Cell Metabolism, Volume *21*

Supplemental Information

LRP5 Regulates Human Body Fat Distribution by Modulating Adipose Progenitor Biology in a Dose- and Depot-Specific Fashion

Nellie Y. Loh, Matt J. Neville, Kyriakoula Marinou, Sarah A. Hardcastle, Barbara A. Fielding, Emma L. Duncan, Mark I. McCarthy, Jonathan H. Tobias, Celia L. Gregson, Fredrik Karpe, and Constantinos Christodoulides

Supplemental Information

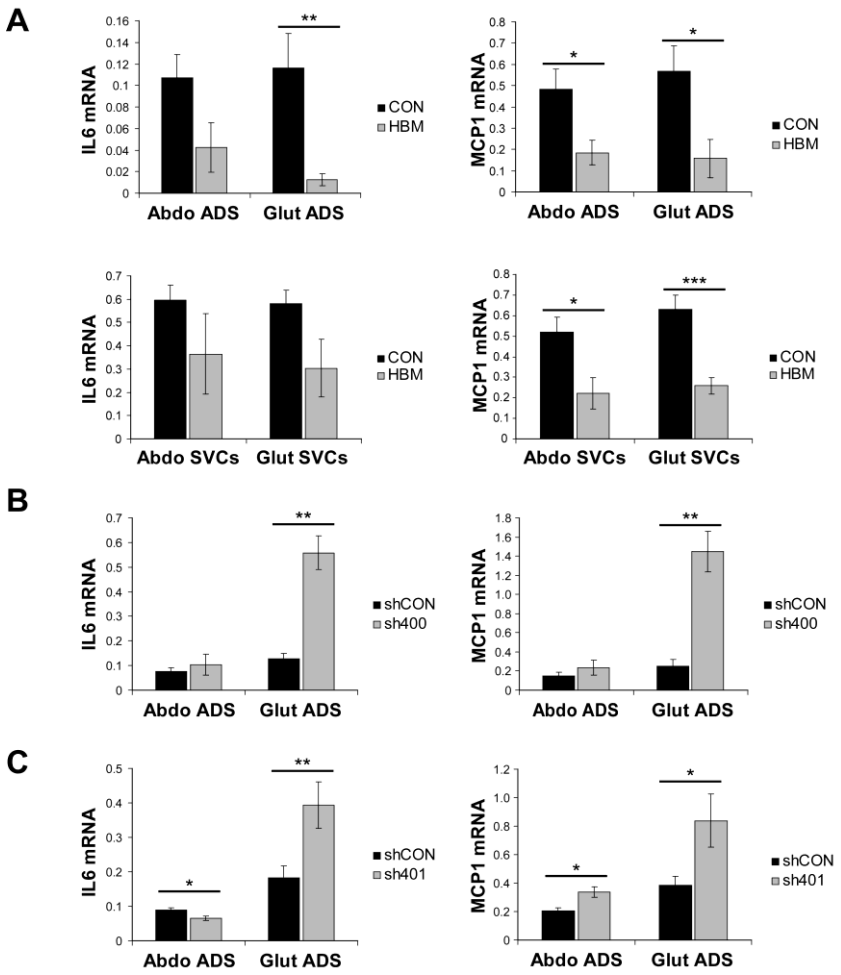

Figure S1 (Relates to Table 1 and Figure 2) Cellular inflammation in fractionated SC WAT of HBM *LRP5* mutation carriers, and *in vitro*-differentiated *LRP5* kd adipocytes. qRT-PCR analyses of *IL6* and *MCP1*, two markers of inflammation, in abdominal and gluteal (**A**) mature adipocytes (ADS) and SVCs isolated from WAT biopsies of subjects with HBM-causing gain-of-function *LRP5* mutations (n=4) and normal controls (n=24 for ADS, n=25 for SVCs), and (**B**) sh400 and (**C**) sh401 cells, and their respective controls, following 14 days of *in vitro* adipogenesis (n=4-7 independent experiments). qRT-PCR data were normalised to *18S*. *p<0.05, **p<0.01, ***p<0.001. Histogram data are expressed as means ± SEM.

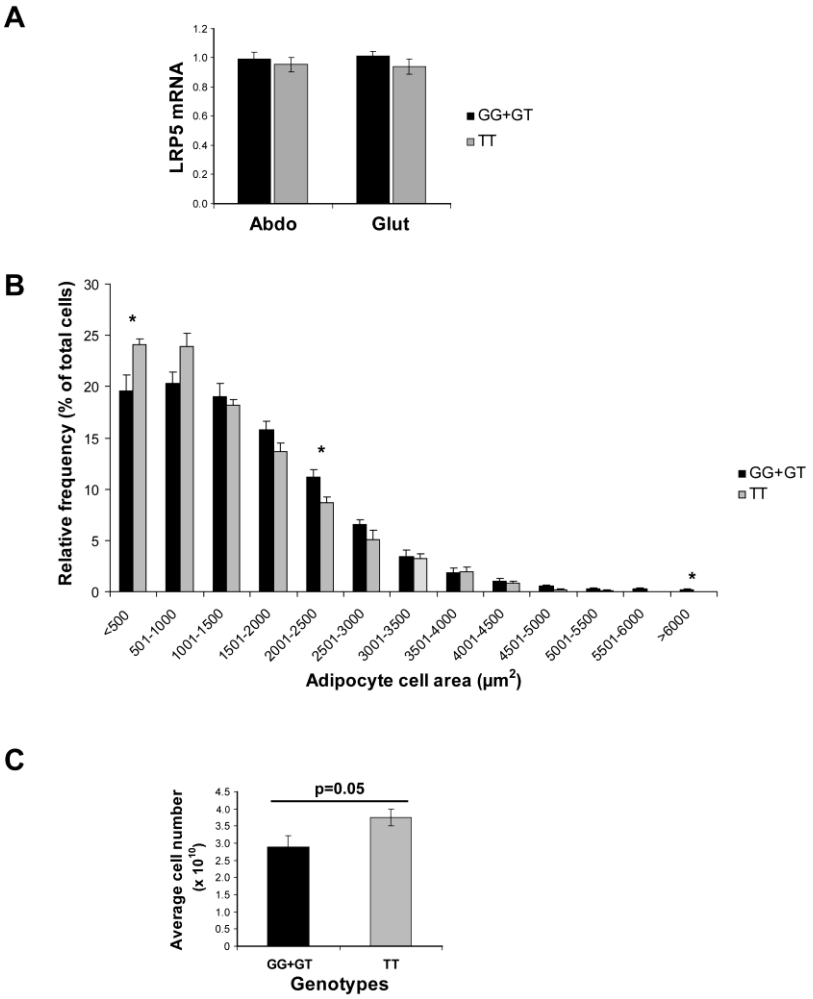

Figure S2 (Relates to Table 3) Comparison of *LRP5* mRNA levels in SC abdominal and gluteal WAT, and adipocyte cell size and number in abdominal SC WAT of subjects grouped according to their rs599083 genotype. (**A**) *LRP5* mRNA levels in SC abdominal and gluteal WAT from carriers of the low BMD-associated, minor allele (GG and GT; n=12 lean, 10 obese) *vs*. individuals homozygous for the common allele (TT; n=7 lean, 8 obese) at rs599083. qRT-PCR data were normalised to *PPIA* and *PGK1*. (**B-C**) Relative frequency of adipocytes of different sizes (cross sectional area, μm^2) (**B**), and estimated total adipocyte number in the android region (**C**) in carriers of the low BMD-associated allele (GG and GT; n=13) *vs.* individuals homozygous for the common allele (TT; n=5) at rs599083. >250 adipocytes were measured for each biopsy. Subject characteristics are shown in **Table S3**. *p<0.05. Histogram data are expressed as means ± SEM.

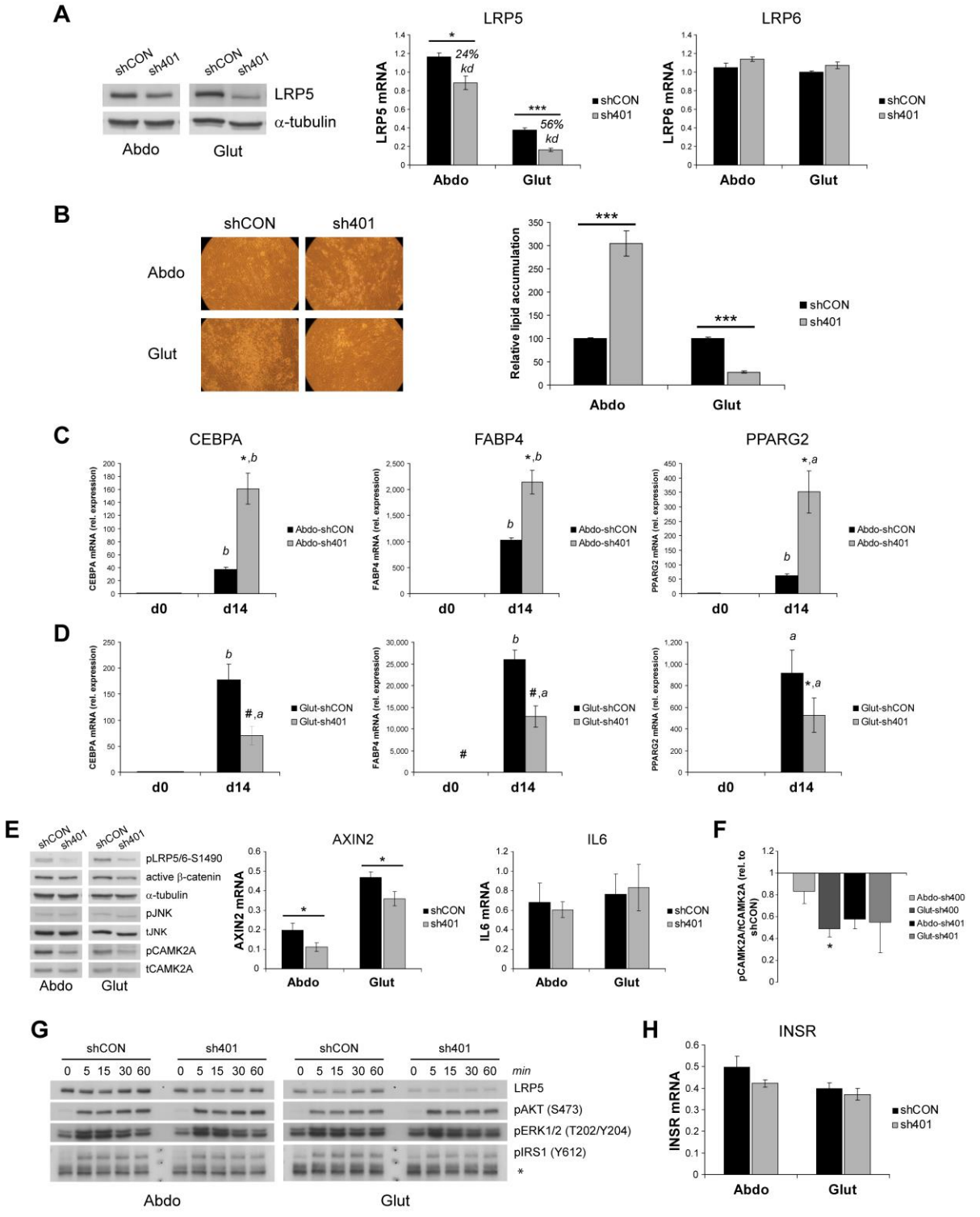

Figure S3 (Relates to Figures 2 and 3) Effects of *LRP5* kd in immortalised abdominal and gluteal SVCs on adipogenesis, and WNT and insulin signalling pathways. (**A**) *LRP5* kd was confirmed by Western blot and qRT-PCR analyses. *LRP5* mRNA levels were reduced by an

average of 24% and 56% in Abdo-sh401 and Glut-sh401 SVCs, respectively, compared with their respective control cells. *LRP6* gene expression was not significantly altered by *LRP5* kd with sh401. shCON=control, sh401=*LRP5* kd cells. α-tubulin was used as a Western blot loading control. *p<0.05, ***p<0.001. (**B**) Representative micrographs of shCON and sh401 abdominal and gluteal precursors at day 14 of adipogenic differentiation. Histogram shows relative lipid accumulation as assessed by AdipoRed assay (n=22-24). ***p<0.001. (**C-D**) Relative mRNA expression of adipogenic genes *CEBPA*, *FABP4* and *PPARG2* in (**C**) abdominal, and (**D**) gluteal cells at baseline (d0) and day 14 (d14) of adipogenic differentiation. shCON *vs*. sh401 cells: *p<0.05, $^{\#}$p<0.01; d0 *vs.* d14 cells: ^{a}p<0.05, ^{b}p<0.01. (**E**) Western blots for phospho-LRP5/6 (Ser1490) (pLRP5/6-S1490), active β-catenin, pJNK, and pCAMK2A, and qRT-PCR analyses of *AXIN2* and *IL6*, in shCON and sh401 abdominal and gluteal SVCs. α-tubulin, total-JNK (tJNK) and total CAMK2A (tCAMK2A) were used as loading controls for Western blots. *p<0.05. (**F**) Protein densitometry of pCAMK2A/tCAMK2A in Abdo-sh400, Glut-sh400, Abdo-sh401 and Glut-sh401 SVCs. Densitometry data are shown relative to their respective shCON levels. N=3 independent experiments. *p<0.05. (**G**) Representative Western blots of confluent shCON and sh401 abdominal and gluteal SVCs stimulated with 100 nM insulin for indicated length of time. *non-specific band, used as loading control. (**H**) Insulin receptor (*INSR*) mRNA levels in control and sh401 abdominal and gluteal SVCs. qRT-PCR data were normalised to *18S*. Histogram data are expressed as means ± SEM. N=4 independent experiments.

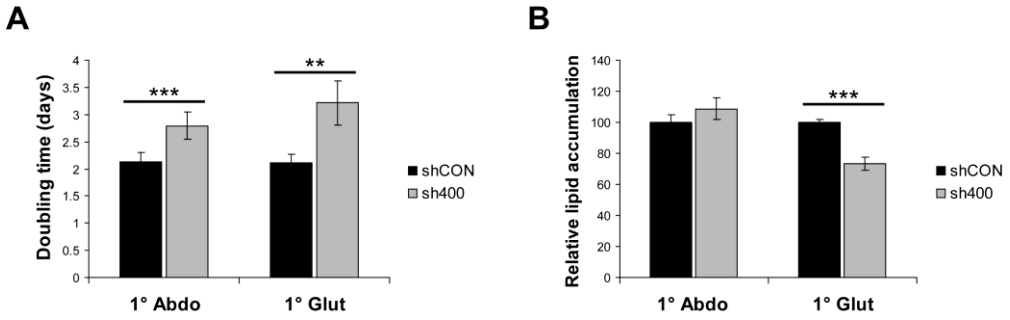

Figure S4 (Relates to Figure 2) Effects of *LRP5* kd in primary SC abdominal and gluteal SVCs on proliferation and adipogenesis. (**A**) Doubling time of control (shCON) and *LRP5* kd (sh400) abdominal and gluteal primary SVCs (n=11). (**B**) Relative lipid accumulation in differentiation day 21 shCON and sh400 primary abdominal and gluteal cells (n=16). **p<0.01, ***p<0.001. Histogram data are expressed as means ± SEM.

Table S1. (Relates to Table 2) Anthropometry and DXA-derived measures of body fat distribution of HBM subjects with gain-of-function *LRP5* mutations *vs.* the rest of the (non-*LRP5*) HBM cohort.

	Unadjusted Means ± SD		**Adjusted for gender, age and BMI Mean (95% CI)**	
	***LRP5* HBM (n=6)**	**non-*LRP5* HBM (n=134)**	***LRP5* HBM**	**non-*LRP5* HBM**
Age (yrs)	43.0 ± 14.5	60.1 ± 14.0		
BMI (kg/m^2)	27.7 ± 4.3	30.8 ± 6.0		
Tissue legs, % fat	39.5 ± 11.8	40.5 ± 9.6	41.2 (36.6, 45.7)	36.4 (35.4, 37.4)[a]
Tissue android, % fat	37.6 ± 14.7	48.6 ± 8.3	40.0 (35.2, 44.9)	46.3 (45.2, 47.4)[a]
Android:leg fat ratio (%)	19.0 ± 4.8	31.9 ± 10.8	21.9 (14.5, 29.3)	34.1 (32.4, 35.7)[b]
Android:total fat ratio (%)	7.1 ± 1.6	9.3 ± 1.6	7.6 (6.5, 8.6)	9.5 (9.3, 9.8)[c]
Central:peripheral fat mass ratio (g/g)	15.0 ± 3.9	22.8 ± 7.4	16.7 (11.8, 21.6)	24.6 (23.5, 25.7)[b]

Non-*LRP5* HBM group of 134 represents all HBM cases (indexes/relatives/spouses) with BMD Z-score of: (a) ≥+3.2 for total hip and ≥+1.2 for L1, or (b) ≥ +3.2 for L1 and ≥+1.2 for total hip (Gregson et al., 2013). 4 (66.7%) *LRP5* HBM and 112 (83.6%) non-*LRP5* HBM subjects were female. The p-values represent the strength of evidence against the H_o that non-*LRP5* HBM have same variable measures as *LRP5* HBM. [a]p<0.05, [b]p<0.01, [c]p<0.001. Abbreviations: HBM, high bone mass; CI, confidence interval; BMI, body mass index.

Table S2. (Relates to Table 1) Additional anthropometry and plasma biochemistry data from HBM patients with *LRP5* gain-of-function mutations *vs*. age-, gender-, and BMI-matched OBB controls.

	S1	Controls for S1	S2	Controls for S2	S3	Controls for S3	S4	Controls for S4	S5	Controls for S5	S6	Controls for S6
n		129 (77[a])		74 (42[a])		105 (65[a])		51 (33[a])		55 (33[a])		102 (59[a])
Age (yrs)	45	44.8 (44.3-45.2)	**68**	49.1 (48.9-49.3)	**50**	48.0 (47.7-48.4)	**28**	31.9 (31.5-32.2)	38	38.1 (36.9-39.3)	**34**	34.6 (34.1-35.1)
Gender	F	F	F	F	F	F	M	M	F	F	M	M
Waist circumference (cm)	**89**	86 (84-87)	**92**	87 (85-88)	84	85 (84-86)	**74**	81 (80-82)	**109**	104 (102-106)	**105**	97 (96-98)
Hip circumference (cm)	105	104 (103-105)	**99**	105 (104-106)	**99**	102 (101-103)	90	93 (89-97)	**124**	118 (117-120)	**116**	105 (104-106)
WHR	**0.85**	0.82 (0.81-0.83)	**0.93**	0.82 (0.81-0.84)	0.85	0.83 (0.82-0.85)	**0.82**	0.85 (0.84-0.86)	0.88	0.88 (0.86-0.90)	0.91	0.92 (0.91-0.93)
Plasma glucose (mmol/liter)	**4.6**	5.1 (5.1-5.2)	5.2	5.1 (5.0-5.2)	**5.4**	5.2 (5.1-5.3)	**4.4**	5.2 (5.1-5.3)	**4.6**	5.3 (5.1-5.4)	**5.0**	5.4 (5.3-5.5)
Plasma insulin (mU/liter)	**5.1**	12.8 (12.0-13.6)	13.0	13.4 (12.1-14.6)	**11.9**	12.9 (12.2-13.7)	**5.1**	11.4 (10.3-12.5)	**9.9**	18.9 (16.7-21.1)	**9.8**	16.6 (15.2-17.9)

Data for controls are means (95% CI). Values below/above the 95% CI in bold. *n*, number of age-, gender-, and BMI-matched controls. [a]number of controls with DXA measurements. Subjects from Kindred 1 (S1, S2) and Kindred 2 (S3, S4) carry the A242T mutation, those from Kindred 3 (S5, S6) carry the N198S mutation. NB. We were unable to match subjects S2 and S4 for age. Abbreviations: HBM, high bone mass; CI, confidence interval; WHR, waist-to-hip ratio.

Table S3. (Relates to Table 3) Anthropometry of **Figure S2B-C** subjects, grouped according to their rs599083 genotype.

	GG+GT	**TT**	***P***
n	13	5	
Gender	8M, 5F	3M, 2F	
Age (yrs)	40.5 ± 6.1	45.4 ± 1.5	0.1
BMI (kg/m^2)	33.8 ± 4.9	36.5 ± 3.0	0.1
Android fat mass (g)	4116 ± 1493	4754 ± 970	0.1

Abbreviations: BMI, body mass index. Data are means ± SD. *P* = p-value, two-tailed Mann Whitney test.

Table S4. (Relates to Table 3) Association studies between rs599083 and anthropometric measures of body fat distribution of subjects from the Oxford Biobank, adjusted for age, gender, and BMI.

	rs599083 **EA=G, EAF=0.34**		
Trait	***p-value***	***β***	***N***
Waist circumference (cm)	0.5	-0.005	5,573
Hip circumference (cm)	0.8	-0.006	5,572
WHR	0.7	0.005	5,571

Abbreviations: BMI, body mass index; WHR, waist-to-hip ratio; EA, effect allele; EAF, effect allele frequency. *P*-value, empirical p-value. β = standardised beta value. *N* = number of subjects. Data from 2,468 men and 3,105 women.

Table S5. (Relates to Table 3) Partial correlations between anthropometric and DXA-derived measures of body fat distribution of subjects from the Oxford Biobank, adjusted for age, gender, and BMI.

Traits	***rho***	***N***
WHR *vs.* android/gynoid	0.54	3,394
WHR *vs*. android/leg	0.49	3,394
Waist *vs*. android fat (g)	0.54	3,394
Waist *vs*. visceral fat (g)	0.46	3,381
Hip *vs*. gynoid fat (g)	0.53	3,395
Hip *vs*. leg fat (g)	0.46	3,395

Abbreviations: BMI, body mass index; WHR, waist-to-hip ratio. *rho,* Spearman's correlation coefficient. *N* = number of subjects. Data from 1,477 men and 1,918 women.

Table S6. (Relates to Figure 1) Anthropometry and plasma biochemistry of 20 lean and 20 obese male and female subjects from the Oxford Biobank.

	Lean	**Obese**	***P***
n	20	20	
Age (yrs)	44.3 ± 5.6	43.0 ± 4.3	0.363
BMI (kg/m^2)	23.4 ± 1.4	33.6 ± 5.3	<0.001
Waist circumference (cm)	83.0 ± 7.9	110.2 ± 14.0	<0.001
Hip circumference (cm)	97.6 ± 3.6	115.4 ± 9.2	<0.001
Plasma glucose (mmol/liter)	5.1 ± 0.5	5.4 ± 0.6	0.147
Plasma insulin (mU/liter)	8.5 ± 2.9	17.2 ± 8.5	<0.001
Triglycerides (mmol/liter)	1.2 ± 1.1	1.7 ± 0.8	<0.001
Plasma HDL cholesterol (mmol/liter)	1.3 ± 0.3	1.1 ± 0.3	0.013

Data are means ± SD. *P* = p-value for lean *vs.* obese subjects, two-tailed Mann Whitney test. 50% of lean and 50% obese subjects were women. Abbreviations: BMI, body mass index; HDL, high-density lipoprotein.

Table S7. (Relates to Figure 1) Anthropometry and plasma biochemistry in pre- and post-menopausal females recruited according to waist circumference.

	WC<80cm	**WC≥80**	***P***
n	23	24	
Age (yrs)	51.0 ± 9.4	50.8 ± 8.8	0.89
BMI (kg/m^2)	23.2 ± 1.8	26.2 ± 2.3	<0.0001
Waist circumference (cm)	76.0 ± 2.9	88.3 ± 6.7	<0.0001
Hip circumference (cm)	94.8 ± 4.0	101.5 ± 6.1	0.0001
Plasma glucose (mmol/liter)	4.8 ± 0.3	5.0 ± 0.4	0.04
Plasma insulin (mU/liter)	9.9 ± 3.5	11.0 ± 2.7	0.1
Triglycerides (mmol/liter)	809 ± 304	893 ± 439	0.6
Plasma HDL cholesterol (mmol/liter)	1.8 ± 0.4	1.6 ± 0.3	0.06

Data are means ± SD. *P* = p-value for women with WC<80cm *vs.* women with WC≥80cm, two-tailed Mann Whitney test. Abbreviations: WC, waist circumference; BMI, body mass index; HDL, high-density lipoprotein. Ten women per group were pre-menopausal.

Table S8. (Relates to Figure 1) Partial correlations (Spearman's) of *LRP5* mRNA levels in SC abdominal and gluteal WAT with measures of body fat distribution, plasma chemistry and markers of inflammation in the pre- and post-menopausal female cohort (n=47), adjusted for age, BMI, and menopause status.

	Abdominal		**Gluteal**	
Traits	***rho***	***P***	***rho***	***P***
Android fat[a]	0.17	0.3	-0.34	**0.02**
Gynoid fat[a]	0.28	0.07	0.17	0.3
Android:gynoid	0.09	0.6	-0.39	**0.01**
Visceral fat[b]	0.16	0.3	-0.31	**0.04**
Subcut. fat[b]	0.36	**0.02**	0.01	0.9
VAT:SCAT	-0.06	0.7	-0.31	**0.04**
WHR	-0.16	0.3	-0.20	0.2
Waist (cm)	-0.04	0.8	-0.29	0.06
Hip (cm)	0.30	**0.05**	-0.06	0.7
Insulin[c]	-0.19	0.2	-0.57	**<0.0001**
HOMA-IR	-0.12	0.4	-0.53	**<0.0001**
Log CRP[c]	-0.05	0.7	-0.32	**0.04**
MCP1[d]	-0.12	0.4	-0.35	**0.02**
CD68[d]	0.09	0.6	-0.33	**0.03**

Abbreviations: BMI, body mass index; subcut., subcutaneous; VAT, visceral adipose tissue; SCAT, subcutaneous adipose tissue; HOMA-IR, Homeostasis Model Assessment-estimated Insulin Resistance; CRP, C-reactive protein; MCP1, monocyte chemotactic protein 1. Measurements determined by [a]DXA/[b]MRI/[c]plasma chemistry/[d]qRT-PCR. Significant p-values in bold.

Supplemental Experimental Procedures

High bone mass (HBM) subjects carrying *LRP5* mutations: The HBM study is a UK-based multi-centered observational study of adults with unexplained HBM identified by screening 335,115 DXA scans from 13 UK DXA databases from which 258 HBM probands with BMD Z-score ≥+3.2 were identified. All participants were clinically assessed by one doctor using a standardized structured history and examination questionnaire, after which total-body Lunar prodigy DXA scans were performed (Gregson et al., 2013; Gregson et al., 2012). Written informed consent was collected for all, in line with the Declaration of Helsinki. Participants were excluded if they were aged <18 years, pregnant, or unable to provide written informed consent for any reason. This study was approved by the Bath Multicenter Research Ethics Committee (REC) and at each NHS Local REC. Cases with HBM *LRP5* mutations were identified by targeted sequencing of exons 2-4 of *LRP5* (i.e. the sites of previously described HBM cases). Primers are available upon request (Duncan et al., 2009). Six autosomal dominant HBM cases from 3 kindreds participated in the follow-on study. All subjects underwent basic anthropometric measurements, fasting blood sampling and a DXA scan. Lunar measured body composition parameters were compared between 6 *LRP5* HBM cases and 18 matched non-*LRP5* HBM controls.

Genotyping: Subjects were genotyped using TaqMan® SNP Genotyping Assays as previously described (Vasan et al., 2011).

Isolation, culture, and differentiation of human SVCs: The SVC layer containing preadipocytes was separated from mature adipocytes following collagenase (Roche)-digestion (1 mg/ml in Hanks' buffered salt solution) of WAT biopsies, and cultured in Dulbecco's modified Eagle's medium nutrient mixture F-12 Ham (DMEM-F12) supplemented with 10% foetal calf serum (FCS), 2 mM L-glutamine, 0.25 ng/ml fibroblast growth factor, 100 units/ml penicillin and 100 μg/ml streptomycin, as previously described (Collins et al., 2010).

For differentiation experiments, SVCs were grown to confluence in 6-well plates, and stimulated for 14 days with a standard adipogenic medium (DMEM-F12 containing 2mM glutamine, 17 μM pantothenate, 100 nM human insulin, 0.1 μM 3,3',5-triiodo-L-thyronine, 33 μM biotin, 10 μg/ml human transferrin and 1 μM dexamethasone). For the first 5 days, 250 μM 3-isobutyl-1-methylxanthine and 4 μM troglitazone were added to the adipogenic medium. For quantitative measurements of intracellular lipids, SVCs were grown to confluence and differentiated in type I collagen-coated 96-well plates for 16 days, then assayed using the AdipoRed adipogenesis assay reagent (Lonza) and a CytoFluor Multi-well Plate Reader series 4000 (PerSeptive Biosystems), according to manufacturers' instructions.

RNA isolation and qRT-PCR: Total RNA was extracted from WAT samples using TRIzol® reagent (Invitrogen), and from isolated mature adipocytes and cultured SVCs using the RNeasy Mini Kit (Qiagen). Between 0.5 and 1 μg of total RNA from WAT samples and SVCs, and 20-200ng total RNA from mature adipocytes, were reverse-transcribed into cDNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). Quantitative PCR assays were performed as previously described (Neville et al., 2011). Expression values were calculated by the ΔCT transformation method ($\Delta CT = \text{efficiency}^{[\text{calibrator Ct} - \text{sample Ct}]}$) and normalized to *PGK1* and *PPIA* in the case of tissue samples (Neville et al., 2011), and to *18S* in the case of mature adipocytes and SVCs.

Western Immunoblotting: Whole cell lysates were prepared in ice-cold lysis buffer containing 50 mM Tris pH8.0, 250 mM NaCl, 5 mM EDTA, 0.5% Igepal CA-630, 10 mM sodium fluoride, 1 mM sodium orthovanadate and protease inhibitors (Complete EDTA-free, Roche). Equal amounts of protein were resolved by SDS-PAGE, transferred onto polyvinylidene fluoride membrane (Biorad) and immunoblotted with the following antibodies according to the manufacturer's instructions: anti-LRP5 (D80F2) rabbit mAb (#5731; Cell Signalling), anti-phospho-LRP5/6 (Ser1490) rabbit pAb (#2568; Cell Signalling), anti-active

β-catenin (8E7) mouse mAb (05-665; Merck Millipore), anti-phospho-JNK (Thr183/Tyr185) rabbit pAb (#9251; Cell Signalling), anti-total JNK mouse mAb (sc-7345; Santa Cruz Biotechnology), anti-phospho-CaMKIIα (Thr286) rabbit pAb (sc12886-R; Santa Cruz Biotechnology), anti-total CaMKII rabbit pAb (sc-9035; Santa Cruz Biotechnology), anti-phospho-AKT (Ser473) rabbit pAb (#9271; Cell Signalling), anti-phospho-Erk1/2 (Thr202/Tyr204) rabbit pAb (#9101; Cell Signalling), anti-phospo-IRS1 (Tyr612) rabbit pAb (#09-432; Millipore), anti-α-tubulin rabbit pAb (ab15246; Abcam) and HRP-conjugated goat anti-actin pAb (sc-1616; Santa Cruz Biotechnology); followed by the appropriate horseradish peroxidise-conjugated secondary antibodies (DAKO) and detection by enhanced chemiluminescence (GE Healthcare). Densitometric analysis was performed using ImageJ (NIH, USA).

Measurement of adipocyte size and number: Adipocyte cell size measurements were performed as follows: Briefly, 5-µm sections of paraffin-embedded WAT biopsies were cut, dewaxed and stained with hematoxylin-eosin. Sections were photographed under 10x magnification and adipocyte cross-sectional area was determined using Adobe Photoshop CS2 9.0.2 (Adobe Systems, San Jose, CA) and the Reindeer image processing toolkit (Reindeer Games, Gainesville FL).

Mean adipocyte volume and weight were calculated for each individual using the following formulae:

$$V = \frac{\sum_{i=1}^{n} \left(\frac{\pi \times d^3}{6}\right)}{n}, \; w = V \times 0.915$$

V = mean cell volume (µm^3), d (real cell diameter) = histological cell diameter (µm) $\times$ 1.1 (Ashwell et al., 1976), w = mean weight of a single adipocyte (µg), n = total adipocyte number, density of fat cell triglycerides = 0.915 g/ml. Adipocytes were assumed to be

spheres. Abdominal adipocyte number was estimated by dividing DXA-derived android fat mass by mean adipocyte weight.

Supplemental References

Ashwell, M., Priest, P., Bondoux, M., Sowter, C., and McPherson, C.K. (1976). Human fat cell sizing--a quick, simple method. J Lipid Res *17*, 190-192.

Duncan, E.L., Gregson, C.L., Addison, K., Brugmans, M., Pointon, J.J., Appleton, L.H., Tobias, J.H., and Brown, M.A. (2009). Mutations in LRP5 and SOST are a rare cause of high bone mass in the general population. Bone *44*, S340-S341.

Gregson, C.L., Paggiosi, M.A., Crabtree, N., Steel, S.A., McCloskey, E., Duncan, E.L., Fan, B., Shepherd, J.A., Fraser, W.D., Smith, G.D., et al. (2013). Analysis of body composition in individuals with high bone mass reveals a marked increase in fat mass in women but not men. J Clin Endocrinol Metab *98*, 818-828.

Neville, M.J., Collins, J.M., Gloyn, A.L., McCarthy, M.I., and Karpe, F. (2011). Comprehensive human adipose tissue mRNA and microRNA endogenous control selection for quantitative real-time-PCR normalization. Obesity (Silver Spring) *19*, 888-892.

Vasan, S.K., Neville, M.J., Antonisamy, B., Samuel, P., Fall, C.H., Geethanjali, F.S., Thomas, N., Raghupathy, P., Brismar, K., and Karpe, F. (2011). Absence of birth-weight lowering effect of ADCY5 and near CCNL, but association of impaired glucose-insulin homeostasis with ADCY5 in Asian Indians. PLoS One *6*, e21331.