LDL receptor-related proteins 5 and 6 in Wnt/β-catenin signaling: Arrows point the way

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Summary

Wnt signaling through the canonical β-catenin pathway plays essential roles in development and disease. Low-density-lipoprotein receptor-related proteins 5 and 6 (Lrp5 and Lrp6) in vertebrates, and their *Drosophila* ortholog Arrow, are single-span transmembrane proteins that are indispensable for Wnt/β-catenin signaling, and are likely to act as Wnt co-receptors. This review highlights recent progress and unresolved issues in understanding the function and regulation of Arrow/Lrp5/Lrp6 in Wnt signaling. We discuss Arrow/Lrp5/Lrp6 interactions with Wnt and the Frizzled family of Wnt receptors, and with the intracellular β-catenin degradation apparatus. We also discuss the regulation of Lrp5/Lrp6 by other extracellular ligands, and LRP5 mutations associated with familial osteoporosis and other disorders.

Supplemental data available online

Introduction

Signaling by the Wnt family of secreted growth factors has key roles in development and disease (Wodarz and Nusse, 1998; Veeman et al., 2003). Since the discovery of *Wnt1* as an oncogene that causes mouse mammary tumorigenesis (Nusse and Varmus, 1982), the Wnt gene family, which includes 19 members in the human genome, has been found in all animal species examined. Wnt proteins regulate many stages of development, from patterning of the embryo and generation of tissues and cell types, to regulation of cell movements, polarity, axon guidance and synapse formation (Nusse, 2003; Packard et al., 2003; Strutt, 2003; Veeman et al., 2003). Defective Wnt signaling plays major roles in diseases such as cancer (Bienz and Clevers, 2000; Polakis, 2000) and osteoporosis (Patel and Karsenty, 2002). Therefore, the investigation of Wnt signal transduction is crucial for understanding development and disease.

The interaction of Wnt proteins with their receptors on the cell surface is the first step in transducing the extracellular signal into intracellular responses. The first identified Wnt receptors were members of the Frizzled (Fz) family of seven-pass transmembrane receptors (Wodarz and Nusse, 1998), 10 of which are encoded in the human genome. In addition to Fz proteins, the canonical Wnt/β-catenin signaling pathway requires single-span transmembrane proteins that belong to a subfamily of low-density-lipoprotein (LDL) receptor related proteins (LRPs): vertebrate Lrp5 and Lrp6, and their *Drosophila* ortholog Arrow (Pinson et al., 2000; Tamai et al., 2000; Wehrli et al., 2000), which are the focus of this review.

We will discuss the structure and function of Arrow/Lrp5/Lrp6, their interactions with Wnt, Fz and the intracellular β-catenin signaling apparatus, their biogenesis and modulation by extracellular antagonists, and, finally, the roles of LRP5 mutations in human diseases. Because of their related biochemical properties in Wnt signaling, we will often, unless otherwise specified, use Lrp5/Lrp6 to refer Lrp5 and Lrp6 together in the discussion. For an overview of Wnt signaling, including accounts of various Wnt transduction pathways and components, readers may refer to many excellent reviews (Wodarz and Nusse, 1998; Adler, 2002; Huelsken and Behrens, 2002; Nusse, 2003; Strutt, 2003; Veeman et al., 2003).

Canonical Wnt/β-catenin signaling

β-catenin phosphorylation and degradation

The outcome of the most intensively studied Wnt pathway – the canonical Wnt/β-catenin signaling pathway – relies to a large extent on the regulation of the stability/abundance of the β-catenin protein (Fig. 1). It is widely accepted that in this Wnt pathway β-catenin associates with, and acts as an obligatory nuclear co-activator for, the TCF/LEF (T cell factor/Lymphoid enhancer factor) family of transcription factors (Bienz and Clevers, 2003; Cong et al., 2003; Tolwinski and Wieschaus, 2004). In the absence of a Wnt ligand, the level of cytosolic β-catenin is kept low as a result of its amino-terminal phosphorylation-dependent ubiquitination/proteosome degradation. When β-catenin is low, TCF/LEF is associated with transcriptional co-repressors and suppresses Wnt-responsive gene expression (Fig. 1A). Upon Wnt stimulation, β-catenin phosphorylation and degradation is inhibited, and the accumulation of β-catenin promotes its association with TCF/LEF, leading to the activation of Wnt-responsive transcription (Fig. 1B).

β-catenin phosphorylation is thus a crucial regulatory step in this Wnt pathway. β-Catenin phosphorylation involves the sequential actions of casein kinase 1 (Ck1) and glycogen synthase kinase 3 (Gsk3), and takes place in a protein complex assembled by the scaffolding protein Axin and the tumor suppressor protein Apc, the *adenomatous polyposis coli* gene product (Fig. 1A). Phosphorylated β-catenin is recognized by

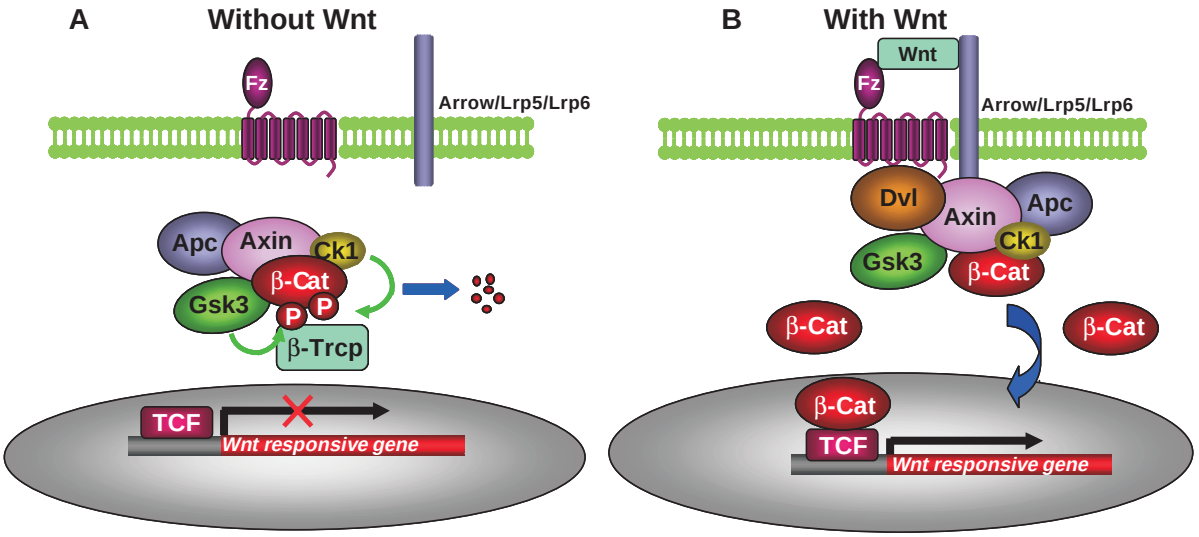


Fig. 1. A simplified prevailing view of Wnt/β-catenin signaling (see Box 1 for some alternative views). (A) Without Wnt, the scaffolding protein Axin assembles a protein complex that contains Apc, Gsk3, Ck1 and β-catenin. In this complex, β-catenin is sequentially phosphorylated by Ck1 and Gsk3. Phosphorylated β-catenin is recognized by β-Trcp, which is a component of an ubiquitin-ligase complex that conjugates β-catenin with ubiquitin. Poly-ubiquitinated β-catenin is degraded by the proteosome. TCF/LEF-associated co-repressors, such as Groucho (Cavallo et al., 1998), and Axin-associated Diversin (Schwarz-Romond et al., 2002), PP2A (Hsu et al., 1999) and other proteins (Kikuchi, 1999) are omitted for simplicity. (B) In the presence of Wnt, β-catenin phosphorylation and degradation is inhibited. Accumulated β-catenin forms a nuclear complex with the DNA-bound TCF/LEF transcription factor, and together they activate Wnt-responsive genes. This signaling cascade is perhaps initiated by a Wnt-induced Fz-Lrp5/Lrp6 co-receptor complex, which recruits Axin to the plasma membrane through Lrp5/Lrp6-Axin association. Fz-associated Dishevelled (Dvl) protein may bind Axin and inhibit Axin-Gsk3 phosphorylation of β-catenin, either directly or indirectly via Dvl-associated proteins. Lrp5/Lrp6-Axin binding may also promote Axin degradation. Either or both of these events can lead to β-catenin accumulation. This description represents one of several possibilities. The composition of the Axin complex upon Wnt stimulation is not well defined. Gsk3-binding protein (GBP/Frat) (Farr et al., 2000; Salic et al., 2000), and nuclear β-catenin-associated Legless/Bcl9 and Pygopus (Belenkaya et al., 2002; Kramps et al., 2002; Parker et al., 2002; Thompson et al., 2002) are omitted for simplicity. Modified with permission from He (He, 2003).

the F-box protein β-Trcp, which is a part of an ubiquitin-ligase complex that conjugates β-catenin with ubiquitin for proteosome degradation (Polakis, 2002; Wu et al., 2004). This phosphorylation-degradation process is often perturbed in human colorectal cancers and other tumors, either by a loss of functional Apc or Axin, or by β-catenin mutations that prevent its phosphorylation and degradation, thereby resulting in constitutive Wnt/β-catenin signaling (Bienz and Clevers, 2000; Polakis, 2000).

How Wnt inhibits β-catenin phosphorylation is not well defined, but may be achieved by any of, or combinations of, the following (Fig. 1B): (1) degradation of the Axin protein (Willert et al., 1999; Yamamoto et al., 1999; Mao et al., 2001b; Tolwinski et al., 2003); (2) alteration of the composition of the Axin complex (such as by dissociation of Gsk3 or β-catenin from Axin) (Kishida et al., 1999a; Willert et al., 1999; Li et al., 1999; Itoh et al., 2000); (3) binding of the Gsk3 binding protein (GBP) to Gsk3 (Farr et al., 2000; Salic et al., 2000); and (4) inhibition of Gsk3 (or Ck1) kinase activity (Cook et al., 1996). A recent genetic study in *Drosophila* (Tolwinski et al., 2003), however, suggested that Wnt/β-catenin signaling can occur in a Gsk3-independent manner (Box 1). Another cytoplasmic protein Dishevelled has a crucial but poorly understood role in the inhibition of the Axin/Gsk3 complex, and is discussed in more detail in later sections.

Fz proteins are Wnt receptors

The Wnt-Fz ligand-receptor relationship is best characterized in *Drosophila*. Studies using cultured *Drosophila* cells indicate that two members of the Fz family, Dfz1 and Dfz2 (Fz and Fz2 – FlyBase), are Wingless (Wg, *Drosophila* Wnt1) receptors, which bind Wg with high affinities (K_d=10^{-8} M and 10^{-9} M for Dfz1 and Dfz2, respectively) (Bhanot et al., 1996; Rulifson et al., 2000). Fly mutants lacking both *Dfz1* and *Dfz2*, but not mutants lacking either, have severely defective Wg signaling (Bhat, 1998; Kennerdell and Carthew, 1998; Bhanot et al., 1999; Chen and Struhl, 1999; Muller et al., 1999), providing unambiguous evidence that, in many contexts, Dfz1 and Dfz2 are redundant Wg receptors.

Studies in nematodes, *Xenopus* and mammalian cells support the Wnt-Fz ligand-receptor relationship (Sawa et al., 1996; Yang-Snyder et al., 1996; He et al., 1997; Rocheleau et al., 1997; Thorpe et al., 1997; Hsieh et al., 1999). However, the specificity of Wnt-Fz interactions remains largely unresolved, particularly in vertebrates, because of difficulties in producing soluble Wnt proteins, the large numbers of Wnt and Fz genes, and the potential multitudes of Wnt-Fz interactions and functional redundancies. Nevertheless, recent success in purification of the first Wnt protein, mouse Wnt3a, may help to pave the way for comprehensive biochemical studies of Wnt-Fz interactions in vitro (Willert et al., 2003).

Arrow/Lrp5/Lrp6: primary structure and function

Arrow/Lrp5/Lrp6 and Wnt/β-catenin signaling

The roles of Arrow and Lrp6 in Wnt signaling were discovered via genetic studies. *Drosophila* mutants lacking *arrow*

Box 1. Is inhibition of Gsk3 phosphorylation of β-catenin required for Wnt signaling?

Although most experimental data support the model that Wnt signals via inhibition of β-catenin phosphorylation-degradation, this dogma appears to be challenged by a genetic study in *Drosophila* (Tolwinski et al., 2003). Using a mutant β-catenin (Armadillo) with weaker activity, these authors showed that Wnt/Wg signaling can occur in Gsk3/Zw3 mutant embryos, probably through promoting Axin protein degradation. Together with other data (Tolwinski and Wieschaus, 2001), a model was proposed in which Axin anchors β-catenin in the cytoplasm; Wnt-induced degradation of Axin releases β-catenin, allowing it to accumulate in the nucleus for signaling (Tolwinski et al., 2003). Although an anchoring role for Axin is possible and may complement its role as a scaffolding protein for β-catenin phosphorylation, one caveat is that the Axin protein level may be significantly lower than that of cytosolic β-catenin (Lee et al., 2003), implying that Axin may only be sufficient to anchor a small fraction of β-catenin. A key issue that remains to be clarified is whether the *Gsk3/zw3* mutant embryos (*zw3*$^{m11-1}$) used in the study retain residual Gsk3/Zw3 activities (Ruel et al., 1993), or whether other Gsk3-like molecules in the fly genome may have compensated for Gsk3/Zw3 in the presence of the weak β-catenin allele.

Another issue that requires further study is whether β-catenin phosphorylation by Gsk3 inhibits β-catenin 'specific activity' (i.e. activity per molecule) independent of its protein level. Some experiments in *Xenopus* embryos/extracts (Guger and Gumbiner, 2000), and in an in vitro reconstituted TCF/LEF transcription assay (Tutter et al., 2001), implied that β-catenin that cannot be phosphorylated (because of mutated/deleted serine/threonine residues) has higher specific activity than the wild-type β-catenin. However, the technical difficulty in dissociating increased β-catenin activity from increased protein level makes such an interpretation less clear-cut.

phenotypically resemble the *wg* mutant (Wehrli et al., 2000), and mutant mice lacking *Lrp6* exhibit composite phenotypes similar to mutations of several individual Wnt genes (Pinson et al., 2000). In *Xenopus* embryos, dominant-negative Lrp6 blocks signaling by several Wnt proteins, whereas overexpression of Lrp6 cooperates with Wnt and Fz to activate Wnt/β-catenin signaling (Tamai et al., 2000). Furthermore, Arrow and Lrp6 are required for cells to respond to Wnt, and act upstream of known intracellular Wnt signaling components in *Drosophila* and *Xenopus* (Tamai et al., 2000; Wehrli et al., 2000), pinpointing a role for Arrow/Lrp6 in Wg/Wnt signal reception. A recent genetic study in mice indicates that Lrp5 also has a role in Wnt signaling (Kelly et al., 2004).

Unlike Fz, which is required for multiple Wnt pathways (Strutt, 2003; Veeman et al., 2003), Arrow and Lrp6 appear to be specifically required for Wnt/β-catenin signaling. In *Drosophila*, Dfz1, but not Dfz2, plays a central role in planar cell polarity (PCP) determination (reviewed by Adler, 2002; Strutt, 2003), whereas *arrow* mutants exhibit normal PCP (Wehrli et al., 2000). This indicates that Arrow is not required for Dfz1 PCP function. Similarly, in *Xenopus*, blocking Lrp6 function has little effect on gastrulation movements (Semenov et al., 2001), which are regulated by a Wnt11/Fz pathway analogous to Dfz1/PCP signaling (Heisenberg et al., 2000; Tada and Smith, 2000; Wallingford et al., 2000).

Lrp5/Lrp6: redundancy and Wnt specificity

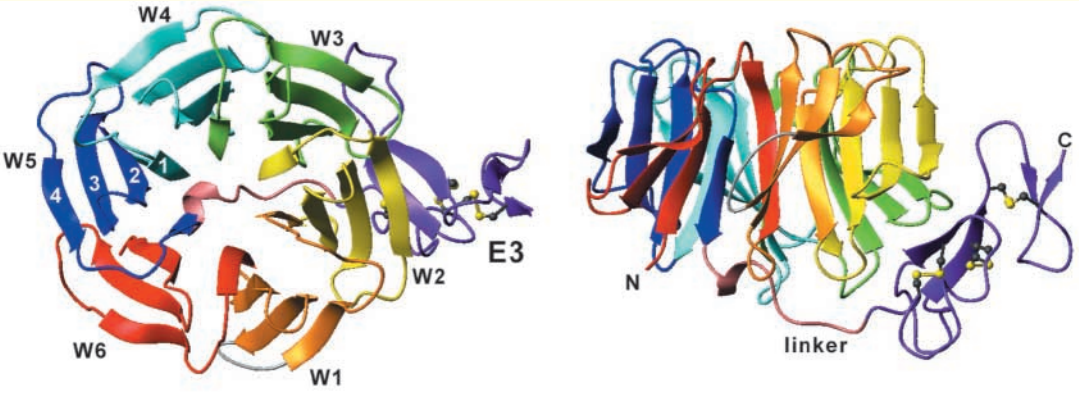
Lrp5 and Lrp6 are highly homologous, and are widely co-expressed during embryogenesis and in adult tissues (Dong et al., 1998; Hey et al., 1998; Kim et al., 1998; Pinson et al., 2000; Houston and Wylie, 2002; Kato et al., 2002; Fujino et al., 2003; Kelly et al., 2004). *Lrp6*$^{-/-}$ mice are perinatal lethal and exhibit mid/hindbrain defects, posterior truncation and abnormal limb patterning, which resemble the defects of mice mutant for *Wnt1*, *Wnt3a* and *Wnt7a* (Pinson et al., 2000). *Lrp5*$^{-/-}$ mice have normal embryogenesis, grow to adulthood and are fertile, but show osteoporosis (Kato et al., 2002) and some metabolic abnormalities (Magoori et al., 2002; Fujino et al., 2003). Thus, the Lrp6 loss-of-function phenotype is much more severe than the Lrp5 loss-of-function phenotype, indicating that Lrp6 has a more influential role than Lrp5 during embryogenesis. There is a hint of functional redundancy between Lrp5 and Lrp6, as defects in *Lrp6*$^{-/-}$ embryos are less severe than those observed in individual Wnt mutants (Pinson et al., 2000). Indeed, *Lrp5*$^{-/-}$*; Lrp6*$^{-/-}$ double mutant mice die during gastrulation (much earlier than *Lrp6*$^{-/-}$ mice); they lack the primitive streak and nascent mesoderm (Kelly et al., 2004), and thus phenotypically resemble the *Wnt3* mutant (Liu et al., 1999). Thus, genetic studies in mice indicate that Wnt3 requires either Lrp5 or Lrp6 in order to function, whereas Wnt1, Wnt3a and Wnt7a rely on Lrp6 primarily and Lrp5 to some extent. Whether signaling by other Wnt proteins requires Lrp5 and/or Lrp6 cannot be inferred at the moment due to the early lethality of double *Lrp5*$^{-/-}$*; Lrp6*$^{-/-}$ mutants and the degree of redundancy between the two proteins. It seems unlikely that Lrp5 and Lrp6 are involved in signaling by distinct Wnt proteins during development (note that *Lrp5*$^{-/-}$ mice have normal embryogenesis), although more genetic studies are needed to clarify this issue. It is also unclear whether, in *Drosophila*, Arrow is required for signaling by other Wnt proteins besides Wg.

An allelic series of compound mutants reveal the following order of severity of developmental abnormalities: *Lrp5*$^{+/-}$ (normal)<*Lrp6*$^{+/-}$<*Lrp5*$^{-/-}$<*Lrp5*$^{+/-}$*; Lrp6*$^{+/-}$<*Lrp5*$^{-/-}$*; Lrp6*$^{+/-}$<*Lrp6*$^{-/-}$<*Lrp5*$^{+/-}$*; Lrp6*$^{-/-}$<*Lrp5*$^{-/-}$*; Lrp6*$^{-/-}$ (Kelly et al., 2004). This is likely to reflect the severity of loss of Wnt/β-catenin signaling, and supports the view that Lrp5 and Lrp6 share significant overlapping functions but that Lrp6 plays a more crucial role, at least during embryogenesis. This is consistent with observations that overexpression of Lrp6 exhibits significantly stronger activity than Lrp5 in *Xenopus* (Tamai et al., 2000) and mammalian cells (Holmen et al., 2002). It is possible that Lrp6 has a higher affinity for Wnts or a stronger signaling efficacy than Lrp5, or both.

Arrow/Lrp5/Lrp6: members of the LDLR family

Arrow/Lrp5/Lrp6 is a subfamily of the LDL receptor (LDLR) family (Fig. 2), which has diverse roles in metabolism and development (Herz and Bock, 2002). Human LRP5 was isolated through its homology to LDLR (Dong et al., 1998; Hey et al., 1998; Kim et al., 1998). Human LRP6 was identified by its homology to LRP5 (Brown et al., 1998). Arrow/LRP5/LRP6 are type I single-span transmembrane proteins with 1678, 1615 and 1613 amino acid residues, respectively. LRP5 and LRP6 share 73% and 64% identity in extracellular and intracellular domains, respectively, whereas Arrow is equally related (40% identical) to LRP5 and LRP6

Box 2. Architecture of Arrow/Lrp5/Lrp6 extracellular domains



Structure of a prototypic YWTD β-propeller-EGF-like domain of the LDLR. (Left) View down the central axis of the six-bladed propeller. Each blade (W1 to W6, in different colors) contains four β-strands (1 to 4). E3 represents the EGF-like domain, which packs tightly against and forms side chain interactions with the propeller. (Right) Side view. Reproduced with permission from Jeon et al. (Jeon et al., 2001).

The Arrow/Lrp5/Lrp6 extracellular domains have three basic domains: the YWTD (tyrosine, tryptophan, threonine and aspartic acid)-type β-propeller domain, the EGF (epidermal growth factor)-like domain, and the LDLR type A (LA) domain. These are defining features of the LDLR family. The YWTD-type β-propeller domain has six YWTD repeats of 43-50 amino acid residues each (the conserved YWTD residues are located at the beginning of each repeat), and forms a six-bladed β-propeller structure (Springer, 1998; Jeon et al., 2001). Arrow/Lrp5/Lrp6 have four YWTD β-propeller domains that are each followed by an EGF-like domain, which comprises approximately 40 amino acids with conserved cysteine residues; these domains are then followed by three LA domains. In the LDLR, LA domains constitute LDL binding sites, and their intramolecular interaction with the β-propeller-EGF-like domain controls LDL binding with, and release from, the LDLR (Rudenko et al., 2002). Whether an analogous intramolecular interaction exists in Arrow/Lrp5/Lrp6 is unknown. The β-propeller-EGF-like domains in Lrp5/Lrp6 appear to bind extracellular ligands.

(Box 2, Box 3, Fig. S1 at http://dev.biologists.org/supplemental/). Indeed, LRP6 substitutes for Arrow during Wg signaling in cultured *Drosophila* cells (Schweizer and Varmus, 2003), and constitutively activated Arrow (discussed later) activates Wnt/β-catenin signaling in mammalian cells and Xenopus embryos (Tamai et al., 2004).

Arrow/Lrp5/Lrp6: Wnt co-receptors?

The simplest model to account for the role of Arrow/Lrp5/Lrp6 in Wnt signaling is that Arrow/Lrp5/Lrp6 are Wnt co-receptors. Nevertheless, a few issues concerning this view remain:

(1) Do Arrow/Lrp5/Lrp6 bind Wnt? Co-immunoprecipitation (co-IP) experiments suggest that Lrp5/Lrp6 can bind Wnt1, Wnt3a, Wnt4 and *Xenopus* Wnt8 (Xwnt8; Table 1) (Tamai et al., 2000; Mao et al., 2001b; Kato et al., 2002; Itasaki et al., 2003; Liu et al., 2003), although Wnt-Lrp5/Lrp6 binding affinities have not been reported, perhaps because of a weaker binding between Wnt and Lrp5/Lrp6 than that between Wnt and Fz. The first two YWTD β-propeller plus EGF-like domains of Lrp6 (Fig. 2; see also Fig. 6) are involved in binding Xwnt8 (Itasaki et al., 2003), and, in mammalian cells, these domains are required for Lrp6 to cooperate functionally with Wnt/Fz (Mao et al., 2001a). Additionally, an artificially-created secreted Lrp5 extracellular domain blocks Wnt signaling, presumably by competing away Wnt ligands (Mao et al., 2001b; Gong et al., 2001) (Fig. 3). We note that Wnt proteins are palmitoylated (i.e. covalently modified by a lipid moiety) and are, therefore, lipoproteins (Willert et al., 2003; Nusse, 2004). It may make sense intuitively that they are

Table 1. Summary of various Wnt-Arrow/Lrp5/Lrp6 binding studies

	Wnt tested	Wnt binding detected	References
Lrp5	Wnt1	Yes	Mao et al., 2001b* Kato et al., 2002†
	Wnt4	Yes	Kato et al., 2002†
Lrp6	Wnt1	Yes	Tamai et al., 2000*
	Wnt3a	Yes	Liu et al., 2003†
	Xwnt8	Yes	Itasaki et al., 2003*
Arrow	Wg	No	Wu and Nusse, 2003*,‡

Various groups employed different binding assays in studying Wnt-Arrow/Lrp5/Lrp6 interactions, which appear to be weaker than Wnt-Fz interactions (M.S. and X.H., unpublished), and the binding affinities have not been reported.

*One assay involves the production of conditioned medium (CM) containing either the secreted Arrow/Lrp5/Lrp6 extracellular domain or Wnt/Wg, then incubation of the two CM together and subsequent co-IPs. Note that CM contains bovine serum proteins and other secreted proteins made by the cultured cells, and thus is not a pure source of proteins of interest.

†Another assay involves co-transfection of Wnt plus Lrp5/Lrp6, either the full length or just the extracellular domain, followed by co-IPs using total cell extracts. One cautionary note here is that Wnt and Lrp5/Lrp6, because in part of their high cysteine content, may exhibit some misfolded proteins in the secretory pathway when overexpressed (see Box 4). Thus total cell lysates may contain Lrp5/Lrp6 and/or Wnt aggregates that pose problems for co-IPs. This is less a problem when using CM, as it is generally believed that proteins secreted into the CM (or presented on the plasma membrane) have been properly matured through the secretory route, i.e. have been correctly folded and processed.

‡The third assay tests the binding of the Arrow extracellular domain (provided by CM) to cells expressing a membrane-tethered form of Wg.

Box 3. Features of Arrow/Lrp5/Lrp6 intracellular domains

Arrow/Lrp5/Lrp6 intracellular domains (see Fig. S1 at http://dev.biologists.org/supplemental/) have 209, 207 and 218 amino acid residues, respectively, and are rich in prolines and serines (15-20% each). They lack any recognizable catalytic motifs and share no sequence similarity with other LDLR proteins. Identifiable are scattered conserved regions, including five reiterated PPP(S/T)P motifs (see Fig. S1), which are the Axin-binding sites and are essential for Lrp6 signaling function (Tamai et al., 2004). These motifs also have some similarity with the consensus binding site for Src homology 3 (SH3)-domains (Dong et al., 1998), found in many scaffolding/signaling molecules (Pawson and Nash, 2003). Two of these, (V/Y)PPPPTP and (Y/F/L)PPPPSP (see Fig. S1), are a good match for the consensus binding site (F/W/Y/L)PPPP for EVH1 (Ena/Vasp homology 1) domains, which are present in the Ena/Vasp family of signaling proteins involved in actin cytoskeletal regulation (Niebuhr et al., 1997). Whether these regions serve as SH3 or EVH1 binding sites in Arrow/Lrp5/Lrp6 function is unknown.

Noticeably absent in the intracellular domains of Arrow/Lrp5/Lrp6 is the NPxY motif (x, any amino acid), which is present in all other LDLR proteins (Fig. 2) and mediates interactions with the endocytic apparatus (for receptor internalization) and/or cytoplasmic signaling/scaffolding proteins in signal transduction (Herz and Bock, 2002). However, other types of potential internalization motifs, such as a di-leucine (LL or IL) motif and an YRxY (aromatic–x–x–aromatic/large hydrophobic) sequence, can be found in Arrow/Lrp5/Lrp6 (Brown et al., 1998) (see Fig. S1), but whether they play roles in Arrow/Lrp5/Lrp6 endocytosis is unknown.

ligands for Lrp5/Lrp6, which, as members of the LDLR family, may be specialized in binding lipoproteins. However, in *Drosophila*, Wg binding to Arrow has not been demonstrated (Wu and Nusse, 2002) (Table 1).

(2) Does Arrow/Lrp5/Lrp6 associate with Fz? Some evidence suggests so. mFz8CRD, which is the cysteine-rich domain of mouse Fz8, and which binds Wnt (Hsieh et al., 1999), exhibits a Wnt1-dependent association with both the Lrp5 and Lrp6 extracellular domains in vitro (Tamai et al., 2000; Semenov et al., 2001), leading to the proposal that Wnt induces a complex formation between Fz and Lrp5/Lrp6 (Fig. 1). Indeed, forced proximity of Dfz2 and Arrow, by fusing the Arrow intracellular domain with the Dfz2 cytoplasmic tail (Fig. 3), activates β-catenin signaling in a Wg-independent manner (Tolwinski et al., 2003), implying that Wg brings Arrow and Dfz2 together. However, attempts to detect a complex of Wg, Dfz2CRD and the Arrow extracellular domain have not so far been successful (Wu and Nusse, 2002).

Further experiments will be needed to substantiate any Fz-Lrp5/Lrp6 association. First, can Wnt-Lrp5/Lrp6 affinities be measured? Second, can an in vitro Wnt-Fz-Lrp5/Lrp6 (extracellular domains) complex be observed for Wnt and Fz proteins other than just Wnt1 and Fz8? Third, can a complex between wild-type Fz and Lrp5/Lrp6 be detected at the plasma membrane? Finally, although the simplest version of the co-receptor model is that Lrp5/Lrp6 and Fz bind independently to Wnt, it remains to be examined whether Fz binding to Wnt enhances the Wnt-Lrp5/Lrp6 interaction, and/or vice versa. Answering these questions may not be simple, however, because Wnt-Fz specificity itself is not well understood, and, with the possible exception of a few Wnts, it is not clear which

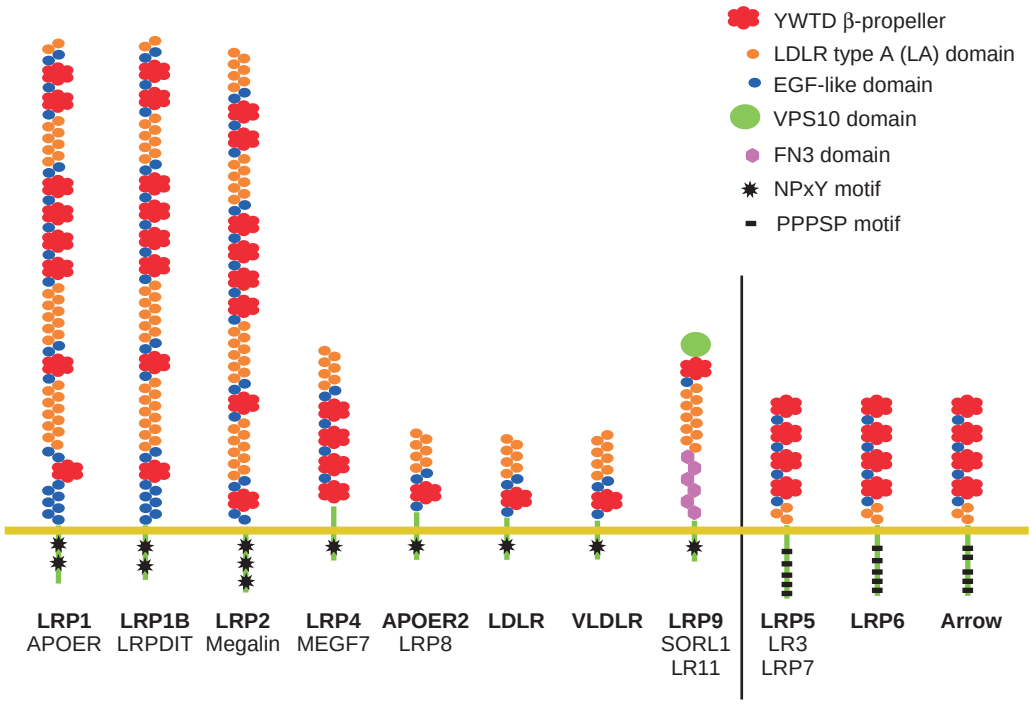


Fig. 2. Schematic diagram of Arrow/LRP5/LRP6 and other members of the LDLR family. All proteins depicted are of human origin except for Arrow (*Drosophila*), and are conserved in vertebrates; some have invertebrate homologs (Herz and Bock, 2002). YWTD (Tyr, Trp, Thr and Asp)-type β-propeller domains, LDLR type A (LA) domains and EGF (epidermal growth factor)-like domains are shared by all LDLR family proteins, although the domain arrangements vary. Arrow/LRP5/LRP6 are highly homologous and have the same extracellular domain arrangements. Intracellularly they do not have the NPxY [Asp, Pro, X (any amino acid) and Tyr] motif, but instead each have five copies of PPP(S/T)P (P, Pro; S/T, Ser or Thr) motifs. Besides Arrow/LRP5/LRP6, VLDLR (very low-density lipoprotein receptor) and APOER2 (apolipoprotein E receptor 2, also called LRP8) have established signaling roles by acting as receptors for the secreted signaling molecule Reelin. Other members of the LDLR family participate in lipoprotein/cholesterol uptake, steroid hormone uptake, regulation of cell surface protease activity and Ca^{2+} homeostasis, or are less characterized (reviewed by Herz and Bock, 2002). Many members have multiple names. The commonly used names are listed in bold.

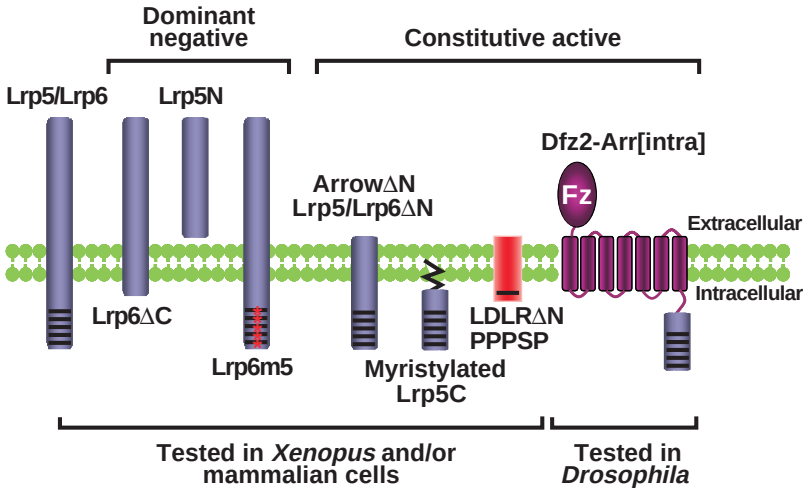


Fig. 3. Schematic diagram of various Arrow/Lrp5/Lrp6 mutant proteins and their signaling properties. The wild-type Lrp5/Lrp6 is depicted on the left. Black bars in the intracellular domain represent PPPSP motifs. Lrp6ΔC (without most of the intracellular domain), Lrp5N (secreted extracellular domain) and Lrp6m5 (alanine substitution of the S/T residue in all five PPP[S/T]P motifs) are dominant-negative reagents that can block canonical Wnt signaling. The following receptor mutants are constitutively active, i.e. they can activate β-catenin signaling in the absence of Wnt: (1) Arrow/Lrp5/Lrp6ΔN (without the extracellular domain but anchored on the membrane); (2) myristoylated Lrp5C (intracellular domain anchored to the plasma membrane via a form of lipid modification); (3) a single PPPSP motif linked to a truncated LDLR; and (4) Dfz2-Arr[intra], which is a fusion of the Arrow intracellular domain with the Dfz2 carboxyl-terminal tail. The Lrp5/Lrp6 intracellular domain that is not anchored to the plasma membrane is inactive. For reasons unclear at the moment, the Arrow intracellular domain designed to anchor to the plasma membrane via myristoylation is inactive in fly embryos, although its protein expression has not been examined (Tolwinski et al., 2003).

of the 19 Wnts and 10 Fzs engage Lrp5/Lrp6-dependent Wnt/β-catenin signaling.

Mechanisms of Arrow/Lrp5/Lrp6 signaling

Arrow/Lrp5/Lrp6 have a key signaling role

Given that Wnt/β-catenin signaling requires both Fz and Arrow/Lrp5/Lrp6, an obvious question to ask is what role do these two distinct receptors play in Wnt signal transduction? A mutant Lrp6 protein lacking the intracellular domain is completely inactive, and in fact blocks Wnt and Fz signaling in a dominant-negative fashion (Tamai et al., 2000) (Fig. 3). Conversely, mutant Arrow/Lrp5/Lrp6 proteins that lack the extracellular domain (but are anchored on the membrane), referred to here as Arrow/Lrp5/Lrp6ΔN (Fig. 3), activate β-catenin signaling constitutively in mammalian cells (Mao et al., 2001a; Mao et al., 2001b; Liu et al., 2003) and in *Xenopus* embryos (Tamai et al., 2004), suggesting that Arrow/Lrp5/Lrp6 have a signaling capacity that is normally suppressed by the extracellular domain. The Lrp5 intracellular domain anchored to the plasma membrane via myristylation (a covalent lipid modification that targets proteins to the plasma membrane) is also constitutively active (Fig. 3) (Mao et al., 2001b). Thus, Arrow/Lrp5/Lrp6 is a key signaling receptor for the Wnt/β-catenin pathway.

Arrow/Lrp5/Lrp6 bind Axin

An important insight into the function of Lrp5 came from the finding that the Lrp5 intracellular domain binds Axin in both yeast two-hybrid and co-IP assays (Mao et al., 2001b), an observation that has been extended to Arrow (Tolwinski et al., 2003) and Lrp6 (Liu et al., 2003; Tamai et al., 2004). As mentioned previously, Axin is a scaffolding protein that contains binding sites for Apc, β-catenin, Gsk3, Ck1 and possibly other proteins (Polakis, 2002; Kikuchi, 1999) (Fig. 4). Axin nucleates this Axin complex, resulting in β-catenin phosphorylation and degradation (Fig. 1). Thus, the binding between the Arrow/Lrp5/Lrp6 intracellular domain and Axin permits the Wnt co-receptors to directly control β-catenin phosphorylation and degradation. The domain of Axin involved in binding Arrow/Lrp5 has only been mapped via the yeast two-hybrid assay and remains poorly defined (Fig. 4). The DIX domain of Axin is necessary, but not sufficient, for the Axin-Arrow/Lrp5 interaction, whereas the RGS domain may be inhibitory to it (Mao et al., 2001b; Tolwinski et al., 2003).

Lrp6 phosphorylation and LRP-Axin association

Wnt stimulation of mammalian cells induces the Lrp5-Axin association within minutes (Mao et al., 2001b). In *Drosophila* embryos, Axin-GFP is recruited to the plasma membrane only in Wg-responsive cells, and this may occur through Arrow, although direct evidence is lacking (Cliffe et al., 2003). How does Wnt promote the Arrow/Lrp5-Axin interaction?

A recent study discovered that a PPP(S/T)P motif, which is reiterated five times in Arrow/Lrp5/Lrp6 intracellular domains (Box 3, see Fig. S1), is the minimal module that is necessary and sufficient for Lrp6 signaling function in mammalian cells and *Xenopus* embryos (Tamai et al., 2004). When a single PPPSP motif is transferred artificially to a truncated LDLR protein (which has no role in Wnt/β-catenin signaling) it becomes phosphorylated and can fully activate Wnt/β-catenin signaling (Tamai et al., 2004) (Fig. 3). Importantly, Axin preferentially binds to the phosphorylated PPPSP motif, whose phosphorylation in Lrp6 is rapidly induced by Wnt (Tamai et al., 2004). It was thus proposed that Wnt activates Lrp6 signaling by inducing Lrp6 phosphorylation at the PPP(S/T)P motifs, which serve as inducible docking sites for Axin, thereby recruiting Axin to the plasma membrane (Fig. 5). This model is also likely to apply to Arrow and Lrp5, which share conserved PPP(S/T)P motifs (see Fig. S1 at http://dev.biologists.org/supplemental/). The phosphorylation-dependent activation of Lrp6 and its inducible recruitment of Axin is reminiscent of other types of transmembrane signaling, such as that by tyrosine kinase receptors and cytokine receptors (Pawson and Scott, 1997).

These results suggest the existence of at least two unidentified components that control Wnt activation of Arrow/Lrp5/Lrp6: a kinase that phosphorylates the PPPSP

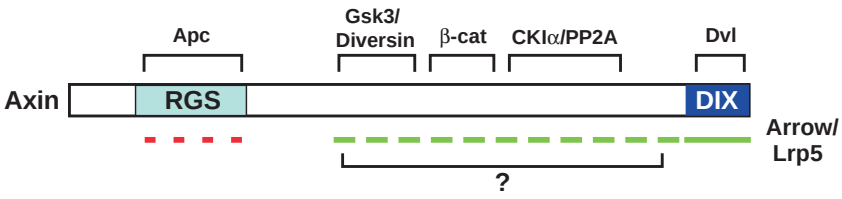


Fig. 4. Schematic diagram of Axin, showing its binding sites for various interacting proteins, including Arrow/Lrp5. How Axin binds to Arrow/Lrp5 was mapped only via yeast two-hybrid assays and is not well defined. Axin-Lrp6 binding has not been mapped. Known binding sites for various Axin-binding proteins are depicted with square brackets on top. For Axin-Arrow/Lrp5 binding, the DIX domain and possibly the domains preceding it appear to be required (marked by the green line), whereas the RGS domain may be inhibitory (marked by the red line) (Mao et al., 2001b; Tolwinski et al., 2003). The broken green or red line indicates that the domain boundary has not been defined by mapping. The question mark indicates some ambiguity/inconsistency of the two mapping studies.

motif and a phosphatase that dephosphorylates it. As Wnt induces Lrp6 phosphorylation (Tamai et al., 2004), Wnt may activate the PPPSP kinase or inhibit the PPPSP phosphatase. Alternatively, Wnt may regulate the access of Lrp6 to this kinase or phosphatase, perhaps by inducing a 'conformational change' in Lrp6. Indeed, a recent study suggests that the oligomerization of Lrp6 may keep Lrp6 in an inactive state, and Wnt binding to Lrp6 may induce a conformational change that activates the Lrp6 oligomer (Liu et al., 2003).

Gsk3 and Lrp5/Lrp6-Axin binding

In mammalian cells, Gsk3 overexpression enhances the Lrp5/Lrp6-Axin interaction in co-IP experiments (Mao et al., 2001b; Liu et al., 2003). This finding is puzzling as Gsk3 antagonizes Wnt/β-catenin signaling. In *Drosophila* mutant embryos lacking *Gsk3/zw3*, Wg recruitment of Axin to the plasma membrane and Wg signaling to Axin can occur (Cliffe et al., 2003; Tolwinski et al., 2003), implying that the Arrow-Axin association is not defective in the absence of Gsk3. Then why does Gsk3 overexpression enhance Lrp5/Lrp6-Axin interaction in mammalian cells? One possibility may be that Lrp5/Lrp6 signaling destabilizes Axin (see below), whereas Gsk3 can phosphorylate and stabilize Axin (Willert et al., 1999; Yamamoto et al., 1999). An increase in the level of Axin following Gsk3 overexpression may explain 'enhanced' Lrp5/Lrp6-Axin binding. Another possibility is that Lrp5/Lrp6 preferentially interact with Axin phosphorylated by or complexed with Gsk3 (Mao et al., 2001b). Additional explanations may include that Gsk3 overexpression mimicks the action of the PPPSP kinase.

Consequences of Arrow/Lrp5/Lrp6-Axin binding

How does Arrow/Lrp5/Lrp6 activation and binding to Axin initiate β-catenin signaling? One possibility is that Arrow/Lrp5/Lrp6 binding to Axin promotes Axin degradation. Indeed, Wnt stimulation (Willert et al., 1999; Yamamoto et al., 1999), Wg overexpression (Tolwinski et al., 2003), and Lrp5ΔN overexpression (Mao et al., 2001b), all induce Axin degradation in mammalian and *Drosophila* cells (see also Cliffe et al., 2003). These studies demonstrated reductions in Axin protein level after 2-4 hours of Wnt stimulation (or longer when transfection or transgenic experiments are involved). However, β-catenin stabilization is detectable within 30 minutes of Wnt stimulation and can thus occur before an obvious reduction in levels of Axin (Willert et al., 1999). One explanation, according to a recent theoretical and experimental analysis (Lee et al., 2003), is that a slight decrease in Axin protein level may have a significant effect on β-catenin phosphorylation and degradation.

It is also possible that Arrow/Lrp5/Lrp6 binding inhibits the activity of the Axin complex, by altering its component composition. This may be important in the early phase of Wnt signaling when Axin degradation is insignificant. In any event, one should keep in mind that, in mammalian cells, Wnt/β-catenin signaling induces the expression of an Axin homolog, Axin2 (also known as Axil/Conductin) (Behrens et al., 1998; Yamamoto et al., 1998; Yan et al., 2001; Jho et al., 2002; Leung et al., 2002; Lustig et al., 2002). Thus, a reduction in the Axin protein level is likely to be accompanied by an increase in the level of Axin2 during Wnt signaling, thereby complicating the Axin degradation scenario. How Arrow/Lrp5/Lrp6 promotes Axin degradation or inhibits Axin function is unknown, but their recruitment of Axin to near the plasma membrane appears essential. Indeed, the Lrp5 intracellular domain, although capable of binding Axin, is incapable of signaling in mammalian cells unless it is anchored to the plasma membrane (Mao et al., 2001b).

Fz and Dishevelled: an unresolved mystery

Overexpression of Arrow/Lrp5/Lrp6ΔN, or even a single PPPSP motif (tethered to the LDLR; Fig. 3), constitutively activates the β-catenin pathway (Mao et al., 2001a; Mao et al., 2001b; Liu et al., 2003; Tamai et al., 2004), probably in a Wnt- and Fz-independent manner. However, this is difficult to verify as a cell completely lacking Fz proteins may not exist. Wnt and Fz might normally function to activate the signaling activity of Arrow/Lrp5/Lrp6. Given the activated nature of the truncated Arrow/Lrp5/Lrp6ΔN, a scenario in which Wnt/Fz induces post-translational cleavage of Arrow/Lrp5/Lrp6 is attractive yet lacks experimental evidence. If Arrow/Lrp5/Lrp6 were to be activated in this way, the cleavage would have to occur extracellularly because an Lrp5/Lrp6 intracellular domain that is not anchored to the membrane is inactive (Mao et al., 2001b).

Fz function remains a mystery. Fz is thought to have a signaling role because its intracellular regions are required for Wnt/β-catenin signaling (Umbhauer et al., 2000). Fz proteins also play a key part in β-catenin-independent signaling, such as in the PCP pathway, in Ca^{2+}/PKC (protein kinase C) signaling, and perhaps in other pathways (Adler, 2002; Strutt, 2003; Veeman et al., 2003). Although some Fz functions, such as PKC activation, can be blocked by pharmacological inhibitors of the trimeric G proteins, whether Fz function during Wnt/β-catenin signaling relies on G proteins remains debatable (reviewed by Malbon et al., 2001). A protein that is required for most, if not all, Fz functions is Dishevelled (Dsh in *Drosophila* and *Xenopus*, and Dvl1-3 in mammals), another mysterious protein that is genetically defined downstream of Fz in both Wnt/β-catenin and PCP pathways (Boutros and

Mlodzik, 1999), and that may also be required for Fz activation of PKC (Sheldahl et al., 2003). Dsh/Dvl is a modular scaffolding protein that contains a DIX domain (which also exists in Axin), a PDZ domain (a domain discovered in PSD, Discs-large, and ZO1 proteins) and a DEP domain (a domain discovered in Dishevelled, Egl-10, and Pleckstrin proteins) (Boutros and Mlodzik, 1999; Wharton, 2003). Dsh/Dvl is recruited to the plasma membrane upon overexpression of a number of different Fz proteins (Axelrod et al., 1998; Boutros et al., 2000; Rothbacher et al., 2000; Umbhauer et al., 2000), and may bind directly to the Fz carboxyl-terminal region via the PDZ domain (Chen et al., 2003; Wong et al., 2003). However, Fz recruitment of Dsh/Dvl to the plasma membrane does not correlate fully with the activation of Wnt/β-catenin signaling (Axelrod et al., 1998; Rothbacher et al., 2000; Umbhauer et al., 2000), and Dsh is not localized near the plasma membrane in Wg-responsive cells in fly embryos (Axelrod et al., 1998; Axelrod, 2001; Cliffe et al., 2003).

Thus, it remains unclear how Fz and Dsh/Dvl fit into the scenario in which Arrow/Lrp5/Lrp6 binding to Axin initiates β-catenin signaling. Three models can be proposed. Dsh appears to be epistatic to, or downstream of, Arrow, because Dsh overexpression activates β-catenin signaling in *arrow* mutants (Wehrli et al., 2000) and the constitutively active Dfz2-Arrow fusion protein is inactive in *dsh* mutants (Tolwinski et al., 2003). In addition, Dsh/Dvl can associate with and inhibit Axin (Fagotto et al., 1999; Kishida et al., 1999b; Li et al., 1999; Smalley et al., 1999; Salic et al., 2000). Therefore, one scenario, referred to here as the 'co-recruitment' model, is that Fz and Arrow/Lrp5/Lrp6 recruit Dsh/Dvl and Axin into the co-receptor complex, respectively, thereby bringing Dsh/Dvl and Axin into proximity for effective Axin inhibition or degradation (Fig. 5A). However, a lack of correlation between Dsh/Dvl plasma membrane localization and Wg/Wnt signaling poses difficulties for this model, although it is possible that a small fraction of Dvl/Dsh recruited to the membrane, albeit undetectable, is sufficient for signaling. This model implies that Dvl/Dsh has a key role in Axin inhibition or degradation, and could account for the finding that overexpression of Dsh activates β-catenin signaling in *arrow* mutant flies (i.e. via inhibiting or degrading Axin in the cytoplasm).

The second scenario, referred to here as the 'vesicle-transport' model (Cliffe et al., 2003) (Fig. 5B), is based on the observations that Dsh/Dvl and Axin, upon overexpression, are co-localized in intracellular 'dots' that may represent 'vesicles' (Axelrod et al., 1998; Fagotto et al., 1999; Kishida et al., 1999; Smalley et al., 1999; Axelrod, 2001; Cliffe et al., 2003), and that Axin recruitment to the plasma membrane requires Dsh (Cliffe et al., 2003). This model proposes that Dsh/Dvl, through association with vesicles and Axin, shuttles Axin to the plasma membrane, where it becomes associated with Arrow/Lrp5/Lrp6. This view is consistent with the observation that the Dvl DIX domain, which is essential for Dsh/Dvl function in β-catenin signaling, harbors phospholipid-binding activity and mediates vesicle association (Capelluto et al., 2002), but it does not easily explain how, in *Drosophila*, Dsh overexpression activates β-catenin signaling in *arrow* mutants.

The third scenario, which perhaps can be referred to as a 'parallel signaling' model (Fig. 5C), implies that Fz-Dsh/Dvl-Axin and Arrow/Lrp5/Lrp6-Axin represent two parallel branches: overactivation of either branch is sufficient to activate β-catenin signaling, whereas simultaneous activation of both is required under physiological conditions. This model,

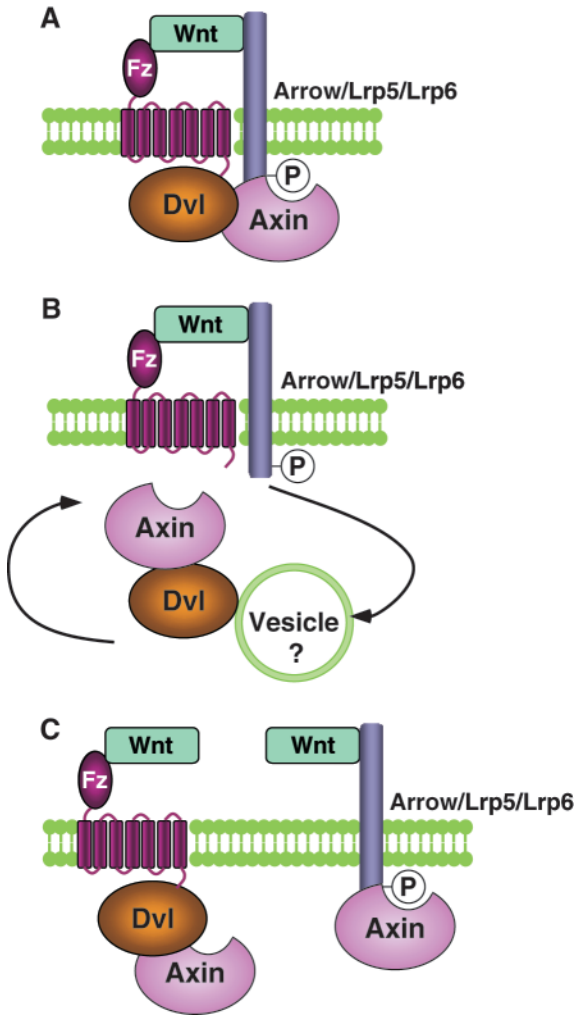


Fig. 5. Three models of how Fz and Dsh/Dvl may function in relation to Arrow/Lrp5/Lrp6-Axin interaction. In all three models, Wnt-induced Arrow/Lrp5/Lrp6 phopshorylation on the PPP(S/T)P motifs provides docking sites for Axin. (A) The co-recruitment model. Wnt-induced Fz-Lrp5/Lrp6 complex co-recruits Dvl and Axin into the co-receptor complex via Fz-Dvl and Lrp5/Lrp6-Axin interactions. This proximity of Dvl and Axin, which can interact with each other, causes functional inhibition or degradation of Axin by Dvl, either directly or via Dvl-associated proteins. (B) The vesicle transport model. Axin is shuttled to the plasma membrane to its docking sites in Arrow/Lrp5/Lrp6 via Dvl-mediated 'vesicular-type' transport, which relies on the ability of Dvl to bind Axin and phospholipids/vesicles. In this and the above co-recruitment model, Dsh/Dvl is downstream of and required for Arrow/Lrp5/Lrp6 function. (C) The parallel signaling model. Fz-Dvl-Axin and Arrow/Lrp5/Lrp6-Axin are two parallel and independent branches. Activation of β-catenin signaling requires both branches under physiological conditions, but can occur when either branch is overactivated, for example, following overexpression of Dvl or Arrow/Lrp5/Lrp6ΔN. This model accounts for the possibility that Dsh/Dvl may not be required for overexpressed Arrow/Lrp5/Lrp6 to activate β-catenin signaling.

which can explain why Dsh overexpression bypasses Arrow function, is based on observations that Lrp5/Lrp6ΔN signaling does not seem to be affected by depletion of Dvl/Dsh proteins in mammalian and *Drosophila* cells [from short interfering RNA (siRNA) or RNA interference (RNAi) experiments] (Li et al., 2002; Schweizer and Varmus, 2003). These observations apparently contradict the finding in *Drosophila* embryos that signaling by the constitutively active Dfz2-Arrow fusion protein (Fig. 3) requires Dsh (Tolwinski et al., 2003), although it is possible that the mechanisms by which Lrp5/Lrp6ΔN and the Dfz2-Arrow fusion protein become constitutively active may be different. However, depletion of the three Dvl proteins or Dsh through siRNA/RNAi is unlikely to be complete, rendering the interpretation less straightforward. Whether ArrowΔN, which is constitutively active in mammalian cells and *Xenopus* embryos (Tamai et al., 2004), can activate β-catenin signaling in *dsh* mutant flies will be a key test. Given the possible Fz-Lrp5/Lrp6 and Dsh/Dvl-Axin interactions, these two parallel branches, if they exist, may nonetheless operate in physical proximity.

Finally, because Wnt activates Lrp6 signaling by inducing Lrp6 phosphorylation at the PPP(S/T)P motif (Tamai et al., 2004), it could be possible that Fz/Dsh signaling acts by activating or recruiting the PPPSP kinase to phosphorylate Lrp6. We consider this scenario to be less likely as Dsh overexpression can activate β-catenin signaling in the absence of Arrow function (Wehrli et al., 2000).

Wnt/β-catenin signaling in worms

Wnt signaling is essential for many aspects of nematode development. However, some Wnt pathways in worms are organized differently to those in *Drosophila* and vertebrates (Korswagen, 2002). Nonetheless, a canonical Wnt/β-catenin signaling pathway controlling neuronal migration was discovered in worms that involves Wnt, Fz, Dsh, Axin, Apc, Gsk3 and β-catenin in a similar way as in flies and vertebrates (Korswagen et al., 2002). Perplexingly however, no Arrow/Lrp5/Lrp6 homologs have been identified in the worm genome, although other Lrp genes (such as Lrp1) exist. Thus either a functional homolog of Arrow/Lrp5/Lrp6 has yet to be discovered, or nematodes use other means for Wnt/Fz/Dsh to activate β-catenin signaling in the absence of Arrow/Lrp5/Lrp6-Axin interaction. This latter possibility shares some resemblance to the 'parallel signaling' model discussed above.

Regulation of Arrow/Lrp5/Lrp6

Dickkopf and Wise

Lrp5/Lrp6 are subjected to modulation by secreted antagonistic/modulatory ligands in vertebrates and by other types of regulations (Box 4). Two families of such ligands have been identified: the Dickkopf (Dkk) family and the Wise family, which antagonize Wnt/β-catenin signaling through interactions with Lrp5/Lrp6. Dkk and Wise homologs have not been found in invertebrate genomes.

The Dkk family

Wnt signaling is required for posterior patterning in vertebrates; thus, inhibition of Wnt signaling permits anterior development (Niehrs, 1999). *Xenopus* Dkk1 was isolated as a head-inducing molecule and behaves as an antagonist for Wnt signaling (Glinka et al., 1998). Genetic analysis of *Dkk1*$^{-/-}$ mice, which lack head formation, is consistent with this view (Mukhopadhyay et al., 2001). Distinct from several families of secreted Wnt antagonists that bind Wnts, including the sFRP (secreted Frizzled-related protein) family, Wif1 (Wnt inhibitory factor 1) and *Xenopus* Cerberus (Semenov and He, 2003), Dkk1 does not bind Wnt but is a high affinity ligand for Lrp6 (K_d=0.3-0.5 nM) and Lrp5 (Bafico et al., 2001; Mao et al., 2001a; Semenov et al., 2001). Dkk1 disrupts the Fz-Lrp5/Lrp6 complex formation induced by Wnt1 in vitro (Semenov et al., 2001), suggesting that Dkk1 inhibits Wnt signaling by preventing Fz-Lrp5/Lrp6 complex formation. The Dkk1-Lrp5/Lrp6 antagonistic relationship is supported by mouse genetic studies (MacDonald et al., 2004). Thus, reducing the dosage of Lrp5 or Lrp6 can significantly rescue phenotypes associated with a loss of Dkk1 function, and vise versa. For example, while *Dkk1*$^{-/-}$ mutant mice lack head formation and die during embryogenesis, *Dkk1*$^{-/-}$*; Lrp6*$^{+/-}$ mice have extensive head development and can survive to postnatal stages (MacDonald et al., 2004).

By inhibiting Lrp5/Lrp6, Dkk1 appears to be a specific antagonist for Wnt/β-catenin signaling (Semenov et al., 2001), and is thus distinct from sFRPs, Wif1 and Cerberus, which may antagonize multiple Wnt pathways (Semenov and He, 2003). Of the two conserved cysteine-rich domains of Dkk1 (see Fig. S2 at http://dev.biologists.org/supplemental/), the carboxyl one is essential for its binding to Lrp6 and its antagonization of Wnt signaling, whereas the amino terminal one may exert some undefined regulatory roles (Brott and Sokol, 2002; Li et al., 2002; Mao and Niehrs, 2003). Dkk1 may interact with a region encompassing the third and fourth YWTD β-propeller-EGF-like domains of Lrp6, which is distinct from the Wnt-binding region (Mao et al., 2001a; Itasaki et al., 2003) (Fig. 6).

In *Xenopus* and mammals, the Dkk family includes Dkk1, Dkk2, Dkk3 and Dkk4 (see Fig. S2 at http://dev.biologists.org/supplemental/), which exhibit distinct and dynamic expression patterns (Glinka et al., 1998; Monaghan et al., 1999) and may have distinct properties. Dkk1 and Dkk4 are antagonists for Wnt signaling (Krupnik et al., 1999; Brott and Sokol, 2002; Mao and Niehrs, 2003), whereas Dkk2 can, paradoxically, inhibit or activate (albeit weakly) β-catenin signaling, depending on the experimental assays employed (Wu et al., 2000; Brott and Sokol, 2002; Li et al., 2002). Whether Dkk2 can function as a Wnt agonist in vivo remains to be seen. Dkk3 neither binds Lrp5 or Lrp6, nor affects Wnt signaling (Krupnik et al., 1999; Mao and Niehrs, 2003).

Kremen: a Dkk1 co-receptor?

Dkk1 also binds to vertebrate Kremen (Krm) 1 and Krm2, two related single-pass transmembrane proteins (Mao et al., 2002) (see Fig. S2 at http://dev.biologists.org/supplemental/). In mammalian cells, either Krm1 or Krm2 can cooperate with Dkk1 in the inhibition of Wnt-Fz-Lrp6 function (Mao et al., 2002). *Drosophila* has no Dkk or Krm homologs, although ectopic expression of vertebrate *Dkk1* and *Krm2* together, but not either of these genes alone, results in inhibition of Wg signaling (Mao et al., 2002). In addition, antisense knockdown of both Krm1 and Krm2 (but not either individually) in *Xenopus* results in deficient head development, similar to phenotypes of embryos with no or reduced Dkk1 function

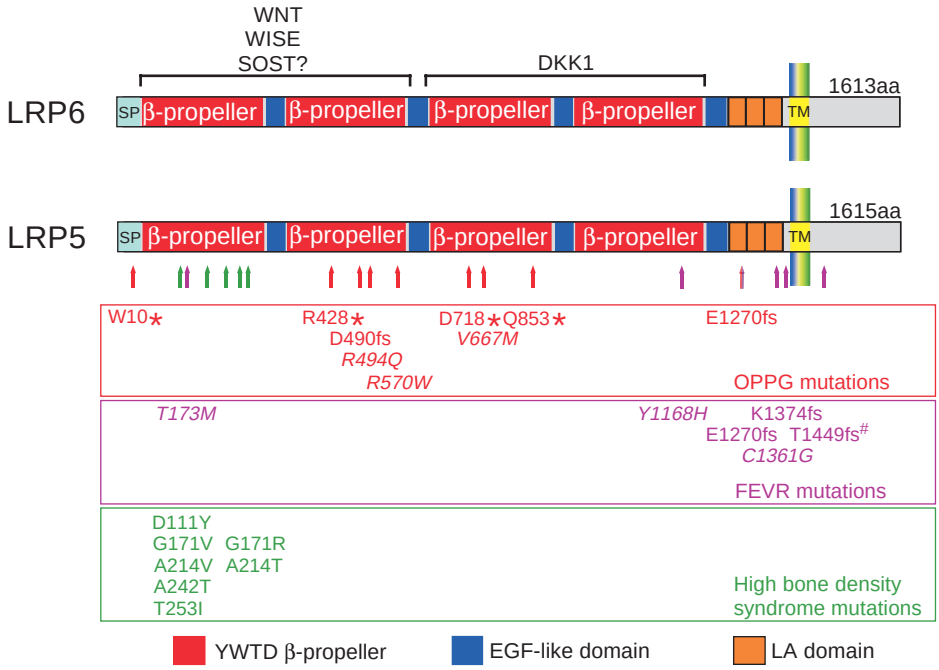


Fig. 6. Schematic representation of possible LRP6 domains involved in Wnt, Dkk and Wise binding, and of LRP5 mutations in human diseases. (Top) LRP6. Domains involved in Wnt, Wise and Dkk1 binding have only been mapped for LRP6 and are marked. Whether SOST binds LRP5/LRP6 is unknown. (Bottom) LRP5 mutations associated with osteoporosis-pseudoglioma (OPPG) syndrome, autosomal-dominant familial exudative vitreoretinopathy (FEVR), and various high bone density diseases are shown in red, purple and green, respectively. Arrows indicate mutation locations. *, nonsense mutation; fs, frame-shift mutation. OPPG is autosomal recessive, and the nine mutations indicated are from homozygous offspring of consanguineous families (Gong et al., 2001). FEVR discussed here is an autosomal-dominant form, possibly due to haploinsufficiency. Whether the three OPPG and three FEVR missense mutations (italicized) are loss-of-function mutations remains to be tested. T1449fs# in FEVR should be treated as hypothetical because the mutation occurs at a splice donor site in an intron. Note that the mutations associated with high bone density diseases, which are autosomal dominant because of probable 'gain of function', are all missense mutations and are clustered in the first YWTD β-propeller domain. SP, signal peptide; TM, transmembrane domain.

(Davidson et al., 2002). Thus, Krm1 and Krm2 appear to have redundant roles in Dkk1 function. Because Dkk1 can stimulate Lrp6 internalization upon Krm2 overexpression (Mao et al., 2002), it was proposed that Dkk1, by binding both Lrp6 and Krm, induces Lrp6 internalization from the cell surface, thereby attenuating Wnt signaling. Perplexingly however, the Krm intracellular domain is neither conserved nor required for any of these functions (Mao et al., 2002), which raises the question of how can Krm have a key function in Lrp6 internalization? This internalization model is different, although not mutually exclusive, from a model in which Dkk1 functions by preventing Fz-Lrp6 complex formation (Semenov et al., 2001).

The Wise family

Wise was identified in *Xenopus* embryo assays as a secreted molecule with dual properties somewhat similar to Dkk2. Wise is an antagonist for Xwnt8/β-catenin signaling, but on its own can weakly activate β-catenin signaling (Itasaki et al., 2003). Wise binds to the Lrp6 extracellular domain in co-IP experiments, in particular to the first two YWTD β-propeller-EGF-like domains (Fig. 6), the same region that Wnt appears to bind, and can compete with Xwnt8 for Lrp6 binding (Itasaki et al., 2003). Wise belongs to a large family of secreted 'cysteine-knot' domain-containing proteins (see Fig. S2 at http://dev.biologists.org/supplemental/), which include members that bind and antagonize BMPs (bone morphogenetic proteins) (Hsu et al., 1998; Pearce et al., 1999; Piccolo et al., 1999). Indeed, Wise was also isolated as a BMP inhibitor (Laurikkala et al., 2003). Thus, Wise appears to be a multi-functional inhibitor for both Wnt/β-catenin and BMP signaling. This property is somewhat similar to the *Xenopus* protein Cerberus, which antagonizes signaling by Wnt, BMP and Nodal (Piccolo et al., 1999).

LRP5 in human diseases

Bone density disorders

WNT signaling is not only essential for embryogenesis, but also for postnatal development and tissue homeostasis. This is illustrated by LRP5 mutations that underlie familial osteoporosis, high bone density syndromes and ocular disorders (Gong et al., 2001; Boyden et al., 2002; Little et al., 2002). Children with autosomal-recessive osteoporosis-pseudoglioma syndrome (OPPG) have low bone mass and are prone to bone fractures (Gong et al., 2001). Most of these children suffer a loss of LRP5 function due to nonsense or frame-shift mutations in the LRP5 extracellular domain (Gong et al., 2001) (Fig. 6). Remarkably, several groups of autosomal-dominant bone disorders, characterized by high bone density traits, are also associated with LRP5 mutations, which are missense in nature and clustered in the first β-propeller region of LRP5 (Boyden et al., 2002; Little et al., 2002; Van Wesenbeeck et al., 2003) (Fig. 6). This is reflected in mice. *Lrp5*$^{-/-}$ mice exhibit low bone density and frequent bone fractures reminiscent of OPPG patients (Kato et al., 2002), and transgenic mice expressing LRP5 (G171V), a mutation from high bone density patients, had increased bone mass (Babij et al., 2003). Thus, loss-of-function mutations of LRP5 lead to low bone densities whereas 'gain-of-function' mutations cause high bone mass. These studies identify LRP5 as a central player and an ideal therapeutic target in bone mass regulation and in associated diseases such as osteoporosis (Patel and Karsenty, 2002). Nevertheless, several key issues remain unresolved:

(1) Does WNT signaling regulate bone mass during

Box 4. Regulation of Arrow/Lrp5/Lrp6 expression and membrane trafficking

In *Drosophila*, *arrow* expression, like that of *Dfz1* and *Dfz2* (Bhanot et al., 1996; Cadigan et al., 1998; Muller et al., 1999), is itself inhibited by Wg signaling (Wehrli et al., 2000), although the significance of this regulation is unclear. In mouse osteoblasts, Bmp signaling enhances *Lrp5* and *Lrp6* mRNA expression (Gong et al., 2001). Thus Wnt signaling can be modulated by extracellular stimuli that regulate *Lrp5/Lrp6* expression.

In order for Arrow/Lrp5/Lrp6 maturation and trafficking to the plasma membrane where they function, the *Drosophila* Boca protein and its mouse homolog Mesd, which are chaperones residing in the endoplasmic reticulum (ER), are required (Culi and Mann, 2003; Hsieh et al., 2003). *Boca* mutants exhibit phenotypes associated with loss of Wg signaling, whereas $Mesd^{-/-}$ mice phenotypically resemble $Wnt3^{-/-}$ mutants and $Lrp5^{-/-}$; $Lrp6^{-/-}$ double mutants. In *Drosophila* cells lacking Boca function, and in mammalian cells overexpressing Lrp5/Lrp6, Arrow/Lrp5/Lrp6 are mostly misfolded in the ER and exhibit inappropriate disulfide bond formation (these results serve as a cautionary reminder when overexpressed Lrp5/Lrp6 is being studied). Mesd and Lrp5/Lrp6 co-expression enables most Lrp5/Lrp6 proteins to fold correctly and reach the cell surface. Boca and Mesd are thus chaperones specific for Arrow/Lrp5/Lrp6 (and some other LDLR proteins) (Culi and Mann, 2003; Hsieh et al., 2003).

development? LRP5 is required for the proliferation of osteoblasts (Gong et al., 2001; Kato et al., 2002), and may inhibit osteoblast apoptosis (Babij et al., 2003). Wnt/β-catenin signaling can promote mouse osteoblastic development in vitro (Gong et al., 2001), and several Wnt genes, including *Wnt1*, are expressed during bone accrual (Kato et al., 2002). Therefore, LRP5 probably mediates WNT signaling in bone mass regulation, but whether LRP5 mediates other types of extracellular signals during bone growth has not been determined.

(2) Does LRP6 regulate bone mass? As discussed earlier, LRP6 is the more crucial WNT co-receptor and shares significant overlapping function with LRP5 during embryogenesis (Kelly et al., 2004). It is therefore somewhat surprising that even heterozygosity of LRP5 reduces bone density in mice and men (Gong et al., 2001; Kato et al., 2002), and that LRP6 does not compensate for this LRP5 function. However, it is possible that LRP6 is not expressed in osteoblasts during postnatal stages, or that the ligand for bone accrual binds preferentially to LRP5.

(3) How do LRP5 mutations associated with high bone density traits, such as G171V, cause LRP5 hyperactivation? A study in mammalian cells suggested that the G171V mutation does not affect WNT-LRP5 signaling, but instead prevents DKK1 inhibition of WNT-LRP5 signaling (Boyden et al., 2002), implying that loss of DKK inhibition of LRP5 may underlie increased LRP5 signaling and high bone densities. A few caveats remain. First, whether DKK1 or any other DKK is expressed during bone accrual has not yet been examined. Second, whether LRP5 high bone density mutations affect WNT or DKK binding has not been examined. These mutations are all clustered in the first YWTD β-propeller domain, which appears to be involved in binding to WNT but not DKK (Fig. 6). Third, it remains unknown whether G171V and other high bone density LRP5 mutations, which are in different blades of the propeller, affect LRP5 activity by the same mechanism.

It is of interest to discuss here sclerosteosis, another rare autosomal-recessive bone disorder characterized by skeletal overgrowth and high bone density. Sclerosterosis is a progressive bone dysplasia associated with loss-of-function mutations of a secreted protein referred to as SOST (Balemans et al., 2001; Brunkow et al., 2001). Intriguingly, SOST is most related to WISE (38% identical; see Fig. S2 at http://dev.biologists.org/supplemental/), which antagonizes LRP6 via binding to the region containing the first and second YWTD β-propeller domains (Fig. 6) (Itasaki et al., 2003). Although SOST, like Wise, can also bind and antagonize BMP (Laurikkala et al., 2003; Kusu et al., 2003), it is tempting to speculate that SOST binds and antagonizes LRP5 in bone growth regulation, and that LRP5 mutations associated with high bone densities prevent/reduce SOST-LRP5 interaction.

Ocular disorders

OPPG patients also suffer, in addition to low bone mass, from severe disruption of ocular structures due to a failure of regression of the primary vitreal vasculature (the temporary capillary networks that normally regress during development) (Gong et al., 2001). This phenotype is recapitulated in $Lrp5^{-/-}$ mice, possibly because of a lack of capillary endothelial apoptosis in the eye (Kato et al., 2002). This may be due to defects in ocular macrophages, which express Lrp5 and are required for the induction of capillary cell death (Kato et al., 2002).

Another hereditary ocular disorder, the autosomal-dominant form of familial exudative vitreoretinopathy (FEVR), which is characterized by the premature arrest of retinal angiogenesis/vasculogenesis, is also associated with *LRP5* mutations (Toomes et al., 2004). Loss-of-function mutations in one *LRP5* chromosomal copy are associated with this disease (Fig. 6), presumably due to haploinsufficiency (Toomes et al., 2004). These patients, like the obligate OPPG carriers (parents of OPPG patients) (Gong et al., 2001), also exhibit low bone mass (Toomes et al., 2004). Interestingly, some autosomal-dominant FEVR families harbor loss-of-function mutations in the frizzled 4 (*FZD4*) gene (Robitaille et al., 2002), which, like *LRP5*, is located in the chromosomal 11q13 region. Therefore, FEVR is associated with a deficiency in either LRP5 or FZD4 function, providing genetic evidence outside *Drosophila* that LRP5 and FZD cooperate in the same signaling pathway. In summary, LRP5 (and FZD) function is important for multiple stages of retinal angiogenesis and associated diseases, presumably because it mediates signaling by a WNT (or non-WNT) ligand.

Cholesterol and glucose metabolism

LRP5 is also involved in lipid metabolism. LRP5 binds apolipoprotein E (APOE), and *LRP5* expression is upregulated in the liver of $Ldlr^{-/-}$ mice (Kim et al., 1998). Indeed, $Lrp5^{-/-}$ mice fed on a high-fat diet exhibit increased plasma cholesterol levels relative to normal mice (Fujino et al., 2003), and mutants for both *ApoE* and *Lrp5* ($ApoE^{-/-}$; $Lrp5^{-/-}$) show hypercholesterolemia, impaired fat tolerance and advanced

atherosclerosis (Magoori et al., 2003). The role of LRP5 in cholesterol metabolism appears analogous to the classical LDLR function and is probably WNT independent (Magoori et al., 2003).

Concluding remarks

Although the role of Arrow/Lrp5/Lrp6 in Wnt/β-catenin signaling during development is established, the underlying molecular mechanism remains relatively poorly defined. Unresolved questions concern how Wnt molecules interact, functionally and/or physically, with Fz and Arrow/Lrp5/Lrp6, how Wnt activates Arrow/Lrp5/Lrp6 by phopshorylation to regulate the Axin complex, and how Fz receptors and Dsh/Dvl proteins operate. Such studies are likely to identify additional signaling components at the plasma membrane or intracellularly that will bridge major gaps in our understanding of transmembrane signaling by Wnt proteins.

Although Dkk and Wise families of Lrp5/Lrp6 ligands/antagonists are only found in vertebrates, the high similarity between the entire Arrow and Lrp5/Lrp6 extracellular domains implies that other evolutionarily conserved ligands may yet be discovered. An area that requires more investigation is the cell biological aspect of Wnt signaling, including the biogenesis, trafficking, localization and endocytosis of Fz and Arrow/Lrp5/Lrp6 during Wnt signaling. On the translational side, the specific function of LRP5 in bone mass regulation provides an ideal therapeutic target for treatment of bone disorders, but this will critically rely on our understanding of LRP5, its putative ligand (a WNT or non-WNT), and antagonists such as DKK, WISE or others during bone accrual. In addition, the roles of LRP5 in lipid metabolism and other physiological regulations, such as in enhancing insulin secretion (Fujino et al., 2003), may have significant medical implications. In this regard, molecules that can stimulate LRP5 expression or activity may be used as therapeutics for osteoporosis, high cholesterol associated diseases and diabetes.

Note added in proof

It was recently reported that Axin has a nuclear-cytoplasmic shuttling role in the regulation of β-catenin subcellular localization (Cong and Varmus, 2004).

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