Published in final edited form as:

Trends Endocrinol Metab. 2013 January ; 24(1): 31–39. doi:10.1016/j.tem.2012.10.003.

Lrp5 and Lrp6 in Development and Disease

Danese M. Joiner¹, Jiyuan Ke², Zhendong Zhong¹, H. Eric Xu², and Bart O. Williams¹

¹Center for Skeletal Disease Research, Laboratory of Cell Signaling and Carcinogenesis, Van Andel Research Institute, 333 Bostwick NE, Grand Rapids, MI 49503, USA

²Center for Structural Biology and Drug Discovery, Laboratory of Structural Sciences, Van Andel Research Institute, 333 Bostwick NE, Grand Rapids, MI 49503, USA

Abstract

Low-density lipoprotein-related receptors 5 and 6 (LRP5/6) are highly homologous proteins with key functions in canonical Wnt signaling. Alterations in the genes encoding these receptors or their interacting proteins are linked to human diseases and as such they have been a major focus of drug development efforts to treat several human conditions including osteoporosis, cancer, and metabolic disease. Here, we discuss the links between alterations in Lrp5/6 and disease, proteins that interact with them, and insights gained into their function from mouse models. We also highlight current drug development related to Lrp5/6 as well as how the recent elucidation of their crystal structures may allow for further refinement of our ability to target them for therapeutic benefit.

Keywords

Lrp5/6; Wnt/ β -catenin; Development; Crystal Structure; Disease

Overview of Lrp5 and Lrp6

Wnt/ β -catenin signaling is a key regulator of tissue development and homeostasis. Abnormal signaling affects tissue growth and function and can progress to a variety of debilitating and terminal diseases. There is an interest in fully understanding Wnt/ β -catenin signaling at the cell and molecular level and recent crystal structure insights have elevated the likelihood of rapidly developing therapeutics. Wnts (See glossary) bind to a member of the Frizzled family of seven-transmembrane proteins and to either Low-Density Lipoprotein Related Receptors 5 (LRP5) or LRP6 leading to down-regulation of GSK-3 activity and the initiation of the canonical Wnt/ β -catenin signaling cascade. Lrp5 and Lrp6 were originally identified based on their homology to the low-density lipoprotein receptor [1]. The two proteins, which share approximately 71% homology, are transmembrane cell surface receptors involved in receptor-mediated endocytosis of lipoprotein and protein ligands (reviewed in [2]). An overview of Wnt/ β -catenin signaling is shown in Figure 1, and Box 1 outlines proteins that interact with and/or modulate Lrp5/6. In addition, several putative β -catenin-independent functions have also been reported (see Box 2). Lrp5 and 6

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Corresponding Author: Bart O. Williams, Bart.Williams@vai.org, Address: 333 Bostwick Ave NE Grand Rapids MI, 49503.

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contain large extracellular domains including four β -propeller motifs followed by three Type 1 low-density lipoprotein (LDL) ligand binding domains (Figure 2).

TEXT BOX 1

Other Proteins that Interact with and/or Regulate Lrp5/Lrp6

R-spondin

R-spondins (roof plate-specific spondin, RSPO) are secreted proteins that function as Wnt/ β -catenin pathway agonists by signaling through LGR4, LGR5, and LGR6 [3]. R-spondin may mediate the membrane availability of Wnt receptors via modulation of the ZNRF3 E3 ubiquitin ligase (Figure 1) [4].

Kremen

Kremen (kringle-containing transmembrane protein) was identified as a putative co-receptor of DKK1, and it may inhibit Wnt/ β -catenin signaling by enhancing DKK1-induced LRP5/6 internalization [5]. Mice deficient for both Kremen1- and Kremen2 develop HBM associated with increased β -catenin signaling [6]. Osteoblast-specific kremen-2 transgenic mice have osteopenia [7]. In terms of molecular mechanisms, there are in vitro and in vivo findings that require reconciliation. In HEK293 cells, Dkk1 inhibits Wnt signaling even without interacting with kremen [8]. In *Xenopus*, kremen promotes LRP6 cell-surface localization and LRP6 signal transduction during the neural crest development [9].

Complement C1q

The complement system is part of the innate immune system and is composed of a number of small serum proteins that augment the ability of the cell-mediated and humoral immune systems in defending the host against pathogens. A component of this system, Complement C1q, was found to activate Lrp6-mediated signaling by binding to Frizzled and cleaving a portion of the extracellular domain of Lrp6 in a Complement C1s-dependent manner [10], creating a truncated form of Lrp6 that signals in a Wnt-independent manner. Given that increased Wnt signaling in many tissues and increased serum C1q levels are both positively correlated with aging [10], inhibition of this proteolytic process provides a novel therapeutic target for aging-related processes.

Apolipoprotein E

Lrp5/6 contain three copies of the LDL type A repeat that, in the LDL receptor, mediates binding to lipoproteins [11]. Consistent with this, Lrp5 can associate with apolipoprotein E [12] and several laboratories have reported roles for these proteins in endocytosis and regulation of lipoprotein metabolism [13, 14] This, along with the evidence for roles of Lrp5 and Lrp6 in coronary artery disease and diabetes (see below), suggests that further research into how Lrp5 and Lrp6 interact with lipoproteins may be fertile ground for novel insights into their functions.

Biglycan

The small leucine-rich proteoglycan, biglycan directly interacts with both Wnt and Lrp6 to enhance Wnt-induced β -catenin signaling [15]. It was speculated that the interaction of Lrp6 with this secreted protein may serve to regulate the availability of Wnt ligand in some contexts.

Lrp4

Recent similarities between Lrp5/6 and Lrp4 have been elucidated. Lrp4 may potentially bind to similar effectors including sclerostin, Dkk1, and/or Wise [16].

Phosphoregulation Lrp5/6

Activation of the Wnt receptor complex leads to increased levels of phosphatidylinositol 4,5-bisphosphate resulting in phosphorylation of Thr1479 and Ser1490 in the carboxyl-terminus of Lrp6 [17]. This creates a binding site for the Axin protein, leading to stabilization and subsequent activation of β -catenin (see Figure 1). Several studies have identified putative kinases that mediate this process (reviewed in [18]).

TEXT BOX 2

β -catenin-independent Lrp5/6 signaling

The circulating hormone Parathyroid hormone (PTH) directly targets bone, kidney, and intestine to regulate calcium metabolism. Several mechanisms have been reported by which PTH modulates Wnt/ β -catenin signaling [80–83]. One potential mechanism is via a complex formed between LRP6 and PTH/PTH1 Receptor [84] can promote phosphorylation of the cytoplasmic tail of LRP6. In these studies, the activation of PKA (but not Wnt) was required for the phosphorylation of LRP6 in response to PTH [84].

cAMP is increased after activation of heterotrimeric G protein-coupled receptors (GPCRs), and rapid synthesis of cAMP involves activation of the transmembrane enzyme adenylyl cyclase (AC) by the α_s subunit of the G protein that is associated with the GPCR [85]. Knockdown of LRP6 inhibits cAMP production in response to the GPCR ligands isoproterenol (a β -adrenergic receptor agonist), adenosine, and glucagon. Isoproterenol and PTH(1-34) stimulate the accumulation of cAMP *in vitro*. Immunoprecipitation has confirmed the binding of the intracellular domains of both LRP5 and LRP6 to $G\alpha_s$, which inhibits the β -catenin destruction complex by binding to axin [86]. Deletion of the PKA phosphorylation site Thr¹⁵⁴⁸ in the intracellular domain of LRP6 reduces interaction with $G\alpha_s$. Mutations in the PKA and GSK-3 phosphorylation sites disrupt the localization of $G\alpha_s$ at the plasma membrane and reduce the amount of cAMP produced in response to PTH [85].

Stabilization of β -catenin was reported after α -adrenergic receptor stimulation in cardiomyocytes and after prostaglandin E₂ receptor stimulation in colon cancer cells [86, 87]. GPCRs such as prostaglandin F₂ receptor FP_B, M1 acetylcholine muscarinic receptor, lysophosphatidic acid receptors, and the prostaglandin E₂ receptor can also activate β -catenin [86, 88–91]. The glucagon receptor (GCGR) is a class B GPCR which, when activated, increases hepatic glucose production and activates the cAMP/protein kinase A (PKA), protein kinase C (PKC), and mitogen-activated protein kinases (MAPK) pathways [92, 93]. Treatment with the glucagon agonist GCG1-29 increased β -catenin activity in primary cells and cell lines in an Lrp5/6- and PKA-dependent manner. Immunoprecipitation and bioluminescence resonance energy transfer assays also demonstrate the interaction between the Wnt co-receptor LRP5 and GCGR [94]. Finally recent evidence in mice deficient for Lrp6 suggests Lrp6 may inhibit non-canonical signaling and activate JNK [95, 96].

Here, we discuss the association of these genes with human disease and summarize the proteins that interact with LRP5/6 to regulate their functions including Wnt, Dkk, Sost, Wise/CTGF, and Norrin. We also discuss insights gained into the functions of these molecules via the use of genetically engineered mouse models and structural studies, and review recent progress in targeting these molecules to treat human disease.

Alterations in LRP5 and/or LRP6 Associated with Human Disease

Alterations in bone mass

Mutations in LRP5/6 have been linked to a number of human diseases. A loss-of-function mutation in LRP5 was shown to cause Osteoporosis pseudoglioma (OPPG), a rare syndrome associated with premature, generalized osteoporosis leading to bone fracture [19]. Further support for the role of LRP5 in bone growth was provided when two groups independently reported that a point mutation in LRP5 (G171V) was present in affected individuals displaying an autosomal dominant high-bone-mass (HBM) trait [19]. This point mutation encodes for a protein that can no longer be inactivated by DKK1 or Sost (see below) thus affecting LRP5 function and promoting high bone mass. Subsequent work has identified numerous additional mutations in LRP5 associated with alterations in human bone mass [19].

Alterations in Eye Vascularization

Some patients with OPPG are born with a severe disruption of ocular structure called phthisis bulbi, and persistent hyperplasia of the primary vitreous has been observed in children with milder eye involvement [20]. In addition, another disease with abnormal retinal vascularization, familial exudative vitreoretinopathy (FEVR), is also associated with mutations in LRP5 [21].

Alzheimer's disease

Alzheimer's disease is a progressive neurodegenerative disorder characterized by a deficit in cognitive processes, and it is the most common form of age-associated dementia [22]. A single nucleotide polymorphism (SNP) in exon 18 of *LRP6* was associated with Alzheimer disease in two brain bank data series [23]. Haplotype tagging SNPs with a set of seven allelic variants of *LRP6* revealed a possible risk haplotype, which included a highly conserved coding sequence SNP, I-1062→V in exon 14 [23].

Coronary artery disease

Coronary artery disease due to atherosclerosis results in myocardial infarction and is the leading cause of death worldwide [24]. Epidemiological studies demonstrate specific risk factors including smoking, hypertension, high low-density-lipoprotein (LDL) cholesterol, high triglycerides, low high-density-lipoprotein (HDL) cholesterol, and diabetes mellitus. Information on the molecular mechanisms that unify their association has developed only recently [24]. A causal link between a mutation in LRP6 (R611C) and early coronary artery disease was observed. Detailed studies on patients carrying this mutation demonstrated they had high serum LDL cholesterol and triglycerides, hypertension, high fasting blood glucose levels, a prevalence of diabetes, and low bone density [25].

Calcific Aortic valve disease

Wnt/ β -catenin signaling plays an important and complex role in cardiac differentiation and development [26]. Calcific aortic valve disease, which involves calcification, is the most common pathologic disease in the United States and Europe, and its prevalence and incidence has increased due to rapidly aging populations [27]. Bone matrix protein expression in the aortic valve and vasculature in the presence of hypercholesterolemia is dependent on LRP5. Increased LRP5 histological staining and whole tissue protein levels have been observed in degenerative human mitral valve and calcified aortic valve specimens [28–30].

Degenerative joint disease

A large literature has focused on the complex associations between mutations in Wnt receptors and osteoarthritis (OA), a disease characterized by degradation of articular cartilage that, if left untreated, eventually results in loss of joint function. Five common single *LRP5* polymorphisms—rs491347, rs583545, rs667126, rs314751, and rs689179—were not correlated with osteoarthritis in Bristol and London populations, but when analyzed as a group, a common haplotype (C-G-C-C-A) was evident and patients with this haplotype have a 1.6-fold increase in OA [31]. *LRP5* and *LRP6* gene expression was found to be up-regulated in hip and knee tissue from human patients with OA [32], but the controls in this study were patients with hip fracture, which could influence the results. Increased *LRP5* mRNA and protein levels have been observed in human osteoarthritic chondrocytes in vitro [33].

Parathyroid tumors

A significant percentage of parathyroid tumors express an internally truncated form of *Lrp5* generated by alternative splicing. Expression of this splice form is associated with activation of β -catenin-dependent signaling and insensitivity to inhibition by DKK1 [34]. The possibility that this could be relevant for a broader spectrum of tissues is suggested by a report showing that this truncated version of *Lrp5* could be detected in human breast cancer samples [35].

Insights Gained from Genetically Engineered Mouse Models

Global Knockout phenotypes

Mice carrying germline inactivating mutations in *Lrp5*, model the aspects of OPPG in that they develop low bone mass and have abnormal eye vascularization [19]. They also display delays in mammary development, and are resistant to MMTV-Wnt1-induced mammary tumorigenesis [36]. Loss of *Lrp5* is also associated with defects in cholesterol and glucose metabolism and simultaneous deletion of *Lrp5* and Apolipoprotein E resulted in severe hypercholesterolemia, impaired fat tolerance, and advanced atherosclerosis [37, 38]. The absence of *Lrp6* leads to neonatal lethality associated with phenotypes that display similarity to several seen in mice lacking specific Wnts [39]. Defects have also been reported in the skeleton [40], central nervous system [41–43], mammary [44], and anomalies of the heart including double-outlet right ventricle (DORV), outflow tract cushion hypoplasia, and ventricular septal defect [45]. The presence of at least one copy of *Lrp5* or *Lrp6* is required for posterior patterning of the epiblast, with mice carrying compound mutations in both genes clearly showing genetic interaction between the two genes in several tissues [40, 46].

Spontaneous mutations in murine *Lrp6* have also been identified. For example, the mutation in the ringelschwanz gene is a hypomorphic point mutation in *Lrp6* (R886W). Mice homozygous for this mutation have multiple abnormalities including defects in the skeleton and neural tube [47, 48]. Another spontaneously arising point mutation in *Lrp6* underlies neural tube defects observed in the crooked tail (cd) mice which can be attenuated by prenatal supplementation with folic acid [49, 50].

Conditional Deletions of *Lrp5* and *Lrp6*

Because of the strong links between *Lrp5* and human bone disease, much of the initial emphasis in this area has been concentrated on determining the roles of these genes within the osteoblast lineage. An initial report on mice carrying a conditional deletion induced within the osteoblast lineage using Collagen1A1-cre indicated that such mice do not develop any defects in skeletal development [51]. Furthermore, this same report indicated that mice carrying a conditional deletion of *Lrp5* within the intestine display reduced bone mass

associated with increased levels of serotonin in the serum. A model in which Lrp5 functions within the intestine to control bone mass was further supported by experiments in which specific expression of the G171V allele of Lrp5 within the intestine caused increased bone mass while osteoblast-specific expression had no effect. However, more recent work has challenged this Lrp5/serotonin model by showing that mice carrying inactivating mutations in Lrp5 within the osteoblast lineage did, in fact, display decreased bone mass [52]. Furthermore, alterations of Lrp5 expression within the intestine did not alter bone mass [52]. Another study found that while loss of either Lrp5 or Lrp6 alone did not significantly alter intestinal differentiation, simultaneous deletion of both genes within the intestine resulted in a progressive loss of cells associated with a premature differentiation of crypt precursor cells and early perinatal lethality [53].

Attention has also been focused on examining the roles of these genes in regulating mesenchymal stem cell differentiation. The simultaneous deletion of both Lrp5 and Lrp6 in the murine embryonic mesenchyme results in embryos with reduced body size, misshaped skull and limbs, shortened skeletal elements, and defects in craniofacial, axial, and appendicular skeleton ossification [54]. Mice carrying a conditional deletion of Lrp6 have also been generated to examine mechanisms related to craniofacial abnormalities and other birth defects [55].

Therapeutic Developments Targeting Lrp5/Lrp6 or Interacting Partners

A representative list of the various agents that target Lrp5/6 in development for clinical trials is shown (Table 1). Below, we discuss in more detail three specific examples of these agents.

Anti-DKK1

Pharmacological inhibition of Dkk1 using monoclonal anti-Dkk1 antibody is an approach to stimulating osteogenesis in patients suffering from low bone mass. Ovariectomized (OVX) mice treated with RH2-18, human monoclonal anti-DKK1 antibody, had significantly higher whole-femur and lumbar spine BMD, as well as higher trabecular bone volume fraction and thickness, relative to vehicle-treated controls [56]. Antibody-based inhibition of Dkk1 has also shown promise in a mouse model of multiple myeloma. Prior work suggests that elevated Dkk1 levels in the bone marrow plasma and peripheral blood from patients with multiple myeloma correlates with gene expression patterns of Dkk1 and is associated with the presence of focal bone lesions [57]. Stimulated osteoblastogenesis and reduced osteoclastogenesis was reported in myelomatous bones after Dkk1-neutralizing antibody treatment in mice, but there was no effect on tumor burden [58, 59]. The inhibition of Dkk1 in a mouse model with a neutralizing rat monoclonal antibody to mouse Dkk1 reverses the bone destructive pattern of rheumatoid arthritis to the bone-forming pattern of OA [60, 61].

Anti-Sclerostin

PTH and its amino-terminal fragment teriparatide, PTH(1-34), are the only therapies for osteoporosis that stimulate bone formation, but they require daily injections, have a two-year use limit, and carry a US Food and Drug Administration mandated “black box warning”. There is an increasing focus on alternative strategies that stimulate osteogenesis, and sclerostin is an attractive candidate [62]. Two doses of sclerostin antibody in cynomolgus monkeys increased trabecular bone volume, bone formation, and mineral density, and sclerostin antibody treatment also improved bone mass and bone formation in a rat model of postmenopausal osteoporosis and in OVX rats pretreated and co-treated with the bisphosphonate drug alendronate [63–65]. Protective effects of sclerostin antibody treatment against bone resorption due to disuse has been demonstrated in a hindlimb-immobilization

rat model, and increased bone formation, mass, and strength was reported in normal aged rats [66, 67]. Chronic inflammation can also lead to bone loss, and many patients with inflammatory bowel diseases like ulcerative colitis and Crohn's disease have low BMD [68]. Significant increases in femoral BMD, trabecular bone volume fraction, cortical thickness, maximum load, and ultimate strength were observed in a colitis female mouse model treated with sclerostin antibody [68].

The first use of sclerostin antibody in humans demonstrated safety, increased concentration of bone turnover markers, and increased lumbar and hip BMD in healthy men and postmenopausal women ages 45–59 [69]. Treatment with sclerostin antibody is also associated with improved fracture healing. In a rat closed femoral fracture model and a cynomolgus monkey fibular osteotomy model, systemic administration of sclerostin antibody increased bone mass, strength, and bone in the fracture gap; it also reduced incomplete unions, cartilage in the callus, fracture gap size and area, and fibrovascular tissue [70]. Sclerostin antibody treatment increased the peak pull-out force, cancellous bone mineral density, and trabecular thickness in a rat model of metaphyseal healing [71].

LRP6 antibodies

Groups from Novartis and Genentech used the extracellular domain of LRP6 to screen human synthetic antibody libraries [72, 73]. Both groups were able to identify two classes of anti-LRP6 antibodies that either inhibit or enhance Wnt/ β -catenin signaling. Further analysis suggested that most of the Wnts can be categorized into two groups that activate canonical Wnt signaling, one binding to the E1-E2 domain of LRP6 and represented by Wnt1, and another binding to the E3-E4 domain, represented by Wnt3a. An antibody that recognizes either domain may antagonize those Wnts binding to the same domain of LRP6. At the same time, this antibody may potentiate signaling by Wnts that bind to a different region, through crosslinking of LRP6 molecules. Both groups were able to show that the antagonizing antibody could inhibit in vivo growth of Wnt-driven allograft tumors. These antibodies may be very useful for treating diseases caused by aberrant Wnt/ β -catenin signaling activation.

Structural Insights into Lrp5/6 Function and Regulation

How Wnt proteins or some known inhibitors of LRP5/6, such as DKK1 and SOST, bind to the LRP5/6 extracellular domain (ECD) to regulate Wnt signaling are key questions. Answering these questions will facilitate drug discovery efforts aimed at targeting the LRP5/6 ECD in order to develop Wnt pathway modulators. Recently, several groups reported crystallographic and EM studies on the LRP6 ECD and its interaction with DKK1 [74–77]. The ECD of LRP5/6 has a modular structure with four tandem six-bladed β -propeller domains each connected by an EGF-like domain, and followed by three LDLR type A domains (Fig. A). Following the nomenclature used by Cheng *et al.* [74], we refer to the first β -propeller and EGF-like domain pair as E1, and the second to fourth pairs as E2-E4 (Fig 2). Crystal structures of LRP6 E1E2 [74] and LRP6 E3E4 [74, 75] in apo form have recently been reported. Each β -propeller domain has six blades with each blade consisting of four antiparallel β -strands. The top surface of E1–E4 is concave, resembling an amphitheater. Sequence comparisons show that β -propeller domains 1–3 have high sequence homology, but their homology with the β -propeller domain 4 is low. The top surfaces of E1, E2, and E3 are similar; they all have a hydrophobic patch surrounded by negatively charged residues. The top surface of E4 lacks the hydrophobic patch and is more positively charged.

The binding sites for Wnt proteins, inhibitors and antibodies are located on the top surface of E1 and E3 (Figure 3A). Two β -propeller domains in each structure are roughly parallel to each other. The EGF-like domain of approximately 40 residues packs tightly against the bottom of the preceding β -propeller. The structures of E1E2 and E3E4 have the same β -

propeller-to-EGF interdomain orientation. The structures are consistent with the idea that LRP6 E1E2 and E3E4 are each a rigid folding unit [78].

The structure of LRP6 E1E4 as examined by electron microscopy (EM) suggested that the orientation between E1E2 and E3E4 is somewhat flexible due to a short linker between them [74]. Chen et al. reported the LRP6 ECD structure as determined by EM at 25 Å to be a skewed planar horseshoe-like structure with three sides. In this case, the LRP6 ECD includes the three C-terminal LDLR type A domains. Further examination with the E1E2 and E3E4 structures showed some extra density at the center that may be accounted for by the LDLR type A domains [75].

The structure of LRP6 E1E4 has also been studied by small-angle X-ray scattering (SAXS) [76]. SAXS data were collected on LRP6 E1E4 in complex with either Fab135, an antibody fragment recognizing β -propeller 1 domain, or the full length DKK1 protein. The data showed that the complex structures have a twisted L-shape, with E1, E2, and E3 being roughly coplanar and E4 forming a short arm. This structure illustrated that E1, E2, and E3 have similar heights with respect to the membrane, allowing easy access of Wnt ligands or DKK1 protein. However, since the structures were determined in complex with either Fab135 or DKK1 protein, they may represent a partially opened conformation for the LRP6 ECD. DKK1 has N-terminal (DKK1_N) and C-terminal (DKK1_C) domains which can both bind to the LRP5/6 ECD. DKK1_N binds specifically to the LRP6 E1 domain, and DKK1_C binds specifically to the LRP6 E3 domain [76]. Thus, the full-length DKK1 binds to the LRP6 ECD in a bipartite manner. Details are presented in box 3.

TEXT BOX 3

DKK1 and LRP6 interactions

A. DKK1_N and LRP6E1

For the DKK1_N and LRP6E1 interactions, a peptide sequence (NAIK) preceding the first cysteine-rich domain of DKK1 was identified to selectively bind to the LRP6 β -propeller 1 domain [77]. Similar peptide sequences were identified from an antibody and LRP6 E1 complex (NAVK), and in several known inhibitors of LRP5/6, including DKKs, SOST, and WISE [97]. Based on sequence alignment, the consensus sequence is NXI, where X can be Ala, Ser, or Trp. The peptide/LRP6 E1 interaction explains the human LRP5 gain-of-function BMD mutations, which map to the E1 top surface. These mutations affect the peptide binding pocket of LRP5 either directly or indirectly and disrupt the LRP5 E1 interaction with DKK1 and SOST inhibitors while leaving the affinity for Wnt ligands unchanged.

B. DKK1_C and LRP6 E3E4

The determination of the LRP6 E3E4 and DKK1_C complex structure found that DKK1_C has two subdomains, connected by a long loop, each having either two [74] or three [76] antiparallel β -strands (Figure 2). The overall structure is very flat and similar to the DKK2_C structure, as determined by NMR [74]. The long loop (residues 222–231), that appears flexible in the DKK2_C NMR structure, mediates the major interactions with the LRP6 E3 top surface, including the hydrophobic patch formed by residues F836, W850, W767, Y875, M877, Y706, and I681. Residues in the hydrophobic patch are conserved among LRP5/6 propellers 1, 2, and 3, but not 4, which explains why propeller 4 does not bind DKK1_C. Sequence alignment showed that almost all LRP6 E3 residues that interact with DKK1_C are identical in LRP5; thus, DKK1_C probably interacts with LRP5 E3 in a similar manner.

Two sides of DKK1_C contact the top surface of LRP6 E3, forming two interfaces (A and B). Interface A has a number of hydrophobic interactions and charged interactions (Table 2). In particular, the DKK1_C R236 makes a key interaction with E3 D811. A lysine is present in E1 and E2 at the position equivalent to D811 in E3, which explains why DKK1_C does not bind to the top surface of E1 or E2 of LRP6. Finally, there is only a single charged interaction (E185- R1184) between DKK1_C and the LRP6E4 domain (Table 2). Mutations of the DKK1 residues within interface A resulted in greatly reduced Wnt signaling activity, implying that interface A is a physiologically relevant interface.

Interface B consists of mainly polar interactions, and this side of the DKK1 surface has been implicated in Kremen binding [8]. Mutations of the interface B residues had only mild effects on the overall interaction, thus the physiological relevance of interface B observed in the crystal structure remains to be determined.

Chen et al. determined the crystal structure of LRP6 E3E4 and studied the effects of mutation of its top surface residues on Wnt3a signaling and on DKK1 inhibition of Wnt1 and Wnt3a signaling [75]. The data suggest that the W875, M877, Glu708, and R1184 residues are important for both Wnt3a and DKK1 binding. E663 is specifically required for Wnt3a binding, but Y706 is more specific for DKK1 binding. Overall, Wnt3a and DKK1 appear to have overlapping binding surfaces on LRP6 E3E4.

Table 1

The interactions of the LRP6 E3E4 and DKK1_C complex structure

Interface A		Interface B	
DKK1_C	LRP6 E3	DKK1_C	LRP6 E3
Phe205, Trp206	Hydrophobic patch	Lys226	Asp878
Leu231, Ile233, Phe234	Hydrophobic patch	His229	Asp811
Arg236	Asp811	Lys222	Ser749
His204	Glu708	Thr221	Arg792
Glu232	Arg792	His223	Arg751
Ser228	His834	Arg191	Asp830
Arg224	Asn813	Leu231	Tyr875
Val219, Leu260	Pro771, Thr812		
Glu185	Arg1184		

Finally, the recent report of the crystal structure of a Wnt in complex with a frizzled receptor may also reveal insights into Lrp5/6 function. A patch of approximately 10 residues derived from three discontinuous regions on the surface of the Wnt structure that are not involved in binding to Frizzled was identified and speculated to be a potential binding site for Lrp5/6 [79]. The recent reports of the structures of Lrp6 ECDs have allowed new insights into the functions and regulation of these proteins. In particular, the binding sites for DKKs, Sost and Wnt ligands on Lrp5/6 have been mapped to the top concaved surfaces of the β -propeller domains 1 and/or 3. These structures serve as templates for understanding Lrp5/6 protein folding, disease-causing mutations, and for rational design of antibodies to specifically targeting these surface areas. The detailed interactions between Lrp6 E3E4 and DKK1_C will help in designing better antibodies to specifically disrupt their interactions for future research and therapeutic uses. Future directions of the structural studies will be on the full length Lrp6 ECD structure to elucidate any possible domain-domain interactions and the Lrp6 ECD structure in complex with Wnt ligands or other inhibitors.

Concluding Remarks

Since their discovery almost 15 years ago, alterations in Lrp5 and Lrp6 have been linked to a variety of diseases and their critical role in transducing signals from Wnt ligands has made them the topic of numerous studies. Because of this, several biotechnology and pharmaceutical companies have focused on them or their interacting proteins as targets for the treatment of a number of human diseases. The recent breakthroughs in establishing the crystal structures of these proteins uncover new opportunities to further understand and target the functions of these proteins for drug development. Future work focused on deciphering the role of potential lipoprotein interactions related to these proteins and how these may modulate signaling activity will enhance therapeutic strategies for a wide variety of human diseases linked to Wnt/ β -catenin signaling. Given the central regulatory role of these proteins in mediating activation of the Wnt/ β -catenin pathway, it is likely that additional new insights await us as numerous laboratories evaluate the physiological roles of these proteins in normal organogenesis and disease.

Acknowledgments

We would like to thank David Nadziejka for help with editing this manuscript. This work is supported by NIH grants RR024887 and AR053293 (BOW) and GM087413 (HEX). We apologize to those whose work could not be cited due to space limitations.

Glossary

β-catenin	a protein that plays central role in Wnt signaling. While stabilization of the protein and subsequent nuclear localization to activate target gene transcription is the key outcome of Wnt-induced activation of Lrp5/6, β -catenin plays multiple roles in cells. For example, it also is a key component of the adherens junction, serving to link Cadherins to the actin cytoskeleton
β-propellers	Key protein structural motifs composed of twisted β sheets that are radially arranged around a central tunnel. Four six-bladed versions of this motif exist on Lrp5/6, and mediate binding to many of the effectors of Lrp5/6 signaling including Wnts, Dkks, and Sost
Dickkopf-1 (Dkk1)	The founding member of a multigene family of which Dkk1, Dkk2, and Dkk4 are secreted proteins that bind to Lrp5/6 and inhibit Wnt signaling. Dkk1 was originally identified in <i>Xenopus</i> as a molecule that can induce head structures which is the basis for the name (Dickkopf is German for “big head” or “stubborn”)
Familial Exudative Vitreoretinopathy (FEVR)	a genetic eye disease affecting the retina, and characterized by failure of peripheral retinal vascularization
Glycogen synthase kinase 3 (GSK3)	A serine/threonine protein kinase with numerous substrates. In the context of Wnt signaling, this kinase phosphorylates β -catenin and targets it for ubiquitin-dependent proteolysis. There are two genes encoding proteins with this activity, GSK3 α and GSK3 β

Low density lipoprotein-related receptor 5. (Lrp5)	A transmembrane cell surface receptor and member of the LDL receptor family that plays a central role in Wnt signal transduction
Low density lipoprotein-related receptor 6 (Lrp6)	Also a transmembrane cell surface receptor and member of the LDL receptor family, highly homologues to LRP5, playing a central role in Wnt signaling
Norrin	A small secreted protein that binds specifically to Frizzled 4 to activate β -catenin signaling. The gene encoding Norrin, <i>NDP</i> , is inactivated in Norrie Disease, which is characterized by abnormal vascularization of the eye leading to blindness
Osteoporosis pseudoglioma	A syndrome caused by inactivating mutations in LRP5. Patients with this syndrome have extremely low bone mass and inappropriate eye vascularization
Sclerostin	This secreted protein, encoded by the SOST gene, is produced in osteocytes and inhibits Wnt signaling by binding to Lrp5/6 and inhibiting their activation by Wnt proteins
Sclerosteosis disease	A disease characterized by generalized overgrowth of bone tissue, mostly in the cranial bones and in the diaphysis of tubular bones [12]. Sclerosteosis is linked to loss of function of the SOST gene product
Van Buchem disease	A disease also characterized by generalized overgrowth of bone tissue, mostly in the cranial bones and in the diaphysis of tubular bones [12]. It is linked to a 52-kb deletion downstream of the SOST gene that causes down-regulation of SOST expression
Wnt	A family of cysteine-rich, secreted glycoproteins that activate several signaling pathways, including one dependent on Lrp5/6 that activates the β -catenin protein. Mammals have individual Wnt genes. Wnt1, the first such gene identified, was originally identified as an integration site, enriched in mouse mammary tumor virus-associated tumors (int-1). Int-1 is homologous to the <i>Drosophila</i> gene <i>Wingless</i> . The combination of the words Int-1 and Wingless led to the name Wnt-1

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Figure 1. Function and Regulation of Lrp5 and Lrp6 in β -catenin Signaling

In the absence of an upstream signal, Lrp5/6 and Frizzled are inactive (1). This results in the cytoplasmic pool of β -catenin being recruited into a multi-protein destruction complex that includes Axin, APC, Casein kinase 1a (CK1), and Glycogen synthase kinase 3 (GSK3) [99]. This destruction complex facilitates the GSK3-dependent phosphorylation of β -catenin and its subsequent ubiquitinylation (Ub) by the E3 ligase, β -TrCP, which targets β -catenin for degradation via the proteasome (3). Several mechanisms have been identified by which Lrp5/6 availability for signaling is regulated. First, several secreted inhibitors can bind directly to Lrp5/6 and prevent activation by Wnt ligands. These include Dkk1, Sost, and Wise (2). In addition, the E3 ubiquitin transmembrane ubiquitin ligase ZNRF3 (and the related RNF43) normally decreases the stability of the Lrp5/6:Frizzled complex by targeting the complex for degradation (4). However, in the presence of R-spondin proteins, a complex is formed between ZNRF3, R-spondin (Rspo), and the seven transmembrane receptor, LGR4, that results in the membrane clearance of ZNRF3 (4), which consequently leads to increased availability of Lrp5/6:Frizzled receptor and potentiated signaling [4].

Activation of Lrp5/6 can occur by several mechanisms. The best known occurs when a Wnt ligand engages a Frizzled receptor, and Lrp5 or Lrp6 (5) [2]. This results in the phosphorylation of the carboxyl terminus of Lrp5/6, creating a binding site for Axin. The formation of this new complex (“inhibited destruction complex”) results in β -catenin remaining in this complex because of failure to recruit β -TrCP for ubiquitinylation, although GSK3-mediated phosphorylation still occurs. Eventually, this results in the saturation of

inhibited destruction complex with β -catenin (8). The complex is no longer available to bind and target newly synthesized β -catenin for degradation. This leads to increased β -catenin levels in the cytoplasm and nucleus where it is now available to complex with members of the LEF/TCF family to regulate transcription of target genes (9). Norrin can also activate β -catenin signaling in a Frizzled-4 and Lrp5-dependent manner, although no evidence for direct binding of Norrin to Lrp5 has been reported [100]. Finally, recent evidence [10] has found that Complement C1q can bind to Frizzled and activate C1s to cleave Lrp5/6, creating a truncated version of this protein that is constitutively active (7).

Figure 2. LRP5 and LRP6 are Highly Homologous Proteins with Similarly Arranged Structural Motifs
A schematic representation of human LRP6 is depicted with specific domains indicated by the color codes shown. The boundaries between each of the domains are indicated by the numbers listed at the top (relative to the 1613 amino acid full length protein). The levels of homology and identity between human LRP6 and human LRP5 are shown.

Figure 3. Structural Insights into the Function and Regulation of Lrp5/6

(A) Schematic representation created with the Pymol program of the ECD of LRP6 with the four β -propeller domains (green) and four EGF-like repeats (cyan) indicated. Binding sites for specific Wnt ligands, various inhibitors, and antibodies generated to these molecules [72, 73] are also noted. Color schemes are consistent with those shown in Panels B and C. This figure is adapted from Cheng et al. [74] (B) A ribbon diagram of the LRP6 E1 structure in complex with the DKK1 peptide NSNAIKN (PDB code 3SOQ) is shown. The peptide sequence is colored in magenta with the NAIK motif shown as a stick model. (C) The Lrp6 E1E2 structure in complex with the DKK1_C domain is represented by a ribbon diagram (PDB code 3S94). (D) A ribbon diagram depicts the Lrp6 E3E4 structure in complex DKK1_C domain (PDB code 3S2K). The DKK1_C structure is colored in magenta with

disulfide bonds shown in yellow. Only one copy of DKK1_C and Lrp6E3E4 corresponding to the interface A is shown.

Table 1

Wnt Therapeutic Modulators (modified from [98])

Disease/Application	Therapeutic	Pathway Target	Compound	Company	Stage of Development
Extracellular Modulators					
Bone	Anti-Sclerostin	SOST/LRP5	Small Molecule	OsteoGeneX	Lead Optimization/Pre-Clinical
Cancer	Nu206	r-Spondin	Biologic	Nuvelo	Pre-Clinical
Bone/Cancer	LRP6 mAb	LRP6	Biologic	Novartis/Genentech	Discovery
Bone	Wnt Pathway	LRP5	Small Molecule	Galapagos	Discovery
Cancer	CTGF mAb	CTGF	Biologic	Fibrogen	Pre-Clinical
Bone	LRP5 mAb	LRP5	Biologic	Nuvelo	Discovery
Cancer	WAY-316606	SFRP	Small Molecule	Wyeth	Pre-Clinical
Bone	Dkk1 mAb	Dkk1	Biologic	Nuvelo	Discovery
Bone	Sclerostin mAb	SOST	Biologic	Amgen	Phase II
Bone	Small Mole NCI	Dkk	Small Molecule	Enzo Biochem	Pre-Clinical
Cancer	Niclosamide	Frizzled	Small Molecule	UTSW	Discovery
Bone	Sclerostin mAb	SOST	Biologic	Novartis	Pre-Clinical
Bone	Sclerostin mAb	SOST	Biologic	Eli Lilly	Pre-Clinical

Table 2

The interactions of the LRP6 E3E4 and DKK1_C complex structure

Interface A		Interface B	
DKK1_C	LRP6 E3	DKK1_C	LRP6 E3
Phe205, Trp206	Hydrophobic patch	Lys226	Asp878
Leu231, Ile233, Phe234	Hydrophobic patch	His229	Asp811
Arg236	Asp811	Lys222	Ser749
His204	Glu708	Thr221	Arg792
Glu232	Arg792	His223	Arg751
Ser228	His834	Arg191	Asp830
Arg224	Asn813	Leu231	Tyr875
Val219, Leu260	Pro771, Thr812		
DKK1_C	LRP6 E4		
Glu185	Arg1184		