Supporting Information

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SI Materials and Methods

Wnt Activity Reporter Gene Assay. The assay was carried out as previously described (1, 2). In brief, NIH 3T3 cells were seeded at 5×10^4 cells per well in 24-well plates and transfected with 0.1 μg GFP, 0.025 μg LEF-1, 0.075 μg LEF luciferase reporter plasmid, and 0.3 μg LacZ per well using Lipofectamine Plus (Invitrogen). Compounds or conditioned media containing WNT3A or Dickkopf (DKK) 1, control conditioned medium, or purified proteins (WNT3A, DKK1, -2, and -4, and sFRP4 are from R&D Systems) were added 24 h after transfection. Six hours later, cell extracts were collected and luciferase assays were performed. Luminescence intensity was normalized against fluorescence intensity of GFP.

DKK1-Alkaline Phosphatase Binding Assay. Dkk-binding assays were carried out as previously described (3). In brief, NIH 3T3 cells in 24-well plates were transfected with LacZ, Kremen1, low density lipoprotein-related protein (LRP) 5 or its mutant. One day later, cells were washed with cold washing buffer (HBSS containing BSA and NaN_3) and incubated with mouse DKK1- alkaline phosphatase (AP) conditioned medium (CM) on ice for two hours. Then, cells were washed three times with the washing buffer and lysed. The lysates were heated at 65 °C for 10 min, and its AP activity was determined using a Tropix luminescence AP assay kit. DKK1-AP was prepared from transfected HEK293 cells. The specific binding is calculated by subtracting the AP activity bound to cells expressing LacZ from that bound to cells expressing LRP5 or its mutant.

Direct Binding Assay. Biotinylated IIIC3a was synthesized by reaction of bromogallocyanine with aminobiotin. PBS (100 μL) containing 0.25% (wt/vol) BSA with or without 60 μM biotinylated IIIC3a was added into the wells of a streptavidin-coated 96-well plate (Pierce Biotechnology) and incubated overnight. After washing, CM containing AP-fused wild-type or mutant LRP5 extracellular domain was added into each well. The conditioned media were prepared from HEK293T cells 48 h after transfection, concentrated 50-fold using Centricon 100 filters (Millipore), and diluted to 1× by PBS containing 0.25% (wt/vol) BSA. The plate was incubated at room temperature for 1 h and washed three times with PBS containing 0.25% (wt/vol) BSA. AP activity was determined using a Tropix luminescence AP assay kit. The activity of AP specifically binding to IIIC3 was determined by subtracting the background, which is the AP activity in wells without IIIC3a.

For determination of the binding of the LRP5 extracellular domain-AP to DKK1 or SOST protein (R&D Systems), an ELISA 96-well plate was coated with the DKK1 or SOST protein (1 μg/mL) overnight. The binding assay was carried out as described above.

Bio-Layer Interferometer Experiment. The Octet RED instrument (FortéBio) was used to examine the binding of DKK2C to LRP6 third and fourth YWTD repeat domains (E3E4), which were prepared as previously described (4, 5). The 0.5 μg/mL of biotinylated S-tag antibody (Abcam) was incubated for 300 s to attach to the super streptavidin biosensors (FortéBio) and washed with buffer [5% (vol/vol) acetic acid, pH 6.5] for 180 s to remove the excess antibody. We used the 0.7 μM of S-tag DKK2C in 5% (vol/vol) Acetic acid, pH 6.5 and then washed the excess protein. To determine the binding of DKK2C to LRP6, we used the 0.3 nM of LRP6 for the association step for 600 s, and then used the buffer for dissociation step for 600 s. For the competition assays, 15 to ∼88 μM of IIIC3 solution was added into the 0.3 nM of LRP6 E3E4. The apparent K_D (K_D^{app}) value of DKK2C with LRP6 was obtained from seven independent experiments. The inhibition constant (K_I) of IIIC3 compound was calculated by using the equation, $K_D^{app} = K_D(1+[I]/K_I)$, where [I] is the concentration of IIIC3. Data were analyzed to obtain the kinetic constants such as k_{on} (on-rate constant), k_{off} (off-rate constant), and K_D with FortéBio analysis software (v6.4).

Animals. The *db/db* mice (BKS.cg-m$^{+/+}$ Leprdb/J) and Wnt reporter line [B6.Cg-Tg(BAT-lacZ)3Picc/J] were purchased from the Jackson Laboratory. Unless specifically indicated, the mice in all experiments are male. *Dkk2*$^{-/-}$ mice were backcrossed into C57 BL/6J background (more than 10 generations). All procedures were approved by the Yale University Animal Care and Use Committee.

Evaluation of the Effects of Compounds on Bone. IIIC3 was dissolved in DMSO and diluted 1:1,000 into PBS at a concentration of 0.44 mg/mL. For calvarial local injection, IIIC3, control vehicle, or positive controls (b-FGF, 12.5 μg·kg·d; WNT3A, 4 μg·kg·d) were injected into the subcutaneous tissue over the right side of the calvaria of 4-wk-old CD-1 mice three times a day for 5 d using an injection method described previously (6, 7). Calvarias were collected 22 d after the first injection and fixed for sectioning.

Routine Blood-Glucose Measurement, Glucose Tolerance Test, and Insulin Tolerance Test. For routine blood-glucose monitoring, blood samples were collected from tail veins (the fasting states are specified in the figure legends), and their glucose concentrations were determined using an OneTough Ultra blood glucose meter (LifeScan). For glucose tolerance test (GTT), mice were fasted for 16 h, and their blood samples were collected from tail veins for determining the basal blood-glucose concentrations. Then the mice were given an intraperitoneal injection of glucose (1 g/kg of body weight). Blood samples were collected from the tail veins at varying time points. Blood-glucose concentrations were determined using an OneTough Ultra blood glucose meter (LifeScan). Plasma insulin levels were determined using the Mouse Insulin ELISA kit (Linco Research).

For the insulin tolerance test (ITT), mice were fasted for 2 h and blood-glucose concentrations were measured. Then mice were injected with insulin (1 IU/kg) and blood-glucose concentrations were measured at different timepoints.

Basal Metabolic Study and Hyperinsulinemic-Euglycemic Clamp. Four-month-old *Dkk2*$^{-/-}$ mice (n = 8) and their littermate mice (n = 6) were fed with high-fat diet for 6 wk and then subjected to the basal metabolic study and hyperinsulinemic-euglycemic clamp experiment, as previously described (8).

Hepatic Glycogen Concentration Determination. Liver tissues (50 mg) from individual mice were lysed with 1 mL of 30% (wt/vol) KOH saturated with Na_2SO_4 at 95 °C for 30 min. The lysates (0.5 mL) were mixed with 0.6 mL of 95% (vol/vol) ethanol and incubated on ice for 30 min. The samples were centrifuged at $850 \times g$ for 30 min to precipitate glycogen. The supernatants were carefully aspirated, and glycogen precipitates were dissolved in 1 mL H_2O. The dissolved glycogen (4 μL) was added into wells of a 96-well plate with a flat and clear bottom containing 36 μL H_2O and 40 μL of 5% (vol/vol) phenol in each well. The wells were then added 200 μL of 96–98% (vol/vol) H_2SO_4 and incubated at 37 °C

for 15 min. The absorbance at 490 nm was determined using a spectrophotometer. In the same plate, varying concentrations of type XI glycogen (Sigma-Aldrich) were used to construct a standard curve. Glycogen concentrations were calculated based on the standard curve, tissue weight, and dilution factor.

Plasma Glucagon and Glucagon-Like Peptide 1 Determination. For plasma portal glucagon-like peptide 1 (GLP-1) quantification, fasted 8-wk-old mice were subjected to gavage administration of glucose (2 g/kg body weight). Blood samples (100 μL), to which dipeptidyl peptidase IV inhibitor (Millipore) was added, were obtained before gavage (time 0) and 10 min after gavage. Concentrations of plasma GLP-1 were determined by ELISA (Glucagon-Like-Peptide-1 active ELISA kit; Millipore).

For glucagon measurement, mice were fasted overnight and blood was collected by tail bleed. Glucagon levels were determined by RIA (Millipore; GL-32K).

Immunohistological and LacZ Staining. Intestines were fixed in fresh 4% (wt/vol) paraformaldehyde for 1 h at 4 °C and washed three times with a rinsing buffer [100 mM sodium phosphate, pH 7.3, 2 mM $MgCl_2$, 0.01% (wt/vol) sodium deoxycholate, 0.02% (vol/vol) Nonidet P-40], followed by staining [5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 1 mg/mL 5-bromo-4-chloro-3-indolyl β- D-galactoside (X-gal; Sigma)] for 4 wk at room temperature. The samples were fixed again in 10% (vol/vol) formalin overnight and embedded in paraffin.

For immunohistostaining, paraffin-embedded sections (5 μm in thickness) were mounted on glass slides, deparaffinized with xylene, and rehydrated through the ethanol series. The sections were then incubated in antigen retrieval solution (10 μg/mL proteinase K, 50 mM Tris-Cl,pH 8.0, 5 mM EDTA, pH 8.0) at 37 °C for 10 min and treated with 3% (vol/vol) H_2O_2 in methanol for 15 min to eliminate endogenous peroxidase. After blocking endogenous mouse IgG binding sites with the M.O.M mouse Ig blocking reagent (Vector Laboratories; Cat # PK-2200), the sections were further blocked with 5% (vol/vol) normal goat serum in PBS containing 0.1% (vol/vol) Tween-20 for 1 h at room temperature and then incubated with a GLP-1 antibody (Phoenix Pharmaceuticals) or a DKK2 antibody (9) overnight at 4 °C in a humid chamber. The sections were developed using kits from Vector Laboratories and counterstained with Hematoxylin.

Gene Expression Determination by Quantitative RT-PCR. Total RNAs were isolated from 10-mg mouse liver samples using the TRIzol reagent (Invitrogen) and were used for cDNA synthesis with the SuperScript II Reverse Transcription kit (Invitrogen) and random primers. Quantitative RT-PCR analysis was carried out using the iQ SYBR Green Supermix and a MyiQ real-time PCR machine (Bio-Rad). For determination of copy numbers of *Dkk* mRNAs, the standard curve for each target was generated by using varying concentrations of the plasmids carrying the cDNA of the target. The mRNA copy numbers were calculated based on the standard curve, tissue weight, and dilution factor.

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Fig. S1. Structure and activity relationship of DKK antagonists. (*A–D*) IIIC3 and its analogs were tested for their abilities to reverse DKK1 CM-mediated antagonism of WNT3A CM using a Wnt reporter gene assay. (*E*) Effects of IIIC3a on the inhibition of WNT3A (100 ng/mL) by Wnt antagonists (DKK1, 250 ng/mL; DKK2, 620 ng/mL; DKK4, 1 μg/mL; sFRP4, 2 μg/mL). These DKK antagonists inhibited Wnt activity by about 50% in the absence of IIIC3a, which was taken as 100% for the figure. (*F*) Effects of IIIC3a on binding of DKK2-AP to cells expressing LRP5 (diamond) or Kremen1 (square). (*G*) IIIC3 competes with DKK2C for the binding LRP6 E3E4. Biolayer interferometry profile of the super strepavidin sensor with biothylated S-tag DKK2C in Octet with the LRP6 E3E4 (black line). IIIC3 compound disrupts the interaction of Dkk2C and LRP6-E3E4. (*H*) Effects of IIIC3a and biotinylated IIIC3a on DKK2-mediated inhibition of Wnt activity. Data are presented as means ± SD. (*I*) Docking of IIIC3 with LRP6E3. (*Upper Panel*) Most residues located in the active site of LRP6E3 are conserved in LRP5 and LRP6, and interact with IIIC3 similarly. The carbon atoms of IIIC3 are shown in purple. The carbon atoms of LRP6 residues interacting with IIIC3 are colored in gray, while the carbon atoms of the corresponding LRP5 residues in homology model are shown cyan. The potential hydrophilic interactions are labeled by dash. (*Lower panel*) IIIC3 binds to the DKK1C binding site on LRP6E3. IIIC3 competes with PHE 205 and PHE 234 of DKK1C and interrupts the salt bridge between GLU 232 of DKK1C and ARG 792. The carbon atoms of IIIC3 are shown in purple, and the carbon atoms of DKK1C are shown in cyan.

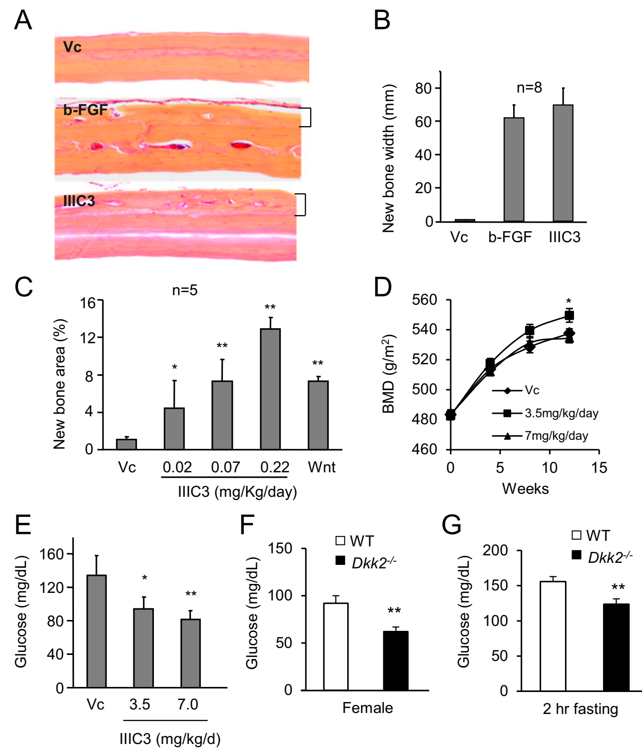

Fig. S2. Compound's effects on bone formation and blood glucose. (*A*–*C*) Effects of IIIC3 on calvarial bone formation. Vehicle control (Vc), b-FGF, IIIC3, or pure WNT3A protein was injected under the skull derma of CD-1 mice. Representative calvarial sections stained with H&E (*A*), and the quantification of new bone formation (*B*); *C* is a separate experiment. **P* < 0.05; ***P* < 0.01 (Student *t* test). (*D* and *E*) Effects of IIIC3 on bone mineral density (BMD) and blood-glucose concentration. C57BL mice (8-wk-old, male) were treated with IIIC3 via intraperitoneal injection daily for 1 wk and their total BMD was determined by dual-energy X-ray absorptiometry at week 4. This treatment and measurement cycle was repeated for three times (*D*). At the end of each injection period, mice were fasted for 16 h and blood-glucose concentrations were determined. Data in *E* are the glucose concentrations after the first injection period. (*F*) Blood-glucose concentrations of female 3-mo-old wild-type and $Dkk2^{-/-}$ mice after 16 h fasting (*n* = 20). (*G*) Blood-glucose concentrations of male 3-mo-old wild-type and $Dkk2^{-/-}$ mice after 2-h fasting (*n* = 12). Data are presented as means ± SEM ***P* < 0.01 vs. wild-type (Student *t* test).

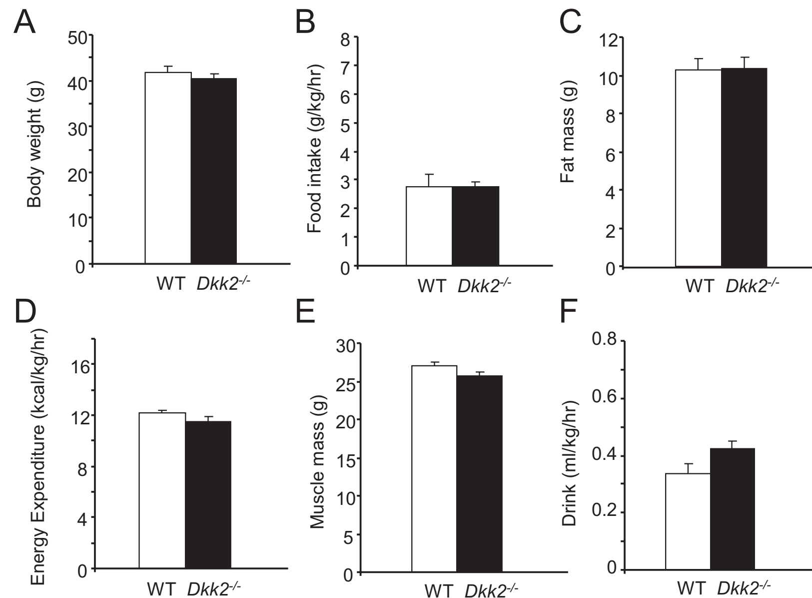

Fig. S3. Effects of DKK2 deficiency on energy balance and body composition. (*A*–*F*) $Dkk2^{-/-}$ mice and wild-type littermates (4-mo-old, male) were fed with high-fat diet for 6 wk and were analyzed by a comprehensive animal metabolic monitoring system (CLAMS; Columbus Instruments) to evaluate food consumption, drink, and energy expenditure. Fat and muscle mass were determined by NMR. Data are presented as means ± SEM (*n* > 6).

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Fig. S4. (*A–D*) Effect of DKK2 deficiency on glucose metabolism-related gene expression in the livers. $^{*}P < 0.05$ (Student *t* test).

Fig. S5. Effects of IIIC3a on glucose metabolism of $Dkk2^{-/-}$ mice. $Dkk2^{-/-}$ mice and wild-type littermates (4-mo-old, male) on chow diet were treated with IIIC3a (1.5 mg·kg·d) or vehicle control (Vc) for 24 d and subjected to a GTT (*A* and *B*) or ITT (*C*). Data are presented as means ± SEM $^{*}P < 0.05$ and $^{**}P < 0.01$ WT+Vc vs. the rest (Student *t* test, $n > 8$).

Fig. S6. Effect of metformin on glucose content and of IIIC3a on hepatic glycogen accumulation. (*A*) *Dkk2*$^{-/-}$ mice were given oral metformin (100 mg/kg) or vehicle (Vc) 30 min before GTT. (*B*) Wild-type littermates (4-mo-old, male) were treated with IIIC3a (1.5 mg·kg·d) or vehicle control (Vc) for 7 d before hepatic glycogen measurement. Data are presented as means ± SEM, $*P < 0.05$ (Student *t* test, $n > 6$).

Fig. S7. Effects of IIIC3a on glucose metabolism of the *db/db* mice. The *db/db* mice (6 wk old) were fed with chow diet and treated with IIIC3a (1.5 mg·kg·d) or vehicle control (Vc) for 4 wk and then subjected to a GTT (*A* and *B*) or ITT (*C*). Data are presented as means ± SEM, $*P < 0.05$ and $**P < 0.01$ (Student *t* test, $n = 6$).

Other Supporting Information Files

Table S1 (DOC)