Frizzled and LRP5/6 Receptors for Wnt/β-Catenin Signaling

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Frizzled and LRP5/6 are Wnt receptors that upon activation lead to stabilization of cytoplasmic β-catenin. In this study, we review the current knowledge of these two families of receptors, including their structures and interactions with Wnt proteins, and signaling mechanisms from receptor activation to the engagement of intracellular partners Dishevelled and Axin, and finally to the inhibition of β-catenin phosphorylation and ensuing β-catenin stabilization.

The Wnt/β-catenin pathway, or canonical Wnt pathway as it is often referred to, is an ancient and conserved signaling cascade involving β-catenin acting as a transcriptional coactivator (Logan and Nusse 2004). The pathway is best understood when considered in a two-state model of OFF (without Wnt) and ON (with Wnt). In the OFF state, cytoplasmic β-catenin is constitutively targeted for degradation by two multidomain scaffolding proteins, Axin and Adenomatous polyposis coli (APC), which facilitate the amino-terminal phosphorylation of β-catenin via the kinases GSK3 and CKIα. Phosphorylated β-catenin is recognized by the E3 ubiquitin ligase β-Trcp and is thus ubiquitinated and degraded by the proteasome, thereby maintaining low levels of free β-catenin in the cytoplasm and nucleus (MacDonald et al. 2009). In the ON state, a Wnt ligand binds to the seven-pass transmembrane receptor Frizzled (FZD) and the single-pass low-density lipoprotein receptor-related protein 5 or 6 (LRP5/6) (He et al. 2004). The Wnt–FZD–LRP5/6 trimeric complex recruits Dishevelled (DVL) and Axin through the intracellular domains of FZD and LRP5/6, resulting in inhibition of β-catenin phosphorylation and thus ensuing β-catenin stabilization. The rise of cytoplasmic and nuclear levels of β-catenin levels promotes β-catenin partnering with the TCF/LEF transcription factors for activation of Wnt-responsive gene expression.

Wnt/β-catenin signaling controls cell proliferation and differentiation and is a key regulatory mechanism for stem cells. As such, mutations in the Wnt pathway cause many diseases including cancer (Clevers 2006). All of the above "core" Wnt/β-catenin signaling components are present in vertebrates and the well-studied fruit fly *Drosophila* and are also encoded in sequenced genomes of radial symmetric Cnidarians *Hydra magnipapillata* and *Nematostella vectensis* (Guder et al. 2006) and of the primitive metazoan sponge *Amphimedon queenslandica*

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(Adamska et al. 2010). Gene loss is prevalent in the genome of nematodes, such as the popular model organism *Caenorhabditis elegans*, which appears to lack an ortholog of LRP5/6 (Phillips and Kimble 2009). As a result, the mechanisms of Wnt receptor activation are likely divergent in nematodes, whose Wnt pathways are discussed in Jackson and Eisenmann (2012). We focus on the vertebrate (and *Drosophila*) Wnt/FZD/LRP5/6 pathway, which is characterized by its absolute requirement for both FZD and LRP5/6 receptors. We note that FZD is also required for other "noncanonical" or "alternative" Wnt pathways that are independent of β-catenin (van Amerongen et al. 2008), and that in some cases such as in planar cell polarity (PCP) signaling in *Drosophila* (Bayly and Axelrod 2011), FZD may signal in a Wnt-independent manner.

WNT–RECEPTOR INTERACTIONS: OUTSIDE THE CELL

FZD Receptors

Calvin Bridges discovered a recessive fly mutant, which he called *frizzled* (*fz*), with irregularly orientated hairs and ommatidia (compound eye) and an occasionally slight reduction in wing size (Bridges and Brehme 1944). It became clear subsequently that *fz* plays an important role in planar cell polarity (PCP) (Bayly and Axelrod 2011). *fz* was shown to encode a protein with an extracellular cysteine-rich domain and a predicted topology of seven transmembrane helices resembling a G-protein-coupled receptor (GPCR) (Vinson et al. 1989). Epistasis and similarities between the *fz* and *dishevelled* (*dsh*) mutants (Wong and Adler 1993) suggested a link of the two genes, although in PCP signaling initially. With the fledgling *wingless* (*wg*, Wnt)–*dsh* (DVL)–*zeste white3* (*zw3*, GSK3)–*armadillo* (*arm*, β-catenin) signaling pathway important for embryonic patterning and wing development (Klingensmith and Nusse 1994; Krasnow et al. 1995), came along the identification of the second fly Frizzled gene *Dfz2* and the finding that Wg is a ligand for Dfz2 and Fz (Bhanot et al. 1996). *fz*(also called *Dfz1*) and *Dfz2* have redundant functions, although *Dfz2* has a more prominent role, in Wg signaling, whereas *fz*/*Dfz1* has a unique role in the PCP pathway (Kennerdell and Carthew 1998; Chen and Struhl 1999; Boutros et al. 2000; Rulifson et al. 2000).

In human, there are 10 FZD genes, numbered FZD1 through 10. Phylogenetic analysis of the mature FZD proteins generates the following five subgroups: FZD1/2/7, FZD3/6, FZD5/8, FZD9/10, and FZD4 (Fig. 1A). *Dfz2* groups closely with FZD5/8 and *fz*/*Dfz1* with FZD3/6. The Hedgehog (another family of secreted signaling protein) pathway protein Smoothened (SMO) is distantly related to FZD (Schulte and Bryja 2007) in their membrane topologies and amino-terminal cysteine-rich domains (CRDs); however, FZD proteins are distinguished from SMO by a conserved juxtamembrane KTxxxW motif in the carboxy tail region necessary for signaling (Fig. 1B).

THE FZD EXTRACELLULAR DOMAIN AND WNT BINDING: THE WNT8-FZD8CRD CO-CRYSTAL STRUCTURE

FZD contains a conserved 120-amino-acid cysteine-rich domain (CRD) at the amino terminus, which is connected to the first transmembrane helix through a variable 70- to 120-amino-acid linker region (Fig. 1B). Wnt/Wg ligands bind to CRD with high affinity (K_d of 1–10 nM) (Hsieh et al. 1999; Rulifson et al. 2000; Wu and Nusse 2002), although fz/Dfz1 PCP signaling may not involve any Wnt ligands. Deletion of the CRD prevents Wnt/Wg binding, but the CRD can be replaced with other heterologous Wnt-binding domains to generate a functional FZD receptor (Povelones and Nusse 2005; Mulligan et al. 2012). Crystal structures of CRDs from mouse FZD8 and Sfrp3 (secreted FZD-related protein 3) reveal a compact, predominantly α-helical regions held in place by disulfide bonds among 10 invariable cysteines (Fig. 1B,C) (Dann et al. 2001).

How a Wnt ligand engages the FZD receptor is revealed by the co-crystal structure of Xenopus Wnt8 in complex with mouse FZD8CRD (Fig. 1D) (Janda et al. 2012). In the complex, Wnt8 forms an unusual structure resembling a human hand with a central "palm" that extends

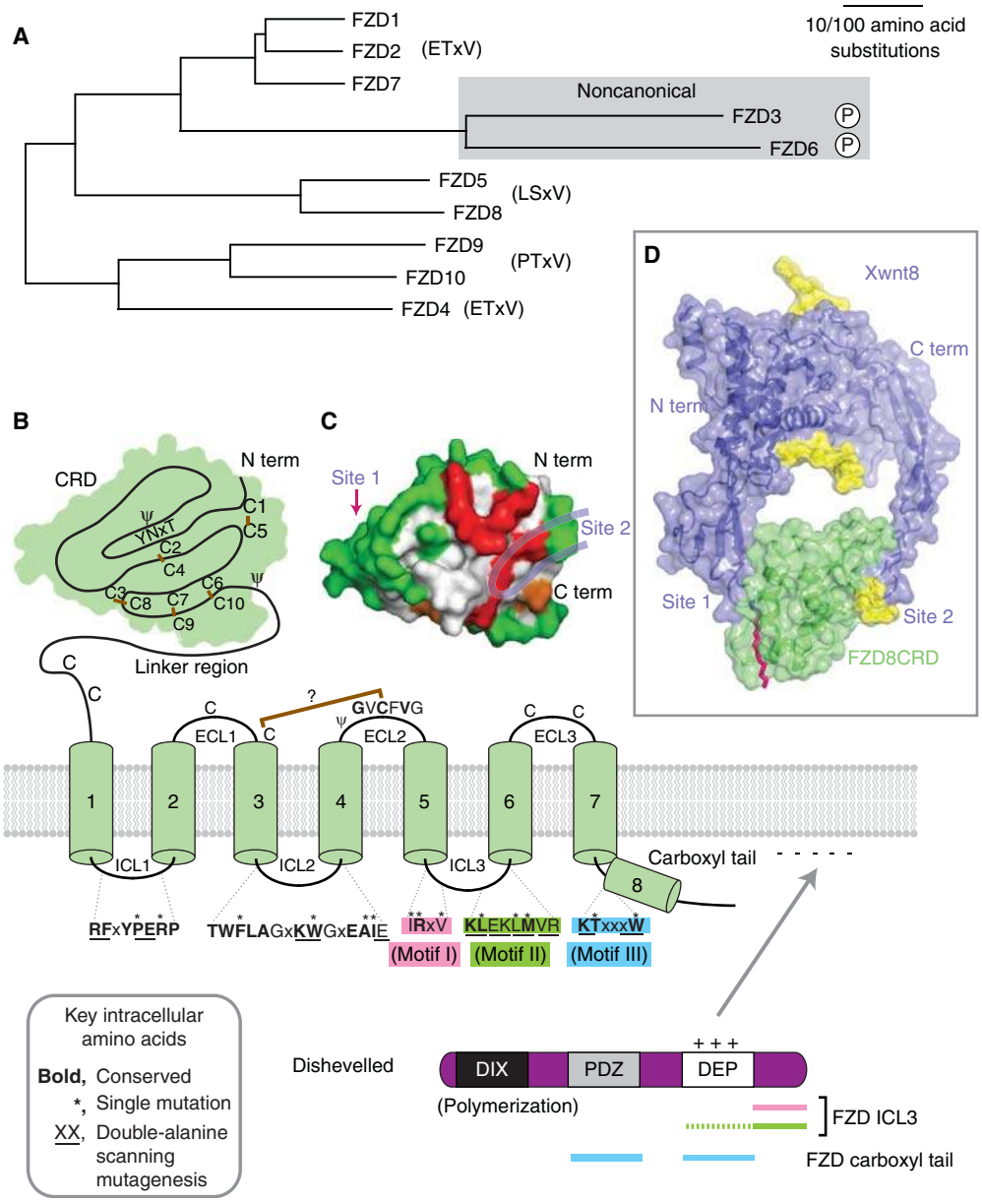

Figure 1. Frizzled (FZD) and Dishevelled (DVL). (*A*) Phylogeny comparing human FZD proteins. (*B*) Topology of a generic FZD on the plasma membrane. The shape of CRD is shaded in green, with the 10 invariable cysteine (C) residues forming five disulfide bonds highlighted. (ψ) Potential *N*-glycosylation sites. Additional conserved cysteine and other residues in the linker and ECL1-3 in the extracellular space are indicated. Conserved residues in ICL1-3 and the carboxy-terminal domain in the intracellular space are also indicated, with invariable residues among all FZD proteins in bold. (* above the letter) Missense mutations found in *Drosophila* Dfz1 (Povelones et al. 2005) and human FZD4 (Robitaille et al. 2002); (underlined) residues tested via double alanine substitution scanning mutagenesis in FZD5 (Cong et al. 2004b). DVL protein is also shown schematically with DIX, PDZ, and DEP domains and their interaction partners highlighted. Two discontinuous regions of FZD ICL3 (motif I [pink]; motif II [green]) bind to the carboxy-terminal region of DVL. The FZD carboxyl tail containing the KTxxxW motif (blue) interacts strongly with the DVL PDZ domain and also the DEP domain, which also shows some interaction with motif II in ICL3. (*See following page for legend.*)

a "thumb" plus an "index finger" to grab/pinch the FZD8CRD globular structure on two opposite sides, without changing FZD8CRD conformation (Fig. 1D). The amino terminal two-thirds of Wnt8 give rise to the palm—which consists mostly of α-helixes and intervening loops—and the thumb that consists of two anti-parallel β-strands and a connecting loop rigidified by a pair of disulfide bonds (Fig. 1D) (Janda et al. 2012). Most strikingly, a fatty acid adduct covalently attached to a serine at the tip of the thumb loop, likely a palmitoleic acid as seen in Wnt3a (Takada et al. 2006), inserts into a hydrophobic groove of FZD8CRD, constituting much of the Wnt8-FZD8CRD binding "site 1" that features extensive hydrophobic interactions between the lipid and apolar residues of FZD8CRD, "lipid-in-groove" fashion (Fig. 1D). The remaining portion of site 1 is contributed by protein–protein contacts between residues of the Wnt8 thumb loop and FZD8CRD (Fig. 1D). The carboxyl one-third of Wnt8 makes up the index finger featuring two anti-parallel β-strands and a long intervening loop, which is also rigidified by several disulfide bonds and engages in hydrophobic contacts within a depression of FZD8CRD, "knob-in-hole" fashion (Fig. 1D). This constitutes Wnt8-FZD8CRD interaction "site 2," which roughly corresponds to a Wnt-binding surface encompassing residues near the second cysteine (C2) of FZD8CRD as suggested by scanning mutagenesis (Fig. 1B,C) (Hsieh et al. 1999; Dann et al. 2001). Both site 1 and site 2 are dominated by hydrophobic contacts (lipid-in-groove and knob-in-hole, respectively), which are mostly mediated by conserved residues of Wnt8 and FZD8CRD, suggesting an explanation for broad or relatively promiscuous specificity of Wnt–FZD relationships, i.e., a single Wnt can often engage multiple FZD proteins and vice versa. However, at both site 1 and site 2, Wnt8 also exhibits protein–protein interactions with FZD8CRD residues that are conserved in some but altered in other FZD proteins, implying certain selectivity on top of broad specificity in Wnt-FZD interactions (Janda et al. 2012).

A YNxT motif at site 2 is conserved in all FZD proteins and is a predicted *N*-glycosylation site, and is indeed glycosylated in the Wnt8CRD crystal (Fig. 1B,C,D). Another predicted *N*-glycosylation site is at the end of the CRD in most FZDs, with the exception of the FZD3/6 group, which has its own unique predicted glycosylation site in extracellular loop 2 (ECL2) (Fig. 1B). *N*-glycans in both Wnt8 and FZD8CRD in the crystal structure are solvent-exposed and do not appear to contribute directly to Wnt–FZD interaction (Fig. 1D) (Janda et al. 2012). FZD *N*-glycosylation appears to be required for receptor maturation and can be regulated by the ER-resident protein Shisa, which binds preferentially to the immature form of FZD for ER trapping and thus antagonizes Wnt signaling (Yamamoto et al. 2005).

Beyond the Wnt–CRD interaction, little is known regarding the function of other FZD extracellular regions. FZD receptors are structurally analogous to GPCRs, and there is evidence that FZDs can interact and signal through G-proteins (see below), although this issue remains debated. Nonetheless, some common GPCR structural elements that are also found in FZD may suggest other potentially important regions. Many small GPCR ligands bind to the extracellular loop regions or between

Figure 1. (*Continued*) (*C*) Crystal structure of FZD8CRD. Residues in red (and to a lesser degree those in orange) are suggested to be a Wnt-binding interface from an alanine scanning mutagenesis (Hsieh et al. 1999; Dann et al. 2001), which partially overlaps with site 2 indentified in the Wnt8–FZD8CRD co-crystal structure. Residues in green when altered did not affect Wnt activity and areas in gray were not tested. Site 1 and site 2, which mediate contacts between Wnt8 and FZD8CRD in the crystal structure, are labeled. A shade of Wnt8 index finger contacting site 2 is sketched. (Panel *C* is derived from Fzd8 1IJY [PDB doi: 10.2210/pdb1ijy/pdb].) (*D*) Wnt8–FZD8CRD co-crystal structure, shown as a ribbon diagram superimposed on surface representation (Janda et al., 2012). Palmitoleic acid adduct from the Wnt8 thumb at site 1 (red); *N*-glycans of Wnt8 and FZD8CRD (yellow). Note that the FZD8CRD in *C* and *D* is viewed in different angles. N term, amino terminal; C term, carboxyl terminal.

the transmembrane helices at the extracellular face (Tebben and Schnur 2011), causing a conformation change for GPCR activation. Ligand binding and relative orientation of the transmembrane helices are influenced by inter- and intraloop disulfide bonds in the extracellular regions/loops of GPCRs. Aside from the 10 invariable cysteines within the CRD, there are additional conserved cysteines in FZD extracellular regions: two in the linker between the CRD and the first transmembrane helix, two in extracellular loop 1 (ECL1), one in ECL2, and two in ECL3 (Fig. 1B). A common extracellular disulfide bond in GPCRs is between the top of transmembrane helix 3 and ECL2 and is thought to be important for orienting and stabilizing the transmembrane helices (Peeters et al. 2010). This disulfide bond is potentially present in all FZDs (Fig. 1B). In addition, the residues flanking the cysteine in ECL2 are conserved (GVCFV) and are predicted to form a β-strand. There are also examples of an intraloop disulfide bond in ECL3 and disulfide bonds connecting with the amino-terminal region (Wheatley et al. 2012). Smo, a distant member of the FZD family, may share these GPCR features in that mutations of cysteines in ECL1 and 2 or a partial deletion of ECL2 renders a constitutively active or less active receptor (Carroll et al. 2012). Furthermore, a fly *fz* allele with a mutation in the ECL2 cysteine causes a partial loss of function (Povelones et al. 2005). Studies are required to elucidate the role of these ECLs in Wnt-induced FZD activation and signaling.

LRP5/6 AND ARROW

Fly mutants for *arrow* were first reported by Nüsslein-Volhard and Wieschaus from the famous genetic screen for embryonic lethal mutants (Nusslein-Volhard et al. 1984). Fly embryo segments normally develop a stripe of anterior denticles that can be distinguished from the naked posterior region of the segment. Mutants for *arrow* contained extra bands of denticles that were more prominent in the midline, causing the denticle stripes to look like arrows. Elimination of maternal and zygotic *arrow* resulted in a phenotype identical to that of *wg* mutants, and molecular cloning revealed that Arrow is homologous to LRP5 and LRP6 (Wehrli et al. 2000), which had been cloned as members of the LDLR family (Brown et al. 1998; Hey et al. 1998). With the evidence that the Lrp6 mouse mutant phenotypically resembles a composite of several Wnt mutants (Pinson et al. 2000), that LRP5 and LRP6 display critical roles in Wnt/β-catenin signaling in *Xenopus* (Tamai et al. 2000), and that Wnt1 can bridge a complex formation between the extracellular domains of FZD and LRP6 (Tamai et al. 2000), LRP5/6 and Arrow were established as coreceptors for the Wnt/β-catenin pathway.

THE LRP5/6 EXTRACELLULAR DOMAIN

LRP5 and LRP6 have more than 1600 amino acids and represent a unique group of the LDLR family. LRP5 and LRP6 proteins are 70% identical, and each is 45% identical to Arrow (He et al. 2004). These single transmembrane receptors have an extracellular domain containing four tandem β-propeller/epidermal growth factor (EGF) repeats followed by three LDLR type A repeats (Fig. 2A). The β-propeller domain was first proposed by Springer, who predicted a layout of a six-bladed propeller, with each blade consisting of four short β-strands and a YWTD motif in strand 2 that acts to stabilize the neighboring β-sheets and is key to the blade structure (Springer 1998). The structure for the single LDLR β-propeller-EGF (PE) unit was solved confirming the predicted six-bladed propeller, which intimately interacts with the EGF repeat (of 50 amino acids) with six cysteines in a disulfide bond pattern of C1–C3, C2–C4, and C5–C6 (Jeon et al. 2001).

Earlier mapping studies divided the LRP5/6 extracellular domain into three segments: β-propeller-EGFs 1 and 2 (P1E1-P2E2), β-propeller-EGFs 3 and 4 (P3E3–P4E4), and the three LDLR type A repeats (Fig. 2A) (He et al. 2004). Deletion of a single β-propeller in LRP5/6 typically interferes with receptor biogenesis, and better stability and recombinant protein production were achieved using tandem pairs of P1E1–P2E2 and P3E3–P4E4, but not P2E2–P3E3 (Liu et al. 2009; Bourhis et al. 2010).

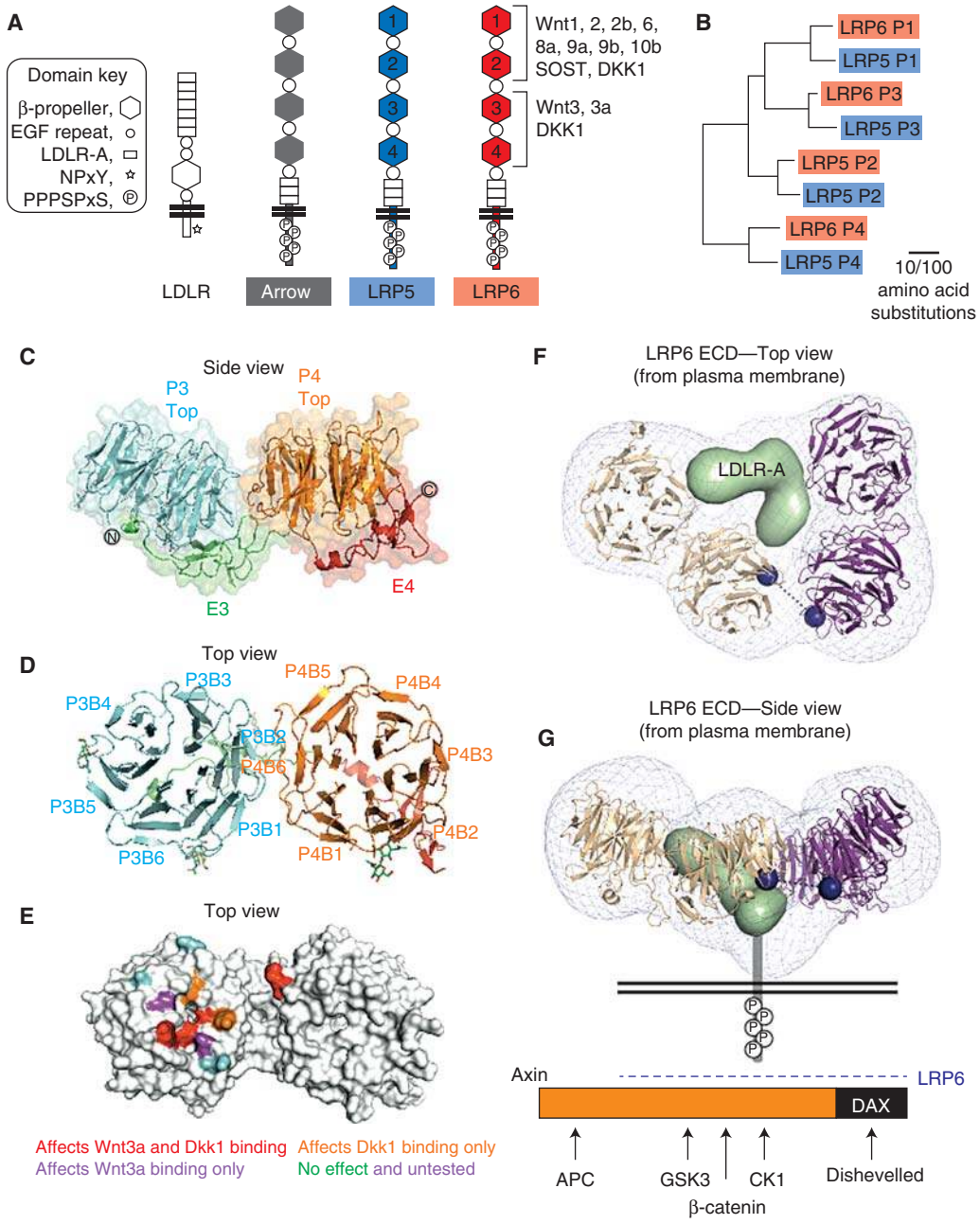

Figure 2. LRP5/6 and Axin. (*A*) Arrow, LRP5, and LRP6 are shown schematically together with LDLR. LRP6 binding to different Wnt proteins and antagonists SOST and DKK1 are shown. (*B*) Phylogeny of the four β-propellers (P1–4) in LRP5 and LRP6. (*C*,*D*) *Side* and *top* views of the atomic structure of LRP6 P3E3–P4E4 (Chen et al. 2011). (*E*) Wnt3a and DKK1 share an overlapping binding surface on the top of LRP6 P3. (*F*,*G*) *Top* and *side* views of a model of the entire LRP6 extracellular domain derived from electron microscopic staining (Chen et al. 2011). Orientation and relationship to the plasma membrane are speculative. Axin is shown schematically. (Dashed line) Indicates the unknown nature of the domain involved in LRP6-binding. Axin domains that interact with DVL, APC, β-catenin, GSK3, and CK1α are indicated. (DAX) The DIX domain of Axin. (*C*–*G*, From Chen et al. 2011; reprinted, with permission, from the author.)

Recent crystal structures of LRP6 P1E1–P2E2 and P3E3–P4E4 highlighted the tandem nature of these repeating units (Fig. 2C,D) (Ahn et al. 2011; Chen et al. 2011; Cheng et al. 2011). Although conforming to the prototypic PE structure of LDLR, extensive interface interactions between P1E1 and P2E2, and between P3E3 and P4E4 were observed, for example, between P3 and P4 and between E3 and P4 (Fig. 2C,D), and these inter-PE interactions were shown to be critical for LRP6 biogenesis and maturation to the plasma membrane. The interface between P2E2 and P3E3 is unresolved but is likely to be different. A low-resolution electron microscopic structure of the entire LRP6 extracellular domain suggested a compact horseshoe platform configuration (Fig. 2F,G), which may be consistent with the notion of a P2E2–P3E3 interface that is distinct from those of P1E1–P2E2 and of P3E3–P4E4 (Chen et al. 2011). A platform configuration of a different kind was also suggested for the LRP6 extracellular domain (lacking the LDLR-A repeats) via a low-resolution small-angle X-ray scattering analysis (Ahn et al. 2011).

The endoplasmic reticulum chaperone protein MESD is required for proper folding of the LRP5/6 extracellular domain (Culi and Mann 2003; Hsieh et al. 2003). MESD also facilitates the folding of other LDLR family members and interacts with multiple β-propellers of LRP5/6 (Culi et al. 2004; Lighthouse et al. 2011). Examination of LRP5/6 via western blotting often reveals two bands, a lower (faster migrating) immature form and a higher fully glycosylated form. Mest (also known as Paternally expressed gene 1, or Peg1), a multispan transmembrane protein that resides in the ER and contains an α/β hydrolase domain, modifies LRP6 glycosylation resulting in less mature LRP6 at the plasma membrane, thereby modulating LRP6 in a manner that appears to be analogous to Shisa inhibition of FZD receptors (Jung et al. 2011).

TWO OR MORE WNT-BINDING SURFACES OF LRP6

Early studies suggested that Wnt1 bridges extracellular domains of a FZD (mFZD8CRD) and LRP6 into a receptor complex via direct binding (Tamai et al. 2000). Consistent with this notion, an LRP6 mutant that lacks the cytoplasmic domain (and is thus membrane bound) or lacks transmembrane plus cytoplasmic domains (and is thus secreted) behaves as a dominant-negative receptor for Wnt/β-catenin signaling (Fig. 3A), presumably via binding to Wnt ligands (Tamai et al. 2000; He et al. 2004). Wnt1 signaling via LRP6 is mediated through the PE domains (P1E1–P4E4) (Mao et al. 2001a). A recent study showed a K_d between the LRP6 extracellular domain and Wnt3a or Wnt9b to be ~10 nM (Bourhis et al. 2010). Unexpectedly, however, Wnt3a and Wnt9b were shown to preferentially interact with P3E3–P4E4 and P1E1–P2E2, respectively, and a Wnt3a–Wnt9b–LRP6 (extracellular domain) complex could be detected in vitro (Bourhis et al. 2010), suggesting a possibility that a single LRP6 may engage two different Wnt proteins simultaneously. Furthermore, functional-blocking monoclonal antibodies (mAbs) against epitopes in P1 and P3, respectively, show a distinct inhibition profile toward different Wnt proteins, presumably via blocking Wnt binding to either P1 or P3 (Ettenberg et al. 2010; Gong et al. 2010). These data infer that many Wnts (Wnt1, Wnt2, Wnt2b, Wnt6, Wnt8a, Wnt9a, Wnt9b, and Wnt10a) interact with P1, whereas Wnt3 and Wnt3a prefer P3 (Fig. 2A). Other Wnts (Wnt7a, Wnt7b, and Wnt10a) could not be assigned into either group because they were not inhibited by the mAbs alone or in combination (Gong et al. 2010), raising the possibility that these Wnts may bind to regions outside P1 and P3. The remaining Wnts have not been tested in these assays. The top surfaces of P1 and P3 (and P2 and P4 as well) do not harbor *N*-glycosylation sites (Ahn et al. 2011; Bourhis et al. 2011; Chen et al. 2011; Cheng et al. 2011) and are likely the Wnt-binding interface. Indeed, missense substitutions of multiple top surface residues of P3 of LRP6, designed based on the crystal structure, diminish or enhance Wnt3a binding and signaling (Fig. 2E) but show minimal effect on signaling by Wnt1 (Chen et al. 2011), which prefers to bind to P1. Therefore, an emerging model is that LRP6, and likely LRP5, engage different Wnts via multiple ligand interfaces

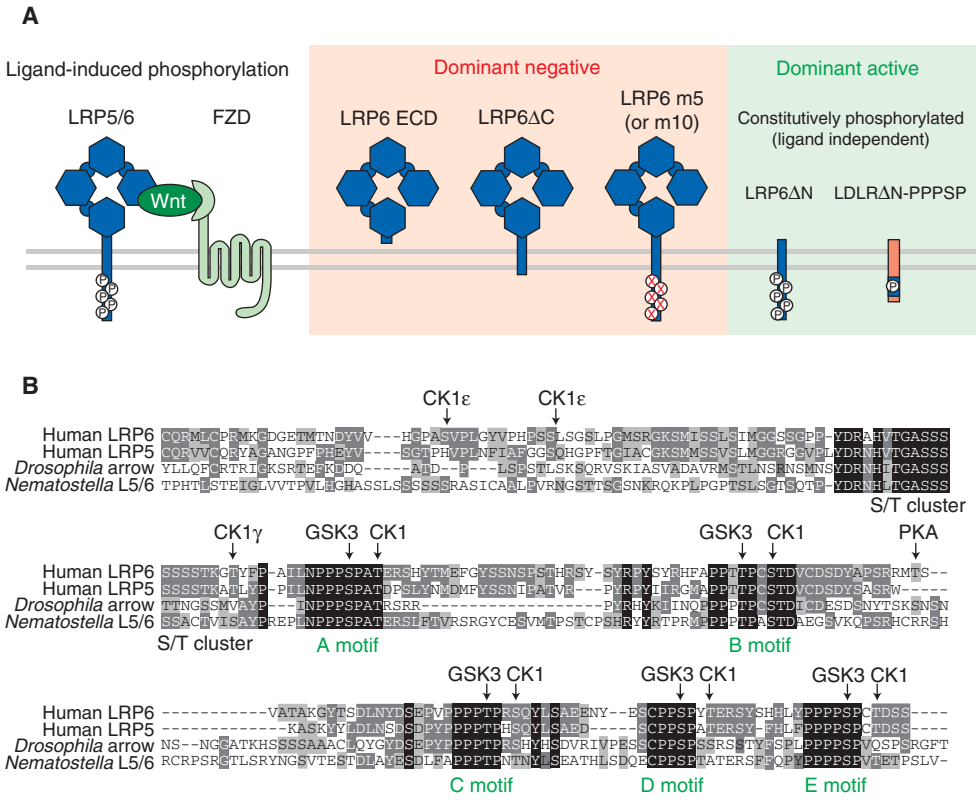

Figure 3. LRP6 phosphorylation and phosphorylation sites. (*A*) Wnt induces LRP6 phosphorylation in the FZD–LRP6 complex. Dominant-negative LRP6 mutants are generated by deleting the cytoplasmic domain or mutating all five PPPSPxS motifs (S to A). Constitutively activated LRP6ΔN and LDLRΔN-PPPSP are constitutively phosphorylated. (*B*) Alignment of the cytoplasmic domain of LRP6, LRP5, Arrow, and the *Nematostella* LRP5/6 homolog. Identified phosphorylation sites by various kinases in LRP6 are indicated.

(Fig. 2A). The LRP6 platform configuration seems to suit this model (Fig. 2F,G). Sequence analysis of LRP5 and LRP6 reveals that P1, P2, and P3 are most related by homology, but P4 is more divergent (Fig. 2B). Further studies of LRP5/6 will be needed to parse out different binding sites for Wnts and other ligands, including whether P2 or P4 also represents a Wnt docking site. The role of three LDLR-A repeats in LRP5/6 remains unknown.

THE WNT–FZD–LRP5/6 COMPLEX

A distinguishing feature of the Wnt/β-catenin pathway is the requirement for both FZD and LRP5/6 receptors (He et al. 2004). Extracellular domains of LRP6 and FZD8 were shown to form a complex in vitro in the presence of Wnt1 (Tamai et al. 2000). A similar trimeric complex was confirmed using recombinant Wnt3a and extracellular domains of FZD8 and LRP6 (Bourhis et al. 2010). These results suggest that Wnts can interact with both receptors simultaneously, perhaps via different parts of the Wnt molecule, although this important question has not been addressed (because of the prior lack of Wnt structural information and the difficulty in generating soluble Wnt protein fragments necessary for mapping studies). The Wnt8–FZD8CRD structure (Janda et al. 2012) will help resolve this issue. Also not addressed is the stoichiometry of the putative Wnt–FZD–LRP6 (or LRP5) complex, which is commonly drawn at a 1:1:1 ratio in models. The

observation in vitro that an LRP6 can simultaneously bind two different Wnt proteins suggests the possibility that various combinations of Wnt–FZD–LRP6 complexes may exist in vivo. An LRP6 platform model (Fig. 2F,G) speculates that β-propeller top surfaces/ligand binding regions may position at roughly the same height relative to the plasma membrane, potentially facilitating engagement of the CRD domain of a single FZD or more FZDs (Ahn et al. 2011; Chen et al. 2011). Interestingly, the Wnt8–FZD8CRD structure suggests a possibility of asymmetric Wnt–FZD oligomers that are formed to fully shield the palmitoleic acid adduct from aqueous solvent (Janda et al. 2012). Whether such asymmetric Wnt–FZD oligomer formation occurs in vivo and has a role in signaling deserves further investigation.

Another limiting factor for understanding the Wnt–FZD–LRP5/6 complex is our poor knowledge of Wnt–FZD and Wnt–LRP5/6 binding specificity and affinity, because most Wnts are not available in soluble forms (Mulligan et al. 2012). FZD8 appears to bind Wnt3a with 2×–3× stronger affinity than LRP6 does (Bourhis et al. 2010), seemingly consistent with some anecdotal experience that Wnt–LRP6 binding appears to be weaker than that of Wnt–FZD (He et al. 2004). In contrast, the reverse is true for Wnt9b, which displays weak binding to FZD8 but binds to LRP6 comparably to how Wnt3a does (Bourhis et al. 2010). It remains possible, however, that Wnt9b may prefer a FZD or FZDs other than FZD8. Some of the questions on the Wnt–FZD–LRP6 complex may have to wait until more recombinant Wnt proteins become available and a high-resolution structure of a Wnt protein in complex with an FZD plus LRP6 is achieved.

WNT RECEPTOR SIGNAL TRANSDUCTION: INSIDE THE CELL

Dishevelled and Axin

FZD and LRP5/6 transduce Wnt signal via engaging downstream cytoplasmic components, among which two scaffolding proteins, Dishevelled and Axin, have prominent roles.

Dishevelled (DVL1-3 in human, Dsh in *Drosophila*, and Xdsh in *Xenopus*) is a multifunctional protein that serves as a hub for canonical and noncanonical Wnt signaling. First identified in *Drosophila*, most fly *dsh* mutants are embryonic lethal because of their loss in wg signaling (Perrimon and Mahowald 1987). DVL proteins are about 700 amino acids in length and contain three main domains of about 80–90 amino acids each: DIX (Dishevelled, Axin), PDZ (Postsynaptic density 95, discs large, zona occludens-1), and DEP (Dishevelled, Egl-10, Pleckstrin) (Fig. 1B). The PDZ domain is essential for binding to a juxtamembrane KTxxxW motif (Umbhauer et al. 2000) in the FZD carboxyl cytoplasmic region (Wong et al. 2003). The first dsh mutant identified, dsh^1, displays a PCP phenotype (Fahmy and Fahmy 1959), and is a missense mutation (K417M) in the DEP domain (Axelrod et al. 1998; Boutros et al. 1998). Further studies have shown that the DEP domain has a positively charged surface that may interact with phospholipids in the plasma membrane (Wong et al. 2000; Simons et al. 2009) and additional surfaces for interacting proteins including the endocytic adaptor protein 2 (AP-2) complex (Yu et al. 2010). A recent study further suggests that DEP plus the carboxyl region of DVL interact with FZD ICL3 (via the so-called motif I and motif II) (Fig. 1B) and the KTxxxW region (so-called motif III) (Fig. 1B), thereby facilitating DVL association with a discontinuous cytoplasmic surface of FZD (Tauriello et al. 2012). Thus, PDZ and DEP domains have roles in recruiting DVL to FZD at the plasma membrane (Fig. 1B). The DIX domain shows an interesting property of head-to-tail polymerization (Schwarz-Romond et al. 2007a). This property correlates well with DVL aggregates under the overexpression condition, and it has been argued that the endogenous DVL may form such aggregates, which are highly dynamic (Schwarz-Romond et al. 2005). DIX oligomerization/polymerization is proposed to provide a DVL platform for dynamic assembly of protein–protein interactions of low avidity, such as between DVL and Axin (Schwarz-Romond et al. 2007b), and is a main underpinning for the receptor

"signalosome" hypothesis (Bilic et al. 2007) (see below). However, some have suggested that the DVL "aggregates" (or "dots" under microscopes) represent endocytic vesicles (Capelluto et al. 2002; Taelman et al. 2010). The simplest model states that DIX and PDZ domains, but not DEP, are required for Wnt/β-catenin signaling, as seen in some overexpression studies. However, evidence exists that the DEP domain influences β-catenin signaling (Wong et al. 2000; Simons et al. 2009; Tauriello et al. 2012). DVL associates with kinases such as CK1ε and CK2, and becomes hyperphosphorylated upon Wnt signaling (Willert et al. 1997; Peters et al. 1999; Kishida et al. 2001; Cong et al. 2004a; Klein et al. 2006). These kinases, in particular CK1ε, have been documented to have activating roles in Wnt/β-catenin signaling, but the role of DVL phosphorylation remains unclear.

Axin was identified as the gene mutated in *Fused* mice (Zeng et al. 1997), which display a dominant phenotype of vertebral fusions or kinked tails, with homozygous mutants showing more severe phenotypes including axis duplication of the tail (Reed 1937). Axin has about 850 amino acids and has a close homolog in vertebrates (Behrens et al. 1998; Yamamoto et al. 1998). Axin is a key negative component of the β-catenin pathway, owing to its scaffolding function in promoting β-catenin phosphorylation and degradation (Behrens et al. 1998; Ikeda et al. 1998; Kishida et al. 1998; Liu et al. 2002). Axin directly binds to β-catenin, GSK3, CK1α, and APC (Fig. 2), and additional proteins in the assembly of the "β-catenin destruction complex," in which β-catenin phosphorylation (and degradation) is performed (MacDonald et al. 2009; Stamos and Weis 2012). In relevance to Wnt receptor and DVL functions, Axin has a carboxy-terminal DIX domain, which was also called for convenience DAX to distinguish it from the DVL DIX domain (Schwarz-Romond et al. 2007b). DAX shows an oligomerization/polymerization property similar to but less dynamic than DIX (Schwarz-Romond et al. 2007a). Studies in mammalian and *Drosophila* models argue that DAX oligomerization is critical for Axin function in β-catenin phosphorylation/degradation (Kishida et al. 1999b; Fiedler et al. 2011). Axin binds directly with DVL and is recruited into DVL aggregates (under overexpression) via, in part, DAX–DIX interaction (Kishida et al. 1999b; Schwarz-Romond et al. 2007b), which may represent one mechanism by which DVL inhibits Axin (Kishida et al. 1999b; Fiedler et al. 2011). But it is unclear whether/how Wnt signaling regulates DVL–Axin interaction.

FZD AND DVL INTERACTION

The characterized FZD–DVL binding involves PDZ interaction with the juxtamembrane KTxxxW motif in the FZD carboxyl terminus (Umbhauer et al. 2000; Wong et al. 2003), DEP interaction with the same or an overlapping KTxxxW motif, and the interaction of the DVL carboxyl region with FZD ICL3 (motif I and motif II) (Fig. 1B) (Tauriello et al. 2012). Thus, multiple DVL domains/regions appear to engage a discontinuous cytoplasmic surface of FZD, and FZD–DVL association, which derives from a sum of multiple relatively weak interactions, may be further stabilized by the DEP–phospholipids (in the plasma membrane) interaction (Fig. 1B). It is unknown how PDZ and DEP may bind to the same or overlapping KTxxxW region, or whether each binding may be exclusive of the other and related to FZD activation status. A common feature of GPCRs is an intracellular helical region in the carboxyl tail, sometimes referred to as helix 8 taking into account the seven transmembrane helices. Crystal structures commonly show helix 8 to be perpendicular to TM7 and parallel with the membrane and often amphipathic to enable an interaction with the lipid membrane. In the case of FZD, a predicted helix 8 begins at the KTxxxW motif and extends for 14 amino acids in most receptors (Fig. 1B). Given the importance of the KTxxxW region for DVL (PDZ and/or DEP) binding (Punchihewa et al. 2009; Tauriello et al. 2012), one potential mechanism for FZD activation would be a Wnt-induced movement of TM7 to expose the key FZD–DVL interaction site, if we assume that Wnt regulates FZD–DVL interaction. It should be noted that FZD–DVL interaction has thus far been studied

either in vitro or under overexpression conditions in vivo, and it remains unknown whether this interaction is under Wnt regulation. However, because Dfz1/Dsh PCP signaling in *Drosophila* appears to be Wnt independent (Bayly and Axelrod 2011), it is conceivable that FZD–DVL interaction can occur without Wnt, at least in some circumstances.

In addition to the helix 8 region, many GPCRs use a region in intracellular loop (ICL) 2 that serves as an interaction site for the Gα protein. However, ICLs are divergent between GPCRs and FZD. Comparison of the 10 FZDs reveals conserved residues in the ICLs (Fig. 1B). The importance of these residues for signaling has been shown by fly Fz/Dfz1 mutants, patient mutations in FZD4, and site-directed mutagenesis studies (Robitaille et al. 2002; Cong et al. 2004b; Povelones et al. 2005; Zeng et al. 2008; Nikopoulos et al. 2010). Some of these findings can potentially be explained by DVL interaction with FZD ICL3 (Tauriello et al. 2012). Whether ICL1 and ICL2 residues are also involved in interaction with DVL, or G-proteins (see below), or unidentified proteins remains to be investigated.

Most FZD receptors, except for the noncanonical FZD3/6 group, contain a carboxy-terminal S/TxV motif (Fig. 1A), which is commonly involved in PDZ binding (Hering and Sheng 2002) and may serve as an accessory site to augment FZD clustering by perhaps PDZ proteins. Phosphorylation of the FZD carboxyl region has been observed and is associated with down-regulation of FZD/PCP signaling, such as Fz/Dfz1 phosphorylation by aPKC around the DVL-interacting SxKTxxSW motif in *Drosophila* (Djiane et al. 2005), and Dvl-dependent FZD3 phosphorylation, which inhibits FZD3 endocytosis and signaling, in axon guidance (Shafer et al. 2011) and possibly in neural crest induction (Yanfeng et al. 2006). However, it is unknown whether phosphorylation regulates FZD function in β-catenin signaling.

LRP6 PHOSPHORYLATION AND AXIN RECRUITMENT

The LRP5/6 cytoplasmic region has about 200 amino acids and contains five signature PPPSPxS motifs named from A to E, which are conserved from invertebrates to human (Fig. 3B). LRP6 mutants that lack the entire cytoplasmic region (LRP6ΔC), or have all five PPPSPxS motifs changed to PPPAPxA (LRP6m10), or have all five PPPSP motifs changed to PPPAP (LRP6m5), behave each as a loss-of-function and, in fact, dominant-negative mutant (Fig. 3A) (Tamai et al. 2000, 2004; Zeng et al. 2005). Conversely, LRP6 (and LRP5) mutants that lack the extracellular domain (LRP6ΔN) behave as constitutively activated Wnt receptors (Mao et al. 2001a,b; Tamai et al. 2004). Most tellingly, a single PPPSPxS motif is sufficient to transfer Wnt signaling activation function to a heterologous receptor (Fig. 3A) (Tamai et al. 2004; MacDonald et al. 2008). These results highlight the critical importance of the PPPSPxS motif.

The PPPSPxS motif is dually phosphorylated in the endogenous LRP6 in response to Wnt but is constitutively phosphorylated in either LRP6ΔN or in any single PPPSPxS motif that is transferred to a heterologous receptor (Fig. 3A) (Tamai et al. 2004; Zeng et al. 2005; MacDonald et al. 2008), correlating fully with the signaling activity. The phosphorylated PPPSPxS motif, but not the unphosphorylated one, is a docking site for Axin (Tamai et al. 2004), explaining, in part, Wnt-induced recruitment of Axin via LRP5/6 to the plasma membrane (Mao et al. 2001b). Axin binding to the phosphorylated PPPSPxS motif is likely direct (Tamai et al. 2004; Zeng et al. 2005; MacDonald et al. 2008, 2011), but it remains unclear which part of Axin is involved in the binding, with the earlier mapping studies implicating a broad segment of Axin (Fig. 2) (Mao et al. 2001b). This critical but unresolved issue has fueled an alternative model in which LRP6–Axin interaction may be indirectly mediated by other factors such as GSK3 (Piao et al. 2008).

LRP6 PHOSPHORYLATION: KINASES AND REGULATION

Of the kinases that have been implicated in LRP6 phosphorylation, the GSK3 and CK1 families are the most prominent in their phosphorylation of PPPSP and xS, respectively (Fig.

3B) (Davidson et al. 2005; Zeng et al. 2005). PPPSP phosphorylation has been mostly studied using Ab1490, which corresponds to motif A (Tamai et al. 2004), and studies of other PPPSP motifs (C and E) suggested similar regulation (MacDonald et al. 2008, 2011). PPPSP is phosphorylated by GSK3, which primes xS phosphorylation by CK1 (Zeng et al. 2005). This dual kinase model contrasts that for β-catenin, whose phosphorylation by CK1α primes phosphorylation by GSK3 (Liu et al. 2002). In many cases such as in β-catenin phosphorylation, GSK3 requires a priming kinase, but in its PPPSP phosphorylation, there does not seem to be a priming requirement (Zeng et al. 2005). GSK3 phosphorylation and activation of LRP6, which also contrasts its phosphorylation and degradation of β-catenin, is supported by two key pieces of evidence: (1) Wnt-induced LRP6 PPPSP phosphorylation does not occur in cells that lack both Gsk3α and Gsk3β; and (2) inhibition of GSK3 by a plasma membrane-tethered inhibitor blocks Wnt signaling, whereas the same inhibitor in the cytoplasm activates Wnt signaling (Zeng et al. 2005, 2008). Such dichotomic roles of GSK3 are not without parallel: in *Drosophila*, protein kinase A (PKA) antagonizes Hedgehog (Hh) signaling by phosphorylating and inhibiting the Ci transcription factor in the absence of Hh but activates Hh signaling by phosphorylating and activating Smo in response to Hh (Jiang and Hui 2008).

FZD is required for Wnt-induced PPPSP phosphorylation by GSK3, and forced FZD–LRP6 association is sufficient to induce PPPSP phosphorylation (Zeng et al. 2008), consistent with the importance of Wnt-induced FZD–LRP6 complex formation in Wnt signaling. Mutations in FZD intracellular loops or the carboxyl tail that prevent FZD–DVL interaction (Wong et al. 2003; Cong et al. 2004b) abolish the ability of FZD to promote LRP6 phosphorylation, implying that DVL is required to mediate FZD regulation of LRP6 PPPSP phosphorylation (Fig. 4), a notion that is supported by Dvl depletion experiments (Zeng et al. 2008). The recent observation in *Drosophila* that Dsh acts upstream of Arrow further corroborates this biochemical relationship (Metcalfe et al. 2010).

Unexpectedly, Axin is also required for Wnt-induced LRP6 PPPSP phosphorylation, and Axin overexpression enhances PPPSP phosphorylation (Tamai et al. 2004; Yamamoto et al. 2006), but only if Axin is not impaired in GSK3 binding (Zeng et al. 2008). These findings led to a model that DVL binding to and recruitment of Axin into the FZD–LRP6 complex initiates GSK3 phosphorylation of LRP6 on PPPSP motifs (Zeng et al. 2008). This model posits that the Axin–GSK3 complex phosphorylates β-catenin in the absence of Wnt but switches to phosphorylate LRP6 under the FZD/DVL control. DIX/DAX-mediated DVL and Axin oligomerization is compatible with this model.

Wnt-induced CK1 phosphorylation of LRP6 presents a more complicated picture. Firstly, there are CK1 phosphorylation sites within and outside the PPPSPxS motif, such as the conserved region that precedes the first PPPSPxS motif and contains residues "YDRxH" and a block of serine and threonine residues, referred to as the S/T cluster (Fig. 3B). This region is likely phosphorylated by CK1, including the nearby T1479 by CK1γ (Davidson et al. 2005). The functional significance of this S/T cluster is unclear, although one study suggests it as a GSK3-binding site upon CK1 phosphorylation (Piao et al. 2008). Secondly, there exist seven mammalian CK1s (α, β, γ1, γ2, γ3, δ, and ε) (Price 2006). CK1α and CK1ε/δ are associated with Axin and DVL, respectively, whereas CK1γ is membrane tethered via isoprenylation (Price 2006; MacDonald et al. 2009), and therefore these CK1s are each in proximity to LRP6 in the receptor complex. xS phosphorylation by CK1 is proceeded/primed by PPPSP phosphorylation (Zeng et al. 2005), and therefore appears to be secondary to regulation of PPPSP phosphorylation by Wnt through FZD, DVL, and Axin, although independent layers of CK1 regulation may exist. Indeed, the transmembrane protein TMEM198 enhances LRP6 signaling and phosphorylation by CK1 through its binding to CK1α, ε, and γ (Liang et al. 2011). Efforts have been taken to distinguish roles of different CK1s in multiple steps in Wnt signaling (Del Valle-Perez et al. 2011), but the available

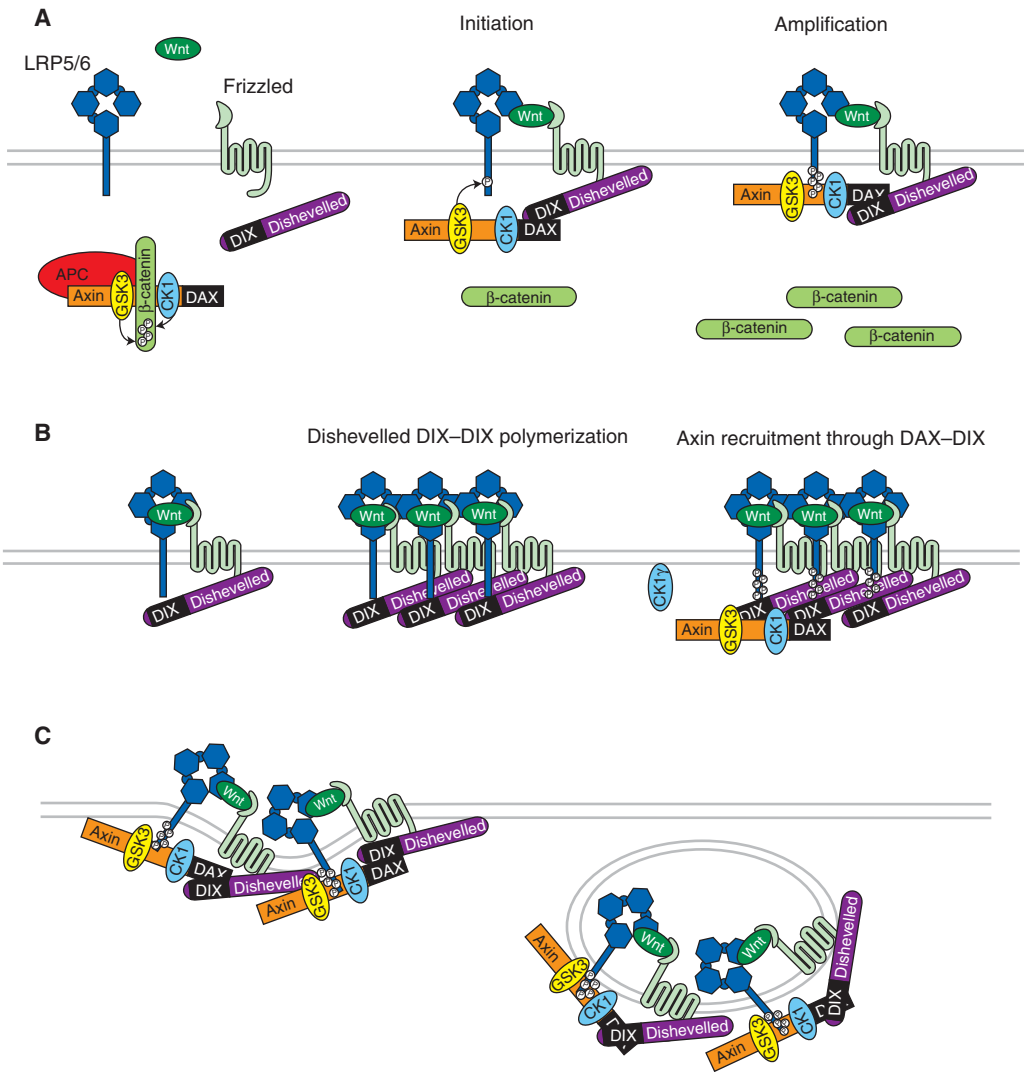

Figure 4. Models for LRP6 phosphorylation and signaling. (*A*) Initiation-amplification model. (*B*) Signalosome model. Only one Axin molecule is drawn for clarity. (*C*) Endosomal signaling model.

evidence suggests overlapping functions of CK1s in LRP6 phosphorylation.

LRP6 PHOSPHORYLATION: ROLES OF PIP2, G-PROTEIN, AND OTHER KINASES

Two lipid kinases, phosphatidylinositol 4-kinase type II (PI4KIIα) and phosphatidylinositol-4-phosphate 5-kinase type I (PIP5KI), which are bound and activated by DVL, mediate Wnt-induced PIP2 (phosphatidylinositol 4,5-bisphosphate) production that promotes LRP6 phosphorylation by GSK3 and CK1 (Pan et al. 2008; Qin et al. 2009). One model suggests that PIP2 enhances LRP6 aggregation/signalosome formation (see below) and thereby phosphorylation (Pan et al. 2008). An alternative mechanism has been suggested by a study of WTX/Amer1, which is an X-linked tumor suppressor in Wilms tumor. WTX binds to Axin and promotes β-catenin degradation in absence of Wnt (Major et al. 2007). However, upon Wnt

stimulation, WTX via its PIP2-binding motif recruits Axin into the Wnt receptor complex, where PIP2 is high, thereby facilitating LRP6 phosphorylation by GSK3 and CK1γ (Regimbald-Dumas and He 2011; Tanneberger et al. 2011). WTX, which is vertebrate specific, thus has dichotomic roles in Wnt signaling, analogous to those for Axin and GSK3.

The FZD topology invites GPCR comparisons, and FZD coupling to trimeric G-proteins in Wnt/β-catenin signaling has received certain support from pharmacological and genetic studies (Ahumada et al. 2002; Katanaev et al. 2005; Liu et al. 2005; Schulte and Bryja 2007), although this remains a debated issue. Although Gαo and Gαq are suggested to regulate Axin–GSK3 interaction (Liu et al. 2005), Gβγ is shown to be in complex with LRP6, DVL, and Axin and to recruit GSK3 to phosphorylate LRP6 (Jernigan et al. 2010). Thus, multiple routes through DVL, G-protein, and WTX promote LRP6 phosphorylation, and DVL is essential in this capacity (Bilic et al. 2007; Zeng et al. 2008).

The SP motif is preferred by the so-called proline-directed kinases, which include GSK3, MAPK, and CDK subfamilies (Manning et al. 2002). PFTK, a CDK, and several MAPKs have been suggested to phosphorylate PPPSP in LRP6 in contexts in which cross-regulation with Wnt signaling may occur. A study showed that the PFTK/CyclinY pair phosphorylates LRP6 in G_2/M of the cell cycle, and thus "poises" LRP6 for Wnt signaling to peak at G_2/M (Davidson et al. 2009). This notion, however, contrasts the conventional view that proliferative signaling pathways commonly act during G_1/S transition (to regulate DNA synthesis). Indeed, a recent study suggests that Wnt signaling is highest at G_1/S but lowest at G_2/M (Hadjihannas et al. 2012). MAPK phosphorylation of LRP6 PPPSP was also suggested and may underlie synergy between FGF and Wnt signaling (Cervenka et al. 2011). This is likely context dependent, however, because antagonism between Wnt and MAPK signaling pathways has been observed in development (Szuts et al. 1997; Freeman and Bienz 2001) and in cancer such as melanoma (Biechele et al. 2012). GRK5/6 kinases, which are mostly associated with down-regulation of GPCR signaling, can phosphorylate LRP6 on PPPSP motifs and other sites in vitro (Chen et al. 2009), although the significance in vivo is unclear. Finally, evidence also exists that PKA can phosphorylate LRP6 at a site between PPPSPxS motifs (Fig. 3B) in response to activation of PTHR, a GPCR, and this event correlates with β-catenin signaling (Wan et al. 2011).

LRP6 SIGNAL INITIATION-AMPLIFICATION, SIGNALOSOMES, AND ENDOSOMAL SIGNALING

Several models have been elaborated based on Wnt-induced LRP6 phosphorylation/activation. FZD/DVL recruitment of Axin–GSK3 initiates PPPSPxS phosphorylation, which, in turn, reinforces Axin interaction, thereby forming a local positive feed-forward loop that promotes further PPPSPxS phosphorylation and enhances further Axin recruitment (Fig. 4A) (MacDonald et al. 2008; Zeng et al. 2008). This feed-forward loop between LRP6 and Axin may act in *cis* (among five PPPSPxS motifs within an LRP6 molecule) or in *trans* (among PPPSPxS motifs of different LRP6 molecules if LRP6 forms higher orders of oligomers), and has inspired the Wnt receptor "initiation- amplification" model (MacDonald et al. 2008; Zeng et al. 2008), which is consistent with transgenic experiments in *Drosophila* suggesting that Dfz2 and Arrow initiate whereas Arrow amplifies Wg signaling (Baig-Lewis et al. 2007). Supporting this model, phosphorylation of an individual PPPSP motif depends profoundly on neighboring PPPSP motifs, and LRP6 requires minimally four PPPSPxS motifs to be quasi-competent for signaling (and LRP6 mutants with combinations of three PPPSPxS motifs are mostly inactive or even dominant negative) (MacDonald et al. 2008; Wolf et al. 2008). Therefore, five PPPSPxS motifs in LRP6 act like an in *cis* amplifier for signaling. Why then is a single PPPSPxS motif sufficient to transfer signaling to a heterologous receptor, LDLRΔN (Tamai et al. 2004; Zeng et al. 2005; MacDonald et al. 2008)? LDLRΔN, and therefore LDLRΔN-PPPSP, fortuitously form on membrane large aggregates

(Bilic et al. 2007; X-J Zhang and X He, unpubl.), which likely constitute in *trans* amplification.

The LRP6 "signalosome" model is based on observations that Wnt induces large-sized LRP6 aggregates in fractionation and immunostaining (Bilic et al. 2007), and that DVL is required for LRP6 phosphorylation in these aggregates in a manner that correlates with DIX-mediated polymerization (Schwarz-Romond et al. 2007a). When LRP6 is overexpressed together with FZD, DVL, Axin, and GSK3, LRP6 aggregates become particularly prominent and likely contain these other components (Fig. 4B) (Bilic et al. 2007; Schwarz-Romond et al. 2007a). Note that these results are consistent with those that led to the "initiation-amplification" model, suggesting that the two models may describe the same receptor activation events from different temporal/molecular and spatial/cellular perspectives, with DVL polymerization underlying and being emphasized in the signalosome model.

Another model, in which phosphorylated/activated LRP6 signals in an endosomal platform, posits that LRP6 and phosphorylation in lipid rafts (a cholesterol-rich microdomain in the plasma membrane) and its subsequent endocytosis through caveolin are both required for signaling (Yamamoto et al. 2006, 2008). Caveolin binds to LRP6 upon Wnt stimulation and is required for phosphorylated LRP6 to recruit Axin (Yamamoto et al. 2006). One may notice significant similarities between lipid raft/endosomal LRP6 versus signalosomes, because methods (gradient fractionation and immunostaining) used to define them are similar, and signalosomes indeed contain caveolin (Yamamoto et al. 2006; Bilic et al. 2007), suggesting that these two models overlap despite different emphases. PIP2 is suggested to promote LRP6 signalosome formation and thus phosphorylation (Pan et al. 2008), but its key roles in receptor endocytosis should be noted (Di Paolo and De Camilli 2006). A new transmembrane Wnt inhibitor, Waif1, binds to LRP6 and prevents LRP6 endocytosis but not its phosphorylation (Kagermeier-Schenk et al. 2011), lending support to this "endosomal signaling" model. Another transmembrane protein, PRR (prorenin receptor), which binds to FZD and LRP6 and also to the vacuolar H^+-adenosine triphosphatase (V-ATPase) complex that acidifies vesicles, is required for LRP6 phosphorylation by CK1 (Cruciat et al. 2010), consistent with the possibility of a vesicular LRP6 signalosome (or endosome) where LRP6 phosphorylation occurs.

An extension of the endosomal signaling model posits that LRP6 endocytosis via caveolin versus clathrin activates and inhibits signaling, respectively (Yamamoto et al. 2008). However, different results exist regarding this issue (Blitzer and Nusse 2006). Another obstacle is that caveolin1$^{-/-}$ mice show apparently higher β-catenin signaling in mammary and intestinal stem cells (Li et al. 2005; Sotgia et al. 2005). In addition, caveolin does not exist in *Drosophila*, and therefore a different mechanism has to be envisioned for Wg signaling. Thus, some cautions are warranted, particularly when perturbations of general endocytic pathways are performed and thus broad cellular events beyond Wnt signaling are affected (Gagliardi et al. 2008). Indeed, manipulating endocytic molecules in *Drosophila* has also yielded contradictory results regarding Wg signaling (Piddini et al. 2005; Rives et al. 2006; Seto and Bellen 2006). Finally, some of the cell biological studies of LRP6 and Wnt signaling have relied on overexpression of LRP6 and other components. Whether the endogenous LRP6 and other Wnt signaling proteins behave similarly is a major caveat that needs to be taken into account.

INHIBITION OF β-CATENIN PHOSPHORYLATION POST RECEPTOR ACTIVATION

How activation of the Wnt receptor complex, in particular phosphorylation of LRP6, leads to inhibition of β-catenin phosphorylation remains not fully understood and debated. An earlier model (Fig. 5A), prior to knowledge of LRP6 phosphorylation, suggests that Wnt signaling via DVL leads to disruption of the Axin complex (Kimelman and Xu 2006; MacDonald et al. 2009). Indeed, upon Wnt stimulation, reduced association of Axin with β-catenin and GSK3 is observed (Kishida et al. 1999a; Li et al.

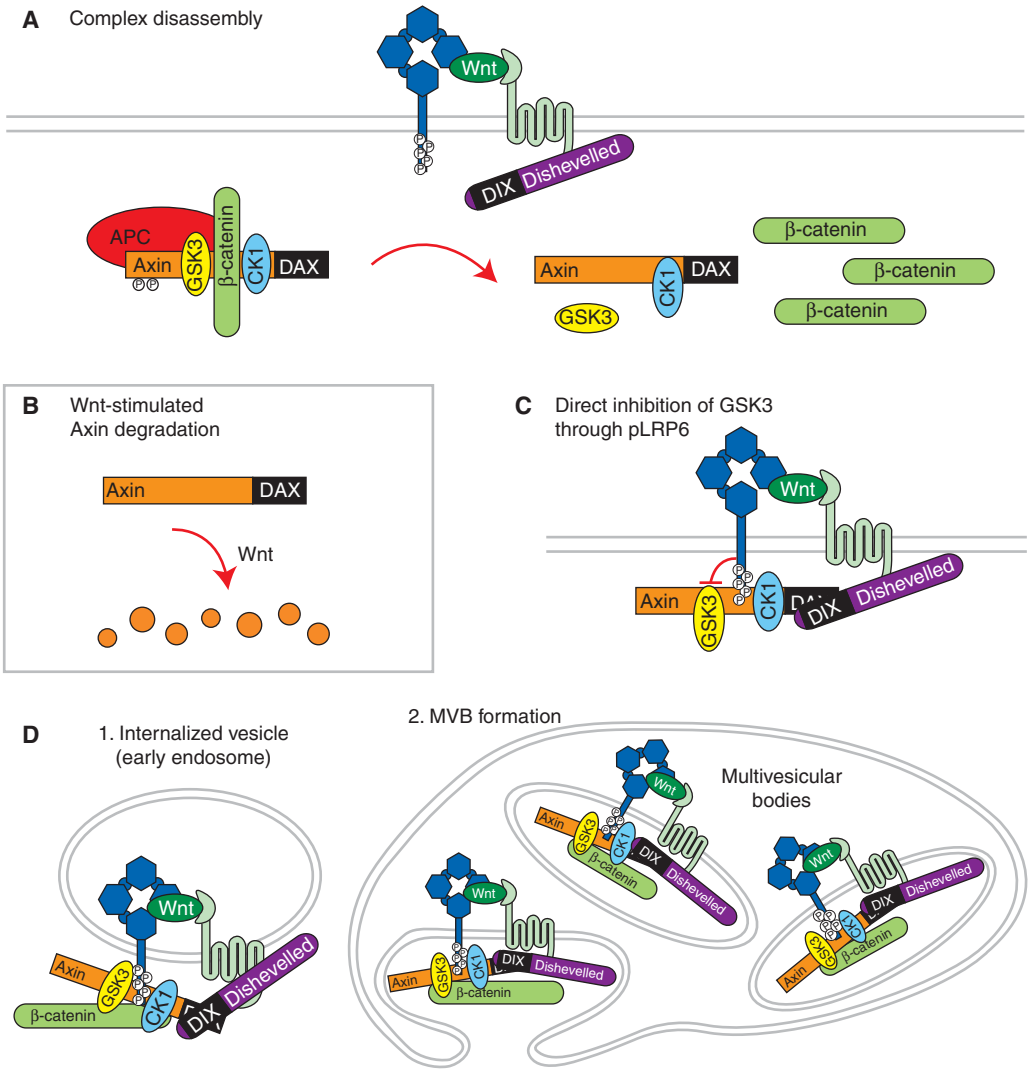

Figure 5. Models for Wnt-induced inhibition of β-catenin phosphorylation. (*A*) Axin complex disassembly. (*B*) Axin protein degradation. (*C*) GSK3 inhibition by phosphorylated PPPSPxS motifs. (*D*) GSK3 inclusion by multivesicular bodies.

1999; Itoh et al. 2000; Liu et al. 2005), and this is accompanied by hypophosphorylation of Axin (Willert et al. 1999; Yamamoto et al. 1999), which itself is a substrate for GSK3 (and CK1) (Ikeda et al. 1998; Jho et al. 1999). This model suggests that displacement from or inhibition of GSK3 in the Axin complex causes Axin hypophosphorylation, which further results in reduced β-catenin binding to Axin and thus β-catenin phosphorylation (Kimelman and Xu 2006). Some of these earlier studies included a role for FRAT/GBP (Frequently Rearranged in Advanced T-cell lymphoma/GSK3-Binding Protein), which is a family of GSK3-binding proteins that displace GSK3 from Axin and thus inhibit GSK3 (Yost et al. 1998; Ferkey and Kimelman 2002; Dajani et al. 2003). Because FRAT also binds to DVL, an extension of the model is that DVL through FRAT displaces GSK3 in the Axin complex, leading to inhibition

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of β-catenin phosphorylation. However, triple knockouts of all three *Frat* genes produced viable and fertile mice, arguing that FRAT is dispensable for Wnt signaling (van Amerongen et al. 2005). This is also true for Wg signaling, because *Drosophila* does not have a FRAT homolog. Although a FRAT-based model is no longer favored, the core part of the "complex disruption" model and data leading to it remain valid and should not be discounted. A recent study showed that protein phosphatase 1 (PP1) is required for Wnt signaling through Axin dephosphorylation that disrupts Axin–GSK3 interaction (Luo et al. 2007), consistent with the suggestion that active Axin dephosphorylation together with inhibition of Axin phosphorylation by GSK3 are involved in Axin complex disruption (Willert et al. 1999).

A study suggests that APC, via an unknown mechanism, is broadly required for GSK3 activity and that Wnt stimulation results in dissociation of APC from the Axin–GSK3 complex, resulting in down-regulation of GSK3 (Valvezan et al. 2012), again consistent with the complex disruption model.

Earlier results showed that overexpression of constitutively activated LRP5 or Arrow resulted in decreased Axin protein levels in mammalian and *Drosophila* systems (Fig. 5B) (Mao et al. 2001b; Tolwinski et al. 2003). However, the Wnt-induced decrease in Axin protein levels lags behind the increase in β-catenin protein levels (Willert et al. 1999; Yamamoto et al. 1999), and thus is unlikely to be the primary mechanism for β-catenin stabilization. However, Axin degradation may be important for long-term or chronic Wnt stimulation. It is worth noting that Tankyrase has been shown to promote Axin PARsylation and degradation (Huang et al. 2009), but Tankyrase-induced Axin degradation does not seem to be under Wnt regulation.

To integrate recent findings of LRP6 phosphorylation and DVL acting primarily through LRP6 phosphorylation, a newer model has emerged in which phosphorylated PPPSPxS motifs directly inhibit GSK3 (Cselenyi et al. 2008; Piao et al. 2008; Wu et al. 2009). This model is based on findings that LRP6 binds to GSK3 (Zeng et al. 2005; Mi et al. 2006) and that phosphorylated recombinant LRP6 cytoplasmic domain and, in fact, phosphorylated PPPSPxS peptides bind to and inhibit β-catenin phosphorylation by GSK3 in vitro and activate β-catenin signaling in vivo (Cselenyi et al. 2008; Piao et al. 2008; Wu et al. 2009). This inhibition mechanism bears apparent similarity to GSK3 inhibition during insulin signaling by its own amino-terminal region, whose phosphorylation (at S21 in GSK3α or S9 in GSK3β by the AKT kinase) creates an inhibitory and binding pseudosubstrate for GSK3 inhibition (Dajani et al. 2001; Frame et al. 2001). This model fits well with the requirement of LRP6 PPPSPxS phosphorylation in signaling and the hypothesis of DVL-dependent signalosomes in recruitment of Axin–GSK3 to the proximity of LRP6 and can account for the specificity of inhibition of GSK3 phosphorylation of β-catenin (but not of other GSK3 substrates outside of the Axin complex). This model is also compatible with the "complex disruption" model, because LRP6 inhibition of GSK3 will also result in Axin hypophosphorylation and thus Axin complex disruption. Implicit in this model is that phosphorylated PPPSPxS motifs may have multiple roles in Wnt signaling: binding to Axin, enhancing mutual PPPSPxS phosphorylation, and binding to and inhibition of GSK3. In this context, it is noted that five PPPSPxS motifs in LRP5/6 are spaced tightly within a region of 120 residues (Fig. 3B), leading to speculation that an LRP6 may only interact with a single Axin molecule at any given time, that is, only one phosphorylated PPPSPxS motif is occupied by Axin, while the remaining ones provide a high local concentration to sustain LRP6–Axin interaction (Wu et al. 2009). In this scenario, LRP6 via its five phosphorylated PPPSPxS motifs can engage Axin and inhibit GSK3 simultaneously, a possibility that is also conceivable if LRP6 aggregates in trans in signalosomes. Related to this issue is whether Axin binds to LRP6 indirectly via GSK3 (Piao et al. 2008), but recent evidence does not support the view of indirect LRP6–Axin interaction (MacDonald et al. 2011). Further studies will be needed to clarify the mechanism of phosphorylated PPPSPxS motifs in Axin and GSK3 binding and GSK3 inhibition.

A drastically different model was proposed recently, in which multivesicular body (MVB) formation after endocytosis of the Wnt receptor wraps GSK3 into the deep interior of the MVB, thereby physically separating GSK3 from its cytoplasmic substrates including β-catenin (Fig. 5D) (Taelman et al. 2010). This "MVB inclusion" model has several features: (1) It requires MVBs to round up most of the cellular GSK3 proteins upon Wnt signaling, because gene deletions of Gsk3α and/or Gsk3β suggest that cells can perform Wnt signaling when two (or less than three) of the four Gsk3 alleles are deleted (Doble et al. 2007). (2) Because GSK3 levels do not overtly decrease during Wnt signaling, it requires a mechanism for GSK3 subsequently to escape MVBs, which normally shuttles the cargo to lysosomes for degradation. (3) It suggests that genes involved in MVB formation are required for Wnt signaling, for which supporting evidence exists in *Xenopus* but not in *Drosophila* (Piddini et al. 2005; Rives et al. 2006; Seto and Bellen 2006; Taelman et al. 2010). (4) It predicts that most GSK3 substrates (in fact, ~20% of cellular proteins) in addition to β-catenin are under Wnt regulation (Taelman et al. 2010), thus departing from the prevailing view that roles of GSK3 in Wnt and other signaling (such as insulin) pathways are insulated from one another. Another unexpected finding is that β-catenin itself promotes inclusion of GSK3 into MVBs (Taelman et al. 2010), implying a new β-catenin function downstream of the Wnt receptors, acting before its role as a transcription coactivator in the nucleus. How to integrate/reconcile this provocative MVB model with the existing data and models remains to be considered (Metcalfe and Bienz 2011), although the possibility of MVB actions in the longer time course of Wnt treatment has been discussed.

CONCLUDING REMARKS

Our understanding of the Wnt receptor complex has progressed tremendously but remains rudimentary. Many unanswered questions linger, including the specificity and stoichiometry of the receptors with Wnt ligands and their antagonists, newly and yet-to-be identified components of the receptor complex, molecular and dynamic interactions that assemble and disassemble the receptor complex and downstream signaling components, and ultimately the atomic structures of some or all of these components in Wnt-OFF and Wnt-ON states. These will represent huge challenges for generations of researchers to accomplish.

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