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 that marks beta-catenin for degradation by the proteasome (Gordon and Nusse, 2006). Although the specificity and initiation of canonical Wnt signaling appears to be determined by a specific interaction between a Wnt ligand and the appropriate FZD receptor, the association of LRP5/6 receptors with the FZD/Wnt receptor complex is critical for the transmission and amplification of the canonical Wnt signal (reviewed in (He *et al.*, 2004)). Wnt binding to the

receptor complex triggers phosphorylation of five PPS/TP-xS motifs in the LRP6 cytoplasmic tail, which subsequently results in the recruitment of the Axin destruction complex (Davidson *et al.*, 2005; Zeng *et al.*, 2005; Bilic *et al.*, 2007; Zeng *et al.*, 2008). How these events lead to inactivation of the destruction complex and subsequent accumulation of beta-catenin still remains controversial. One explanation is that inactivation may involve endocytosis of the LRP6/Axin 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-

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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 LRP6 Δ 1-2 and LRP6 Δ 1-4 were generated by cloning LRP6 nt 1891-4842 or nt 3730-4842 into the vector pIntronIgK.nHA, in which inserts are fused to an N-terminal HA tag and protein secretion and membrane anchorage is guided by an IgKappa signal peptide. LRP6 Δ 2-4 and LRP6 Δ 3-4 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 pIntronIgK.nHA. Human/mouse chimeric constructs LRP6-1a, LRP6-1b, and LRP6-1c derived from the LRP6 Δ 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 LRP6 Δ 3-4 construct using the method of Quick-change site-directed mutagenesis according to manufacturers instructions (Stratagene, La Jolla, CA).

The extracellular regions of LRP6 comprising the four YWTD β -propellers and EGF-like domains were cloned into the pACGP67 baculovirus transfer vector (BD Biosciences, San Jose, CA). A C-terminal hexa-histidine tag was engineered onto the end of the protein. Full-length Dkk1 was subcloned into the same vector, and a C-terminal hexa-histidine tag was also engineered into the vector. For expression, high-five cells were infected with the LRP6 or Dkk1 viruses for protein purification from the supernatant. After 72 h, the cells were spun down and the supernatant was loaded onto a Ni-NTA column (Qiagen, Valencia, CA). 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 16TCF luciferase reporter construct was generated by cloning 16 repeats of a TCF consensus site (AGATCAAGG) (van Beest *et al.*, 2000) into the pTA-Luc vector (Clontech, Mountain View, CA). A geneticin selectable marker was inserted into the vector and used to select a stable HEK-293 clone (A6) 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 h in DMEM containing 0.1% FBS, treated in triplicate for 18 h with indicated proteins, and reporter activity determined using a Veritas Luminometer (Turner Biosystems, Sunnyvale, CA).

HEK293 seeded in 6-well plates and serum starved ON in DMEM with 0.1% FBS were pretreated with mAb135 at indicated concentrations for 30 min at RT, followed by DKK1 treatment (1.5 h at 37°C) and subsequent treatment with Wnt3A (3 h at 37°C). Cytosolic extracts were prepared and analyzed for beta-catenin by Western blotting as described previously (Binnerts *et al.*, 2007).

HEK293 seeded in 6-well plates and serum starved ON in DMEM with 0.1% FBS were treated with mAb135 at indicated concentrations for 10 min at RT followed by treatment with Wnt3A or DKK1 for 4 h at 37°C. Cells were washed with PBS collected into lysis buffer (Invitrogen) and analyzed by Western blotting using anti-LRP6 phospho-serine 1490 antibodies (Cell Signaling Technology, Danvers, MA).

LRP6 Expression, FACS Analysis, and siRNA Experiments

2.5×10^5 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 μ g of each LRP6 plasmid or vector control DNA per well according to the manufacturer's protocol. 48 h post transfection cells were detached with Trypsin/EDTA (Invitrogen) for FACS analysis. Trypsin was neutralized with 10% FBS DMEM media, and cells were counted and washed into FACS Buffer (PBS without $\text{Ca}^{2+}/\text{Mg}^{2+}$ (Invitrogen) containing 0.5% BSA, 0.05% Sodium Azide, and 2 mM EDTA). 5×10^5 cells/ml/sample were stained individually with primary antibodies for 20 min on ice using 1 μ g/ml Anti-HA-PE (Miltenyi Biotech, Auburn, CA), 100 ng/ml hLRP6 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 two times with FACS Buffer and resuspended in 200 μ l for FACS analysis using a FACStar (BD Biosciences).

HEK293 or HEK293T cells were transfected with LRP6 siRNA duplexes (GGCUACAAAUGUUAUCGAtt) or control siRNA complexes (negative control no.1) purchased from Ambion (Austin, TX) using siRNA Lipofectamine 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 YWTD β -propeller+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 His₆ tag. For expression, high-five cells were infected with the LRP6 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 Δ 1-2, and mutant Δ 3-4 were individually incubated with DKK1-his (1 μ g), and proteins were injected in the presence or absence of mAb135 (15 μ g) in a total volume of 10 μ l 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, 0.3M NaCl) using an ÄKTA explorer chromatography system (GE Healthcare). 100 μ l 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

Confocal 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). After blocking for 30 min in Superblock (Pierce, Rockford, IL), 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 μ g/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, two times with a short dissociation of 300 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

Figure 1. mAb135 recognizes endogenous LRP6 with high-affinity. (A) 293T cells were transfected with indicated HA-tagged LRP6 constructs. Transfected cells were stained with anti-HA or mAb135 antibodies and analyzed by flow cytometry. (B) mAb135 was immobilized on an anti-mouse sensor chip surface and its affinity for hLRP6 ECD-Fc determined by BIAcore analysis. (C) HEK 293 cells were transfected with control or LRP6 specific siRNA duplexes (10 nM) and analyzed for endogenous LRP6 levels by Western blotting with anti-LRP6 polyclonal antibodies. Recognition of endogenous LRP6 by mAb135 in siRNA-treated cells was analyzed by FACS analysis.

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

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.

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 mAb135 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 $\approx$ 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 (Figure 3B). Although 100 ng/ml DKK1 was sufficient to completely block Wnt3A activity, mAb135 could restore Wnt3A activity even at excess amounts of DKK1 (3200 ng/ml). In contrast to their lack of effect in enhancing Wnt activity, Fab fragments derived from mAb135 were also able to interfere with DKK1 activity to restore Wnt3A signaling (Figure 3, C and D), indicating that LRP6 dimeriza-

tion is not required for mAb135 dependent DKK1 inhibition. In agreement with the reporter activity, mAb135 treatment also restored Wnt3A dependent beta-catenin accumulation in cells cotreated with DKK1 (Figure 3E).

To further extend these observations, we next evaluated the effect of mAb135 on LRP6 phosphorylation. Wnt3A-dependent phosphorylation of endogenous LRP6 was blocked by DKK1, whereas phosphorylation of LRP6 was restored in the presence of mAb135, confirming that mAb135 binding to LRP6 protects it from inhibition by DKK1 (Figure 3F).

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 β -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 Δ 3-4 and LRP6 Δ 2-4 expressing cells, but not cells expressing LRP6 deletion mutants Δ 1-2 or Δ 1-4, 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

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.

binding site on LRP6 (Figure 4C). Staining with anti-HA mAb confirmed cell surface expression of chimeric constructs (Figure 4D), indicating that they are likely to be correctly folded. Although substitution of regions A or B within propeller 1 with corresponding mouse sequence did not affect mAb135 binding, no binding was detected in a LRP6 chimera with a region C substitution, indicating that mAb135 binds to the C-terminal part of the first propeller domain of LRP6 (Figure 4D). Interestingly, within this region only three amino acids differed among human and mouse LRP6 (Figure 4E: Ile236, Ser243, Asp264). We therefore substituted these individual amino acids within the human sequence with the equivalent mouse residues and analyzed the binding of mAb135. As shown in Figure 4F, we found that only substitution of Ser243 to Asn completely inhibited antibody binding, indicating that Ser243 within the first propeller domain of LRP6 is required for mAb135 binding. Finally, we mutated Asn243 in mouse LRP6 to Ser and analyzed binding of mAb135. As shown in Figure 4G, the Asn to Ser mutation restored binding of mAb135 to mouse LRP6, confirming that Ser243 is critical for recognition by mAb135.

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 coinjected 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

Figure 4. mAb135 maps to an epitope in the first β -propeller domain of LRP6. (A) Diagram of LRP6 propeller domain mutants. (B) HEK293T cells transfected with the indicated LRP6 domain mutants were analyzed for binding of anti-HA mAb or mAb135 by FACS analysis. (C) Diagram of LRP6 propeller domain 1 human/mouse chimeric constructs. (D) HEK293 T-cells were transfected with the indicated human/mouse chimeric LRP6 propeller domain 1 constructs and analyzed for HA and mAb135 binding by FACS analysis. (E) Outline of amino acid differences between human and mouse LRP6 in the C-terminal region of the first propeller domain. (F) HEK293T cells were transfected with indicated LRP6 propeller domain 1 human → mouse substitution mutants and analyzed for anti-HA and mAb135 binding as described above. (G) HEK293T cells were transfected with indicated LRP6 propeller domain 1 mouse → human substitution mutants and analyzed for anti-HA and mAb135 binding.

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, coincubation with mAb135 prevented DKK1 bind-

ing 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 immuno-fluorescence 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

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.

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 6. mAb135 inhibits DKK1-dependent internalization of LRP6. HEK-293 cells were co-transfected with LRP6-HA and Kremen1 constructs, pretreated with mAb135 (5 μ g/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 β -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 β -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 over-expression 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 LRP5 (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 surface. 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 structures 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 LRP5, 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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