Oligomerization of Frizzled and LRP5/6 protein initiates intracellular signaling for the canonical WNT/β-catenin pathway

Received for publication, June 12, 2018, and in revised form, October 24, 2018 Published, Papers in Press, October 25, 2018, DOI 10.1074/jbc.RA118.004434

Yue Hua, Yilin Yang, Qian Li, Xinyu He, Wei Zhu, Jiyong Wang[1], and Xiaoqing Gan[2]

From the Key Laboratory of Metabolism and Molecular Medicine, Ministry of Education, Department of Biochemistry and Molecular Biology, School of Basic Medical Sciences, Fudan University, Shanghai 200032, China

Edited by Xiao-Fan Wang

Upon binding to the canonical WNT glycoproteins, Frizzled family receptors (FZDs) and low-density lipoprotein receptor-related protein 5/6 (LRP5/6) undergo a series of polymerizations on the cell surface that elicit canonical WNT/β-catenin signaling. The hyperactivation of WNT/β-catenin signaling is the major cause of tumorigenesis, but the mechanism in tumors such as hepatoma remains unclear. Here, we observed that WNT3A manifested the hyperactivity in β-catenin–dependent signaling after binding to FZD's competitive inhibitory molecule secreted Frizzled-related protein 2 (SFRP2). To understand the mechanism of FZDs in the presence of SFRP2, we explored how FZDs can bind and activate the LRP5/6 signalosome independently of WNT glycoproteins. Our findings further revealed that oligomerizations of FZDs and LRP5/6 can integrate the cytoplasmic protein Dishevelled into the LRP5/6 signalosome, resulting in a robust activation of ligand-independent β-catenin signaling. We propose that besides WNT-bridged FZD–WNT–LRP5/6 protein complexes, the homo- and hetero-oligomerizations of WNT receptors may contribute to the formation of the LRP5/6 signalosome on the cell surface. Of note, we identified four highly expressed FZDs in the hepatoma cell line HepG2, all of which significantly promoted ligand-independent LRP5/β-catenin signaling. As FZDs are ectopically expressed in numerous tumors, our findings may provide a new perspective on tumor pathologies. Furthermore, the results in our study suggest that the composition and stoichiometry of FZDs and LRP5/6 within the LRP5/6 signalosome may tune the selection of bound WNT glycoproteins and configure downstream WNT/β-catenin signaling.

Signaling elicited by the WNT family of secreted glycoproteins is highly conserved in evolution, innervating cell proliferation, cell specification, and stem cell renewal during embryonic development and tissue homeostasis (1, 2). In most mammals, including humans, WNT family proteins consist of 19 members and are divided into canonical and noncanonical subfamilies. Upon binding of a canonical WNT ligand to low-density lipoprotein receptor-related protein 5/6 (LRP5/6)[3] and Frizzled family receptors (FZDs), intracellular modulators Dishevelled (DVL) and AXIN1 are recruited to the plasma membrane through the intracellular domains of FZDs and LRP5/6, resulting in repression of a "destruction complex" formed by AXIN1, glycogen synthase kinase 3β (GSK3β), and adenomatous polyposis coli protein (APC); hence, β-catenin proteins escape from N-terminal phosphorylation and subsequent proteasome-dependent degradation. Thereafter, stabilized β-catenin proteins enter the nucleus and facilitate transcription of WNT target genes via partnering with T-cell factor/lymphoid-enhanced binding factor 1 family transcription factors (3). Because of the pivotal role of WNT/β-catenin signaling in tumorigenesis and other pathological processes (1, 4), the molecular mechanisms underlying this pathway have been extensively investigated over the past two decades.

A prominent feature of WNT/β-catenin signaling is the requirement for both FZDs and LRP5/6; however, the mechanism underlying the formation of FZDs–LRP5/6 coreceptor complexes remains ambiguous. The paradigm of WNT-bridged FZD–WNT–LRP5/6 trimeric protein complex which has long been considered as an ideal model for the initiation of WNT/β-catenin signaling on the cell surface (1, 5) is now insufficient to explain the architecture of large-sized LRP5/6-FZDs signalosome crucial for WNT/β-catenin activation (6). There have been a large number of studies to illustrate how the LRP5/6-FZDs signalosome is formed. For example, the oligomerization of AXIN1 or DVL has been described as the key molecular basis underlying signalosome formation (7, 8). At the same

This work was supported by National Natural Science Foundation of China (NSFC) Grants 31471317 and 31671466. The authors declare that they have no conflicts of interest with the contents of this article.

[1] To whom correspondence may be addressed: Key Laboratory of Metabolism and Molecular Medicine, Ministry of Education, Dept. of Biochemistry and Molecular Biology, School of Basic Medical Sciences, Fudan University, P. O. Box 240, Shanghai 200032, China. Tel.: 86-21-54237661; E-mail: jiyongwang73@gmail.com.

[2] To whom correspondence may be addressed: Key Laboratory of Metabolism and Molecular Medicine, Ministry of Education, Dept. of Biochemistry and Molecular Biology, School of Basic Medical Sciences, Fudan University, P. O. Box 240, Shanghai 200032, China. Tel.: 86-21-54237661; E-mail: xiaoqinggan@fudan.edu.cn.

[3] The abbreviations used are: LRP5/6, low-density lipoprotein receptor-related protein 5/6; FZD, Frizzled family receptor; SRFP, secreted Frizzled-related protein; DVL, Dishevelled; GSK3β, glycogen synthase kinase 3β; APC, adenomatous polyposis coli protein; CRD, cysteine-rich domain; GPCR, G protein–coupled receptor; LDLR, low-density lipoprotein receptor; HA, hemagglutinin; CM, conditioned medium; co-IP, coimmunoprecipitation; NTR, netrin-related motif; PORCN, protein-serine *O*-palmitoleoyltransferase porcupine; ECD, extracellular domain; LNCAP, lymph node carcinoma of the prostate; CLTC, clathrin heavy chain 1; CAV1, caveolin 1; DPBS, Dulbecco's phosphate-buffered saline; FZD5-N, N terminus of Frizzled-5.

time, the structural analyses of WNT glycoproteins as well as their receptors also provide interesting information regarding the establishment of LRP5/6–FZD signalosome. The 3.25-Å structure of *Xenopus* Wnt8 in complex with mouse FZD8 cysteine-rich domain (CRD) revealed that there may be double binding sites on Wnt8 to grasp the FZD8 CRD (9), raising the possibility of more than one Frizzled receptor binding to one WNT glycoprotein. In contrast, LRP6 appears to engage different subsets of WNT glycoproteins via different β-propeller modules, similarly implying the potency of LRP6 simultaneously binding to two WNT glycoproteins (10–12). Hence, these observations strongly suggest that the arrangement of the LRP5/6 signalosome may be variable rather than a simple oligomerization of trimeric FZD–WNT–LRP5/6 protein complex. In addition, during signalosome formation on the cell surface, whether or not the simultaneously binding of WNT glycoproteins to LRP5/6 and FZDs is essential is still unknown. In fact, there is still a lack of sufficient biochemical and structural evidence for simultaneous binding of a WNT ligand to a Frizzled receptor and an LRP5/6 receptor. Meanwhile, it is also worth noting that the large-sized LRP6 signalosome can also be induced by APC loss in tumor cells regardless of the presence or absence of WNT glycoproteins (13).

Recently, the function of FZDs in the LRP5/6 signalosome has been challenged as several studies have demonstrated that the secreted Frizzled-related proteins (SFRPs), the conserved inhibitors competing with FZDs to bind to WNT glycoproteins, can promote, but not inhibit, ligand-dependent β-catenin signaling. These observations contradict the established mechanism for FZDs in the canonical WNT pathway. Unlike LRP5/6 proteins, which appear to possess intrinsic capability of inducing intracellular β-catenin signaling (14, 15), FZDs themselves barely activate the canonical WNT pathway. In structure, FZDs resemble G protein–coupled receptors (GPCRs) (16, 17); however, the mechanism involved in G protein– and GPCR-dependent intracellular signaling still remains controversial. Therefore, except for the CRD crucial for binding to WNT glycoproteins, the role of other structure modules on FZDs in the formation of the LRP5/6-FZD signalosome and subsequent signaling transduction is not fully understood.

Here, we confirmed that WNT3A could induce β-catenin signaling more strongly after binding to SFRP2. Although the binding of FZD5 to WNT3A was inhibited by SFRP2, the regulation of FZD5 in the canonical WNT/β-catenin pathway appeared to be unaffected by the presence of SFRP2. A previous report has demonstrated that the homo-oligomerization of LRP6 via LDLR repeats is required for the canonical WNT/β-catenin signaling (18). In the current study, we further revealed that the hetero-oligomerization of FZD5 and LRP5 could induce robust activation of β-catenin signaling in a ligand-independent manner and that the oligomerization between the FZD5 extracellular N terminus and the LDLR repeats of LRP5 was critical for this ligand-independent mechanism. Moreover, we also observed the homo-oligomerization of FZD5, which may also be involved in initiation of canonical WNT signals on the cell surface. Furthermore, the state of receptor oligomerization within the LRP5/6 signalosome may be involved in the configuration of the extracellular and intracellular signaling axis for canonical WNT/β-catenin signaling transduction. Therefore, these findings provide novel insight into the architecture of the LRP5/6 signalosome and the underlying mechanisms for fine-tuning the signaling axis according to physiological or pathological changes of cells or tissues.

Results

Competitively binding of SFRP2 to WNT3A leads to hyperactivation of β-catenin signaling

As the SFRPs resemble the ligand-binding CRD of the FZD family and thereby can compete with FZDs for binding to WNT glycoproteins, they have been identified as the largest family of secreted WNT inhibitors. However, a number of recent studies demonstrate that SFRPs, such as SFRP1 and SFRP2, can also act as agonists in canonical WNT/β-catenin–dependent physiologies and pathologies (19–22). To determine the mechanism underlying SFRP2 regulation, purified WNT3A proteins were preincubated with an excess amount of hemagglutinin (HA)-tagged SFRP2-conditioned medium (CM) for 6 h and then subjected to coimmunoprecipitation (co-IP) and β-catenin–responsive TOPFlash reporter assays, respectively (Fig. 1*A*). The result of co-IP experiments showed that ~90% of input WNT3A proteins was precipitated by SFRP2-HA immobilized on beads (Fig. 1*B*), indicating that WNT3A proteins were nearly completely embedded by SFRP2-HA molecules. At the same time, WNT3A proteins embedded by SFRP2 exhibited a much stronger agonistic ability than free WNT3A proteins in the β-catenin–responsive TOPFlash reporter assays (Fig. 1*C*). In addition, we also confirmed this agonistic activity in other cells such as U2OS osteosarcoma cells and C2C12 pluripotent precursor cells and observed similar regulation of SFRP1 in canonical WNT/β-catenin signaling (data not shown). By contrast, when cells were pretreated with vehicle or SFRP2 CM for 6 h and then CM was withdrawn, the subsequent WNT3A-stimulated β-catenin signaling in both vehicle- and SFRP2-pretreated cells was comparable (Fig. 1*D*). The immunofluorescence against β-catenin proteins validated that the presence of SFRP2 could really enhance WNT3A-mediated stabilization of intracellular β-catenin proteins (Fig. 1*E*). Moreover, the presence of SFRP2 significantly accelerated WNT3A-mediated accumulation of cytosolic β-catenin proteins (Fig. 1*F*) and enhanced the sensitivity of cells responding to WNT3A (Fig. 1*G*). Taking these results together, we conclude that SFRP2 elicits the enhanced WNT3A-dependent β-catenin stabilization by competitively binding to WNT3A rather than other cell surface molecules.

SFRP2 regulates the paracrine activity of WNT glycoproteins

The binding of WNT glycoproteins to FZDs is essential to canonical WNT/β-catenin signaling. Hence, several indirect mechanisms have been proposed to explain the unexpected agonistic activity of SFRP1/2, for example via forming a complex with FZDs, inhibiting noncanonical WNT signaling, or promoting the spread of WNT glycoproteins among tissues (23–25). Here, we showed that SFRP2 did bind to WNT3A with a higher affinity (Fig. 2*A*) and could inhibit the interaction of WNT3A with the CRD derived from the N terminus of Frizzled-5 (FZD5-N) (Fig. 2*B*). The result that SFRP2 suppresses

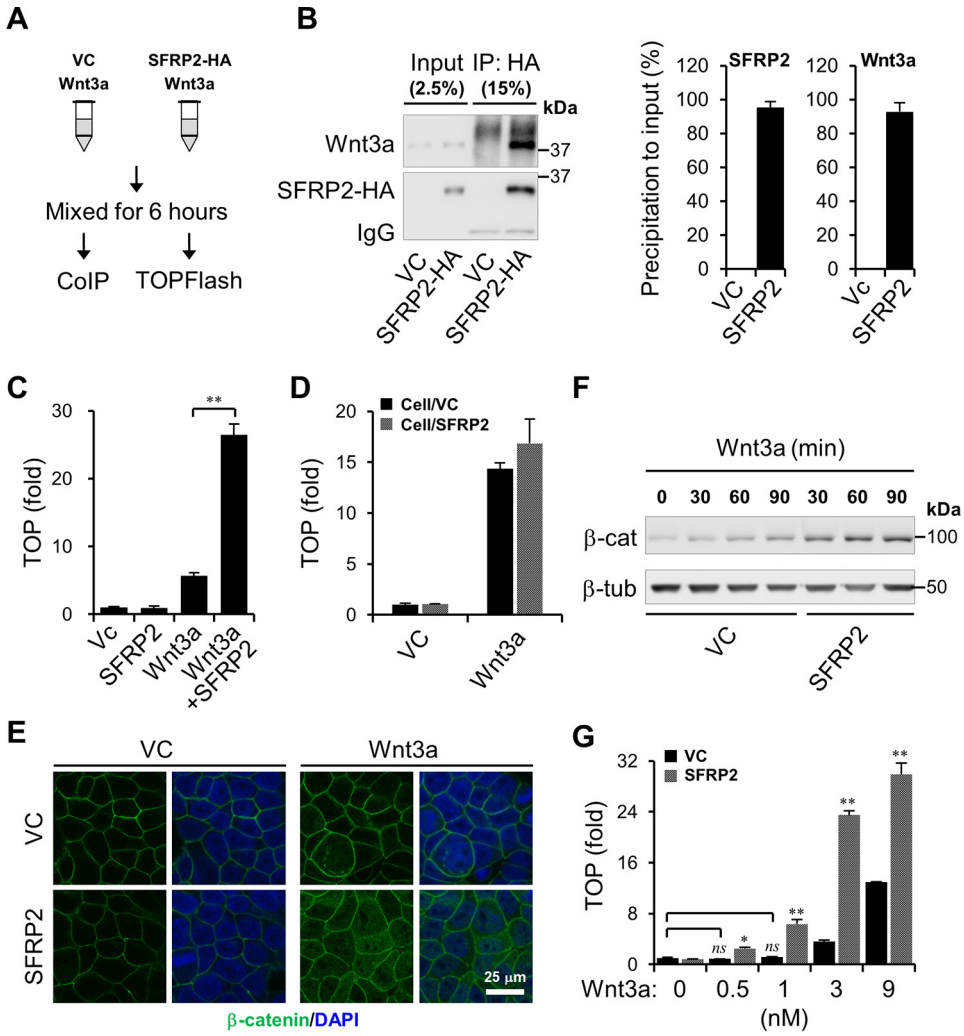

Figure 1. Competitive binding of SFRP2 to WNT3A leads to hyperactivation of β-catenin signaling. *A*, schematic diagram showing the experimental procedure for determining the activity of SFRP2-embedded WNT3A proteins. Purified WNT3A proteins were premixed with vehicle (*VC*) or SFRP2 CM for 6 h and then subjected to coimmunoprecipitation or reporter assays, respectively. *B*, coimmunoprecipitation experiments determined the percentage of SFRP2-embedded WNT3A proteins. Western blot image is a representative of three independent experiments, and the percentage shown in the graph was measured by calculating the ratio of WNT3A proteins in the immunoprecipitations to the input. *Error bars* represent S.D. *C*, HEK293T cells transfected with TOPFlash were stimulated with free or SFRP2-embedded WNT3A for 6 h, and then luciferase activity was examined. *TOP*, β-catenin–responsive luciferase reporter TOPFlash. *Error bars* represent S.D. *D*, the activity of WNT3A/β-catenin signaling was determined in vehicle- or SFRP2 CM-pretreated cells. HEK293T cells transfected with TOPFlash were pretreated with vehicle or SFRP2 CM for 6 h, and then CM was withdrawn. The pretreated cells were subsequently stimulated by WNT3A for an additional 6 h. *Error bars* represent S.D. *E*, immunofluorescence experiments with a β-catenin antibody examined intracellular β-catenin accumulation. *F*, SFRP2 accelerated the accumulation of cytosolic β-catenin (*β-cat*) stimulated by WNT3A. The cytosolic β-catenin proteins were extracted as described under "Experimental procedures." *G*, SFRP2 enhanced the sensitivity of cells responding to WNT3A. *Error bars* represent S.D. *, $p < 0.05$; **, $p < 0.01$; *ns*, not significant; Student's *t* test; $n = 3$. *β-tub*, β-tubulin; *DAPI*, 4′,6-diamidino-2-phenylindole.

but does not participate in WNT3A–FZD5-N protein complexes seems to rule out the possibility of SFRP2–WNT3A–FZD trimeric complexes. Interestingly, we further detected a high ratio (>2.0) of WNT3A:SFRP2 in SFRP2–WNT3A coimmunoprecipitation complexes (Fig. 2*C*), suggesting that the SFRP2–WNT3A complex may not be composed in a one-to-one manner. There have been reports that the C-terminal netrin-related motif (NTR) but not the CRD of SFRP1 is also capable of binding to Wingless (26). However, our result clearly showed that the CRD but not the NTR of SFRP2 is responsible for binding to WNT3A (Fig. 2*D*). Furthermore, deletion of the CRD completely abolished SFRP2-mediated enhancement in WNT3A/β-catenin signaling (Fig. 2*E*). In addition, although the NTR could not bind to WNT3A, its absence impaired SFRP2 regulation, but only moderately (Fig. 2*E*). In conclusion, these results support that CRD-dependent competitive binding to WNT3A is pivotal in SFRP2 regulation. Next, we used the protein-serine *O*-palmitoleoyltransferase porcupine (PORCN) inhibitor LGK974 (27) to block the production of noncanonical WNT glycoproteins. Fig. 2*F* shows that transfected WNT11-mediated suppression of WNT3A/β-catenin signaling was well restored by LGK974 treatment; however, the activity of WNT3A or SFRP2-embedded WNT3A could not be counteracted by LGK974 treatment, strongly ruling out potential inhibition of endogenous noncanonical WNT glycoproteins in SFRP2 regulation. Unlike R-spondin 1, which can facilitate both autocrine and paracrine activities of WNT3A glycoproteins, SFRP2 could only act on paracrine WNT3A proteins (Fig. 2*G*). To further confirm this finding, cells were transfected with WNT1 or WNT3A plasmids and with TOP-

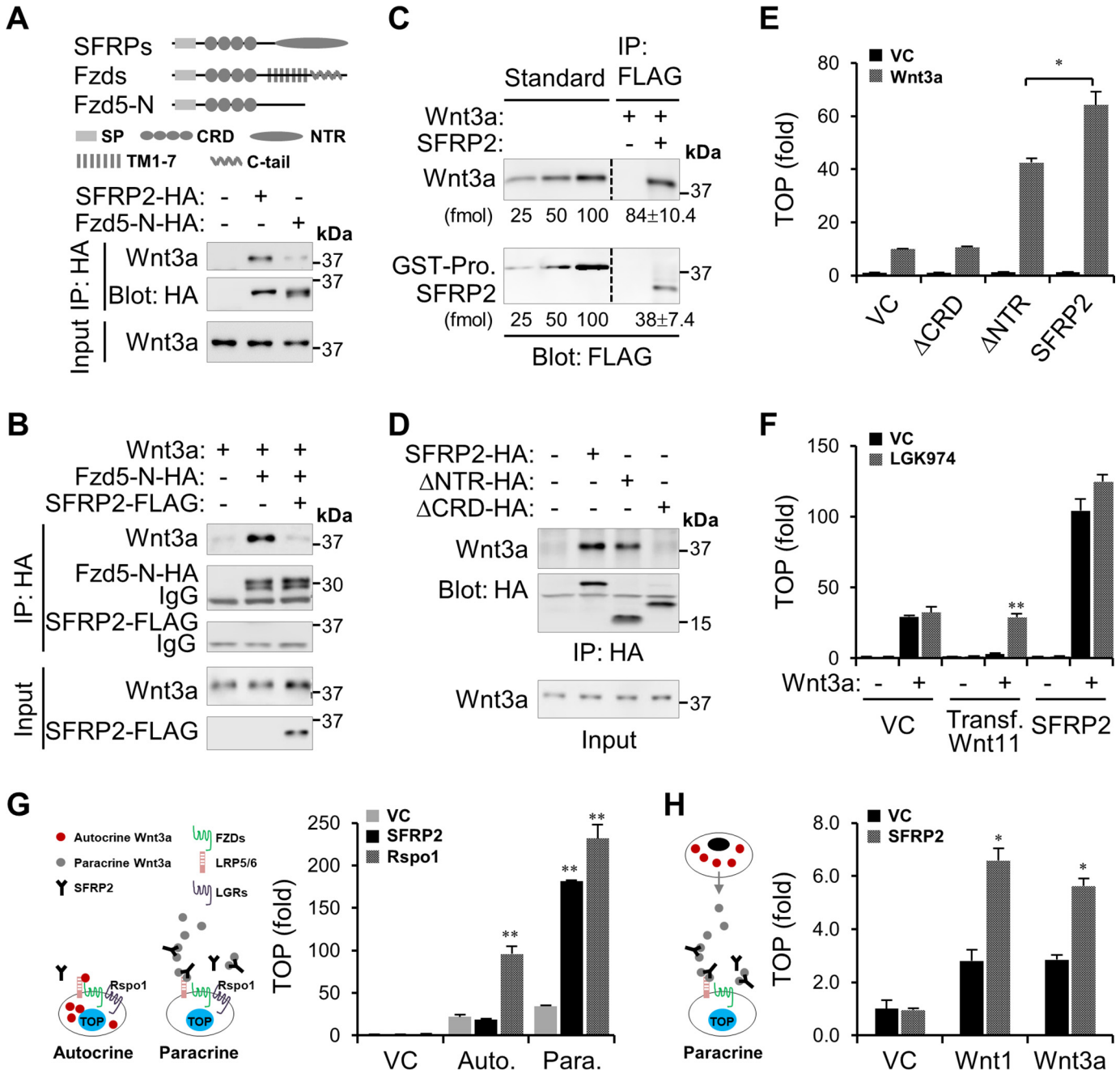

Figure 2. SFRP2 regulates the paracrine activity of WNT glycoproteins. *A*, analysis of binding affinities of SFRP2 and the soluble extracellular FZD5-N for WNT3A. *SP*, signal peptide; *TM1–7*, transmembrane helices 1–7; *C-tail*, carboxyl tail. *B*, SFRP2 suppressed the association of FZD5-N with WNT3A. *C*, the binding ratio of WNT3A to SFRP2 was determined in SFRP2 immunoprecipitation complexes. Briefly, an excessive dose of WNT3A proteins was preincubated with FLAG-tagged SFRP2 for 6 h, and then SFRP2 was pulled down by FLAG M2 antibodies. The standard protein concentration gradients of FLAG-tagged GST-fused proteins and WNT3A (25, 50, and 100 fmol) were used in Western blotting. *D*, coimmunoprecipitation experiments showed that the CRD was sufficient to bind to WNT3A proteins. *E*, the lack of the CRD completely and the lack of the NTR domain weakly inhibited SFRP2 promotion in WNT3A/β-catenin signaling. *Error bars* represent S.D. *F*, inhibition of endogenous noncanonical WNT signaling did not counteract SFRP2 promotion in WNT3A/β-catenin signaling. LGK974 was used at 1 μM. *Transf. Wnt11*, WNT11 plasmids were cotransfected with TOPFlash (*TOP*) reporter; *VC*, vector. *Error bars* represent S.D. *G*, SFRP2 promoted the paracrine activity of the diffused WNT3A proteins. *Auto.*, autocrine; *Para.*, paracrine; *Rspo1*, R-spondin 1–conditioned medium; LGRs, leucine-rich repeat–containing G-protein–coupled receptors. *Error bars* represent S.D. *H*, SFRP2 promoted the paracrine activity of WNT1 and WNT3A. WNT plasmids and TOPFlash reporter were transfected to HEK293T cells separately, and then the transfected cells were cocultured for 24 h. *Error bars* represent S.D. *, $p < 0.05$; **, $p < 0.01$, Student's *t* test; $n = 3$.

Flash reporter separately. The reporter assays showed that both paracrine WNT1 and WNT3A could be similarly enhanced by SFRP2 supplement (Fig. 2*H*). Therefore, these results demonstrate that SFRP2 regulates the paracrine activity of WNT glycoproteins.

Ligand-independent oligomerization of FZD5 and LRP5 activates β-catenin signaling

Given that SFRP2 competes with FZDs to bind to WNT glycoproteins, we then set out to investigate whether the signaling transduction by LRP5/6 or FZDs can be affected by SFRP2.

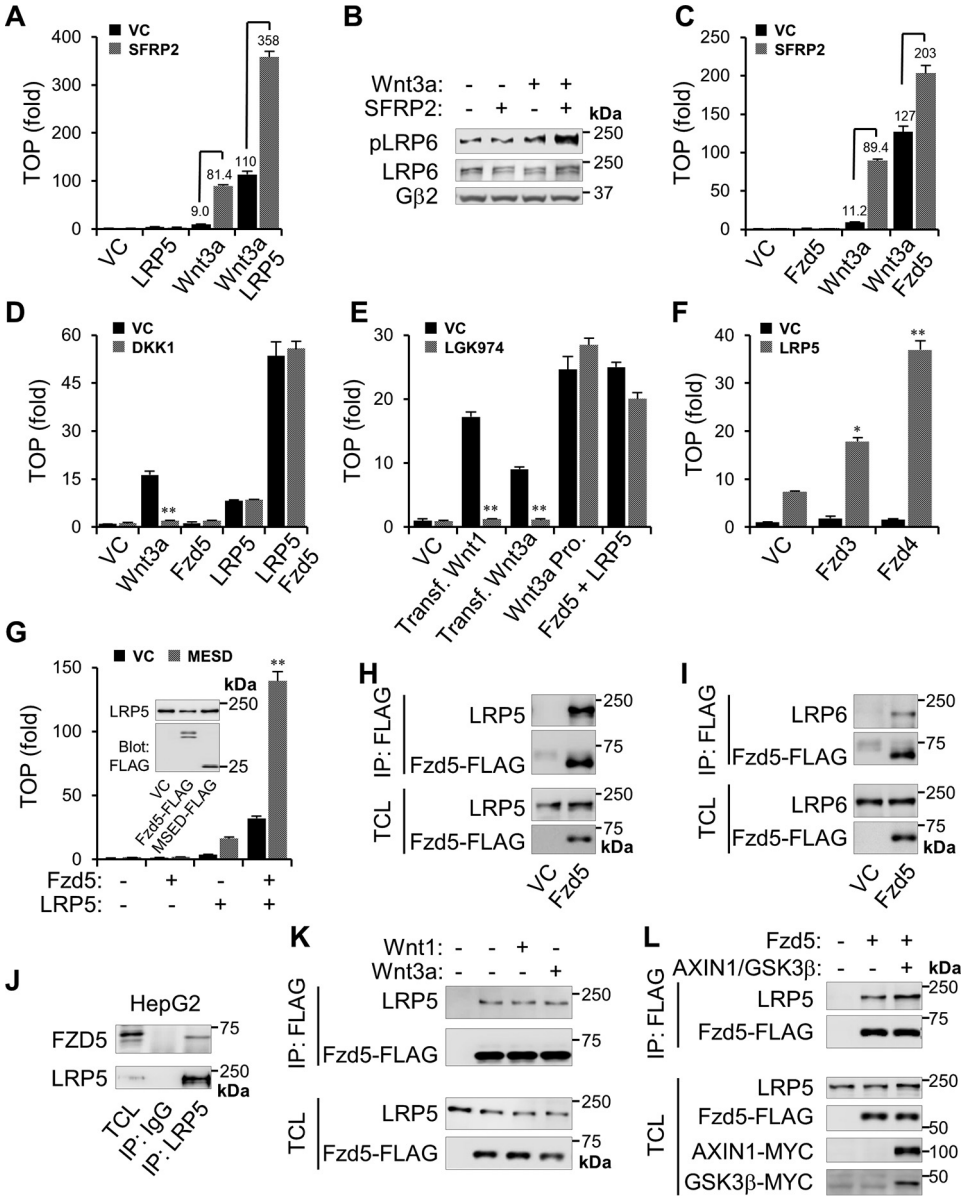

Figure 3. Ligand-independent oligomerization of FZD5 and LRP5 activates β-catenin signaling. *A*, SFRP2 enhanced WNT3A/LRP5 signaling. HEK293T cells were cotransfected with TOPFlash (*TOP*) and LRP5 plasmids for 24 h, and then cells were stimulated with WNT3A with or without SFRP2. *Error bars* represent S.D. *B*, SFRP2 increased WNT3A-stimulated LRP6 phosphorylation (*pLRP6*) (phospho-Ser-1490) in HEK293T cells. *C*, SFRP2 and FZD5 additively augmented WNT3A/β-catenin signaling. *Error bars* represent S.D. *D*, FZD5 directly stimulated LRP5/β-catenin signaling. DKK1 was used at 200 ng/ml. *E*, inhibition of endogenous canonical WNT glycoproteins did not affect FZD5-activated LRP5/β-catenin signaling. *Transf.*, transfected; *Pro.*, protein. *Error bars* represent S.D. *F*, FZD3 and FZD4 stimulated LRP5/β-catenin signaling. *Error bars* represent S.D. *G*, endoplasmic reticulum chaperone MSED enhanced FZD5 regulation in LRP5/β-catenin signaling. *Error bars* represent S.D. *H*, coimmunoprecipitation of FLAG-tagged FZD5 with LRP5 in HEK293T cells. *TCL*, total cell lysates. *I*, coimmunoprecipitation of FLAG-tagged FZD5 with LRP6 in HEK293T cells. *J*, the assay of endogenous interaction between LRP5 and FZD5 was performed in human hepatoma HepG2 cells. *K*, WNT1 and WNT3A could not regulate the interaction between LRP5 and FZD5. *L*, overexpressed AXIN1 and GSK3β slightly increased the interaction between LRP5 and FZD5. *, $p < 0.05$; **$p < 0.01$, Student's *t* test; $n = 3$. *VC*, vector.

SFRP2 could not regulate LRP5 itself but significantly promoted ligand-dependent LRP5/β-catenin signaling (Fig. 3*A*). Moreover, SFRP2 could also augment WNT3A-stimulated LRP6 phosphorylation (Fig. 3*B*). Interestingly, although the binding of FZD5 to WNT3A could be inhibited by SFRP2 (Fig. 2*B*), FZD5-mediated WNT3A/β-catenin signaling was not perturbed by SFRP2, which unexpectedly exhibited a superposition effect on WNT3A/β-catenin signaling rather than the mutual repulsion in the presence of SFRP2 (Fig. 3*C*). Therefore, perturbation of the binding of FZDs to WNT3A by SFRP2 seems not to interfere with signaling transduction modulated by both LRP5/6 and FZDs. We therefore speculate that, in addition to binding to the canonical WNT glycoproteins, there may be other mechanism for FZDs to participate in the activation of the LRP5/6 signalosome. Indeed, overexpression of LRP5 along with FZD5 resulted in a much stronger ability to activate β-catenin signaling than LRP5 alone, but DKK1 had no effect (Fig. 3*D*), suggesting that FZD5-regulated LRP5 activation is independent of the binding of WNT glycoproteins to LRP5. To further exclude the potential regulation of endogenous canonical WNT glycoproteins, we treated cells with PORCN inhibitor LGK974. The activities of the transfected WNT1 or

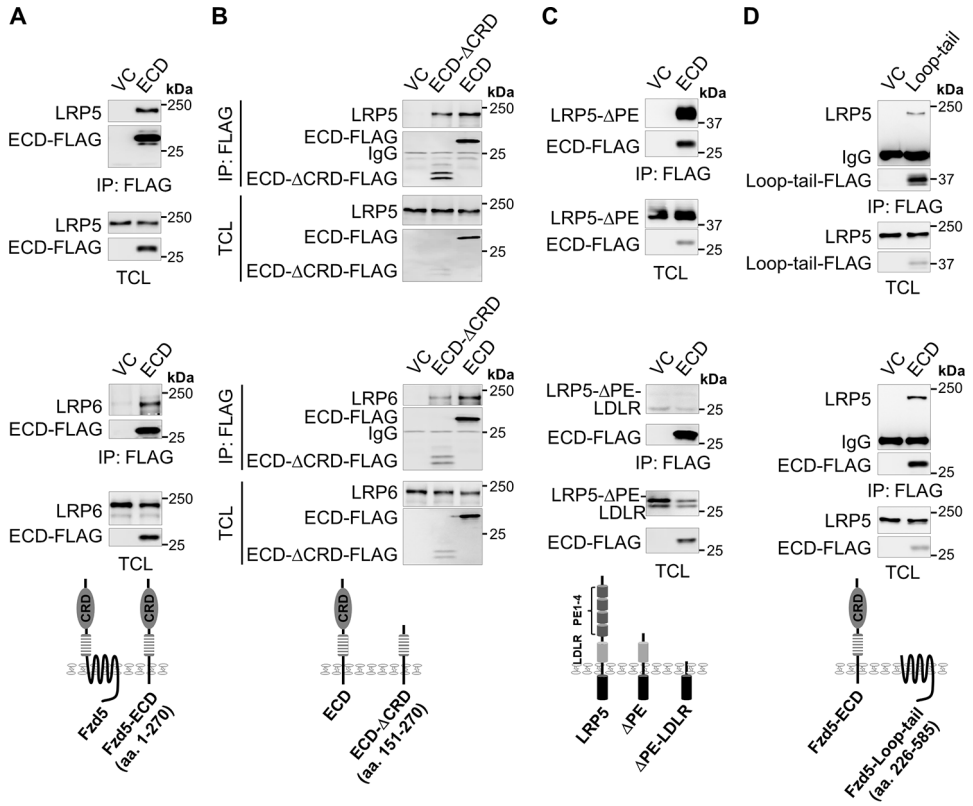

Figure 4. The oligomerization of FZD5 and LRP5 is regulated by multiple sites. *A*, coimmunoprecipitation of FLAG-tagged FZD5-ECD with LRP5 or LRP6 in HEK293T cells, respectively. *B*, the CRD was dispensable for the interaction between FZD5-ECD and LRP5 or between FZD5-ECD and LRP6. *C*, LDLR repeats of LRP5 were required for the interaction between FZD5-ECD and LRP5. *D*, both FZD5-ECD and FZD5-loop-tail interacted with LRP5. *TCL*, total cell lysates; *VC*, vector; *aa.*, amino acids.

WNT3A plasmids, but not WNT3A proteins, were completely suppressed, indicating that LGK974 can efficiently block the production of canonical WNT family glycoproteins (Fig. 3*E*). However, when LGK974 was applied to FZD5-activated LRP5/β-catenin signaling, it did not show inhibition like that in cells transfected with WNT1 or WNT3A (Fig. 3*E*). In addition to FZD5, other members of Frizzled receptors, such as FZD3 and FZD4, could also activate the LRP5 signalosome (Fig. 3*F*). Taking these results together, we conclude that FZDs can activate LRP5/β-catenin signaling independently of the canonical WNT glycoproteins.

Next, we further investigated how FZDs activate the LRP5/6 signalosome. The endoplasmic reticulum chaperone MESD, which regulates LRP5/6 maturation and membrane localization (28), could significantly enhance FZD5-activated LRP5/β-catenin signaling (Fig. 3*G*), suggesting that ligand-independent regulation of FZDs favors plasma membrane–localized LRP5/6. Of note, overexpression of FZD5 did not evidently change the key components within the LRP5/6 signalosome, such as LRP5/6, DVL3, and AXIN1, except for the hyperphosphorylation of DVL3 (data not shown). However, coimmunoprecipitation experiments revealed strong interaction of FZD5 with LRP5 (Fig. 3*H*) or with LRP6 (Fig. 3*I*). The endogenous interaction between FZD5 and LRP5 could be validated in the hepatoma cell line HepG2, which harbors strong constitutive canonical WNT/β-catenin pathway activity (Fig. 3*J*). Importantly, the interaction between LRP5 and FZD5 was free of WNT1 or WNT3A stimulation (Fig. 3*K*) but could be mildly regulated by AXIN1 and GSK3β (Fig. 3*L*), two key modulators for LRP5/6 signalosome activation (6), providing additional evidence for ligand-independent FZD–LRP5/6 signaling transduction.

The oligomerization of FZD5 and LRP5 is regulated by multiple sites

To further access the molecular basis for the interaction of FZD5 and LRP5 in the LRP5/6 signalosome, we first mapped the critical amino acid sequences responsible for FZD5–LRP5/6 interaction. FZD5 is composed of three parts: the extracellular N terminus containing a CRD and a linker sequence between CRD and the first transmembrane helix, six extracellular and intracellular loop sequences, and a cytosolic tail of ~60 amino acid residues. Coimmunoprecipitation experiments revealed that the FZD5 extracellular domain (FZD5-ECD) was sufficient for binding to LRP5 or LRP6 (Fig. 4*A*). Moreover, the interaction of FZD5-ECD with LRP5 and LRP6 was not regulated by the CRD but instead by the linker sequence between CRD and first transmembrane helix (Fig. 4*B*). The extracellular domain of LRP5/6 possesses four β-propeller motifs that alternate with four epidermal growth factor–like repeats (P1E1–P4E4), which are followed by three LDLR type A repeats. Coimmunoprecipitation experiments demonstrated that P1E1–P4E4 of LRP5 are dispensable for the interaction of LRP5 with FZD5-ECD, but LDLR repeats are required (Fig. 4*C*). It has been reported that P1E1–P4E4 of LRP6 bind to the extracellular loop sequences of FZD8 by which LRP6 can suppress noncanoni-

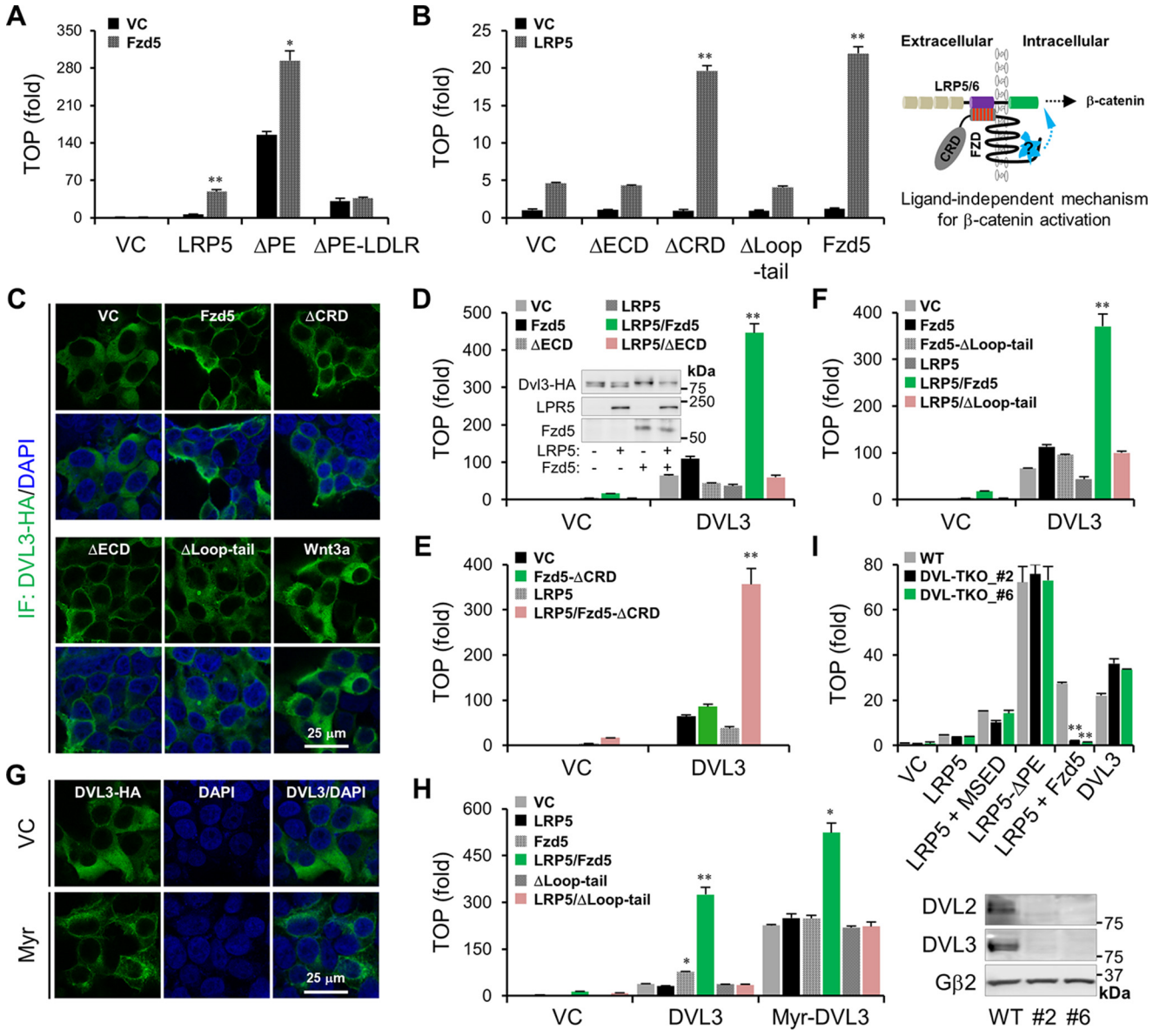

Figure 5. DVL family proteins are required for ligand-independent FZD–LRP5/6 axis. *A*, FZD5 activated LRP5/β-catenin signaling, relying on the LDLR repeats of LRP5. *Error bars* represent S.D. *B*, all of the structural modules of FZD5, except for the CRD, were required to activate LRP5/β-catenin signaling. *Error bars* represent S.D. *C*, immunofluorescence (*IF*) experiments showed the recruitment of DVL3 to the plasma membrane by FZD5. *D*, WT but not ECD-lacking FZD5 regulated the collaboration of DVL3 and LRP5 in β-catenin signaling. The *inset* shows the posttranslational modification of DVL3 in the presence of FZD5 overexpression. *Error bars* represent S.D. *E*, the CRD was dispensable for FZD5 regulation of DVL3 and LRP5 collaboration. *Error bars* represent S.D. *F*, FZD5 lacking its loop and carboxyl tail could not regulate DVL3 and LRP5 collaboration. *Error bars* represent S.D. *G*, immunofluorescence images show the recruitment of myristoylated (*Myr*) DVL3 to the plasma membrane. *H*, the recruitment of DVL3 to the plasma membrane by myristoylation did not mimic the role of the loop and carboxyl tail of FZD5 in regulating DVL3 and LRP5 collaboration. *Error bars* represent S.D. *I*, triple knockout (*TKO*) of *DVL1*, *DVL2*, and *DVL3* led to suppression of FZD5-activated, but not MSED-enhanced or constitutively activate by deletion of P1E1–P4E4 repeats, LRP5/β-catenin signaling. Western blotting showed deficiencies of DVL2 and DVL3 expression in triple knockout cells. *Error bars* represent S.D. *, $p < 0.05$; **, $p < 0.01$, Student's *t* test; $n = 3$. *DAPI*, 4′,6-diamidino-2-phenylindole; *VC*, vector; *TOP*, TOPFlash.

cal WNT/c-Jun N-terminal kinase signaling in metastasis cancer cells (29). Here, we also confirmed that the loop and cytosolic tail modules (FZD5-loop-tail) could still bind to LRP5, but the affinity seemed to be weaker than that of FZD5-ECD (Fig. 4*D*). Taking these results together, we conclude that the FZD5–LRP5 interaction is a multisite-mediated protein interaction.

DVL family proteins are required for ligand-independent FZD–LRP5/6 axis

To investigate the regulatory role of FZD5–LRP5 interaction, we introduced different truncated mutants of FZD5 or LRP5 into HEK293T cells and examined their abilities in ligand-independent β-catenin signaling. Compared with WT LRP5, both LRP5-ΔPE (P1E1–P4E4 repeat deletion) and LRP5-ΔPE-LDLR (extracellular domain deletion) possess strong constitutive activity (Fig. 5*A*); these mutants have been previously reported to be desensitized to the canonical WNT signals (14, 15). However, FZD5 could activate LRP5-ΔPE but not LRP5-ΔPE-LDLR (Fig. 5*A*), demonstrating that the interaction of LDLR repeats with FZD5, but not the interaction of P1E1–P4E4 repeats with FZD5, is required for the ligand-independent FZD–LRP5/6 signaling axis. Meanwhile, deletion of the entire

N terminus, but not just the CRD, abolished FZD5 regulation in LRP5/β-catenin signaling (Fig. 5*B*), further confirming the important role of the linker sequence between the CRD and the first transmembrane helix in FZD5-mediated LRP5 signalosome activation. The interaction between the P1E1–P4E4 repeats of LRP5 and the extracellular loops of FZD5 appears to be dispensable for FZD5 regulation in LRP5/β-catenin signaling as LRP5 lacking the P1E1–P4E4 repeats could still be activated by FZD5 (Fig. 5*A*). However, the loop and carboxyl tail of FZD5 could still contribute to the FZD5–LRP5 signaling axis (Fig. 5*B*), demonstrating that the intracellular signaling axis triggered by these modules of FZD5 may be required for LRP5 signalosome activation.

DVL family proteins act as the key transducer of the canonical WNT/β-catenin pathway, and they can be recruited to the plasma membrane via binding to the intracellular loop and carboxyl tail of FZDs (5, 30). Immunofluorescence images showed that overexpression of FZD5 as well as FZD5-ΔCRD and FZD5-ΔECD did recruit DVL3 to the plasma membrane, but FZD5-Δloop-tail could not (Fig. 5*C*). Although LRP5 and DVL3 themselves could constitutively activate β-catenin signaling independently of WNT glycoproteins, they cooperated poorly until coexpressed with FZD5 (Fig. 5*D*). Importantly, FZD5 lacking the entire N terminus (Fig. 5*D*), not just the CRD (Fig. 5*E*), failed to regulate LRP5 and DVL3 collaboration. In addition, FZD5 lacking its loop and carboxyl tail could not regulate such collaboration (Fig. 5*F*). Of note, the collaboration of myristoylated DVL3, which can be constitutively translocated to the plasma membrane, and LRP5 could not be regulated by FZD5-Δloop-tail mutant (Fig. 5, *G* and *H*). Taking these results together, we concluded that FZD5 facilitates ligand-independent LRP5/6 signalosome activation probably via two key steps. First, the intracellular loop and carboxyl tail of FZD5 binds and recruits DVL family proteins to the plasma membrane. Second, the extracellular N terminus of FZD5 binds to the LDLR repeats of LRP5 and then integrates DVLs and LRP5/6 together. Consistent with this hypothesis, CRISPR/Cas9-mediated triple knockout of DVL family proteins led to a complete suppression of FZD5-activated LRP5/β-catenin signaling (Fig. 5*I*). By contrast, the constitutive activity of LRP5, including WT and with P1E1–P4E4 deletion, was not dependent on DVL family proteins. Hence, FZD5-facilitated ligand-independent LRP5/6 signaling may still adopt the common signaling axis of the canonical WNT pathway.

Ligand-independent FZD–LRP5/6 signaling may be involved in tumorigenesis

Hyperactivation of canonical WNT/β-catenin signaling caused by hereditary or incidental mutations contributes to the occurrence of most tumors (1, 3, 31). However, mutations giving rise to the loss of function of *APC* and *AXIN1* crucial for degrading β-catenin or the gain of function of *CTNNB1* occur only in a few tumor cells. In the present study, we demonstrate that the interaction of FZDs with LRP5/6 can induce robust activation of ligand-independent β-catenin signaling, thus providing an alternative mechanism for activating WNT/β-catenin signaling in tumor cells. Using quantitative RT-PCR and protein Western blotting, we observed that the total expression levels of LRP5 and LRP6 were roughly equivalent in various tumor cells (Fig. 6, *A–C*). However, the expression of Frizzled receptors showed evident tumor-context specificity. Quantitative RT-PCR analyses indicated that FZD1, FZD2, and FZD3 were ubiquitously expressed in most tumor cells examined here, whereas the expressions of other Frizzled receptors appeared to be more distinct but random (Fig. 6*D*). For example, two kinds of cancer cells with different metastatic abilities, Saos2 and U2OS osteosarcoma cells and MCF7 and MDA-MB-231 mammary cancer cells, exhibited no significant difference in expression levels and members of FZD family; however, PC3 prostate cancer cells harbored prominent expression of FZD8 (32) compared with LNCAP cells, which have low metastatic ability (Fig. 6*D*). Interestingly, HepG2 hepatoma cells expresses four Frizzled receptors, FZD3/4/5/9 (Fig. 6*D*), all of which could strongly regulate the collaboration of DVL3 and LRP5 to activate β-catenin signaling (Fig. 6*E*) and recruit DVL family proteins to the plasma membrane (Fig. 6*F*). One of these FZDs especially, FZD9, could dramatically activate the LRP5 signalosome dependent on DVL family proteins (Fig. 6, *E* and *G*). Hyperactivation of WNT/β-catenin signaling has been considered as the major cause for hepatoma genesis; however, the mechanisms still remain unclear (31, 33). In fact, the destruction complex formed by AXIN1, GSK3β, and APC is still functional in HepG2 cells as cytosolic β-catenin proteins were dramatically stabilized when treated with the GSK3β inhibitor CHIR99021 (Fig. 6*H*). However, cytosolic β-catenin stabilization could not be suppressed by DKK1 (Fig. 6*H*), implying that the endogenous canonical WNT glycoproteins were not involved in suppressing GSK3β–AXIN–APC complexes. Therefore, we concluded that ectopic expression of Frizzled receptors (34, 35), such as FZD3/4/5/9, which cause ligand-independent LRP5/6 signalosome activation, might be the putative pathological factor for the constitutive activation of WNT/β-catenin signaling in HepG2 cells.

Receptor oligomerizations configure the canonical WNT/β-catenin signaling mechanism

In addition to heterodimerization with LRP5, FZD5 could also homodimerize with its extracellular N terminus or loop-tail modules (Fig. 7*A*). Moreover, FZD5 homodimerization could not be regulated by WNT3A (Fig. 7*B*) or DVL3 (Fig. 7*C*). The canonical WNT glycoproteins can induce large-sized LRP5/6 signalosomes composed mainly of FZDs, DVLs, and AXIN1 (6). However, the model of ligand-bridged WNT–FZD–LRP5/6 trimeric protein complexes is insufficient to explain how the signalosome is constructed despite the suggestion that homo- and heterodimerization of DVLs and AXIN1 contribute to the formation of the LRP5/6 signalosome (6–8). In fact, in addition to the proposed oligomerization between FZDs and LRP5/6, LRP6 is capable of dimerizing, which has been reported to be required for the initiation of canonical WNT/β-catenin signaling on the cell surface (18). Therefore, these findings prompted us to hypothesize that the oligomerization of FZDs and LRP5/6 may establish the fundamental large-sized LRP5/6 signalosome. The increase in FZD5 protein content, which can alter the stoichiometry of FZDs and LRP5/6 on the cell surface and thus appears to lead to more WNT–

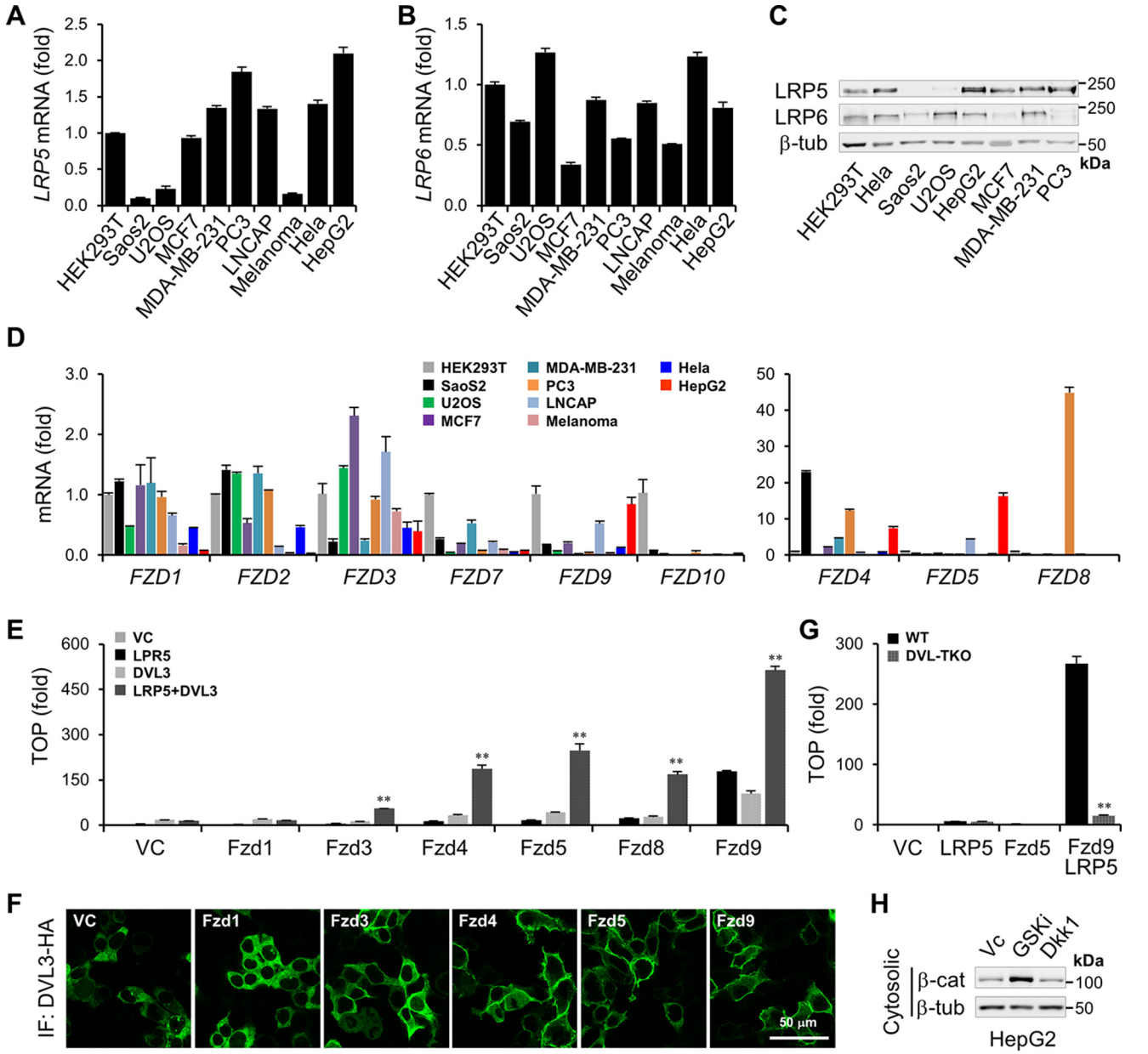

Figure 6. Ligand-independent FZD–LRP5/6 signaling may be involved in tumorigenesis. *A*, quantitative RT-PCR analyses for *LRP5* mRNA in human tumor cells. *Error bars* represent S.D. *B*, quantitative RT-PCR analyses for *LRP6* mRNA in human tumor cells. *Error bars* represent S.D. *C*, Western blot analyses for LRP5 and LRP6 protein contents in human tumor cells. *D*, quantitative RT-PCR analyses for *FZD1–10* mRNA in human tumor cells. *Error bars* represent S.D. *E*, FZD3/4/5/9 regulated LRP5 and DVL3 collaboration in β-catenin signaling. *Error bars* represent S.D. *F*, immunofluorescence (*IF*) experiments show the recruitment of DVL3 to the plasma membrane by FZD3/4/5/9. *G*, *DVL* triple knockout (*TKO*) led to significant suppression of FZD9-activated LRP5/β-catenin signaling. *Error bars* represent S.D. *H*, treatment with GSK3β inhibitor CHIR99021, but not the WNT antagonist DKK1, affected β-catenin (*β-cat*) stabilization in HepG2 cells. *, $p < 0.05$; **, $p < 0.01$, Student's *t* test; $n = 3$. *VC*, vector; *TOP*, TOPFlash; *β-tub*, β-tubulin.

FZD5 rather than WNT–FZD5–LRP5/6 complexes, however, gave rise to enhanced WNT3A/β-catenin signaling (Fig. 7*D*) and increased the sensitivity of cells to WNT3A (Fig. 7*E*). Moreover, the unilateral increase in other FZDs family, such as FZD4, FZD8, and FZD9 (Fig. 7*F*), as well as in LRP5 (Fig. 7*G*) could also enhance WNT3A/β-catenin signaling. These results strongly support the functionality of oligomerized rather than monomerized receptors in the LRP5/6 signalosome.

Interestingly, the receptor complex with LRP5-predominant stoichiometry appeared to favor the paracrine activity of the WNT1 subtype glycoproteins consisting of WNT1, WNT2, WNT2B, and WNT6 (Fig. 7*H*), which have been suggested to bind to the P1E1 and P2E2 repeats of LRP5/6 (10–12). By contrast, WNT3A and WNT3, which bind to the P3E3 and P4E4 repeats of LRP5/6 (12), could be used to form both FZD5- and LRP5-dominant receptor complexes, but they may prefer the FZD5 preponderance (Fig. 7*H*). Taking into account the above results, we conclude that the state of receptor oligomerizations may affect the recognition and binding of the LRP5/6 signalosome to different subtypes of canonical WNT glycoproteins. A recent study demonstrated ligand-independent LRP5/6 signaling in APC-deficient tumor cells where clathrin- instead of caveolin-mediated endocytosis is required (13). It has been reported that clathrin- and caveolin 1–mediated endocytosis

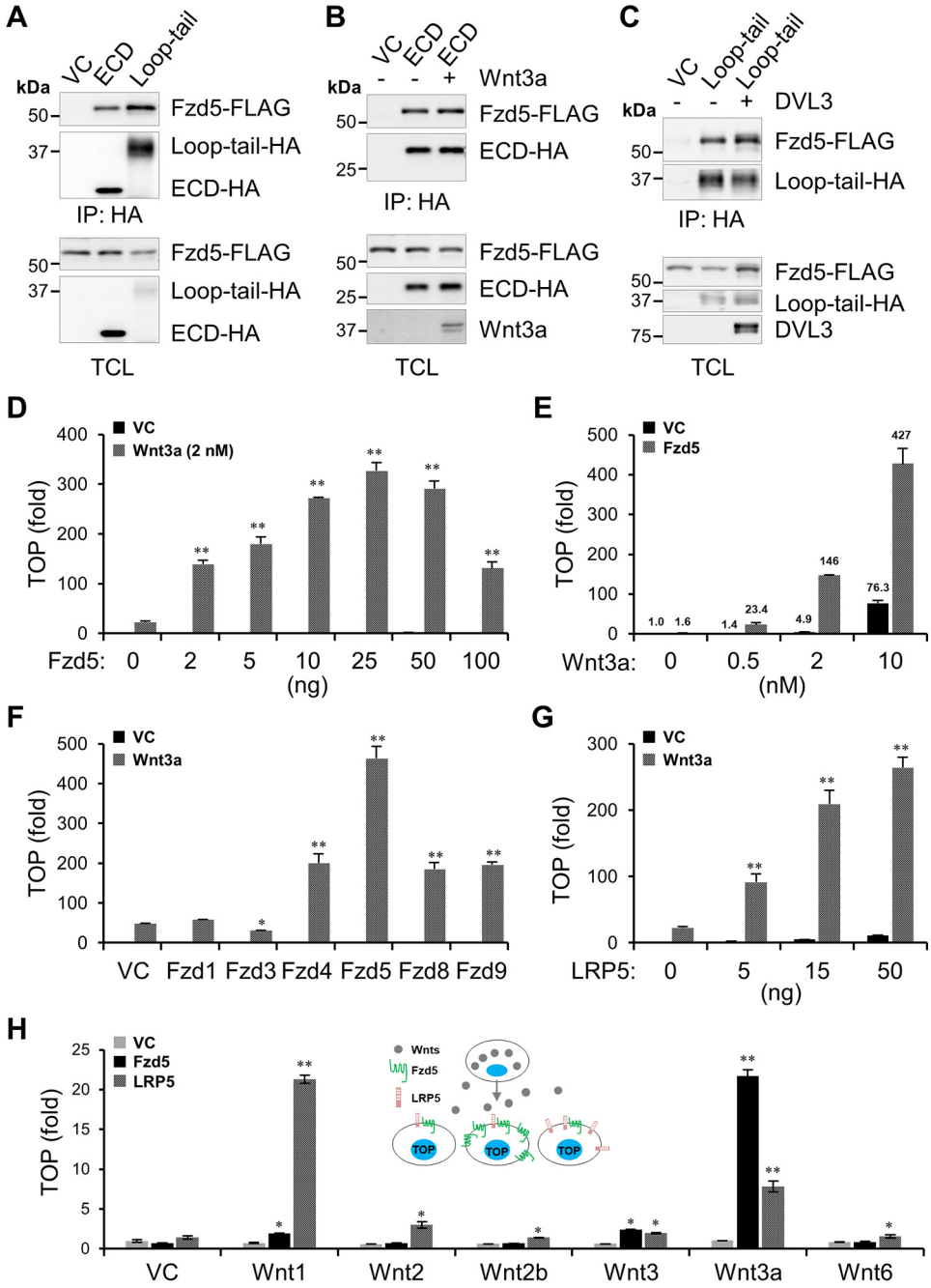

Figure 7. The oligomeric state of receptors regulates the response of cells to WNT glycoproteins. *A*, the homodimerization of FZD5 with its N terminus and loop-tail sequence. *B*, WNT3A did not regulate the homodimerization of FZD5 with its N terminus. *C*, DVL3 did not regulate the homodimerization of FZD5 with its loop-tail sequence. *D*, FZD5 could augment WNT3A/β-catenin signaling in a dose-dependent manner. *Error bars* represent S.D. *E*, increase in FZD5 protein content enhanced cell response to WNT3A. *Error bars* represent S.D. *F*, overexpression of FZDs enhanced WNT3A/β-catenin signaling. *Error bars* represent S.D. *G*, LRP5 could augment WNT3A/β-catenin signaling in a dose-dependent manner. *Error bars* represent S.D. *H*, subtypes of canonical WNT glycoproteins adopted distinct stoichiometries of FZDs and LRP5/6 on the cell surface. *Error bars* represent S.D. *, $p < 0.05$; **, $p < 0.01$, Student's *t* test; $n = 3$. *TCL*, total cell lysates; *VC*, vector; *TOP*, TOPFlash; *β-tub*, β-tubulin.

are both required for the canonical WNT/β-catenin pathway (36–38). To illustrate whether the state of receptor oligomerization affects the preference of the canonical WNT pathway for endocytic pathways, targeted disruption of clathrin heavy chain 1 (CLTC) was established in HEK293T cells (Fig. 8*A*). Consistently, the β-catenin signaling elicited by APC knockdown but not AXIN1/2 double knockdown was impaired in CLTC-deficient cells (Fig. 8, *A* and *B*). Although the basal and constitutive activity of LRP5 was not regulated by CLTC (Fig. 8*C*), FZD5-activated LRP5/β-catenin signaling could be significantly suppressed by CLTC deficiency (Fig. 8*D*). Moreover, WNT1- or WNT3A-stimulated activity of overexpressed LRP5 was unaffected by *CLTC* knockout (Fig. 8, *E* and *F*) but could be inhibited by caveolin 1 (CAV1) knockdown (Fig. 8*G*). Of note, both caveolin 1– and clathrin-mediated endocytosis appeared to be dispensable for β-catenin signaling stimulated by WNT3A and overexpressed FZD5 (Fig. 8, *G* and *H*). Taking these results together, we conclude that receptor oligomerizations can con-

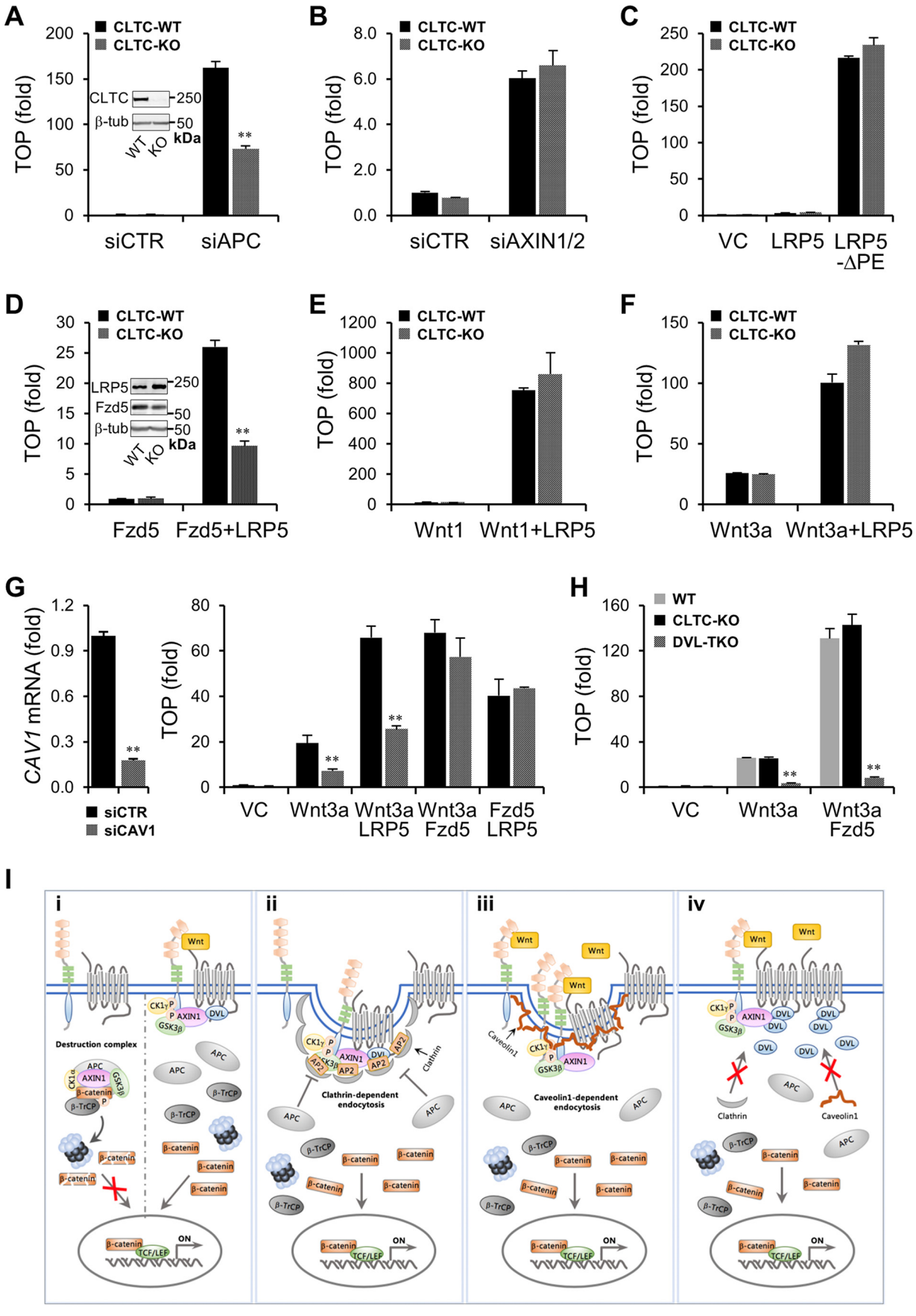
A
CLTC-WT
CLTC-KO
TOP (fold)
CLTC
β-tub
WT
KO
kDa
siCTR
siAPC
B
siCTR
siAXIN1/2
C
VC
LRP5
LRP5 -ΔPE
D
LRP5
Fzd5
Fzd5
Fzd5+LRP5
E
Wnt1
Wnt1+LRP5
F
Wnt3a
Wnt3a+LRP5
G
CAV1 mRNA (fold)
siCTR
siCAV1
VC
Wnt3a
Wnt3a LRP5
Wnt3a Fzd5
Fzd5 LRP5
H
WT
CLTC-KO
DVL-TKO
I
i
ii
iii
iv
Destruction complex
Wnt
APC
AXIN1
DVL
GSK3β
β-catenin
β-TrCP
TCF/LEF
ON
Clathrin-dependent endocytosis
Caveolin1-dependent endocytosis
Clathrin
Caveolin1

figure the extracellular and intracellular canonical WNT/β-catenin signaling mechanism.

Discussion

WNT-bridged FZD–WNT–LRP5/6 trimeric complexes have long been considered as an ideal model to describe the initiation of canonical WNT/β-catenin signaling. However, the discovery of large-sized LRP5/6 signalosomes consisting of FZDs, AXIN1, and DVL (6) excludes the monomeric mode of a FZD–LRP5/6 coreceptor complex on the cell surface. There have been a large number of models illustrating how the LRP5/6 signalosome is orchestrated, for example those involving oligomerization of AXIN1 or DVL (6–8). Nonetheless, the details of LRP5/6 signalosome arrangement are still unclear. Notably, large-sized LRP5/6 signalosomes can also be induced by APC loss in a ligand-independent manner. Therefore, a comprehensive understanding of the mechanism underlying LRP5/6 signalosome assembly will help to elaborate cell context–specific WNT/β-catenin signaling. Here, we surprisingly observed that Frizzled receptors could activate the LRP5/6 signalosome in a ligand-independent manner via receptor hetero-oligomerizations and further propose that the receptor oligomerizations may facilitate LRP5/6 signalosome formation and configure the downstream signaling axis for β-catenin stabilization.

Although FZDs are essential for WNT glycoprotein binding to the LRP5/6 signalosome, the traditional role of FZDs in canonical WNT/β-catenin signaling is now being challenged as SFRP1/2, which competes with FZDs to bind to WNT ligands, has been recently observed to activate ligand-dependent β-catenin signaling (19–22). In the present study, the cooperation of SFRP2 and overexpressed FZD5 in transducing WNT3A/β-catenin signaling prompted us to explore the unexpected role of oligomerization between FZD5 and LRP5/6 in ligand-independent β-catenin signaling pathway. Moreover, the oligomerization of Frizzled family receptors with LRP5/6 may be a universal mode on the cell surface. With the exception of FZD5, other FZDs, including FZD3, FZD4, FZD8, and especially FZD9, can also significantly promote LRP5/β-catenin signaling in a ligand-independent manner. Because ectopic expression of FZDs in tumor cells is a common phenomenon (34, 35), these findings may provide an alternative mechanism to comprehensively understand the molecular basis underlying tumorigenesis.

Importantly, FZDs activate ligand-independent LRP5/6 signalosomes, which still require the intracellular components of canonical WNT/β-catenin signaling. For example, AXIN1 and GSK3β may promote the oligomerization of FZD5 and LRP5, and the presence of DVL family proteins can dramatically enhance FZD5-mediated LRP5/β-catenin signaling. Interestingly, the recruitment of DVL family proteins to the LRP5/6 signalosome requires not only FZD intracellular module transport of DVL proteins to the plasma membrane but also interaction of the FZD extracellular N terminus with the LDLR repeats of LRP5/6. Therefore, the arrangement of the LRP5/6 signalosome requires the coordination of both extracellular and intracellular regulations. Taking into account the homo- and hetero-oligomerization of FZDs and LRP5/6, it is worth noting that the state of receptor oligomerization, such as the composition and stoichiometry of FZDs in the LRP5/6 signalosome, may vary in different types of cells or tissues (39) as the expression levels of FZDs and LRP5/6 are cell context–specific. Even in the same cell, the asymmetrical distribution of FZDs on the cell surface also results in mixed LRP5/6 signalosomes with different FZD composition and stoichiometry. More interestingly, we further demonstrate that the state of receptor oligomerization may determine the extracellular and intracellular signaling mechanisms. First, different subtypes of WNT glycoproteins appear to recognize and bind to the LRP5/6 signalosome with a distinct stoichiometry of FZDs and LRP5/6. Second, ligand-independent signaling facilitates clathrin-mediated endocytosis to activate intracellular β-catenin signaling, whereas canonical WNT signals via LRP5-predominant receptor complexes may favor caveolin-mediated endocytosis. In addition, canonical WNT signals via FZD-predominant receptor complexes do not require any regulation originating from endocytosis.

Due to cell context specificity, pathological ectopic expression of FZDs and LRP5/6, or dysfunctions of molecules like APC, there may be multiple modes of signaling initiation and transduction in the canonical WNT/β-catenin pathway depending on the oligomerization of FZDs and LRP5/6 (Fig. 8*I*). In addition, these multiple modes may also coexist in the same cells. Overall, our presented model provides a new perspective for understanding the complexity and diversity of canonical WNT/β-catenin signaling transduction and an alternative mechanism for tumor pathologies.

Experimental procedures

Antibodies and materials

Protein A/G PLUS-agarose beads were purchased from Santa Cruz Biotechnology (Dallas, TX). Antibodies against HA.11 (16B12; 1:1000; 901514) and MYC (9E10; 1:1000; 626802) were purchased from BioLegend (San Diego, CA).

Figure 8. Receptor oligomerizations configure the canonical WNT/β-catenin signaling mechanism. *A*, CLTC deficiencies suppressed β-catenin signaling caused by APC knockdown. *Error bars* represent S.D. *B*, CLTC did not regulate β-catenin signaling caused by double knockdown of AXIN1 and AXIN2. *Error bars* represent S.D. *C*, CLTC did not regulate basal or constitutive activity of LRP5/β-catenin signaling. *Error bars* represent S.D. *D*, CLTC deficiencies suppressed FZD5-activated LRP5/β-catenin signaling. *Error bars* represent S.D. *E*, CLTC deficiencies did not affect the β-catenin signaling stimulated by Wnt1 and overexpressed LRP5. *Error bars* represent S.D. *F*, CLTC deficiencies did not affect the β-catenin signaling stimulated by WNT3A and overexpressed LRP5. *Error bars* represent S.D. *G*, knockdown of *CAV1* suppressed the β-catenin signaling stimulated by WNT3A and overexpressed LRP5. *Error bars* represent S.D. *H*, *DVL* triple knockout (*TKO*), but not *CLTC* knockout (*KO*), suppressed the β-catenin signaling stimulated by WNT3A and overexpressed FZD5. *I*, the multiple modes of the canonical WNT/β-catenin signaling transduction according to receptor oligomerization. *i* shows the traditional model of canonical WNT/β-catenin signaling. *ii* shows ligand-independent FZD-mediated LRP5/6 signaling via clathrin-mediated endocytosis. *iii* shows the putative mechanism for LRP5/6-predominant receptor oligomerization in ligand-dependent β-catenin signaling, which may rely on caveolin-mediated endocytosis. *iv* shows the putative mechanism for FZD-predominant receptor oligomerization in ligand-dependent β-catenin signaling where clathrin-mediated endocytosis and caveolin-mediated endocytosis may both be dispensable. *TCL*, total cell lysates; *VC*, vector; *TOP*, TOPFlash; *β-tub*, β-tubulin; *CK1α*, casein kinase 1α; *TCF/LEF*, T-cell factor/lymphoid-enhanced binding factor; *β-TrCP*, β-transducin repeat–containing protein.

FLAG M2 (1:1000; F3165) and FZD5 (QC9970; 1:1000; AV41245-50UG) were from Sigma-Aldrich. WNT3A (C64F2; 1:1000; 2721), LRP5 (D80F2; 1:1000; 5731), LRP6 (C47E12; 1:1000; 3395), phospo-LRP6 (Ser-1490) (1:1000; 2568), DVL2 (30D2; 1:1000; 3224) were from Cell Signaling Technology (Danvers, MA). β-Catenin (610154; 1:1000) was from BD Biosciences. Gβ2 (C-16; 1:1000; sc-380) was from Santa Cruz Biotechnology. CLTC (EPR12235; 1:10,000; ab172958) and DVL3 (EP1991Y; 1:1000; ab76081) were from Abcam (Cambridge, UK). Recombinant mouse WNT3A protein (1324-WN) and DKK1 (5897-DK) were obtained from R&D systems (Minneapolis, MN). CHIR99021 was purchased from Stemcell Technologies (Canada), and LGK974 was purchased from MedChemExpress (Monmouth Junction, NJ).

Cell culture

HEK293T, C2C12, and HepG2 cells were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (Life Technologies), 1× GlutaMAX (Life Technologies), and 100 units/ml penicillin and streptomycin. U2OS, Saos2, MCF7, MDA-MB-231, PC3, LNCAP, HeLa, and human melanoma cells were maintained in RPMI 1640 medium (Life Technologies) with 10% fetal bovine serum, 1× GlutaMAX, and 100 units/ml penicillin and streptomycin. All cell lines were cultured at 37 °C in 5% CO_2.

Reporter assays

HEK293T cells were transiently transfected with TOPFlash reporter and GFP using Lipofectamine LTX from Life Technologies according to the manufacturer's instructions. LRP5 (15 ng/well), FZDs (10 ng/well), DVL3 (25 ng/well), and WNTS (25 ng/well) were transfected as indicated; the LacZ plasmid was added to make the total amount of DNA equal (0.25 μg of total plasmids/well in a 48-well plate). Luciferase reporter assays were performed, and the luciferase activities presented were normalized against the levels of GFP expression as described previously (40).

Gene transcription analysis

Total RNAs were extracted from cultured cells by directly adding the TRIzol reagent to the cells. cDNAs were synthesized using the ProtoScript II First Strand cDNA Synthesis kit (New England Biolabs, Ipswich, MA). Quantitative real-time PCR analysis was performed using Power SYBR Green PCR Master Mix (Life Technologies). The following primer pairs were used: human *GAPDH*: forward, ctt tgg tat cgt gga agg act ca; reverse, ttc ccg ttc agc tca ggg atg a; *CAV1*: forward, tta ctt cgc cat tct ctc tt; reverse, agt tga tgc gga cat tgc t; *LRP5*: forward, aac gtg gtc atc tcc ggc ctg gtc tct; reverse, ggg gtc caa ggc gat ggc cct cgg ct; *LRP6*, forward, gca tgt gat tgg ctt gga gaa aaa tt; reverse, cac ttc tcc cca gtc tgt cca gta ca; *FZD1*: forward, ggc tgc acc atc ctc ttc atg at; reverse, gat ggt ctt gat ggc cgg cac a; *FZD2*: forward, gct tcg tgt cgc tct tcc gca t; reverse, acg agc gct ccc agt gct cgc gg; *FZD3*: forward, cat ccc tgc aca ata taa ggc tt; reverse, ttc ttc tca ata gct tca cta c; *FZD4*: forward, gga tgt gca ata att ttc ttg ct; reverse, aat ggt ttt cac tgc ggg gat g; *FZD5*: forward, tcg cca cct tct gga tag gcc tg; reverse, cat ggc cca cga cca gac gca c; *FZD6*: forward, ttc tgg ggg aca agg ata taa gt; reverse, aat ctt cta aca tca att aaa a; *FZD7*: forward, gac gct ctt tac cgt tct cac ct; reverse, acc gtg cgg tag cca tcg tcc g; *FZD8*: forward, tcg ccg gct act cgc agt act t; reverse, aag agg tag atg acc agc ggc g; *FZD9*: forward, tga cgc tca cct ggt tcc tgg ct; reverse, ccg tgc tgg cca cgt agc aaa g; *FZD10*: forward, caa cat gga tta ctg gaa gat c; reverse, gtc ttg gag gtc caa atc cac at.

Protein complex assays

Co-IP experiments were carried out in HEK293T cells. Briefly, cells were transfected with the indicated constructs for 24 h and then lysed in a cell lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.5 mM EDTA, protease inhibitor mixture (Roche Applied Science), and phosphatase inhibitor mixture (Roche Applied Science)). After centrifugation, the supernatants were mixed with the indicated antibodies for 1 h at 4 °C, and the Protein A/G Plus-agarose was added for an additional 1 h. The beads were washed three times, resuspended in 30 μl of SDS loading buffer, and then analyzed by Western blotting.

Immunofluorescence

Cells on coverslips were washed once with Dulbecco's phosphate-buffered saline (DPBS) and then fixed for 20 min in DPBS containing 4% paraformaldehyde at room temperature. Fixed cells were permeabilized by 0.1% Triton X-100 for 5 min and then blocked by 2% BSA for 30 min. Finally, cells were stained with primary antibodies followed by FITC-conjugated secondary antibodies. Immunofluorescence images were captured using Leica SP5.

Assays of β-catenin stabilization

HEK293T cells were stimulated with WNT3A, SFRP2, or in combination for indicated hours. Then the cells were harvest in cold DPBS, and centrifuged. The cell pellet was re-suspended in low salt buffer (10 mM Hepes, pH 7.9, 20 mM NaCl, protease inhibitor mixture and phosphatase inhibitor mixture) swelling for 10 min on ice. The swelled cells were homogenized by a needle for 10 times, and then were centrifuged at 25000g for 30 min at 4 °C. The supernatants were harvested and analyzed by western-blotting.

Knockout cell lines

To generate *DVL* triple knockout cell line (41) and *CLTC* knockout cell line, HEK293T cells were transfected with plasmids encoding Cas9 and guide RNA for 24 h, and then transfected cells were treated with puromycin (2 μg/ml) for additional 24 h. Clones were selected and verified by immunoblotting. The guide RNA sequence for *DVL1/2*, 5′-cta cat tgg ctc cat cat ga-3′; *DVL3*, 5′-acc atg ctt caa tgg ccg gg-3′; *CLTC*, 5′-gat cgc cat tct agc ctt gc-3′.

siCAV1 design and assays

Human *CAV1* siRNA targeted the following sequence: 5′-cac ctt cac tgt gac gaa a-3′. HEK293T cells were transfected with control or *CAV1* siRNA using Lipofectamine RNAiMAX from Life Technologies according to the manufacturer's instructions. After 48 h of transfection, cells were reseeded for the reporter assay.

Statistical analysis

We used two-tailed Student's t tests to evaluate statistical significance. $p < 0.05$ was considered statistically significant. All values are presented as means ± S.D.

Author contributions—Y. H., J. W., and X. G. conceptualization; Y. H., J. W., and X. G. data curation; Y. H., J. W., and X. G. formal analysis; Y. H., J. W., and X. G. investigation; Y. H., Y. Y., Q. L., X. H., W. Z., J. W., and X. G. methodology; J. W. and X. G. supervision; J. W. and X. G. writing-original draft; J. W. and X. G. project administration; X. G. funding acquisition.

Acknowledgments—We thank Y. Huang and J. Li for confocal microscopy technical help.

References

1. Nusse, R., and Clevers, H. (2017) Wnt/β-catenin signaling, disease, and emerging therapeutic modalities. *Cell* **169,** 985–999 CrossRef Medline
2. Miki, T., Yasuda, S. Y., and Kahn, M. (2011) Wnt/β-catenin signaling in embryonic stem cell self-renewal and somatic cell reprogramming. *Stem Cell Rev.* **7,** 836–846 CrossRef Medline
3. MacDonald, B. T., Tamai, K., and He, X. (2009) Wnt/β-catenin signaling: components, mechanisms, and diseases. *Dev. Cell* **17,** 9–26 CrossRef Medline
4. Schinner, S. (2009) Wnt-signalling and the metabolic syndrome. *Horm. Metab. Res.* **41,** 159–163 CrossRef Medline
5. MacDonald, B. T., and He, X. (2012) Frizzled and LRP5/6 receptors for Wnt/β-catenin signaling. *Cold Spring Harb. Perspect. Biol.* **4,** a007880CrossRef Medline
6. Bilic, J., Huang, Y. L., Davidson, G., Zimmermann, T., Cruciat, C. M., Bienz, M., and Niehrs, C. (2007) Wnt induces LRP6 signalosomes and promotes dishevelled-dependent LRP6 phosphorylation. *Science* **316,** 1619–1622 CrossRef Medline
7. Schwarz-Romond, T., Fiedler, M., Shibata, N., Butler, P. J., Kikuchi, A., Higuchi, Y., and Bienz, M. (2007) The DIX domain of Dishevelled confers Wnt signaling by dynamic polymerization. *Nat. Struct. Mol. Biol.* **14,** 484–492 CrossRef Medline
8. Bienz, M. (2014) Signalosome assembly by domains undergoing dynamic head-to-tail polymerization. *Trends Biochem. Sci.* **39,** 487–495 CrossRef Medline
9. Janda, C. Y., Waghray, D., Levin, A. M., Thomas, C., and Garcia, K. C. (2012) Structural basis of Wnt recognition by Frizzled. *Science* **337,** 59–64 CrossRef Medline
10. Ettenberg, S. A., Charlat, O., Daley, M. P., Liu, S., Vincent, K. J., Stuart, D. D., Schuller, A. G., Yuan, J., Ospina, B., Green, J., Yu, Q., Walsh, R., Li, S., Schmitz, R., Heine, H., *et al.* (2010) Inhibition of tumorigenesis driven by different Wnt proteins requires blockade of distinct ligand-binding regions by LRP6 antibodies. *Proc. Natl. Acad. Sci. U.S.A.* **107,** 15473–15478 CrossRef Medline
11. Chen, S., Bubeck, D., MacDonald, B. T., Liang, W. X., Mao, J. H., Malinauskas, T., Llorca, O., Aricescu, A. R., Siebold, C., He, X., and Jones, E. Y. (2011) Structural and functional studies of LRP6 ectodomain reveal a platform for Wnt signaling. *Dev. Cell* **21,** 848–861 CrossRef Medline
12. Bourhis, E., Tam, C., Franke, Y., Bazan, J. F., Ernst, J., Hwang, J., Costa, M., Cochran, A. G., and Hannoush, R. N. (2010) Reconstitution of a Frizzled8·Wnt3a·LRP6 signaling complex reveals multiple Wnt and Dkk1 binding sites on LRP6. *J. Biol. Chem.* **285,** 9172–9179 CrossRef Medline
13. Saito-Diaz, K., Benchabane, H., Tiwari, A., Tian, A., Li, B., Thompson, J. J., Hyde, A. S., Sawyer, L. M., Jodoin, J. N., Santos, E., Lee, L. A., Coffey, R. J., Beauchamp, R. D., Williams, C. S., Kenworthy, A. K., *et al.* (2018) APC inhibits ligand-independent Wnt signaling by the clathrin endocytic pathway. *Dev. Cell* **44,** 566–581.e8 CrossRef Medline
14. Mao, J., Wang, J., Liu, B., Pan, W., Farr, G. H., 3rd, Flynn, C., Yuan, H., Takada, S., Kimelman, D., Li, L., and Wu, D. (2001) Low-density lipoprotein receptor-related protein-5 binds to Axin and regulates the canonical Wnt signaling pathway. *Mol. Cell* **7,** 801–809 CrossRef Medline
15. Liu, G., Bafico, A., Harris, V. K., and Aaronson, S. A. (2003) A novel mechanism for Wnt activation of canonical signaling through the LRP6 receptor. *Mol. Cell. Biol.* **23,** 5825–5835 CrossRef Medline
16. Kilander, M. B., Petersen, J., Andressen, K. W., Ganji, R. S., Levy, F. O., Schuster, J., Dahl, N., Bryja, V., and Schulte, G. (2014) Disheveled regulates precoupling of heterotrimeric G proteins to Frizzled 6. *FASEB J.* **28,** 2293–2305 CrossRef Medline
17. Dijksterhuis, J. P., Petersen, J., and Schulte, G. (2014) WNT/Frizzled signalling: receptor-ligand selectivity with focus on FZD-G protein signalling and its physiological relevance: IUPHAR review 3. *Br. J. Pharmacol.* **171,** 1195–1209 CrossRef Medline
18. Chen, J., Yan, H., Ren, D. N., Yin, Y., Li, Z., He, Q., Wo, D., Ho, M. S., Chen, Y., Liu, Z., Yang, J., Liu, S., and Zhu, W. (2014) LRP6 dimerization through its LDLR domain is required for robust canonical Wnt pathway activation. *Cell. Signal.* **26,** 1068–1074 CrossRef Medline
19. Xavier, C. P., Melikova, M., Chuman, Y., Üren, A., Baljinnyam, B., and Rubin, J. S. (2014) Secreted Frizzled-related protein potentiation versus inhibition of Wnt3a/β-catenin signaling. *Cell. Signal.* **26,** 94–101 CrossRef Medline
20. von Marschall, Z., and Fisher, L. W. (2010) Secreted Frizzled-related protein-2 (sFRP2) augments canonical Wnt3a-induced signaling. *Biochem. Biophys. Res. Commun.* **400,** 299–304 CrossRef Medline
21. Esteve, P., Sandonìs, A., Ibañez, C., Shimono, A., Guerrero, I., and Bovolenta, P. (2011) Secreted frizzled-related proteins are required for Wnt/β-catenin signalling activation in the vertebrate optic cup. *Development* **138,** 4179–4184 CrossRef Medline
22. Sugiyama, Y., Shelley, E. J., Wen, L., Stump, R. J., Shimono, A., Lovicu, F. J., and McAvoy, J. W. (2013) Sfrp1 and Sfrp2 are not involved in Wnt/β-catenin signal silencing during lens induction but are required for maintenance of Wnt/β-catenin signaling in lens epithelial cells. *Dev. Biol.* **384,** 181–193 CrossRef Medline
23. Brinkmann, E. M., Mattes, B., Kumar, R., Hagemann, A. I., Gradl, D., Scholpp, S., Steinbeisser, H., Kaufmann, L. T., and Özbek, S. (2016) Secreted Frizzled-related protein 2 (sFRP2) redirects non-canonical Wnt signaling from Fz7 to Ror2 during vertebrate gastrulation. *J. Biol. Chem.* **291,** 13730–13742 CrossRef Medline
24. Rodriguez, J., Esteve, P., Weinl, C., Ruiz, J. M., Fermin, Y., Trousse, F., Dwivedy, A., Holt, C., and Bovolenta, P. (2005) SFRP1 regulates the growth of retinal ganglion cell axons through the Fz2 receptor. *Nat. Neurosci.* **8,** 1301–1309 CrossRef Medline
25. Mii, Y., and Taira, M. (2009) Secreted Frizzled-related proteins enhance the diffusion of Wnt ligands and expand their signalling range. *Development* **136,** 4083–4088 CrossRef Medline
26. Uren, A., Reichsman, F., Anest, V., Taylor, W. G., Muraiso, K., Bottaro, D. P., Cumberledge, S., and Rubin, J. S. (2000) Secreted frizzled-related protein-1 binds directly to Wingless and is a biphasic modulator of Wnt signaling. *J. Biol. Chem.* **275,** 4374–4382 CrossRef Medline
27. Liu, J., Pan, S., Hsieh, M. H., Ng, N., Sun, F., Wang, T., Kasibhatla, S., Schuller, A. G., Li, A. G., Cheng, D., Li, J., Tompkins, C., Pferdekamper, A., Steffy, A., Cheng, J., *et al.* (2013) Targeting Wnt-driven cancer through the inhibition of Porcupine by LGK974. *Proc. Natl. Acad. Sci. U.S.A.* **110,** 20224–20229 CrossRef Medline
28. Hsieh, J. C., Lee, L., Zhang, L., Wefer, S., Brown, K., DeRossi, C., Wines, M. E., Rosenquist, T., and Holdener, B. C. (2003) Mesd encodes an LRP5/6 chaperone essential for specification of mouse embryonic polarity. *Cell* **112,** 355–367 CrossRef Medline
29. Ren, D. N., Chen, J., Li, Z., Yan, H., Yin, Y., Wo, D., Zhang, J., Ao, L., Chen, B., Ito, T. K., Chen, Y., Liu, Z., Li, Y., Yang, J., Lu, X., *et al.* (2015) LRP5/6 directly bind to Frizzled and prevent Frizzled-regulated tumour metastasis. *Nat. Commun.* **6,** 6906 CrossRef Medline
30. Zeng, X., Huang, H., Tamai, K., Zhang, X., Harada, Y., Yokota, C., Almeida, K., Wang, J., Doble, B., Woodgett, J., Wynshaw-Boris, A., Hsieh, J. C., and He, X. (2008) Initiation of Wnt signaling: control of Wnt coreceptor Lrp6 phosphorylation/activation via frizzled, dishevelled and axin functions. *Development* **135,** 367–375CrossRef Medline

31. Takagi, H., Sasaki, S., Suzuki, H., Toyota, M., Maruyama, R., Nojima, M., Yamamoto, H., Omata, M., Tokino, T., Imai, K., and Shinomura, Y. (2008) Frequent epigenetic inactivation of SFRP genes in hepatocellular carcinoma. *J. Gastroenterol.* **43,** 378–389 CrossRef Medline
32. Murillo-Garzón, V., Gorroño-Etxebarria, I., Åkerfelt, M., Puustinen, M. C., Sistonen, L., Nees, M., Carton, J., Waxman, J., and Kypta, R. M. (2018) Frizzled-8 integrates Wnt-11 and transforming growth factor-β signaling in prostate cancer. *Nat. Commun.* **9,** 1747 CrossRef Medline
33. Laurent-Puig, P., and Zucman-Rossi, J. (2006) Genetics of hepatocellular tumors. *Oncogene* **25,** 3778–3786 CrossRef Medline
34. Chan, K. K., and Lo, R. C. (2018) Deregulation of Frizzled receptors in hepatocellular carcinoma. *Int. J. Mol. Sci.* **19,** E313 CrossRef Medline
35. Zeng, C. M., Chen, Z., and Fu, L. (2018) Frizzled receptors as potential therapeutic targets in human cancers. *Int. J. Mol. Sci.* **19,** E1543 CrossRef Medline
36. Blitzer, J. T., and Nusse, R. (2006) A critical role for endocytosis in Wnt signaling. *BMC Cell Biol.* **7,** 28 CrossRef Medline
37. Yamamoto, H., Komekado, H., and Kikuchi, A. (2006) Caveolin is necessary for Wnt-3a-dependent internalization of LRP6 and accumulation of β-catenin. *Dev. Cell* **11,** 213–223 CrossRef Medline
38. Feng, Q., and Gao, N. (2015) Keeping Wnt signalosome in check by vesicular traffic. *J. Cell. Physiol.* **230,** 1170–1180 CrossRef Medline
39. Schulte, G. (2015) Frizzleds and WNT/β-catenin signaling—the black box of ligand-receptor selectivity, complex stoichiometry and activation kinetics. *Eur. J. Pharmacol.* **763,** 191–195 CrossRef Medline
40. Gan, X. Q., Wang, J. Y., Xi, Y., Wu, Z. L., Li, Y. P., and Li, L. (2008) Nuclear Dvl, c-Jun, β-catenin, and TCF form a complex leading to stabilization of β-catenin-TCF interaction. *J. Cell Biol.* **180,** 1087–1100 CrossRef Medline
41. Gammons, M. V., Rutherford, T. J., Steinhart, Z., Angers, S., and Bienz, M. (2016) Essential role of the Dishevelled DEP domain in a Wnt-dependent human-cell-based complementation assay. *J. Cell Sci.* **129,** 3892–3902 CrossRef Medline