Characterization of the Interaction of Sclerostin with the Low Density Lipoprotein Receptor-related Protein (LRP) Family of Wnt Co-receptors

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**Background:** Sclerostin, an inhibitor of Wnt signaling, binds to the β-propeller domain-containing Wnt co-receptors LRP6 and LRP4.

**Results:** An NXI motif in sclerostin mediates interactions with LRP6 (but not LRP4) and blocks Wnt1 signaling.

**Conclusion:** The sclerostin/LRP6 interaction shares features with the well characterized nidogen/laminin interaction.

**Significance:** NXI motifs are important in mediating interactions with β-propeller containing proteins.

LRP5 and LRP6 are proteins predicted to contain four six-bladed β-propeller domains and both bind the bone-specific Wnt signaling antagonist sclerostin. Here, we report the crystal structure of the amino-terminal region of LRP6 and using NMR show that the ability of sclerostin to bind to this molecule is mediated by the central core of sclerostin and does not involve the amino- and carboxyl-terminal flexible arm regions. We show that this structured core region interacts with LRP5 and LRP6 via an NXI motif (found in the sequence PNAIG) within a flexible loop region (loop 2) within the central core region. This sequence is related closely to a previously identified motif in laminin that mediates its interaction with the β-propeller domain of nidogen. However, the NXI motif is not involved in the interaction of sclerostin with LRP4 (another β-propeller containing protein in the LRP family). A peptide derived from the loop 2 region of sclerostin blocked the interaction of sclerostin with LRP5/6 and also inhibited Wnt1 but not Wnt3A or Wnt9B signaling. This suggests that these Wnts interact with LRP6 in different ways.

In humans, there are 19 members of the Wnt family, which interact to different extents with cell surface receptors that are usually comprised of one of 10 different members of the frizzled family (a family of seven-span receptors) in combination with one of a number of different co-receptors (4, 5). The detailed mechanisms involved in Wnt signaling are complex and not completely understood. It is known that Wnt engagement can trigger a number of different intracellular signaling cascades and it has been suggested that the particular cascade triggered by a Wnt binding to its receptor complex is determined by the co-receptor involved in the initial engagement (6). Two co-receptors known to be involved in Wnt signaling are LRP5 and LRP6; both are members of the low-density lipoprotein receptor-related family and typically promote canonical Wnt signaling (7). The canonical Wnt signaling pathway involves stabilization of cytoplasmic β-catenin and its subsequent translocation into the nucleus; once in the nucleus, β-catenin regulates gene expression by interacting with members of the TCF/LEF family of transcription factors (8).

Low density lipoprotein receptor-related proteins 5 and 6 (LRP5 and -6) are transmembrane proteins whose large extracellular domains are highly related (73% sequence identity) and are predicted to consist of four six-bladed β-propeller domains separated by four EGF domains and followed by three LDL A-type repeats in the membrane proximal region (see Fig. 1A) (9). Both of these receptors have cytoplasmic domains of ~200 amino acids, which contain five copies of a PPPS/TPXS signaling motif that is involved in the recruitment of axin and the inhibition of glycogen synthase kinase 3β (GSK3β), a kinase that phosphorylates β-catenin and targets it for proteasomal destruction (2, 9, 10). The folding of both LRP5/6 is assisted by a specialized chaperone protein called MESD (mesoderm...
development) (11). In the mouse, in the absence of MESD, there is a lack of functional LRP5/6 expression, and this causes a disruption of Wnt signaling, which results in embryonic defects (12). The structure of MESD and the nature of its interactions with LRP5/6 proteins are being actively investigated by several groups (13–15). It was recently reported (16) that a peptide from the carboxyl-terminal region of MESD blocked Wnt3A signaling.

Because the Wnt signaling pathway plays such a key role in development and homeostasis, it is perhaps not surprising that it is closely regulated at many different levels. Inhibitors include a number of secreted factors that can sequester Wnt family members by direct binding or inhibit Wnts by binding competitively to their receptor complexes (2). Such inhibitors include sclerostin, sclerostin-domain containing, and the DKK family of inhibitors (2). The importance of these inhibitors has been emphasized by studies linking changes in their expression to significant defects in development, homeostatic regulation, or cancer prognosis (17–21). Sclerostin is an osteocyte-produced extracellular glycoprotein of 190 amino acids that negatively regulates the anabolic output of bone-forming osteoblasts (22)). The human-inherited condition sclerosteosis is caused by an inactivating mutation in sclerostin (23, 24), and sufferers show lifelong increases in bone formation (25). The bone produced is of normal quality and anecdotally protects sufferers from fractures (26). However, the increased bone formation also causes a number of side effects, including facial palsy and in some cases a life-threatening increase in intracranial pressure (26).

The structure of mouse and human sclerostin, determined by NMR, has been reported by two groups (27, 28). Both groups report that sclerostin has highly flexible amino- and carboxyl-terminal arms with a core region consisting of three loops projecting from a central cystine knot. Two of the loops (designated loops 1 and 3, Veverka et al. (28)) appear more rigid and are formed from twisted anti-parallel β-strands joined by a disulfide bond at their tip. The third loop (designated loop 2) is relatively flexible and has been shown to be the primary binding site for a monoclonal antibody that down-modulates the activity of sclerostin both in vitro and in vivo (28). Sclerostin has been reported to bind to LRP5/6 (29) and point mutations in the amino-terminal β-propeller of LRP5 that are associated with high bone mass reduce the ability of sclerostin to interact with LRP5 (30). This suggests that sclerostin interacts with the amino-terminal region of LRP5/6. Sclerostin has also been shown to bind to another member of the LDL receptor family called LRP4 (31), which is organized differently from LRP5/6 but includes some of the same domain structures including four six-bladed β-propeller domains (9).

The work here reports the crystal structure of the first two propeller domains of LRP6, describes the nature of the interaction of sclerostin with LRP5/6, and shows that the domain is different from the interaction with LRP4. It also describes small peptides that can interfere with the binding of sclerostin to LRP5/6 and shows the effects of these peptides on the canonical signaling of different Wnts.

EXPERIMENTAL PROCEDURES

Molecular Biology—Full-length human cDNA clones encoding human Wnt1, Wnt3A, Wnt9B, sclerostin, LRP4, LRP5, LRP6, and MESD were obtained from Origene. Mutations were introduced using a QuikChange II kit (Agilent Technologies). The numbering of residues in this work is from the start of the mature sequence (see Fig. 1 for sclerostin). The nomenclature used for fragments of LRP6 is as follows: LRP6-Fc contains full extracellular domain of LRP6 fused to human IgG1 Fc, LRP6-E1 contains the first propeller and EGF domain of LRP6, and LRP6-E1E2 contains the first and second propeller and EGF domains of LRP6. Further details of molecular biology methods are provided in the supplemental data.

Canonical Wnt Signaling Assays—Wnt activity assays were performed using HEK293 cells stably transfected with reporter construct (HEK293 Tcf-Luc), which was based on the SuperTopFlash reporter (46) and contained 16× TCF/LEF binding sites upstream of the optimized luciferase reporter present in the pGL4.26 vector (Promega). 5 × 10⁴ cells were seeded into solid white poly-d-lysine-coated 96-well plates in DMEM supplemented with 2 mM L-glutamine, non-essential amino acids, and 0.5% FCS, and allowed to attach before being transiently transfected with a total of 200 ng DNA/well, using Lipofectamine 2000 (Invitrogen). Peptides were dissolved in DMSO and added to wells at the time of transfection; the final concentration of DMSO was 0.3%. Approximately 44 h post-transfection, plates were developed using Steady Glo luciferase substrate (Promega) and read on a luminometer.

FACS Binding Assay—Cells were seeded into poly-d-lysine-coated six-well plates (1.2 × 10⁵/well) and allowed to attach before being transiently transfected with a total of 4 μg DNA per well, using Lipofectamine 2000 (Invitrogen). Cells were harvested non-enzymatically, typically on the day after transfection. For detection of sclerostin binding to cell surface LRP6, 2.2 × 10⁵ cells were labeled with biotinylated human sclerostin for 1 h at 4 °C in FACS buffer (10% FCS, 1% BSA in PBS). In competition experiments, unlabeled proteins, or peptides (dissolved in DMSO, final concentration of DMSO was 1.5%) were added at the same time as biotinylated sclerostin. After washing, cells were stained with streptavidin-PE (Invitrogen) for 45 min at 4 °C. Cells were washed then analyzed using a FACS Calibur (Becton Dickinson).

Immunoprecipitation—Supernatants containing LRP4, -5, or -6 were mixed with sclerostin (or a sclerostin derivative) at the concentration indicated in the figure legends for 1 h at 4 °C, and then Sepharose beads coated with a non-neutralizing anti-sclerostin antibody were added, and tumbling was continued for a further 1 h. Beads were spun down washed, in PBS containing 200 μg/ml BSA and 0.5% Nonidet P-40. Bound protein was eluted from the beads by boiling in sample buffer and analyzed by SDS-PAGE. Further details are provided in the supplemental data.

Purification of LRP6-E1E2—LRP6-E1E2-Fc containing a TEV protease site between the LRP6-E1E2 and the Fc was transiently co-expressed with MESD in CHO cells (in the presence of 5 μM kifunensine when used for crystallography). Superna-
tant was harvested and passed down a protein A column. Following TEV cleavage from the protein A matrix, the material was further purified by gel filtration. In material for crystallization, MESD was eluted prior to TEV cleavage with a pH 4.9 phosphate/citrate buffer, and the material was treated with endoglycosylase H. Further information on protein purification is provided in the supplemental data.

Crystal Structure of N-terminal Region of LRP6—Cubic-looking crystals up to 0.25 mm on an edge were grown in a buffer containing polyethylene glycol. The data were initially processed in space group P3,21. Using the nidogen component of the nidogen/laminin structure (Protein Data Bank code 1NPE), Phaser was used to search for the propeller domains. The building and graphics visualization was done in O and COOT, and refinement cycles were performed with CNX. The final model had residues 1 to 616 of the mature protein, also identified were sugars at residues 281, 433, and 486, and two phosphate ions. The R/Rfree of the structure was 0.24/0.31. The coordinates have been deposited in the Protein Data Bank (code 4DG6). Further information on crystallography is provided in supplemental data.

Mapping of Interaction between Sclerostin and LRP6 by NMR Spectroscopy—Uniformly 15N-labeled sclerostin was prepared as described previously (28) and mixed with an equimolar amount of unlabeled LRP6-E1E2 to prepare a 60\%/H9262 complex. The specific binding of LRP6 to sclerostin was monitored by changes induced in the positions of signals from backbone amide groups of 15N-labeled sclerostin in two-dimensional 15N/1H HSQC spectra, which were collected at 35 °C on 600 MHz Bruker Avance spectrometer equipped with a triple-resonance cryoprobe.

**FIGURE 1. Schematic of domain structure of LRP5 and sequence of sclerostin.** A, circles represent predicted propeller domains, black bars represent predicted EGF domains, and the line represents the transmembrane and cytoplasmic region. B, representation of the sclerostin structure showing the position of the PNAIG sequence in a flexible loop. Loop 1 is shown in orange, loop 2 is shown in blue, loop 3 is shown in green, and interloop regions and unstructured regions are shown in gray. The unstructured N (N-term) and C termini (C-term) have been removed for clarity. C, alignment of β-propeller binding proteins containing PNAIG-like motif.

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FRET Assay—An assay was carried out in 50 mM Tris, pH 7.5, 150 mM NaCl, 0.05% Tween 20, 0.1% ovalbumin terbium-labeled streptavidin was diluted to 25 nM and mixed 1:1 with biotinylated sclerostin at 25 nM. LRP6-E1E2-Fc at 75 nM was mixed 1:1 with DyLight 488-conjugated AffiniPure (F(ab')2 fragment goat anti-human IgG, Fc/fragment-specific (Jackson ImmunoResearch Laboratories) at 30 μg/ml. Assays were performed in 384-well Corning Costar plates (black, low volume). To each well, 10 μl of the terbium-labeled streptavidin/biotinylated sclerostin, 5 μl of test compound (or control), and 10 μl of LRP6D-Fc/Dylight 488 mix were added. The plate was incubated covered in foil for 1 h at room temperature on a shaking platform. Data were collected on an Analyst GT (Molecular Devices) using time resolved fluorescent (Green) FRET low volume plate mode (filters, 330 nm (excitation), 485-10/520-10 (emission)). Data from titration curves were analyzed in XLFit (version 5), and IC50 values were calculated.

Isothermal Calorimetry—LRP6-E1E2 (wild type or N185A) was buffer-exchanged into 100 mM HEPES, pH 7.2, and diluted to the required concentration (LRP6-E1E2 = 41 μM; LRP6(N185A)-E1E2 = 22 μM). Peptide 1 was prepared at 200 μM (2% DMSO matched in all solutions), and all solutions were degassed prior to the experiment. ITC was performed at 25 °C on an Auto ITC200 instrument (GE Healthcare) using a first injection of 0.5 μl followed by 19 μl injections with the syringe spinning at 1000 rpm. Each experiment utilized two-injection series into the cell contents. Data were collected and analyzed automatically using the GE data concatenation tool and Origin (version 7.0) for ITC (one-site fitting model).

Statistical Analysis—Statistical analysis of the data were performed using one-way analysis of variance with a Dunnett’s post hoc test to adjust for multiple comparisons. p values < 0.01 were considered significant.

RESULTS

Ability of Sclerostin to Inhibit Wnt Signaling Is Dependent on Asn-93—To better understand the nature of the interaction between LRP5/6 (predicted domain structure shown in Fig. 1A) and sclerostin, we reviewed the structural information available for other six-bladed β-propeller containing proteins. One of the best characterized protein/protein interactions involving a six-
bladed $\beta$-propeller structure is the interaction of nidogen with laminin. A high-resolution crystal structure of the complex has been reported (32) and shows that a loop from laminin with the amino acid sequence PNAV interacts with an amphitheater-shaped surface on the six-bladed $\beta$-propeller region of nidogen. Careful inspection showed that the PNAV sequence of this loop was highly homologous to part of the flexible loop 2 region of human sclerostin (with the sequence PNAIG, see Fig. 1B), a sequence that is highly conserved in the sclerostin protein from many different species (Fig. 1C). The loop 2 region of sclerostin has also been reported to be the epitope for an antibody that down-modulates sclerostin activity in vitro and leads to increased bone formation in vivo (28). These observations provided circumstantial evidence that the PNAIG sequence in the sclerostin loop 2 region might mediate some of its interaction with LRP5/6. To confirm the functional relevance of the PNAIG sequence, a number of site-directed mutations were made in this region of the human sclerostin sequence, and the

![Diagram](https://example.com/diagram.png)

**FIGURE 3. Different regions of sclerostin interact with LRP5/6 and LRP4.** A, Western blot of the bead eluate from a co-immunoprecipitation using supernatant containing LRP5ECD-His and anti-sclerostin-coated beads. The table below the figure shows the contents of the immunoprecipitation (IP) mixes. The top row shows the input LRP5 (revealed with anti-His), the middle row shows the sclerostin pulled down (revealed with anti-sclerostin), and the bottom row shows the LRP5ECD-His co-precipitated with the sclerostin (revealed with anti-His). B, Western blot of the bead eluate from a co-immunoprecipitation experiment using supernatant containing LRP6ECD-Fc (lanes 1–5) or LRP4ECD-Fc (lanes 7–11). The top row shows the input Fc fusion protein (either LRP6-Fc or LRP4-Fc revealed with anti-Fc), the middle row shows the sclerostin pulled down (revealed with anti-sclerostin), and the bottom row shows the Fc fusion protein co-precipitated with the sclerostin (revealed with anti-Fc). Sclerostin (and N93A sclerostin) were used at 1 $\mu$g/ml. The sclerostin blocking antibody (Scl-Ab1) and control antibody were used at 100 $\mu$g/ml. C and D, biotinylated wild type sclerostin was incubated with LRP6 transfected HEK293 cells and revealed with streptavidin-PE. In both cases, the shaded area shows binding of streptavidin-PE in the absence of biotinylated sclerostin, the solid line shows staining with 0.3 $\mu$g/ml biotinylated sclerostin alone, and the dotted line shows staining with 0.3 $\mu$g/ml biotinylated sclerostin in the presence of 10 $\mu$g/ml unlabeled wild type (C) or N93A sclerostin (D). Ab, antibody.
mutants were assessed for their ability to inhibit Wnt signaling in a cell-based reporter assay. Fig. 2A shows the effect on luciferase expression of co-transfecting genes encoding Wnt1 and sclerostin (or sclerostin with mutations in the PNAIG sequence) into HEK293 cells containing a luciferase gene whose expression is dependent on canonical Wnt signaling. Wnt1-induced luciferase expression was inhibited dramatically by the presence of wild type sclerostin but not inhibited (or only slightly inhibited) by versions of sclerostin in which Asn-93 or Ile-95 (two of the residues in the loop 2 PNAIG sequence) had been replaced by other amino acids (except in the case where the isoleucine was replaced by the structurally related amino acid valine). For unknown reasons, some of the changes at the Asn-93 position appeared to slightly enhance Wnt signaling. Western blot analysis showed that expression of the different sclerostin mutants did not alter Wnt1 expression and that although there was some variation in the expression levels of the different sclerostin mutants, this did not correlate with their ability to inhibit Wnt1 signaling (Fig. 2B). The last three columns in Fig. 2A show that changing the order of the loop 2 PNAIG sequence also destroyed the ability of sclerostin to inhibit Wnt signaling.

Asn-93 of Sclerostin Plays Key Role in Its Ability to Interact with LRP5 and LRP6 but Not LRP4 Extracellular Domains—The tagged full-length extracellular domains of human LRP4 (Fc-tagged), LRP5 (His-tagged), and LRP6 (Fc-tagged) were expressed in mammalian cells in conjunction with MESD, which has been reported to be a chaperone for LRP5/6 molecules (33). Co-immunoprecipitation showed that wild type sclerostin bound to LRP5-His but that sclerostin containing a N93A mutation did not (Fig. 3A). LRP6-Fc behaved in a similar manner as LRP5-His, binding to wild type but not N93A sclerostin (Fig. 3B, tracks 2 and 3). In contrast, LRP4-Fc showed similar levels of binding to both wild type and N93A sclerostin (Fig. 3B, lanes 8 and 9). To further confirm that different sites on sclerostin were involved in its interaction with LRP4 and LRP5/6, a co-immunoprecipitation experiment was performed in the presence of an antibody shown previously to modify the function of sclerostin in vivo (28). Fig. 3B shows that although the antibody blocked binding to LRP6-Fc (lane 5, bottom panel), it did not inhibit binding to LRP4-Fc (lane 1, bottom panel). A surface plasmon resonance (Biacore) study performed with the first two domains of LRP6 fused to human Fc (LRP6-E1E2-Fc) showed that wild type sclerostin bound to the Fc captured fusion protein with an affinity of \( \sim 5 \) nM, whereas the Asn-93 mutant showed no binding in this assay even when used at 20 nM (supplemental Fig. 1).

To investigate whether the Asn-93 residue of sclerostin was important for LRP6 binding in a cellular context, biotinylated wild type sclerostin was incubated with HEK293 cells transfected with the gene for full-length human LRP6. Wild type sclerostin showed clear binding to LRP6 transfected cells but not to untransfected cells (supplemental Fig. 2). This binding was inhibited by a sclerostin function modifying antibody but not by an antibody that does not block the activity of sclerostin in a cell-based assay (supplemental Fig. 3). Fig. 3 shows that the binding of biotinylated wild type sclerostin to the LRP6-transfected cells could be inhibited by excess unlabeled wild type sclerostin (Fig. 3C) but not by sclerostin containing the N93A mutation (Fig. 3D).

**Inhibition of Sclerostin LRP5/6 Interaction**—We investigated the ability of a cyclized synthetic peptide with the same sequence as the sclerostin loop 2 region (peptide 1, see Fig. 1A and Table 1) to inhibit the interaction of sclerostin with LRP5-ECD-His in an immunoprecipitation assay. As a control, the same cyclic peptide with the equivalent of the N93A change was used (peptide 2, Table 1). Fig. 4A shows that although the peptide with the wild type sequence inhibited the interaction of sclerostin with LRP5 when added at a concentration of 500 \( \mu \)M, the peptide with the equivalent of the N93A change did not.

A FRET assay was established using a truncated version of LRP6 (containing the first two propeller and EGF domains of LRP6 fused to human Fc and designated LRP6-E1E2-Fc) binding biotinylated sclerostin. This assay was used to quantify the relative potency of peptides derived from the semi-flexible loop of sclerostin to inhibit the sclerostin/LRP6-E1E2 interaction. Representative titration curves are shown in supplemental Fig. 4. A number of peptides were synthesized that interfered with the binding of sclerostin to LRP6-E1E2-Fc. Table 1 shows the sequences and IC\(_{50}\) values for the peptides assayed in the FRET format. Fig. 4 shows that a series of the peptides (including some very short peptides) that were active in the FRET assay also inhibited the binding of biotinylated sclerostin to LRP6-transfected HEK293 cells, whereas peptides in which the equivalent of the Asn-93 residue had been changed to an alanine (peptides 5, 7) were inactive in the FRET assay and incapable of inhibiting sclerostin binding to LRP6-transfected HEK293 cells.

**Peptides That Block Interaction of Sclerostin with LRP5/6 Inhibit Wnt Signaling**—Using the HEK293 reporter cell line, the effects of the loop 2-derived peptides on Wnt signaling were investigated. Fig. 5 (solid shaded bars) shows that peptides 3 and 4 (but not peptide 5) dose-dependently inhibited Wnt1 induction of luciferase in the reporter cell line. It has been reported previously that expression of a construct containing some of the LRP6 PPSXPXP motifs fused to part of the LDL receptor induced the canonical Wnt signaling pathway in a manner that was independent of exogenous Wnt (34). A similar construct containing two copies of the PPSXPXP motif was transfected into our reporter cell line and induced Wnt-independent luciferase activity. This activity was not inhibited

![Image](http://example.com/image.png)

**TABLE 1**

The sequence (single letter code) and IC\(_{50}\) values (inhibition of sclerostin binding to LRP6-E1E2-Fc in FRET assay) for peptides used in this study.

| Peptide | Sequence | IC\(_{50}\) in FRET assay |
|---------|----------|--------------------------|
| Peptide 1 (native loop 2 sequence) | CGPARLLLPAIIGKWRPSGPDFRC | 6 \( \mu \)M |
| Peptide 2 | CGPARLLLPAIIGKWRPSGPDFRC | >200 \( \mu \)M |
| Peptide 3 (truncated loop 2 sequence) | CLHPAIGKWRPSGPDFRC | 14 \( \mu \)M |
| Peptide 4 | CLNIDIRRGKWC | 0.1 \( \mu \)M |
| Peptide 5 | CLNIDIRRGKWC | >200 \( \mu \)M |
| Peptide 6 | 2ZAIIRR | 11 \( \mu \)M |
| Peptide 7 | 2ZAIIRR | >200 \( \mu \)M |
by any of the peptides tested. Fig. 5A shows that although peptide 3 inhibited Wnt1, it did not inhibit Wnt3A signaling (checked bars), which was consistent with a model proposed by Bourhis et al. (35), suggesting that Wnt1 binds to propeller 1 of LRP6, whereas Wnt3A binds to propeller 3. Even the short linear peptide 6 dose-dependently inhibited Wnt1 signaling but not signaling induced by the PPSPXP receptor fusion (Fig. 5D). For reasons that are not clear, the short control peptide (peptide 7) consistently caused a small increase in signaling from the constitutively active PPSPXP vector (Fig. 5E).

The ability of the peptides used in this study to inhibit Wnt1 signaling was unexpected in the light of a report by Bourhis et al. (36), suggesting that similar peptides did not inhibit Wnt9B.
Binding to LRP6 (which, similar to Wnt1, has been proposed to bind to the first propeller of LRP5/6). To investigate this further, we attempted to stimulate canonical Wnt signaling in our reporter cell line with Wnt9B. The level of stimulation achieved was extremely small but was enhanced by the co-transfection of the gene encoding human frizzled 8 and could be inhibited by sclerostin (supplemental Fig. 5). Although sclerostin inhibited Wnt9B signaling, peptide 3 did not (Fig. 5A, cross-hatched bars). This suggests that Wnt1 and Wnt9B interact with LRP6 in different ways.

**Preparation of First Two N-terminal β-Propeller and EGF (E1E2) Domains of LRP6**—The region of LRP6 necessary for binding to sclerostin was investigated by transiently expressing various LRP6 deletion constructs as Fc fusions and examining their ability to be co-precipitated with sclerostin (supplemental Fig. 6A). A version of LRP6 containing the first two propeller and EGF domains fused to human Fc (LRP6-E1E2-Fc) appeared to interact with sclerostin in a similar manner to the full-length LRP6-ECD-Fc version, whereas a truncation that contained only the first propeller and EGF domains (LRP6-E1-
Fc) appeared to interact weakly with sclerostin. However, the interpretation of this result was complicated by the extensive aggregation shown by the LRP6-E1-Fc. We transiently overexpressed LRP6-E1-Fc, LRP6-E1E2-Fc, and LRP6-ECD-Fc in conjunction with MESD in CHO cells at a large scale to enable the LRP6 truncations to be purified. The Fc fusion proteins were purified by protein A affinity chromatography and analyzed by SDS-PAGE. The LRP6-ECD-Fc and the LRP6-E1E2-Fc material co-purified with a protein that was recognized by an anti-MESD antibody, but the LRP6-E1-Fc did not co-purify with MESD. The failure of the LRP6-E1-Fc to interact with MESD in the same way as the longer forms may explain its increased tendency to aggregate. The addition of excess sclerostin to protein A bound LRP6-E1E2-Fc displaced the bound MESD (supplemental Fig. 6B), which was consistent with previous reports that sclerostin and MESD compete for LRP5/6 binding (37). TEV protease was used to cleave the LRP6-E1E2 material from the protein A resin utilizing a TEV cleavage site that has been introduced between the LRP6 sequence and the Fc region. The putative MESD material co-eluted with the LRP6-E1E2, and its identity was confirmed by mass spectroscopy and amino-terminal sequencing. Following TEV cleavage, the LRP6-E1E2 could still interact with sclerostin in a co-immunoprecipitation assay (supplemental Fig. 6C).

Crystal Structure of N-terminal Region of LRP6—Attempts to get LRP6-E1E2 with bound MESD to form crystals useful for structural determination were unsuccessful. However, after removing the MESD with a mildly acidic wash, crystals were obtained and the structure of the LRP6-E1E2 determined. Fig. 6A shows that as expected the amino-terminal region of LRP6 is composed of a two six-bladed β-propeller domains each followed by an EGF domain. The failure of the LRP6-E1-Fc to interact with MESD in the same way as the longer forms may explain its increased tendency to aggregate. The addition of excess sclerostin to protein A bound LRP6-E1E2-Fc displaced the bound MESD (supplemental Fig. 6B), which was consistent with previous reports that sclerostin and MESD compete for LRP5/6 binding (37). TEV protease was used to cleave the LRP6-E1E2 material from the protein A resin utilizing a TEV cleavage site that has been introduced between the LRP6 sequence and the Fc region. The putative MESD material co-eluted with the LRP6-E1E2, and its identity was confirmed by mass spectroscopy and amino-terminal sequencing. Following TEV cleavage, the LRP6-E1E2 could still interact with sclerostin in a co-immunoprecipitation assay (supplemental Fig. 6C).

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in the asymmetric unit, implying that in those structures the loop regions are flexible.

We identified three crystal forms for the LRP6-E1E2 construct, and they all had very similar architecture. Whereas Cheng et al. (39) describe their structures as rigid, we observed that in the three different crystal forms, there were clear differences in the relative orientations of each of the propellers. When overlaid, there was an apparent shift in the relative orientations of the second propeller with respect to the first. Fig. 6B shows the first propeller aligned, and the variation observed in the position of the second propeller.
This variation is due to the inherent flexibility of the region at the end of the EGF domain, and we would suggest that this flexibility is an intrinsic characteristic of the LRP6 receptor. It is interesting to note that one of the structures in the asymmetric unit deposited by Chen et al. under Protein Data Bank code 3S94 (39) almost exactly overlays one of the conformations we observe (in crystal form 3), but the other adopts yet another conformation that is in between the range of conformations we observe.

Site-directed Mutation of Propeller Domains—Bourhis et al. (36) showed that a key asparagine residue (equivalent to Asn-93) in a peptide derived from sclerostin made an important bidentate interaction with an asparagine residue in the propeller domains of LRP6 (Asn-185). To confirm that the amino-terminal propeller domain of LRP6 was the major site of sclerostin binding, we changed the key asparagine residue in the first propeller to an alanine residue to generate a N185A version of LRP6. As seen in Fig. 7, A and B, the full-length N185A version of LRP6 expressed to similar levels as wild type LRP6 as assessed by binding of an antibody that recognizes a conformational epitope in the N-terminal part of the LRP6. Biotinylated sclerostin bound well to cells transfected with full length LRP6 N185A (Fig. 7, C and D). A small amount of sclerostin binding could be detected if the concentration of sclerostin was increased sufficiently. Having confirmed that the Asn-185 residue was important for sclerostin binding, we carried out an isothermal calorimetry experiment to examine the binding of the peptide 3 to LRP6-E1E2 and LRP6-E1E2 with the N185A change. Fig. 7E shows that the binding of peptide 3 to wild type LRP6-E1E2 could be well fitted using a single binding site model and demonstrated an affinity in the low micromolar range. No binding was detected when the experiment was performed with LRP6(N185A)-E1E2 (Fig. 7F), confirming that peptide 3 interacted with the first but not the second propeller of LRP6.

Involvement of Sclerostin Arms in LRP6 Binding—The structure of sclerostin determined by NMR shows that it has flexible amino and carboxyl-terminal “arms” projecting from the central core region (28). Because sclerostin showed some residual binding to full-length LRP6 containing the N185A mutation, it was of interest to determine whether the flexible arms interacted with the first two propeller domains of LRP6. Uniformly $^{15}$N-labeled sclerostin was mixed with unlabeled LRP6-E1E2 and examined by NMR. The NMR
data clearly showed that the amide proton signals from residues in the flexible arm regions in sclerostin were not affected (apart from the expected line broadening) by addition of unlabeled LRP6-E1E2, as illustrated in Fig. 8, whereas the signals from the residues forming the structured core of sclerostin disappeared entirely from the LRP6-E1E2-bound spectrum. This suggested that the flexible arms did not contribute directly to the interaction with the amino-terminal region of LRP6, which appears to be mediated by the structured core of sclerostin.

Asparagine Residue Is also Involved in Interaction of MESD with LRP6—The data on LRP6 purification presented here are consistent with previous reports that MESD binds to LRP6 in a competitive manner with sclerostin (37), and it has been reported that a 38-amino acid peptide from the C-terminal region of MESD can inhibit Wnt signaling (16). Fig. 9, A and C, shows that a 15-amino acid peptide (KGGGSKEK-NKTKQDK) from the amino terminus of the peptide reported by Liu et al. (16) will compete with sclerostin for binding to LRP6 and also inhibit Wnt1 signaling. The peptide contains a single asparagine residue and when this was changed to an alanine residue it no longer competed with sclerostin for LRP6 binding or inhibited Wnt1 signaling (Fig. 9, B and D).

DISCUSSION

Wnt signaling is an important regulator of many aspects of embryogenesis, homeostasis, and tissue regeneration and is tightly regulated in a variety of different ways. LRP5/6 are six-bladed β-propeller-containing proteins that play an important role in Wnt signaling and are binding partners for a number of Wnt regulatory proteins. Takagi et al. (32) reported that a six-bladed β-propeller domain of nidogen played a key role in the interaction of laminin and nidogen. Here, we have reported the x-ray crystal structure of an amino-terminal portion of the LRP6 receptor and showed that both of the β-propeller domains share similarities with the nidogen β-propeller domain reported by Takagi and co-workers (32). The structure reported here is also consistent with recent reports from Cheng et al. (39) and Chen et al. (40).

Inspection of the semi-flexible loop 2 region of sclerostin revealed a sequence (PNAIG) that was strikingly similar to a sequence in laminin that interacts with the surface of the nidogen β-propeller domain (PNAV). We show here that residues within the PNAIG sequence of sclerostin are important for its ability to function as a Wnt inhibitor. Mutation of the asparagine residue in the PNAIG sequence of sclerostin completely destroyed its ability to inhibit Wnt1 signaling and bind to LRP6-transfected cells. Takagi et al. (32) reported...
that mutation of the analogous asparagine in the laminin PNAV G sequence also has a dramatic effect of the ability of laminin to interact with nidogen. Mutation of the isoleucine in the PNAIG sequence similarly destroyed the ability of sclerostin to inhibit Wnt signaling.

The asparagine residue in the PNAIG motif (Asn-93) is key for the interaction of sclerostin with LR P5/6 but not LR P4. An antibody (Scl-Ab1) that inhibits the interaction of sclerostin with LR P5/6 but not LR P4 has previously been shown to significantly increase bone mass in vivo (28), indicating that blockade of the interaction of sclerostin with LR P5/6 is sufficient to lead to an increase in bone formation.

Data from our crystallization studies show that there is flexibility between the propeller domains of LR P6. In the three crystal forms we obtained, the relationship between the first and second propellers was not a simple translocation of the structure but was composed of a shift plus a rotation. This also was seen in recently published crystal structures of LR P6-E1E2 and LR P6-E3E4 (40). Fig. 6 B shows that the extent of the rotation can vary, but taking one of these relationships as constant, we have built a model of the four propeller and EGF domains of LR P6 receptor (Fig. 6 C). This model suggests that LR P6 can adopt a horseshoe-like conformation and because the extent of the rotation between propellers is not fixed the precise orientation of the individual propellers can be variable (in a cell context this might be regulated by presence or absence of coreceptors/ligands). The horseshoe conformation is consistent with data generated by electron microscopy reported recently by Chen et al. (40).

Conformational changes that alter the relative positions of the first and third propeller domains may help to explain some of the inconsistencies in data on the activity of sclerostin in cell-based assays. We have generated data in HEK293 cells showing that sclerostin can inhibit Wnt1 but not Wnt3A signaling (supplemental Fig. 7), which is consistent with predictions made by Bourhis et al. (35). However, we have previously found that sclerostin did inhibit Wnt3A in MC3T3 cells (28). Li et al. (41) and Ellies et al. (42) reported that sclerostin inhibited Wnt3A signaling in HEK293 cells, whereas van Be zooijen et al. (43) and Krause et al. (44) showed that sclerostin could antagonize Wnt3A signaling in Lys-483 cells. These observations are consistent with sclerostin (which has been reported to bind to the first β-propeller domain LR P5/6, (45) only being capable of inhibiting Wnt3A signaling (Wnt3A binds to the third propeller domain of LR P6, (35)) when LR P6 adopts certain conformationa. A flexible horseshoe-like structure might mean that in some (but not all) conformations of LR P6 the first and third propeller domains of LR P6 are in close enough proximity for sclerostin to inhibit both Wnt1 and Wnt3A signaling. The precise orientation of the individual propellers in a cellular context might be regulated by growth conditions or by the presence or absence of co-receptors or ligands. This could explain why different groups report very different data on the ability of sclerostin to inhibit Wnt3A signaling.

A peptide based on part of the loop 2 region of sclerostin (peptide 3) was found to inhibit the binding of sclerostin to LR P6 but not to inhibit either Wnt3A or Wnt9B signaling (Fig. 5 A). Surprisingly, this peptide did block Wnt1 signaling, suggesting that although Wnt9B and Wnt1 are both believed to bind to propeller 1 of LR P6, they use distinct binding sites. The sclerostin loop 2 binding site must overlap (or be close to) the Wnt1 binding site in propeller 1 because even a very short peptide (peptide 6) blocks both sclerostin binding and Wnt1 signaling. Interestingly, sclerostin inhibits signaling from both Wnt9B and Wnt1 (supplemental Figs. 5 and 7) possibly because of steric effects associated with its larger size.

We have previously suggested a role for the highly flexible amino- and carboxyl-terminal arms of sclerostin in stabilization of interactions with target proteins (28). Both arm regions exhibit a similar degree of sequence conservation as the remaining parts of sclerostin, which is consistent with them having functional importance. However, data presented here show that any function of the arms is not mediated through an interaction with the amino-terminal region of LR P6 region. NMR data for sclerostin bound to LR P6-E1E2 showed that the high flexibility of these regions is retained in the complex, and the binding is exclusively mediated by the central structured core of sclerostin. It remains possible that the arms interact with more membrane proximal regions of LR P6 or with other molecules such as frizzled receptors that are involved in Wnt signaling.

The work presented here supports a key role for the NXI motif in the interaction of sclerostin with LR P5/6 but not LR P4. It also shows that a sclerostin-derived NXI containing peptide can inhibit Wnt1 but not Wnt9B or Wnt3A canonical signaling.

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