Supporting Information

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SI Materials and Methods

Generation of Human LRP6 Antibodies from the HuCAL GOLD Library. The HuCAL GOLD antibody library was used for selection of LRP6 specific antibodies. In this phage-display library, human Fab fragments are coupled to the phage-gIII protein through a disulfide bond a method called CysDisplay (1). LRP6-specific antibodies were identified by whole cell-based panning and recombinant protein-based panning using protocols described previously (2). For whole-cell panning, HEK293 cells stably expressing LRP6ΔC-GFP and MESD were incubated with pools of HuCAL GOLD phage. After multiple washes, phages were eluted and adsorbed with HEK293 cells stably transfected with LRP5/6 shRNA to minimize the generation of unspecific cell binders. For recombinant protein-based panning, Fc-LRP6 proteins (R&D Systems) were captured by antihuman IgG-Fc coated on Maxisorp plates and incubated with pools of HuCAL GOLD phage. After several rounds of selection, the pool of phagemids, as amplified in *Escherichia coli*, and Fab-encoding fragments were excised as a pool and cloned onto the expression vector pMORPHX9_Fab_FS for transformation of TG1-F^-. Clones from whole-cell panning were screened in FACS on HEK293-LRP6ΔC cells and counterscreened on HEK293-LRP5/6 shRNA cells. Clones from Fc-capture panning were screened in an Fc-capture ELISA setup. Fab fragments with confirmed binding were expressed and purified. Conversion into IgG format was done by subcloning variable domain fragments of heavy and light chains from Fab expression vectors into the pMORH2 vector series. Anti-LRP6 IgGs were expressed in HEK293 cells and subsequently purified.

Plasmids. SuperTopFlash (STF) reporter was generated by inserting 12 TCF-binding sites into pTA-Luc (Clontech). Full-length and truncated LRP6 and LRP5 were fused with a signal peptide and Flag epitope at the amino termini and cloned into a mammalian expression vector under the control of the cytomegalovirus (CMV) promoter.

STF Luciferase Reporter Assay. HEK293 cells were grown in DMEM supplemented with 10% FBS at 37 °C with 5% CO_2. Cells were seeded into a 96-well tissue-culture plate (Costar) at 3×10^4/well and transfected with 0.1 ng/well Wnt-expression plasmid, 50 ng/well STF reporter, and 0.5 ng/mL phRL-SV40 (Promega) mixed with 0.2 µL/well FuGene6 (Roche). Four hours after transfection, antibodies were diluted in PBS and added to the transfected cells. After 18 h of incubation, Firefly luciferase and Renilla luciferase activities were measured using DualGlo Luciferase reagent (Promega). Renilla luciferase was used to normalize transfection efficiency. To obtain robust STF activation, Frizzled 8 was cotransfected with Wnt2, Wnt6, Wnt7a, Wnt7b, and Wnt10b, Frizzled 10 was cotransfected with Wnt9a, and Frizzled 5 was cotransfected with Wnt10a.

FACS Assay. For the FACS-based competition assay, anti-LRP6 Fab fragments and the negative-control Fab fragment were biotinylated using the ECL protein biotinylation module (GE Healthcare) according to the manufacturer's instructions. The biotinylated Fab fragments were used for FACS staining on HEK293-hLRP6ΔC-GFP cells at a constant Fab concentration (20 nM final concentration) and were competed with a 100-fold molar excess of unlabeled Fab. Cells were incubated with the Fab dilutions for 1 h at 4 °C on a plate shaker. After washing the cells one time with FACS buffer, they were incubated with PE-conjugated Streptavidin (Dianova) for 1 h at 4 °C on a plate shaker in the dark. Cells were washed two times with FACS buffer, and fluorescence was measured using FACS Array (BD). Similarly, unbiotinylated anti-LRP6 Fab fragments were competed with a 100-fold molar excess of the LRP6-binding protein Wnt antagonist Sclerostin (SOST), and binding of the Fab fragments to the cells was monitored by PE-conjugated antihuman IgG antibody (Dianova). For FACS-based epitope-mapping assay, HEK293 cells were cotransfected with MESD and N-terminal Flag-tagged full-length or truncated LRP6-expression constructs, and they were stained with anti-LRP6 Fab fragments, anti-Flag antibody (Sigma), or polyclonal anti-LRP6 antibodies (R&D Systems) and subjected to FACS analysis.

Immunoblotting Assay. Total cell lysates were prepared in RIPA buffer (50 mM Tris·HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA). Lysates were normalized for protein concentration, resolved by SDS/PAGE, transferred onto nitrocellulose membranes, and probed with the indicated antibodies. pT1479 LRP6 antibody requires generation of membrane extracts to achieve satisfactory results. To generate membrane extracts, cells were lysed in hypotonic buffer (10 mM Tris·HCl, pH 7.5, 10 mM KCl) by performing four freeze–thaw cycles, and the insoluble membrane fraction was isolated and solubilized using RIPA buffer. Protease inhibitor mixture (Sigma) and 1× phosphatase inhibitor mixture (Upstate) were added into the lysis buffers. Commercial antibodies used in the Western blot assay include rabbit anti-LRP6, rabbit anti-pT1479 LRP6, and rabbit anti-pS1490 LRP6 antibodies (Cell Signaling Technology).

MMTV-Wnt1 and MMTV-Wnt3 Xenografts. Tumors from MMTV-Wnt1 transgenic FVB mice were harvested and serially passaged as tumor fragments s.c. in female nude mice. MMTV-Wnt3 tumors were obtained from an MMTV insertional mutagenesis screen. Tumors carrying a single integration in the Wnt3 gene were harvested and passaged as tumor fragments in the mammary fat pad of nude mice before being serially passaged as tumor fragments s.c. in nude mice. For the MMTV-Wnt1 xenograft experiments, mice were randomized into groups with eight mice per group 11 or 19 d postimplant and dosed every 3 d (B2-IgG) or every 7 d (A7-IgG). For the MMTV-Wnt3 xenograft experiment, mice were randomized into three groups with eight mice per group 15 d postimplant and dosed two times a week with different antibodies at indicated doses and schedules. Tumors and body weight were measured two times a week. Tumor volume (TV) was calculated using the formula: TV (mm^3) = [length (mm) × width $(mm)^2$]/2, where the length and width are the longest and shortest diameter, respectively, of each tumor.

1. Rothe C, et al. (2008) The human combinatorial antibody library HuCAL GOLD combines diversification of all six CDRs according to the natural immune system with a novel display method for efficient selection of high-affinity antibodies. *J Mol Biol* 376:1182–1200.

2. Rauchenberger R, et al. (2003) Human combinatorial Fab library yielding specific and functional antibodies against the human fibroblast growth factor receptor 3. *J Biol Chem* 278:38194–38205.

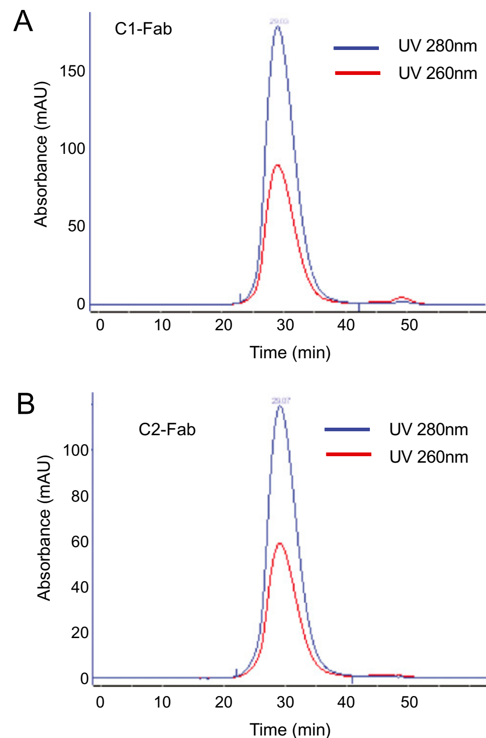

Fig. S1. Analysis of C1-Fab and C2-Fab using analytical size-exclusion chromatography. C1 (*A*) and C2-Fab (*B*) Fab fragments were analyzed by size-exclusion chromatography using Superdex 75 PC. Results indicate that both Fab fragments are over 99% monomeric.

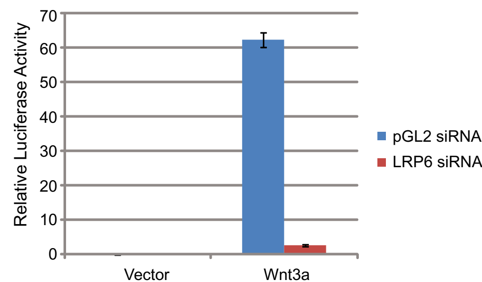

Fig. S2. LRP6 is essential for Wnt-induced STF activation in HEK293 cells. HEK293 cells were transfected with control pGL2 siRNA or LRP6 siRNA and then, transfected with STF reporter and empty vector or Wnt3a-expression plasmid. STF reporter activity was measured 24 h after STF transfection.

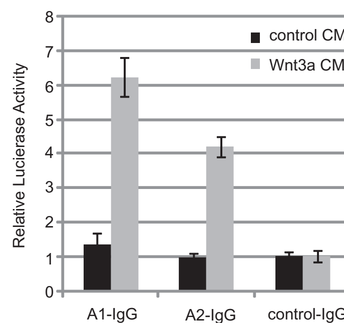

Fig. S3. Wnt1-specific antagonistic anti-LRP6 IgGs do not induce STF activation without Wnt treatment. HEK293 cells transfected with STF reporter were treated with indicated anti-LRP6 IgGs at 10 μg/mL in the presence of control- or Wnt3a conditioned medium. Luciferase-reporter activities were normalized against cells treated with control antibody.

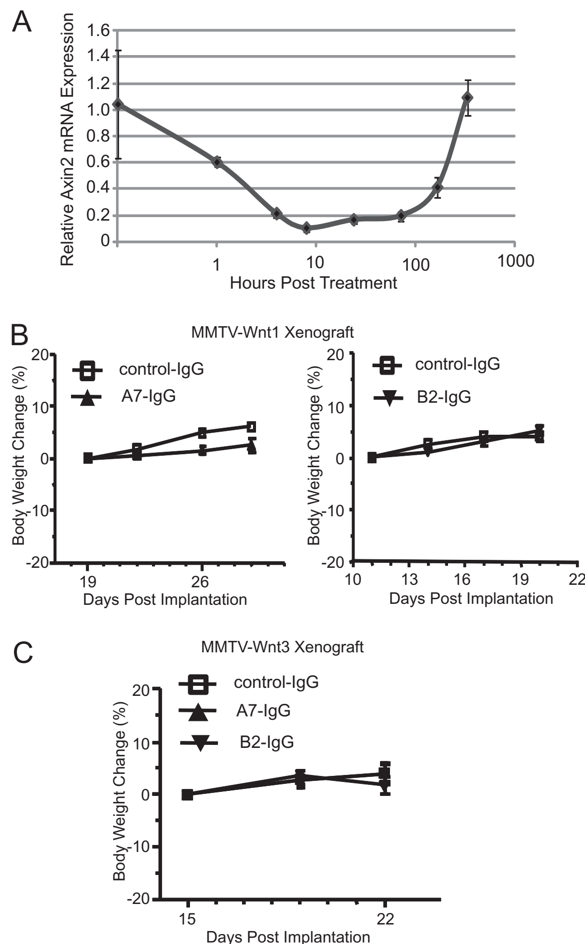

Fig. S4. Wnt1 or Wnt3a class-specific LRP6 antibody does not affect mouse body weight. (*A*) Wnt1 class-specific anti-LRP6 antibody inhibits Wnt signaling in MMTV-Wnt1 xenografts. Nude mice implanted with MMTV-Wnt1 tumors were dosed i.v. with a single dose of 5 mg/kg A7-IgG. The mRNA level of Axin2 was normalized to tumors from untreated mice. Time is plotted at the log scale to show the inhibition of Axin2 expression by anti-LRP6 antibody at early time points. (*B*) Wnt1 class-specific LRP6 antibody does not affect body weight in MMTV-Wnt1 xenograft experiments. Mice bearing established MMTV-Wnt1 xenografts were treated with either Wnt1 class-specific LRP6 antibody (A7-IgG) at 4 mg/kg every 7 d or with Wnt3a class-specific LRP6 antibody (B2-IgG) at 10 mg/kg one time every 3 d. IgG served as negative control in both experiments. Body weight was measured every 3 d. (*C*) Wnt3a class-specific LRP6 antibody does not affect body weight in MMTV-Wnt3 xenograft experiment. Mice bearing established MMTV-Wnt3 tumor xenografts were treated with IgG control or Wnt3a class-specific anti-LRP6 antibody (B2-IgG) at 10 mg/kg two times a week or Wnt1 class-specific anti-LRP6 antibody (A7-IgG) at 3 mg/kg. IgG served as negative control. Body weight was measured two times a week.