The First Propeller Domain of LRP6 Regulates Sensitivity to DKK1


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The Wnt coreceptor LRP6 is required for canonical Wnt signaling. To understand the molecular regulation of LRP6 function, we generated a series of monoclonal antibodies against the extra cellular domain (ECD) of LRP6 and selected a high-affinity mAb (mAb135) that recognizes cell surface expression of endogenous LRP6. mAb135 enhanced Wnt dependent TCF reporter activation and antagonized DKK1 dependent inhibition of Wnt3A signaling, suggesting a role in modulation of LRP6 function. Detailed analysis of LRP6 domain mutants identified Ser 243 in the first propeller domain of LRP6 as a critical residue for mAb135 binding, implicating this domain in regulating the sensitivity of LRP6 to DKK1. In agreement with this notion, mAb135 directly disrupted the interaction of DKK1 with recombinant ECD LRP6 and a truncated form of the LRP6 ECD containing only repeats 1 and 2. Finally, we found that mAb135 completely protected LRP6 from DKK1 dependent internalization. Together, these results identify the first propeller domain as a novel regulatory domain for DKK1 binding to LRP6 and show that mAb against the first propeller domain of LRP6 can be used to modulate this interaction.

INTRODUCTION

The canonical Wnt signaling pathway has been clearly established as a critical pathway during development and disease (Logan and Nusse, 2004). In the adult, Wnt signaling appears to play an important role in regulating tissue maintenance and repair, including the regulation of intestinal crypt epithelial cell proliferation (Korinek et al., 1998; Reya and Clevers, 2005; Radtke et al., 2006) and bone homeostasis (Johnson et al., 2004; Baron et al., 2006; Baron and Rawadi, 2007). Canonical Wnt signaling is initiated upon binding of a Wnt ligand to a Frizzled (FZD) receptor and one of two coreceptors, LDL receptor related proteins 5 or 6 (LRP5/6). Activation of the receptor complex leads to the accumulation of beta-catenin and subsequent initiation of beta-catenin/T-cell factor (TCF)-mediated gene transcription, by inactivation of a glycogen synthase kinase-3 (GSK-3)/Casein kinase-1/Axin containing beta-catenin destruction complex followed by the release of beta-catenin (Yamamoto et al., 2006; Khan et al., 2007). In addition, recent findings suggest that inactivation of the destruction complex may involve direct inactivation of GSK-3 by the phosphorylated PPS/TP-xS motif (Piao et al., 2008).

The activity of LRP5/6 receptors is regulated by the secreted LRP5/6 binding inhibitors DKK1 and 2 that prevent Wnt dependent interaction of LRP6 with FZD receptors. In addition, DKK1 has been shown to remove LRP5/6 from the cell surface by targeting the receptors for internalization by Kremen receptors in certain cell types and tissues such as bone (Mao et al., 2001; Semenov et al., 2001; Mao et al., 2002; Semenov et al., 2008; Ellwanger et al., 2008). LRP5 and -6 are large (200 kDA) type I transmembrane proteins consisting of four subunits, each comprising a conserved YWTD repeat beta-propeller fold and a conserved EGF like domain, followed by three LDL receptor type A repeats, a transmembrane region, and cytoplasmic tail. Whereas the first two propeller domains have been reported to be required for cooperation with Wnt, the third domain has been shown to be required for interaction with DKK1 (Mao et al., 2002; He et al., 2004; Zhang et al., 2004).

The importance of LRP5 and -6 for Wnt signaling is highlighted by deletions or mutations in patients and mice. De-
letion of LRP6 causes severe developmental defects, most notably in the brain, limb, and eye, which appear a composite of individual Wnt mutations (Pinson et al., 2000). In contrast, deletion of LRP5 causes a restricted bone loss phenotype, which mimics the osteoporosis-pseudoglioma syndrome in patients with mis-sense mutations or deletions in LRP5 (Gong et al., 2001). The low bone mass phenotype of LRP5-deficient mice is exacerbated by crossing these mice into an Lrp6+/− background (Holmen et al., 2004), indicating that both LRP5 and -6 are important regulators of bone density.

Because LRP6 is an important regulator of canonical Wnt signaling, we generated monoclonal antibodies (mAbs) against the extracellular domain (ECD) of LRP6 and used them to dissect the functional domains required for LRP6 function. Here, we describe a mAb (mAb135) specific to the first WYTD β-propeller domain (propeller) of LRP6 and provide data implicating this region in the regulation of receptor function and DKK1 interaction.

MATERIALS AND METHODS

Reagents and Constructs

LRP6 deletion constructs LRP6Δ1-2 and LRP6Δ1-4 were generated by cloning LRP6 nt 1891-4842 or nt 3730-4842 into the vector plntrongknHa/H, in which inserts are fused to an N-terminal HA tag and protein secretion and membrane anchorage is guided by an IgKappa signal peptide. LRP6Δ2-4 and LRP6Δ3-4 were generated by stitching PCR, assembling nt 58-975 and 3730-4842 or nt 58-1884 and 3730-4842, respectively, followed by cloning of the resulting PCR products into plntrongknHa/H. Human/mouse chimeric constructs LRPs1-1a, LRPs1-1b, and LRPs1-1c derived from the LRPs1-3-4 construct were generated by replacing the original human LRP6 nt 58-459, 460-699, or 700-972 with the corresponding homologous mouse LRP6 sequence by stitching PCR. LRP6 single point mutation constructs used for fine epitope mapping were derived from the LRPs1-3-4 construct using the method of Quickchange site-directed mutagenesis according to manufacturers instructions (Stratagene, La Jolla, CA).

The extracellular regions of LRPs comprising the four WYTD β-propellers and EGF-like domains were cloned into the pACGP67 baculovirus transfer vector (BD Biosciences). A C-terminal hexa-histidine tag was engineered onto the end of the protein. Full-length Dkk1 was cloned into the same vector with a C-terminal His tag. For expression, high-five cells were infected with the LRPs6 or Dkk1 viruses. After 72 h, the cells were spun down and the supernatant loaded onto a Ni2+-NTA column (Qiagen). The eluate was subsequently loaded onto a Superdex 200 column for additional purification.

Luciferase Reporter Assays, Cytosolic Beta-Catenin Assay, and LRP6 Phosphorylation Assay

A 16TCT luciferase reporter construct was generated by cloning 16 repeats of a TCF consensus site (AGATCAAAGG) (van Beest et al., 2000) into the pTA-Luc vector (Clontech, Mountain View, CA). A genicelink selectable marker was inserted into the vector and used to select a stable HEK-293 clone (A) exhibiting minimal basal reporter activation. HEK-293 A6 cells were seeded in 96-well plates in Dulbecco’s Modified Eagle Medium (DMEM) containing 10% FBS (Invitrogen, Carlsbad, CA), starved for 8 hi nDMEM (A6) containing 10% FBS (Invitrogen) for transfection in 6-well plates and allowed to attach overnight.

LRP6 Expression, FACS Analysis, and siRNA Experiments

2.5 × 10⁶ HEK 293T cells were seeded in DMEM containing 10% FBS (Invitrogen) for transfection in 6-well plates and allowed to attach overnight. FugeneHD (Roche, Indianapolis, IN) was used to transfect 2 μg of each LRP6 plasmid or vector control DNA per well according to the manufacturer’s protocol. 48 h post transfection cells were treated with Trypsin/EDTA (Invitrogen) for FACS analysis. After fixation and permeabilization, cells were stained with antibody, FBS in DMEM media, and cells were counted and washed into FACS Buffer (PBS without Ca2+ /Mg2+ (Invitrogen) containing 0.5% BSA, 0.05% Sodium Azide, and 2 mM EDTA). 5 × 10⁶ cells/ml/sample were stained individually with primary antibodies for 20 min on ice using 1 μg/ml Anti-HA-PE (Miltenyi Biotech, Auburn, CA), 100 ng/ml anti-LRP6 mAb135 and 100 ng/ml Mouse IgG2a Isotype Control (BD Biosciences). Cells were washed with FACS Buffer and incubated with secondary antibody (PE labeled Goat Anti-Mouse Ig; BD Biosciences) at a 1/50 dilution. Cells were washed twice with FACS Buffer and resuspended in 200 μl for FACS analysis using a FACStar (BD Biosciences).

HEK293 or HEK292T cells were transfected with LRP6 siRNA duplexes (GCCUACAAUGUUCACUGA) or control siRNA complexes (negative control no.1) purchased from Ambion (Austin, TX) using siRNAlo- Lectinamine RNAiMAX transfection reagent (Invitrogen). 48 h posttransfection cells were analyzed by FACS using mAb135 as described above, or by western analysis using polyclonal anti-LRP6 antibodies (Cell Signaling Technologies, Danvers, MA).

Binding Studies with Recombinant LRP6 and DKK1 Proteins

LRP6 extracellular constructs containing the four WYTD β-propeller and EGF-like domain repeats, or deletions of the first two or last two repeats, were cloned into the pACGP67 baculovirus transfer vector (BD Biosciences). A C-terminal hexa-histidine tag was engineered onto the end of the protein. Full-length Dkk1 was subcloned into the same vector with a C-terminal His tag. For expression, high-five cells were infected with the LRPs6 or Dkk1 viruses. After 72 h, the cells were spun down and the supernatant loaded onto a Ni2+-NTA column (Qiagen). The eluate was subsequently loaded onto a Superdex 200 column for additional purification.

Five micrograms of recombinant His tagged LRP6 ECD, LRP6 deletion mutant Δ1-2, and mutant Δ3-4 were individually incubated with DKK1-bis (1 μg), and proteins were injected in the presence or absence of mAb135 (15 μg) in a total volume of 10 μl onto a Superose 6 PC 3.2/30 column (GE Healthcare, Piscataway, NJ) and run at a flow rate of 0.05 ml/min in running buffer (50 mM Tris pH 8.0, 0.33 M NaCl) using an AKTA explorer chromatography system (GE Healthcare). 100 μl fractions were collected and analyzed by Western blotting using anti-DKK1 polyclonal antibodies (R&D systems). Molecular weight standards (GE Healthcare) were run in the same manner for comparison.

LRP6 Internalization Assays

Confoocal microscopy analysis was used to analyze the cellular distribution of LRP6 in HEK-293 cells transfected with LRP6-HA and Kremen1. Cells were seeded onto 12-mm glass coverslips coated with poly-D lysine (BD Biosciences, Bedford, MA). The following day cells were transfected with LRP6-HA and Kremen1 plasmids using Fugene 6 reagent (Roche). Cells were serum starved overnight 24 h post transfection and were pretreated with mAb135 at 37°C for 1 h followed by treatment with Dkk1 at 37°C for 10 min. Cells were washed three times with ice-cold PBS, fixed in 4% paraformaldehyde in PBS for 20 min on ice, and permeabilized in permeabilization buffer (50 mM NaCl, 300 mM sucrose, 3 mM MgCl2, 10 mM Pipes pH 6.8, and 0.5% TX-100) on ice. Blocking buffer was a Superblock (Pierce) and cells were incubated with Alexa-594 conjugated anti-HA-mAb (Invitrogen). Stained specimens were mounted in anti-fade reagent (Prolong Gold anti-fade reagent, Invitrogen) and examined using a Zeiss confocal laser-scanning microscope LSM510 with 63X objective (Zeiss, Thornwood, NY).

Real-Time Binding Analysis

Surface plasmon resonance was carried out on a Biacore (Piscataway, NJ) system as described previously (Katsamba et al., 2006). Briefly, mAbs were diluted to 2 μg/ml and then captured on the biosensor surface using an anti-mouse mAb. Antigen (hLRP6 ECD-Fc) was diluted to a starting concentration of 46 nM and tested for binding to the mAb samples using a threefold dilution series. Each of 5 concentrations was tested twice except the highest concentration which was tested 5 times in total. DKK1 dissociation was monitored for 30 s and then 3 times with a dissociation of 60 min. The data sets from the dissociation experiments were globally fit with the association experiments to determine binding constants for the interactions. The analysis was carried out in 10 mM Hepes, 150 mM NaCl, 1.4 mM edTA, 0.05% Tween-20, pH 7.4 buffer at 25°C.

RESULTS

Generation of mAbs Against the Extracellular Domain of LRP6

The beta-propeller domains 3 and 4 in LRP6 have been implicated in DKK1 interaction, whereas beta-propeller
domains 1 and 2 are required for Wnt responsiveness (Mao et al., 2001; Itasaki et al., 2003). To further analyze the regulation of LRP6 function, we generated monoclonal antibodies (mAbs) against the human LRP6 extracellular domain (ECD). LRP6 mAbs were screened by ELISA for specific interaction with recombinant LRP6 ECD and for recognition of cell-surface expressed LRP6 by flow cytometry. Of a series of mAbs that specifically recognized human LRP6, we selected one mAb (mAb135) for further characterization. As shown in Figure 1A, mAb135 selectively recognized human and cynomolgous LRP6 overexpressing cells, but not cells overexpressing mouse LRP6. In addition, mAb135 recognized cell surface expression of endogenous LRP6 in HEK 293T cells as shown by FACS analysis (Figure 1, A and C). To confirm the specificity, we used LRP6 specific siRNA duplexes to knockdown endogenous LRP6 expression. As shown in Figure 1C, transfection of LRP6 specific siRNA into HEK293 cells abrogated cellular binding of mAb135, confirming that mAb135 specifically recognizes endogenous LRP6.

We next analyzed the direct binding of mAb135 to hLRP6 ECD by surface plasmon resonance and determined mAb135 affinity. As shown in Figure 1C, mAb135 bound hLRP6 with a \( K_d \) of 0.7 nM, indicating that mAb135 binds human LRP6 with high-affinity.

**mAb135 Enhances Wnt3A Signaling**

To investigate the effect of mAb135 on Wnt signaling, we used a TCF luciferase reporter assay in HEK-293 cells stably expressing a 16TCF luciferase reporter (Binnerts et al., 2007). HEK-293 cells were incubated with recombinant Wnt3A in the presence or absence of increasing amounts of mAb135. As shown in Figure 2A, mAb135, but not isotype control mAb, increased Wnt3A-dependent TCF reporter activity (up to sevenfold) in a dose dependent manner, indicating that a direct interaction of mAb135 with LRP6 can enhance the magnitude of the Wnt3A response. Similarly, increasing amounts of mAb135 not only increased the amplitude of the Wnt3A signal but also lowered the minimal dose of Wnt3A required for induction of TCF reporter activation, indicating that mAb135 renders cells more sensitive to Wnt3A signaling (Figure 2B). Interestingly, these effects were not observed when cells were incubated with Fab fragments of mAb135 (Figure 2, A and B), suggesting that enhancement of Wnt3A signaling by mAb135 requires antibody mediated dimerization of LRP6.

To confirm these findings, we also analyzed the effect of mAb135 on Wnt3A-dependent accumulation of cytosolic beta-catenin, by treating HEK-293 cells with different doses of Wnt3A. As shown in Figure 2C, cotreatment with mAb135 increased Wnt3A dependent beta-catenin accumulation at all
Wnt3A doses tested, confirming that interaction of mAb135 with LRP6 enhances the amplitude of Wnt signaling.

mAb135 Protects LRP6 From DKK1 Inhibition

Because we previously found that HEK293 cells express low levels of endogenous DKK1 (Binnerts et al., 2007), one explanation for the enhancement of Wnt signaling observed with mAb135 could be that mA135 protects LRP6 from endogenous DKK1. It was previously shown that the Wnt inhibitor DKK1 interacts with the third and fourth propeller domains of LRP6 to couple LRP6 with Kremen and subsequently target the receptor complex for internalization. To further understand the molecular mechanism of action of mAb135, we therefore tested whether mAb135 affects DKK1 activity. As previously reported (Mao et al., 2001; Mao et al., 2002, Binnerts et al., 2007), DKK1 completely inhibited Wnt3A dependent reporter activation. However, in the presence of mAb135 and DKK1, Wnt3A dependent reporter activation was not only restored, but enhanced ~eight to tenfold (Figure 3A) relative to cells treated with Wnt3A alone. Furthermore, the effect of mAb135 on DKK1 inhibition was even more significant on cells that were incubated with increasing amounts of DKK1 in the presence of a fixed amount of mAb135 IgG, isotype control IgG (10 µg/ml), or mAb135 Fab (3.3 µg/ml) and analyzed for reporter activation. (C) HEK 293 cells were treated with increasing amounts of Wnt3A in the absence or presence of mAb135 (10 µg/ml) and analyzed for cytosolic beta-catenin by Western blotting.

Figure 2. mAb135 enhances Wnt signaling. (A) HEK 293 A6 stable 16TCF reporter cells were treated with media control or Wnt3A (200 ng/ml) in the presence of increasing concentrations of mAb135 IgG, mAb135 Fab, or isotype control IgG. (B) HEK 293 A6 cells were treated with increasing amounts of Wnt3A in the presence of a fixed amount of mAb135 IgG, isotype control IgG (10 µg/ml), or mAb135 Fab (3.3 µg/ml) and analyzed for reporter activation. (C) HEK 293 cells were treated with increasing amounts of Wnt3A in the absence or presence of mAb135 (10 µg/ml) and analyzed for cytosolic beta-catenin by Western blotting.

mAb135 Binding Maps to the First Propeller Domain of LRP6

To investigate how mAb135 regulates LRP6 activity, we first delineated the mAb135 binding site within LRP6. A series of HA-tagged LRP6 deletion mutants lacking different YWTD-EGF-propeller domains were constructed as schematically described in Figure 4A, transfected into HEK293T cells and cell surface binding of mAb135 to these mutants was analyzed by flow cytometry. As shown in Figure 4B, all LRP6 mutants were expressed to similar levels, as detected by staining with anti-HA antibodies. However, mAb135 detected full-length LRP6 (FL), LRP6 Δ3-4 and LRP6 Δ2-4 expressing cells, but not cells expressing LRP6 deletion mutants Δ1-2 or Δ1-4, indicating that the first YWTD-EGF β-propeller domain in LRP6 is required for mAb135 binding.

Because mAb135 does not cross react with the mouse LRP6 orthologue, we generated mouse/human LRP6 chimeric propeller domain 1 constructs to further map the
mAb135 Fab (D) (3.3 µg/ml), and analyzed for phosphorylated LRP6 by Western blotting with phospho-Ser1490 specific antibodies.

(F) HEK293 cells were treated with media control, Wnt3A (50 ng/ml), or Wnt3A plus DKK1 (400 ng/ml) in the presence or absence of mAb135 (10 µg/ml), and analyzed for reporter activity. (E) HEK293 cells were treated with media control, Wnt3A (50 ng/ml), or Wnt3A plus DKK1 (400 ng/ml) in the presence or absence of mAb135 (10 µg/ml) and analyzed for cytosolic beta-catenin by Western blotting. (F) HEK293 cells were treated with media control, Wnt3A (50 ng/ml), or Wnt3A plus DKK1 (400 ng/ml) in the presence or absence of increasing amounts of mAb135 and analyzed for phosphorylated LRP6 by Western blotting with phospho-Ser1490 specific antibodies.

Figure 3. mAb135 antagonizes DKK1. HEK293 A6 cells were incubated with increasing amounts of mAb135 IgG (A), mAb135 Fab (B), or isotype control antibody before treatment with Wnt3A (200 ng/ml) in the presence or absence of DKK1 (400 ng/ml). HEK293 A6 cells were treated with Wnt3A (200 ng/ml) and increasing amounts of DKK1, after addition of mAb135 IgG or isotype control IgG (C) (10 µg/ml), or mAb135 Fab (D) (3.3 µg/ml), and analyzed for reporter activity. (E) HEK293 cells were treated with media control, Wnt3A (50 ng/ml), or Wnt3A plus DKK1 (400 ng/ml) in the presence or absence of mAb135 (10 µg/ml) and analyzed for cytosolic beta-catenin by Western blotting. (F) HEK293 cells were treated with media control, Wnt3A (50 ng/ml), or Wnt3A plus DKK1 (400 ng/ml) in the presence or absence of increasing amounts of mAb135 and analyzed for phosphorylated LRP6 by Western blotting with phospho-Ser1490 specific antibodies.

mAb135 Competes with DKK1 for LRP6

Our results implicate propeller domain 1 in the regulation of DKK1 function. This contrasts with earlier data indicating that domain 3 plays a dominant role in DKK1 binding (Mao et al., 2002; Zhang et al., 2004). To further understand the molecular mechanism of DKK1 inhibition by mAb135, we analyzed the interaction of recombinant DKK1 with the recombinant extracellular domain of LRP6 (LRP6 ECD). Complex formation of purified proteins was analyzed using size exclusion chromatography and the effect of mAb135 on DKK1 binding to LRP6 ECD was evaluated. Figure 5A shows the migration of DKK1 when injected into the column alone. DKK1 migrated at an apparent molecular weight of 40 to 50 kDa, slightly larger than its predicted molecular weight of 30 kDa but clearly monomeric. In contrast, when DKK1 was co-injected with LRP6-ECD, a portion of DKK1 comigrated with LRP6 ECD at a molecular weight of ~200 kDa (Figure 5A), indicating an interaction between LRP6 and DKK1 proteins. Complex formation between the LRP6 ECD and DKK1 was prevented by preincubating LRP6 ECD with a fivefold molar excess of mAb135. Under these conditions...
DKK1 eluted as a monomer (Figure 5A), indicating that mAb135 protects LRP6 from DKK1 inhibition by interfering with binding of DKK1 to LRP6.

To further dissect binding of DKK1 to LRP6 and the effect of mAb135 on this interaction, we next analyzed protein complex formation of purified LRP6 propeller domain deletion mutants and DKK1, in the presence or absence of mAb135. As shown in 5B, a LRP6 mutant containing propeller domains 3–4 (LRP6 dom 3–4) migrated as a monomer of 60 kDa and bound to DKK1 as evidenced by comigration of the injected proteins, in agreement with published data that the third propeller domain of LRP6 contains a binding site for DKK1 (Zhang et al., 2004; Chen et al., 2008).

As expected, coincubation with mAb135 did not inhibit the interaction with DKK1, as this mutant does not contain the mAb135 epitope. We next analyzed migration of the LRP6 mutant containing propeller domains 1 and 2 (LRP6 dom 1–2) and found that in contrast to the LRP6 dom 3–4 mutant, this mutant migrated broadly, with peaks at ~80 kDa and ~160 kDa, suggesting exchange between monomeric and dimeric species. LRP6 dom 1–2 comigrated with DKK1 upon coinjection, indicating interaction between the proteins and supporting the existence of an additional DKK1 binding site within the first or second propeller domain of LRP6. Interestingly, coinjection with mAb135 prevented DKK1 binding to this mutant, suggesting that mAb135 inhibits binding of DKK1 to LRP6 by interfering with this novel DKK1 binding site located in propeller domain 1 or 2.

**mAb135 Antagonizes DKK1-Dependent Internalization of LRP6**

To assess the functional consequence of the reduction of DKK1 binding to the LRP6 by mAb135, we next monitored DKK1-dependent internalization of LRP6 by immunofluorescence microscopy, as previously described (Mao et al., 2002; Binnerts et al., 2007). 293 cells were cotransfected with HA-tagged LRP6 and Kremen1 proteins, and cell surface localization was analyzed using anti-HA antibodies (Figure 6). As previously reported, LRP6 was mostly localized to the cells surface in untreated cells, whereas treatment with DKK1 for 30 min caused LRP6 to internalize and localize into distinct intracellular punctae (Mao et al., 2002; Binnerts et al., 2007). In contrast, pretreatment with mAb135 prevented DKK1-dependent internalization of LRP6 and restored normal LRP6 cells surface levels.

**DISCUSSION**

The LRP6 receptor is a key regulator of canonical Wnt signaling, but the molecular mechanism of action is not fully...
understood. In this study we identified and characterized a novel antibody (mAb135) that interferes with DKK1 function and enhances canonical Wnt signaling. Using cellular and biochemical assays we have demonstrated that mAb135 inhibits DKK1 function by interfering with its binding to LRP6. We have also mapped the binding of mAb135 to the first LRP6 repeat domain, implicating this region in regulating binding of DKK1. It is unclear whether this occurs through direct competition for a DKK1 binding site in repeat 1 or whether the antibody influences the overall conformation of the LRP6 ECD such that binding of DKK1 to repeat 3, which is known to be essential for its inhibitory activity, is prevented.

The effect of mAb135 on the sensitivity of LRP6 to DKK1 is reminiscent of the effect of mutations in the first propeller domain of LRP5. Point mutations in LRP5 identified in human patients desensitize LRP5 to DKK1 and lead to enhancement of Wnt signaling in bone and subsequent high bone mass (HBM) phenotypes (Boyden et al., 2002; Little et al., 2002; Babij et al., 2003; Ai et al., 2005). These findings also point to the importance of the first WYTD-EGF/β-propeller domain in regulating sensitivity of LRP5/6 to DKK1. Interestingly, all HBM mutations in LRP5 identified to date as well as the critical residue (Ser 243) required for mAb135 binding in LRP6, map to loops located on the upper face of the first WYTD-EGF β-propeller domain (Bhat et al., 2007).

Figure 5. mAb135 inhibits interaction of recombinant DKK1 and LRP6 ECD proteins. Interaction of recombinant DKK1-his protein with full-length recombinant His-tagged LRP6 ECD (LRP6 ECD) (A) or recombinant LRP6 ECD domain mutants containing WYTD beta-propeller domains 1–2 or 3–4 (LRP6 Dom 1–2 and LRP6 Dom 3–4) (B) were evaluated by analyzing the mobility of DKK1 with or without LRP6 on an analytical Superose 6 size exclusion column, in the presence or absence of a fivefold molar excess of mAb135. Fractions were analyzed for the presence of DKK1 by Western blotting and the elution profile of DKK1 compared with the profile of molecular weight standards. In several experiments, we observed that free DKK1 bound the column and can be eluted with urea, therefore the recovery of free DKK1 was varied as presented in B.

Figure 6. mAb135 inhibits DKK1-dependent internalization of LRP6. HEK-293 cells were co-transfected with LRP6-HA and Kremen1 constructs, pretreated with mAb135 (5 μg/ml) for 1 h followed by treatment with recombinant DKK1 (200 ng/ml) for 10 min. Distribution of immunostained HA-tagged LRP6 was evaluated by confocal microscopy.
From these data it is tempting to speculate that an additional DKK1 binding site may be located on a binding interface located on top of the first WYTD-EGF beta-propeller domain of LRP6. This notion is supported by structural studies on the lipoprotein receptor (LDLR), which showed that a similar region on the surface of the single WYTD-EGF beta-propeller domain in LDLR is involved in inter- and intramolecular interactions of this receptor (Rudenko et al., 2002).

Previous studies have demonstrated DKK1 binding to propeller domains 3, but not propeller domains 1 and 2 using Alkaline-phosphatase (AP)-tagged DKK1 and cells overexpressing LRP6 (Mao et al., 2002; Zhang et al., 2004). We adopted the same assay conditions and were unable to block DKK1-AP binding to cells over expressing LRP6 with mAb135 (data not shown). These results suggest that overexpression conditions may change LRP6 receptor dynamics and subsequently affect DKK1 and Wnt responsiveness. In agreement with this notion, it has previously been demonstrated that overexpression of LRP6 can activate downstream signaling by organization of LRP6 into higher molecular weight clusters (Bilic et al., 2007; Zeng et al., 2008), suggesting that overexpressed multimerized LRP6 represents an activated state of the receptor, which may be less sensitive to DKK1 inhibition. It is also possible that the addition of the large AP moiety to DKK1 has unforeseen consequences on the binding of mAb135, for example acting by steric exclusion to prevent binding of the antibody. Our data indicate that the first propeller domain of LRP6 regulates DKK1 dependent inhibition of LRP6. A simple explanation for our findings is that DKK1 binds to LRP6 domains 1 and 2, likely with lower affinity than to domains 3 and 4, and that cooperative interaction of DKK1 with all of the four propeller domains is required for inactivation of LRP6. Interestingly, a recent molecular modeling study of DKK2 binding to LRPS (Chen et al., 2008) suggested that DKK2 could interact with WYTD domains 1 or 2, although it appeared to bind propeller domain 3 with the highest affinity. If DKK1 interacts directly with domain 1, mAb135 may prevent DKK1 binding by direct competition for a binding site. Alternatively, our results would also be consistent with mAb135 stabilizing an active conformation of LRP6 that is unable to bind to DKK1. The ability of LDLR-like receptors to adopt multiple conformations is suggested by studies of the LDL receptor, which revealed that internalization of LDL occupied receptor into endosomes causes the receptor to adopt a new conformation in which the N-terminal LDL repeats interact with the membrane proximal beta-propeller domain, effectively displacing LDL from the receptor (Rudenko et al., 2002).

Because the effect of mAb135 on DKK1 function is similar to the effect of high bone mass mutations in LRPS, it will be interesting to determine whether treatment with mAb135 will have a positive impact on bone density in animals, as LRP6 has also been implicated in regulating bone density (Holmen et al., 2004). The hypothesis that mAb135 may have therapeutic applications in bone is further supported by the recent finding that Kremen receptors are critical regulators of bone density (Ellwanger et al., 2008), because we found that mAb135 can antagonize Kremen/DKK1-dependent internalization of LRP6.

Together, our results have revealed novel insights into the regulation of LRP6 function and have validated mAb135 as a novel agent for manipulating LRP6 activity, which may provide therapeutic opportunities for treatment of DKK1 dependent diseases.

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