding (Fab) protein has been reported to recognize only $FZD_7$ among all FZDs.[110] Using flow cytometry, $FZD_7$–Fab can be used to enrich human embryonic stem cells (hESCs) expressing high $FZD_7$. A prolonged $FZD_7$–Fab treatment reduced the expression of pluripotency markers in hESCs and disrupted the Wnt-3a-induced Wnt pathway in these cells.[110] Interestingly, the $FZD_7$–Fab treatment did not directly counteract the interaction between Wnt-3a and $FZD_7$, but led to $FZD_7$ degradation, which then disabled the receptor that binds Wnt-3a and transduces the Wnt pathway.[110] Fernandez *et al.*[110] speculate that $FZD_7$–Fab induced the internalization of $FZD_7$, hence quenching Wnt pathway activation by Wnt-3a/$FZD_7$.[110] $FZD_7$ internalization has also been observed upon treatment of Wilm's tumor with different anti-$FZD_7$ Ab, leading to signal shutdown and growth inhibition.[93] Thus, these studies report another potentially attractive mechanism: $FZD_7$ signaling shutdown through receptor internalization. Mechanistically, a very important and potentially targetable step in this process is mediated by the E3 ubiquitin ligases Rnf43 and Znrf3, which have been reported to regulate Wnt signaling negatively by promoting the degradation of FZD–LRP complexes.[111]

Lately, the development of surrogate Wnt molecules has been seen as a highly active approach to trigger the Wnt pathway artificially. Wnt proteins are difficult to produce and to purify because they have multiple post-translational modifications and because of the resulting hydrophobicity. Consequently, researchers have attempted to develop soluble Wnt surrogates. Chen *et al.*[48] developed a soluble Wnt surrogate by linking two scFv antibodies to a FZD CRD (FZD binder) and LRP6 (LRP binder). By adapting the FZD-binder scFv for each FZD subtype (termed as F2 for $FZD_{1/2/7}$ and F3 for $FZD_{5/8}$), researchers achieved selective Wnt activation upon treatment with the surrogate.[48] Interestingly, by using the FZD binding arms alone, the surrogates could be turned into FZD subtype-selective antagonists.[48]

The most advanced antibody against $FZD_7$ is vantictumab (OMP-18R5), a fully humanized monoclonal antibody that binds $FZD_{1/2/5/7/8}$.[67] Vantictumab interacts with the discontinuous epitope that spans a 'cleft' region that is apparent in the reported crystal structure of mouse $FZD_8$.[67] In a cell-based assay, vantictumab blocked most β-catenin signaling in response to Wnt-3a.[67] *In vivo*, vantictumab exhibited an anticancer effect against BC, CRC, lung cancer, pancreatic cancer, and gastric cancer.[44,67] Vantictumab also demonstrated synergistic anticancer effects upon combination with several standard chemotherapeutic agents, such as taxol in non-small cell lung cancer and BC models, irinotecan in colon cancer models, and gemcitabine in pancreatic cancer models.[67,112] Vantictumab also suppressed tumor recurrence following treatment with high-dose chemotherapies. Despite these impressive pre-clinical profiles, the development of vantictumab has been halted in clinical trial phase 1b due to bone toxicity.[113–114]

## Discussion

### *The need for selective Wnt pathway inhibition in cancer*

Despite significant progress in oncology, there is still a large unmet need for novel anticancer therapies. Better understanding of the disease at the molecular level has shifted drug discovery from cytotoxic drugs towards targeted treatments and immunotherapies. Despite these advances, cancer is still a major health burden, and drug resistance that plagues current therapies urges the development of novel therapeutic options.[49] The Wnt pathway is an example of a therapeutic opportunity waiting to be fully unlocked as a cancer target.

The first generation of Wnt pathway inhibitors confirmed the importance of this pathway in cancer, but showed that non-selective inhibition of the pathway is linked to on-target side effects.[6] Such first-generation drugs include porcupine inhibitors, which are pan-Wnt pathway inhibitors that prevent the secretion of functional Wnt proteins. Porcupine inhibitors produce dose-dependent adverse effects, such as loss of bone volume and density, within four weeks of exposure in mice treated with two structurally distinct inhibitors, LGK974 and ETC-159.[115] Administration of alendronate overcame the bone toxicity of ETC-159 in mice, although success of this strategy remains to be confirmed further in clinical studies.[115] Another example of the toxicity of Wnt pathway inhibitors involves tankyrase (TNKS) inhibitors. TNKS regulates the stability and turnover of Axin, a component of the β-catenin destruction complex. Despite showing promising anticancer activity in pre-clinical studies, the current TNKS inhibitors exhibit multiorgan toxicity, particularly GI tract toxicity.[116–117] Hence, the next generation of Wnt pathway inhibitors must tackle the challenge of targeting cancer-relevant Wnt signaling sub-pathways while sparing physiologically important sub-systems. Potential targets to fulfill this profile are the FZD proteins. FZDs have a defined tissue-specific profile (Fig. 1) and are key to the initiation of multiple Wnt signaling branches.

Vantictumab was initially designed to target $FZD_7$ but later demonstrated cross-reactivity with four other FZDs. Extensive pre-clinical studies in various types of cancer allowed vantictumab to enter clinical trials, with potential efficacy shown in phase 1 clinical studies.[113–114] Vantictumab in combination with nab-paclitaxel and gemcitabine has been evaluated in a phase 1b clinical trial for untreated metastatic pancreatic cancer, improving the median overall survival from 8.5 months to 10.2 months.[113] Another recent phase 1b clinical trial has studied a combination of vantictumab and paclitaxel in patients with locally advanced or metastatic HER2–negative BC.[114] Promising clinical activity of the vantictumab– paclitaxel combination was observed in this trial, in which the overall response rate of patients who received up to two prior lines of chemother-



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apy was comparable to paclitaxel in the first-line setting. However, the lack of FZD specificity was the likely cause of the unfortunate failure of vantictumab in phase I clinical studies, in which an increase in the frequency of bone fractures was attributed to the treatment.[113–114] Indeed, three of the FZDs targeted by vantictumab, $FZD_1$, $FZD_4$, and $FZD_8$, are important for bone homeostasis. Adjustment of vantictumab dose and supplementation with zolendronic acid alleviated the bone fragility, but bone toxicity remains a significant concern within the overall toxicity profile of vantictumab.[113] This case underlines the importance of developing a FZD-selective antagonist in order to achieve selective Wnt pathway inhibition in cancer.

### *An $FZD_7$-selective inhibitor for selective Wnt pathway inhibition in cancer*

$FZD_7$ serves as a validated target in various types of cancer. $FZD_7$ overexpression has been reported in TNBC, gastric cancer, HCC, and ovarian cancer.[53,70–71,77–78,84] In addition to these cancers, $FZD_7$ also regulates tumorigenesis in several other cancers, including CRC, pancreatic cancer, and melanoma.[18,62,66,88–89,92] $FZD_7$ affects cancer growth and metastasis *in vitro* and *in vivo*. The branches of the Wnt pathway that are activated by $FZD_7$ are cancer-type dependent. For example, $FZD_7$ relays both β-catenin-dependent and -independent Wnt pathways in TNBC, CRC, ovarian cancer, and pancreatic cancer.[18,23,52–53,56,62,64,66,84–85,92] Meanwhile, $FZD_7$ initiates mainly the β-catenin-dependent Wnt pathway in HCC and the β-catenin-independent pathways in melanoma.[87–89] This highlights the diverse role of $FZD_7$ in carcinogenesis.

Other intertwined aspects of carcinogenesis that are regulated by $FZD_7$ are cancer stemness and chemoresistance. In normal development and physiology, $FZD_7$ is required for the maintenance of hESCs and adult intestinal stem cells.[19,43,110] Recent studies report the importance of $FZD_7$ in maintaining cancer stemness in various cancers, including TNBC, gastric cancer, ovarian cancer, and esophageal cancer.[56,70,84,98] CSCs contribute to cancer chemoresistance as they are frequently in a quiescent state with a low proliferation rate, which shields them from conventional cytotoxic chemotherapeutic agents that target highly proliferative cancer cells.[59] Furthermore, CSCs often express chemoresistance-mediating drug-efflux pumps, such as ABCG2 and MDR1, whose protein expression is regulated by $FZD_7$.[59,82,92] Studies highlight that $FZD_7$ knockdown decreased the stemness properties of TNBC, gastric cancer, ovarian cancer, and esophageal cancer cells, as well as sensitizing those cancer cells to cytotoxic chemotherapeutic agents.[56,70,84,98] Hence, inhibition of $FZD_7$ becomes a viable option for depleting the CSC niche and overcoming chemoresistance.

Deep sequencing of cancer genomes reveals that 4.2% of all tumor sequences deposited in the COSMIC database show activating mutations in *GNAS*, locus encoding Gαs, thereby highlighting the oncogenic potency of Gαs.[118] Accumulating evidence shows the involvement of Gαs in the carcinogenesis of various cancers, including endocrine tumors, BC, and lung cancer.[119–121] Hence, there is a compelling need to evaluate the $FZD_7$–Gαs signaling axis in various $FZD_7$-related cancers.

As Wnt pathway inhibitors are usually jeopardized by on-target toxicity, it is valid to ask whether $FZD_7$-selective inhibition could avoid such toxicity. Using mice as the animal model, a crucial non-redundant role has been established for $FZD_7$ in the maintenance of intestine and atrial stomach epithelium.[19,43] Contrasting these results are the *$FZD_7$*-knockout mice that are viable and fertile with no overt intestinal phenotype under basal, non-challenging conditions. These two seemingly contradictory results demonstrate that loss of $FZD_7$ in a developing organism is compensated by other mechanisms, whereas in adults, $FZD_7$ is indispensable. This unoptimistic perspective is somewhat alleviated by the observation that, following injury, $FZD_7$ downregulation did not prevent, but rather delayed, intestinal regeneration.[19] Recent studies using selective biomolecules that inhibit different FZD subtypes highlighted the redundant role of $FZD_5$ and $FZD_7$ in the maintenance of GI survival *in vitro* and homeostasis *in vivo*, with $FZD_5$ inhibition showing a more detrimental effect on the GI tract.[47–48] No significant GI track toxicity was reported for vantictumab, which targets both $FZD_5$ and $FZD_7$,[67,113–114] although this might be attributed to low exposure in the GI tract due to the intravenous administration of the drug. These studies highlight the prospect of the use of $FZD_7$-selective antagonists to deplete cancer cells, with fewer adverse events compared to the use of pan-Wnt pathway inhibitors.

### *Challenges and perspectives in developing $FZD_7$-selective inhibitors*

Several challenges still hamper the development of $FZD_7$-selective inhibitors, either small molecules or biomolecules. The first challenge is the lack of structural information, both for the full-length $FZD_7$ structure and the ternary complex with a Wnt and co-receptor(s). The recent first co-crystal structure of a small molecule, carbamazepine, interacting with $FZD_8$ (PDB ID: 6TFB) has shed light on the novel binding site for small molecules in the CRD.[122] The development of biomolecules such as antibodies might overcome the insufficiency of $FZD_7$ crystal structures.

A second challenge is the lack of proximal and/or specific assays to identify and characterize $FZD_7$ inhibitors. A recent development adapted BRET and fluorescence resonance energy transfer (FRET) technologies to FZDs and resulted in the identification of SAG1.3, a SMO-targeting molecule that acts as a partial low-potency agonist of $FZD_6$ and $FZD_7$ but not $FZD_4$, thus opening an interesting prospect of developing this scaffold to target FZDs.[26]

It is also necessary to evaluate and distinguish functional FZD selectivity, and how this translates to Wnt sub-pathway(s) inhibition. Although functional *in vitro* assays, such as the TopFlash assay, are key to assessing the inhibition of the Wnt pathway upon treatment, different cell lines express a different set of FZDs. Therefore, it is difficult to interpret the outcome of standard cell-based assays and to judge whether a given inhibitor exerts its Wnt inhibitory activity by specifically antagonizing $FZD_7$. A *$FZD_{1\text{-}10}$* knockout cell line (*$FZD^{-/-}_{1\text{-}10}$*) has recently been developed using iterative CRISPR mutagenesis.[123] This cell line is a remarkable starting point for screens to identify $FZD_7$-selective inhibitors. It is now possible to express individual FZD proteins (e.g. $FZD_7$) in these $FZD^{-/-}_{1\text{-}10}$ cells and use them to perform a functional assay, such as the TopFlash assay, to assess



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the selectivity of the candidate molecules for different FZDs. This work will pave the way towards the discovery and characterization of therapeutics that act as selective inhibitors of $FZD_7$.

## Conclusions

$FZD_7$-selective inhibition has emerged as a highly attractive avenue for the second generation of Wnt-inhibitors. Numerous studies corroborate the importance of $FZD_7$ in the tumorigenesis of various cancers because of its importance in regulating cancer growth, metastasis, CSCs, and chemoresistance. In normal physiology, $FZD_7$ plays a role in the regeneration of the GI tract upon injury, but seems to work in a redundant manner with other FZDs. Despite the remaining challenges, the recent development of *in vitro* assays to evaluate selectivity, paralleled by insights into the structural details of the FZD family, will be pivotal in identifying $FZD_7$-selective inhibitors. It is a matter of time before we see novel small molecules or biologics that have with better $FZD_7$-selectivity profiles. Such $FZD_7$-selective inhibitors have highly promising therapeutic potential as monotherapies or in combination with standard of care treatments.

## Conflicts of Interest

The authors declare that they have no conflict of interests.

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KEYNOTE (GREEN)

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