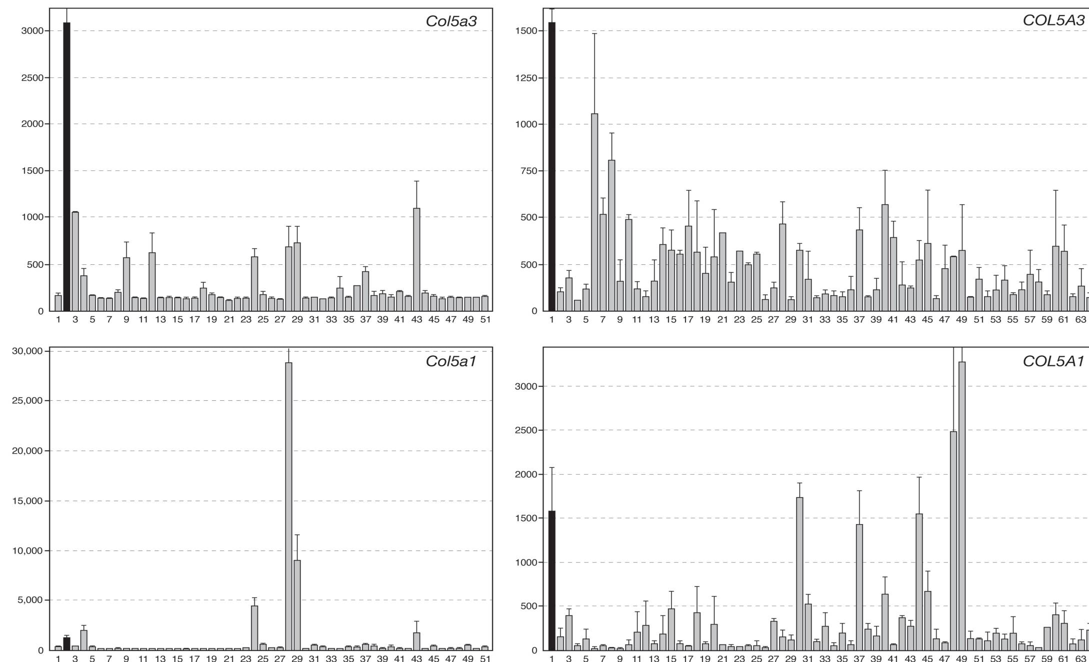
**Supplemental Data**

**$\alpha$3(V) collagen ablation in mice induces diabetes-related symptoms ivia effects on pancreatic islets and peripheral tissues, by Guorui Huang *et al*.**




Supplemental Fig. 1. Microarray analyses of comparative expression levels of *Col5a3* and *Col5a1* in mouse and of *COL5A3* and *COL5A1* in human cells and tissues. **Mouse**: 1, brown fat; 2, adipose tissue; 3, adrenal gland; 4, bone; 5, bone marrow; 6, amygdala; 7, frontal cortex; 8, preoptic nerve; 9, trigeminal nerve; 10, cerebellum; 11, cerebral cortex; 12, dorsal root ganglia; 13, dorsal striatum; 14, hippocampus; 15, hypothalamus; 16, main olfactory epithelium; 17, olfactory bulb; 18, lower spinal cord; 19, upper spinal cord; 20, substantia nigra; 21, blastocysts; 22, fertilized egg; 23, lactating mammary gland; 24, ovary; 25, placenta; 26, prostate; 27, testis; 28, umbilical cord; 29, uterus; 30, oocyte; 31, heart; 32, large intestine; 33, small intestine; 34, liver; 35, lung; 36, lymph node; 37, skeletal muscle; 38, vomeronasal organ; 39, salivary gland; 40, tongue; 41, pancreas; 42, pituitary; 43, epidermis; 44, spleen; 45, stomach; 46, thymus; 47, thyroid; 48, trachea; 49, bladder; 50, kidney; 51, retina. **Human**: 1, adipocyte; 2, adrenal cortex; 3, adrenal gland; 4, whole blood; 5, bone marrow; 6, amygdala; 7, cingulate cortex; 8, prefrontal cortex; 9, bronchial epithelial cells; 10, caudate nucleus; 11, cerebellum; 12, cerebellum peduncles; 13, fetal brain; 14, globus pallidus; 15, hypothalamus; 16, medulla oblongata; 17, occipital lobe; 18, olfactory bulb; 19, parietal lobe; 20, pons; 21, spinal cord; 22 subthalamic nucleus; 23, temporal lobe; 24, thalamus; 25, whole brain; 26, PB-BDCA4+ dendritic cells; 27, BM-CD 105+ endothelial cells; 28, DRG; 29, ovary; 30, placenta; 31, prostate; 32, testis; 33, testis - germ cell; 34, testis - interstitial; 35, Leydig cell; 36, seminiferous tubule; 37, uterus; 38, uterus corpus; 39, atrioventricular node; 40, heart; 41, appendix; 42, fetal liver; 43, liver; 44, fetal lung; 45, lung; 46, lymph node; 47, skeletal muscle; 48, cardiac myocytes; 49, smooth muscle; 50, salivary gland; 51, tongue; 52, pancreas; 53, pancreatic islets; 54, ciliary ganglion; 55, superior cervical ganglion; 56, trigeminal ganglion; 57, pituitary; 58, skin; 59, thymus; 60, fetal thyroid; 61, thyroid; 62, tonsil; 63, trachea; 64, kidney. Expression levels are relative hybridization in arbitrary units (2).

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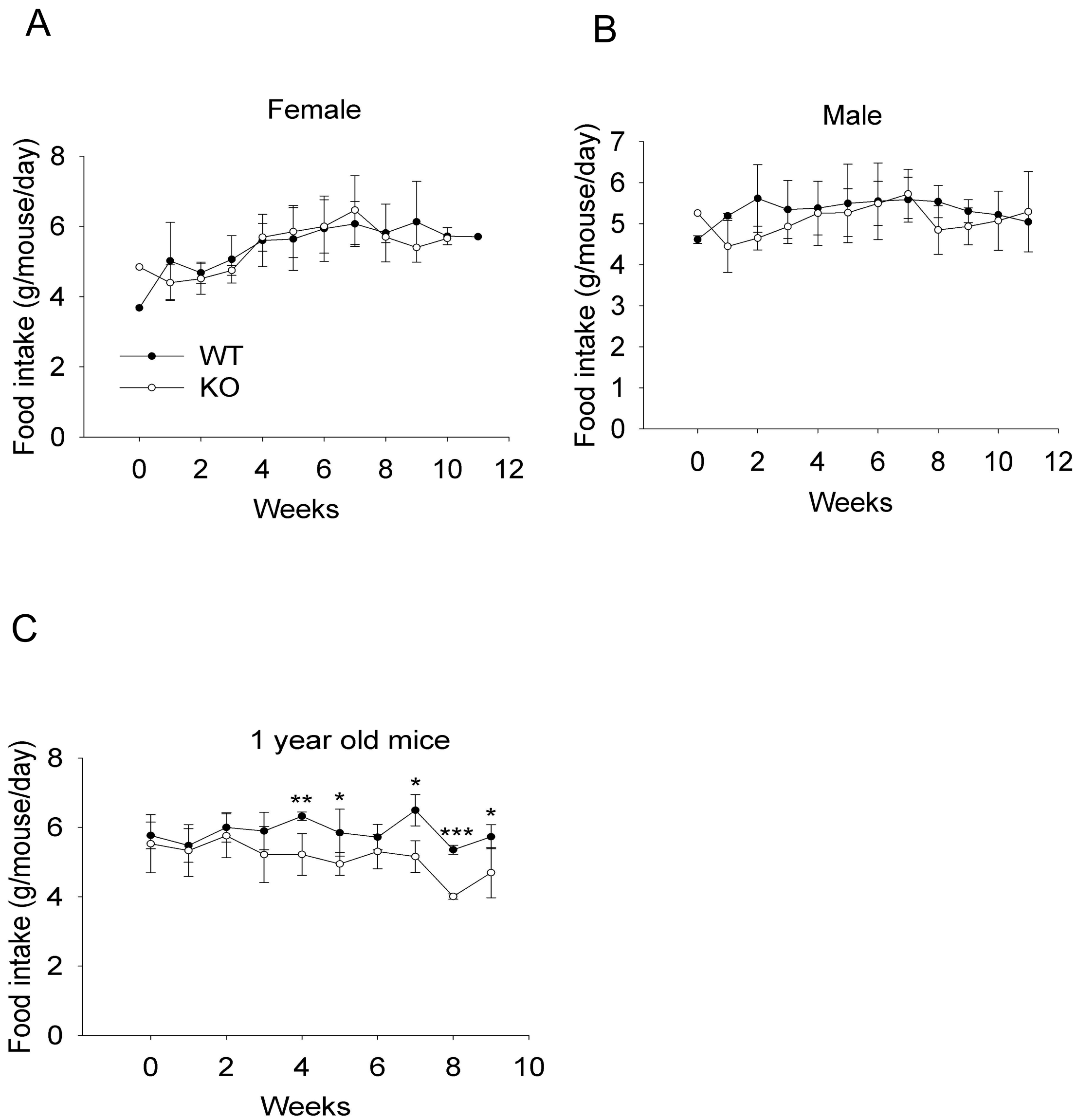
Supplemental Fig. 2




Supplemental Fig. 2. Feeding study. 6-week-old (A and B) or 1-year-old mice (C) were housed two-per-cage and given a regular chow diet (Harlan Teklad Rodent Diet 8604) for a 3-month period, during which time food intake was assessed by determining the difference in food weight during each 7-day interval. Food intake was assessed as average daily food intake (g) per mouse and analyzed using a two-tailed Student's t-test. Food intake was not significantly different between younger wild type and *Col5a3*-/- mice (A and B), but was significantly different at a number of time points for the 1-year-old mice. One, two and three asterisks denote data points with $P < 0.05$, 0.01, and 0.001, respectively.

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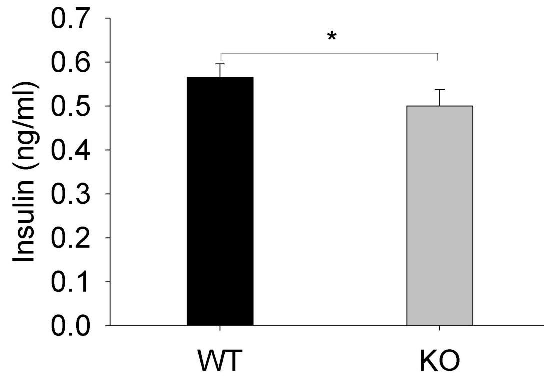
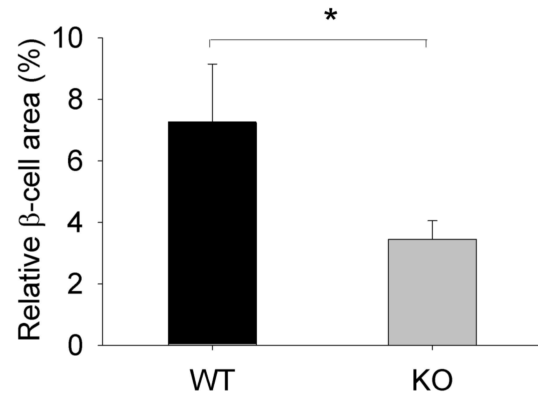
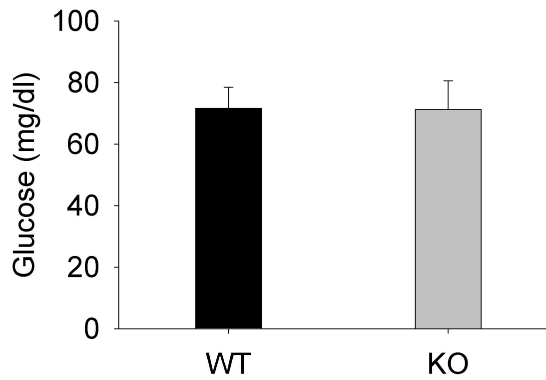
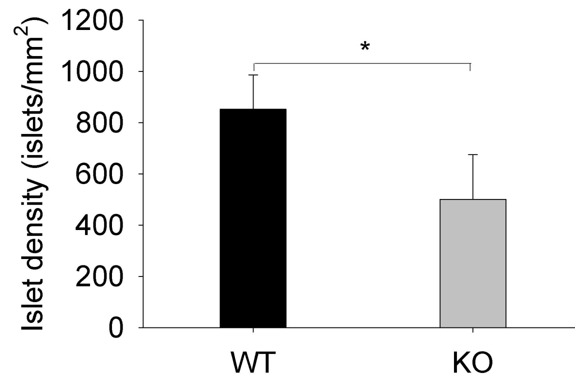
Supplemental Fig. 3



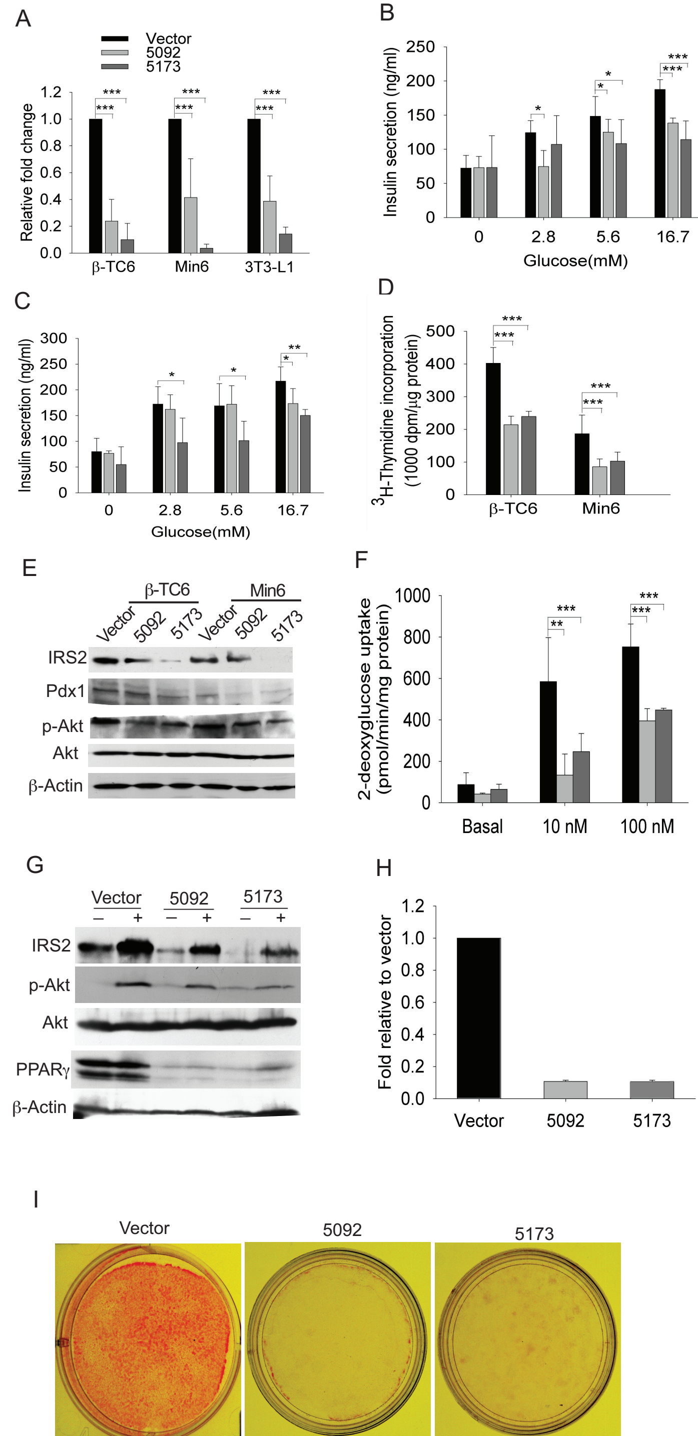



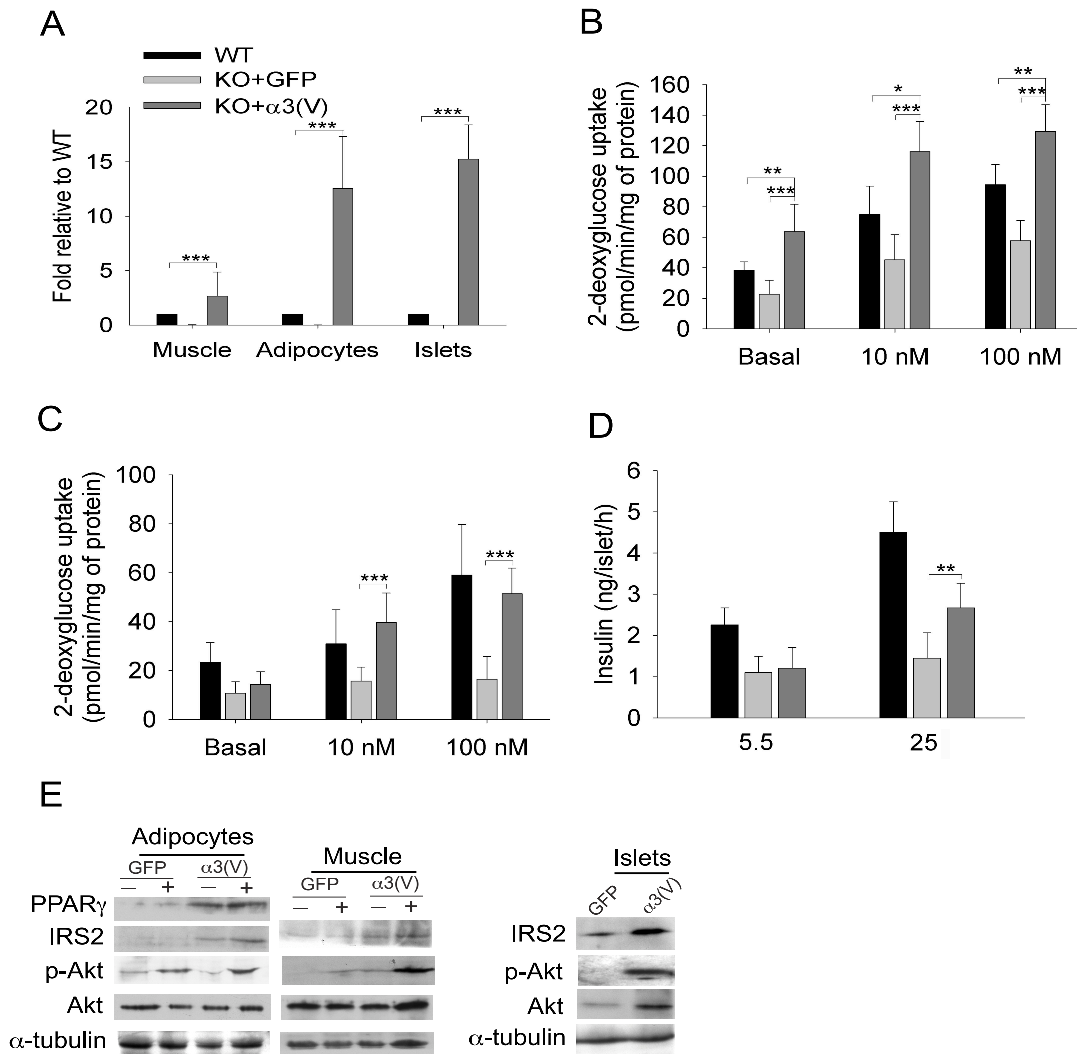




Supplemental Fig. 3. Newborn Col5a3-/- mice have (A) plasma insulin levels, (B) relative area of β-cells, and islet density (D), significantly lower than wild type; but plasma glucose levels indistinguishable from those of wild type counterparts.

Huang et al. Supplemental Fig. 4

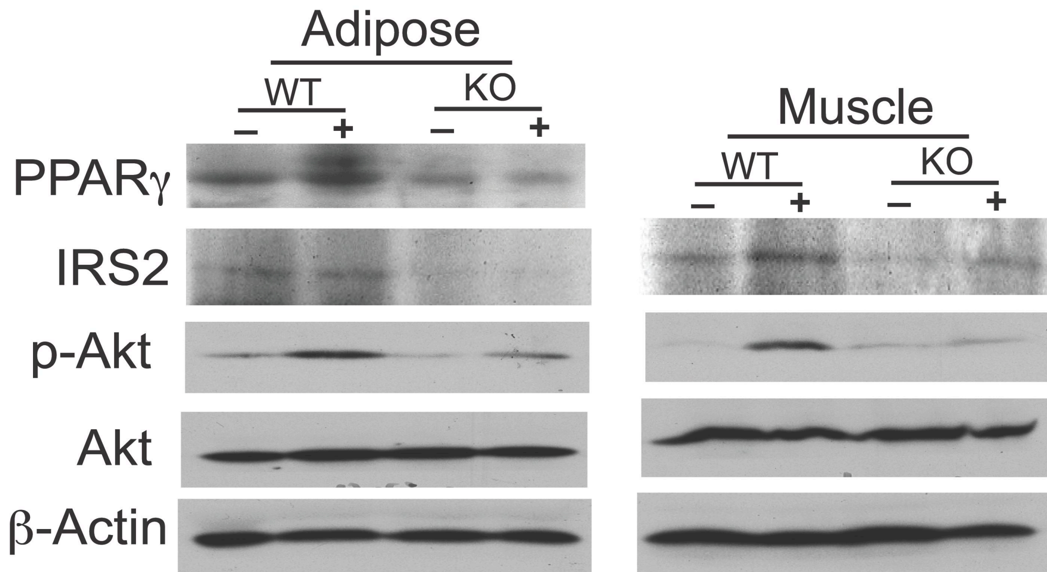



Supplemental Fig. 4. *siRNA knockdown of Col5a3 in cell lines*. (A) quantitative real time PCR shows relative levels of *Col5a3* RNA in cells infected with either lentiviral vector containing siRNA template oligonucleotide 5092 or 5173, or with empty vector, as a control. Levels of insulin secretions are shown for (B) β-TC6 (B) or (C) Min6 cells infected with 5092, 5173, or control vector. (D) Levels of $^{3}$H-thymidine incorporation are compared for β-TC6 and Min6 cells infected with 5092, 5173, or control vector. (E) Immunoblots are shown of extracts of β-TC6 and Min6 cells infected with 5092, 5173, or control vector. Blots were stained with antibodies to IRS2, Pdx1, Akt, or phospho-Akt (Ser 473). Blots were also stained with antibodies to β-actin, as a loading control. (F) Levels of 2-deoxyglucose uptake are compared for 3T3-L1 cells infected with 5092, 5173, or control vector and assayed 8 days subsequent to induced adipocytic differentiation. (G) Immunoblots are shown of extracts of 3T3-L1 cells infected with 5092, 5173, or control vector and assayed 8 days subsequent to induced adipocytic differentiation. Staining employed antibodies to IRS2, Pdx1, Akt, phospho-Akt (Ser 473), or PPARγ. Blots were also stained with antibodies to β-actin, as a loading control. Quantitation via isopropanol extraction (H) and representative photographs (I) and are shown of oil red O staining, comparing relative degrees of maturation of 3T3-L1 cells infected with 5092, 5173, or control vector and assayed 8 days subsequent to induced adipocytic differentiation.




Supplemental Fig. 5. *Metabolic effects and Col5a3-/- phenotypic rescue resulting from α3(V) overexpression*. (A) quantitative real time PCR shows relative levels of *Col5a3* RNA in muscle strips, adipocytes and islets infected with lentiviral vector containing cDNA encoding either pro-α3(V) or GFP. Adipocytes isolated from epididymal fat pads (B) and strips of soleus muscle (C) were incubated in the absence (basal), or presence of 10 or 100 mM insulin, prior to incubation in the presence of 2-deoxy-D[2,6-$^{3}$H]glucose and 1 mM 2-deoxyglucose, and subsequent ascertainment of uptake. (D) Islets were incubated in the presence of 5.5 or 25 mM glucose, followed by ELISA quantification of insulin secretion. Eighteen islets were assayed per mouse (3 islets per tube, 3 tubes per each concentration of glucose, so that each assay was performed in triplicate). (E) Representative immunoblots are shown of extracts of adipocytes, muscle strips, and isolated islets infected with lentiviral vector containing cDNA encoding either pro-α3(V) or GFP, and incubated in the absence (-) or presence (+) of 100 nM insulin. Blots were stained with IRS2, p-Akt, Akt, or α-tubulin antibodies. The latter was a loading control. Adipocyte blots were also stained with antibodies to PPARγ.

# Huang et al. Supplemental Fig. 6




Supplemental Fig. 6. Decreased levels of insulin/IGF-1 signaling components and PPARγ in the peripheral tissues of 1-year-old *Col5a3*-/- mice. Immunoblots are shown of extracts of epididymal fat pads and soleus muscle isolated from wild type (WT) and *Col5a3*-/- (KO) mice, that had been injected either with PBS (-) or insulin (+). Blots were stained with antibodies to IRS2, phospho-Akt (Ser 473), or Akt. An adipose blot was also stained with antibody specific to PPARγ. Bots were also stained with antibodies to β-actin, as a loading control.

**Supplemental methods:**

*Generation of Col5a3-/- mice*. A targeting vector was created by inserting 129/Sv genomic DNA fragments into pPNT (2). The 5' homology arm contains 12 *Col5a3* collagenous (COL1) domain exons and a junctional exon encoding the final COL1 Gly-X-Y triplet, and the beginning of the C-propeptide. Bunt-ending a *Nar*I site in the junctional exon caused a frame-shift, changing sequence RRRSV.... to RR**ADL**-STOP, with just 3 residues (boldface) in the new reading frame prior to a stop codon. The 3' homology arm contains the final *Col5a3* exon. In the targeted allele, the *neo* cassette replaces 1.2-kb of genomic sequences including 3 C-propeptide exons. R1 ES cells (3) were electroporated, G418/gancyclovir selected, and Southern blots of *AflII* cut genomic DNA were hybridized to 5' or 3' external probes to confirm correct targeting. For wild type alleles, 5' and 3' probes hybridized to 8- and 6.3-kb bands, respectively. For null alleles, both probes hybridized to ~15-kb bands. ES cells from two independently targeted lines with normal karyotypes were separately employed to produce chimeric embryos (4) and germ line transmission was via mating male chimeras to ICR females. To follow wild type allele expression, total RNA was extracted from 15.5-dpc embryos with TRIzol (Invitrogen), followed by RT-PCR with *Col5a3* primers 5'GCTGAGGTGCATGGCCTGCGCAGGC3' (forward) and 5'GAACTTCTTCCCTCGGCGGAAGGTGC3' (reverse), and GAPDH control primers. To compare α3(V) protein levels, 15.5 dpc embryos were Dounce homogenized in 4x Laemmli buffer, 1 mM PMSF, 1 mM *N*-ethylmaleimide, 1 mM *p*-aminobenzoic acid, 10 mM EDTA. 5% β-mercaptoethanol was then added, and boiled samples were subjected

to immunoblotting with anti-pro-α3(V) antibody (5), kindly provided by David Carey (Weis Center for Research, Danville, PA) or with anti-β-actin, as a loading control.

*Food Intake Study*. 6-week-old female (WT, n=10; KO, n=8) or male (WT, n=10; KO, n=12) mice or 1-year-old mice (WT=12, KO=6) were housed two-per-cage and given a regular chow diet (Harlan Teklad Rodent Diet 8604) for a 3-month period, during which time food intake was assessed by determining the difference in food weight during each 7-day interval. Food intake was assessed as average daily food intake (g) per mouse and analyzed using a two-tailed Student's t-test.

*siRNA knockdown of Col5a3 in cell lines*. Two DNA template oligonucleotides corresponding to target sites 5092-5112 (oligonucleotide 5092, 5'-GATCCGGGAAGAGTTGTCCTTCAACCCTCGAGGGTTGAAGGACAACTCTTCCC TTTTTTC-3' hybridized to 5'-TCGAGAAAAAAGGGAAGAGTTGTCCTTCAACCCTCGAGGGTTGAAGGACAACTCTTCCCG-3') and 5173-5193 (oligonucleotide 5173, 5'-GATCCGCGAAGACCCTCTTTGAATTCCTCGAGGAATTCAAAGAGGGTCTTCGC TTTTTTC-3' hybridized to 5'-TCGAGAAAAAAGCGAAGACCCTCTTTGAATTCCTCGAGGAATTCAAAGAGGGTCTTCGCG-3') in the open reading frame of mouse *col5a3* were inserted between the BamHI and XhoI sites downstream of the H1 promoter of a previously decscribed lentiviral vector (6) (a kind gift from Y. Shi), and resulting plasmids were confirmed by DNA sequencing. Lentiviral vector DNAs and packaging vectors (VSVG and Δ8.9) were then co-transfected into 293T cells. Supernatants containing lentiviruses were

harvested three times during a 24 hr period after transfection, followed by purification using ultracentrifugation and titering of lentiviruses. After that, targeted cell lines were infected with oligomer-containing virus or empty virus, as a control, and GFP-positive cells were purified by FACS sorting, and then cultured.

β-TC6 cells were cultured in DMEM containing 15% heat-inactivated FBS, 100 mg/L streptomycin sulfate, 1 mM L-glutamine. Min6 cells were cultured in the same medium supplemented with 50 μM 2-mercaptoethanol. For insulin secretion, cells were seeded on 24-well plates and cultured 2 days in the media described above. Cells were then preincubated 30 min in HEPES-balanced Krebs-Ringer bicarbonate buffer (10 mM HEPES pH 7.4, 119 mM NaCl, 4.74 mM KCl, 1.19 mM $MgSO_4$, 1.19 mM $KH_2PO_4$, 2.54 mM $CaCl_2$) plus 0.5% BSA and 5 mM glucose, and then were incubated 1.5 h in the same buffer with various concentrations of glucose. Secreted insulin was measured by ELISA and amounts of secreted insulin were normalized to cellular protein content.

3T3-L1 cells were cultured and induced to differentiate, as described above. On day 8 (full differentiation), differentiated 3T3-L1 adipocytes were stained with oil red O, which detects triglyceride accumulation. After photographing stained culture plates, the oil red O was extracted with 100% isopropanol and the OD was determined at 520 nm via spectrophotometry. The OD of extracts of oil red O-stained non-differentiated cultures, representing background, was subtracted from this value.

*Lentivirus rescue experiments*. To prepare an α3(V) lentivirus expression vector, oligonucleotides 5'-CACGCGTCTTAAGTCTAGAGC-3' and 5'-GGCCGCTCTAGACTTAAGACGCGTGACGT-3' were annealed, and then inserted into the pGEM-T vector (Promega) between the AatII and NotI sites, thus adding MluI,

AflII, and XbaI restriction sites. The resulting construct was then restricted with XbaI and NotI, as was a construct consisting of full-length murine pro-α3(V) cDNA contained within the pCEP vector. Bands were gel purified, and the pro-α3(V) cDNA with XbaI and NotI overhangs was then ligated between the XbaI and NotI sites of the modified pGEM-T vector. The full-length pro-α3(V) band was then cut from the resulting construct with Mlu I and Sal I, and ligated between the MluI and SalI sites of lentiviral vector pWPT-GFP, which simultaneously removed GFP sequences from the vector. Either the resulting pWPT-α3(V) vector or control vector pwPT-GFP was then co-transfected with packaging vectors psPAX2 and pMD2.G into 293T cells (vectors pWPT-GFP, psPAX2, and pMD2.G are from the laboratory of D. Trono, University of Geneva, Geneva Switzerland). After 24 h, supernatants containing lentiviruses were harvested three times at 12-hr intervals, and concentrated by ultracentrifugation at 47,000 X g for 4 hrs. For rescue experiments, three-month old mice were anesthetized and received bilateral multiple injections into soleus muscles of either pWPT-α3(V) or pWPT-GFP, totaling 100 μl of concentrated virus, suspended in PBS plus 8 μg/ml polybrene (Sigma-Aldrich), per animal. 48 h later, soleus strips were dissected and glucose uptake assays were done as described in Methods. Adipocytes and islets were isolated as described in Methods, and then cultured in 6-well plates in media containing virus and polybrene. 48 h later, glucose uptake in transfected adipocytes and insulin secretion in transfected islets were assayed as described in Methods. Transfected samples (skeleton muscle, adipocytes and islets) were also extracted with TRIzol for Real-time quantitative PCR, or prepared for Western blot analysis. Real-time PCR was performed on an ABI 7300 instrument with power SYBR Green Master Mix (Applied Biosystems). Relative expression levels

were determined by real-time RT-PCR from a standard curve of serial dilutions of cDNA samples and were normalized to GAPDH expression. *Col5a3*-specific primers were 5'-AAAGGGCAAAGGGAAGAAAA-3' (forward) and 5'-CAATCGTGACAGTGGTGGTGA-3' (reverse). GAPDH control primers have been described previously (7).

*DEXA analysis*. DEXA analysis was performed using a PIXIMUS unit (GE Healthcare).

*Immunohistochemistry*. P10 inguinal fat pads were fixed in 4% paraformadelhyde/PBS and paraffin embedded. 10 μm sections were deparaffinized, and rehydrated through an ethanol gradient and PBS. Autofluorescence was quenched with 1% $NaBH_4$. Antigen retrieval was via autoclaving sections in 10 mM Na-Citrate, pH 4, and sections were treated with 1 mg/ml hyaluronidase (Type IV-S, Sigma) in 0.1 M NaAc, pH 5.5, 145 mM NaCl. Sections were then blocked with 5% fish skin gelatin (Sigma) in PBS. Anti-α3(V) antibodies diluted 1:500 in blocking solution were applied to sections and incubated overnight at 4ºC, followed by incubating with Alexa Fluor 555 donkey anti-rabbit secondary antibodies diluted in blocking solution, and mounting in IMMU-MOUNT (Thermo Shandon).

Soleus muscle and epididymal fat pad were fixed in 10% buffered formalin (Sigma), and either frozen in OCT or embedded in paraffin, sectioned and then immunostained as described (38). Paraffin embedded sections were deparaffinized prior to immunostaining. For immunostaining, sections were treated with PBS, 50 m*M* glycine to quench nonspecific binding sites. Samples were then permeabilized and blocked with buffer B1 (1% BSA, 3% normal goat serum, 0.1% saponin in PBS), followed by

overnight incubation at 4 °C with primary antibody diluted in buffer B1. Samples were washed with buffer B2 (0.1% saponin in PBS), incubated 1 h at room temperature with secondary antibody, washed again with buffer B2 and then mounted. Guinea pig anti-human insulin antibody and goat anti-rabbit FITC secondary antibody were from Invitrogen, caveolin 1 antibody was from Santa Cruz, GLUT4 antibody was from Abcam, and donkey anti–guinea pig secondary antibodies and rabbit anti-glucagon antibody were gifts from Alan Attie (University of Wisconsin-Madison). For some experiments, pancreatic islets were isolated, formalin fixed, and immunostained, as above. Fluorescence photomicroscopy was with a Zeiss Axiophot 2 microscope.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