**Investigating the Roles of Wnt Signaling in Mature Adipocyte Function**

by

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WNTLESS

WNT-ER IS          COMING

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## DEDICATION

For my parents, Drs. Milan and Indrani Bagchi, who instilled in me the
virtues of perseverance and commitment,
and whose unfaltering support and love encourage me to
strive for excellence always.

This accomplishment is as much yours as it is mine.

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Above all, thank you to my parents, who instilled in me a love of learning, filled me with the spirit of ambition, commitment and creativity, and who have always believed in me, even when I didn't believe in myself – this dissertation would not be possible without your wisdom, encouragement, and unconditional love. Whatever I am, it is because of you.

To all those who remain unnamed but who nonetheless have a hand in the successful realization of this goal of mine, thank you from the bottom of my heart – it truly takes a village.

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When circulating energy exceeds the storage capacity of WAT, such as in obesity or lipodystrophy, lipids accumulate in ectopic locations, including liver and muscle. Ectopic storage of lipids causes metabolic dysfunction, including insulin resistance and hepatosteatosis.

The intrinsic cellular and metabolic properties of different adipocytes are shaped by the specific niches in which they reside. Important regional differences in the developmental, molecular, and functional profiles of four major types of adipocytes (subcutaneous, visceral, brown, marrow) are depicted.

(**A**) Wnts are lipid-modified secreted glycoproteins (~30-40 kDa) characterized by a signal peptide sequence (yellow), varying numbers of N-glycosylation sites (N-glyc; purple), and two conserved lipid modifications: palmitic acid (C16:0) at Cys77 and palmitoleic acid (C16:1) at Ser209. Palmitoleoylation (C16:1) at Ser209 is required for the interaction between Wnts and their dedicated chaperone protein, Wntless; palmitoylation (C16:0) at Cys77 is required for functionality of secreted Wnts. (**B**) Wntless is an evolutionarily conserved transmembrane protein required for intracellular trafficking and secretion of lipidated Wnts. Wntless (~62 kDa) is predicted to have seven transmembrane domains (TMD; red), a signal peptide sequence (yellow), an endocytosis motif (green), and a hydrophobic lipocalin domain (teal) thought to be the site of interaction with Wnts.

After Wnt proteins are synthesized in the endoplasmic reticulum (ER), they undergo significant post-translational modifications, including N-glycosylation and lipidation. The ER acyltransferase Porcupine catalyzes addition of palmitic (C16:0) and palmitoleic (C16:1) acid moieties at conserved Cys77 and Ser209 residues, respectively. Palmitoleoylation (addition of C16:1) is required for interaction of Wnts with their chaperone protein, Wntless. Wntless binds to and chaperones Wnt proteins from the ER through the trans-Golgi network, and to the plasma membrane in secretory vesicles. Once Wnts are secreted, Wntless is transported back via the retromer complex to the Golgi for reuse or to lysosomes for degradation.

(**A**) β-catenin is comprised of a central 12-unit Armadillo repeat domain (ARM) flanked on either side by distinct N- and C-terminal domains. The N-terminal region consists of conserved serine and threonine residues that are sequentially phosphorylated by CK1α and GSK3β; phosphorylation of these sites promotes binding to β-TrCP, subsequent ubiquitination, and proteasomal degradation. (**B**) The ARM region forms a superhelix featuring a positively charged groove that serves as a platform for interactions with various β-catenin binding partners, including E-cadherin, Axin, APC, and TCF/LEF proteins. These partners share overlapping binding sites in the ARM groove, and thus typically cannot bind simultaneously.

In the absence of extracellular Wnts (inactive; left panel), β-catenin is bound by a destruction complex comprised of Axin, APC, GSK3β, CK1α, and PP2A. CK1α and GSK3β sequentially phosphorylate free cytosolic β-catenin, targeting it for ubiquitination by βTrCP and subsequent proteasomal degradation. When canonical Wnts bind to their frizzled receptors and LRP co-receptors (active; right panel), they trigger an intracellular signaling cascade that leads to hypo-phosphorylation, stabilization, and accumulation of cytosolic β-catenin. β-catenin can then translocate to the nucleus, displace Groucho/TLE repressors, and coactivate TCF/LEF transcription factors to mediate Wnt target gene expression.

Activation of canonical Wnt/β-catenin signaling in mesenchymal stem cells suppresses adipogenesis and promotes osteoblastogenesis. Wnt signaling suppresses adipocyte differentiation by inhibiting expression of PPARγ and C/EBPα, the central regulators of adipogenesis.

**CHAPTER II: Methodology for Dissection of Diverse Murine Adipose Depots**

Adipocytes are located throughout the body in discrete depots, as well as singly and in small clusters associated with vascular and lymphatic structures. Although adipocytes throughout the body have overlapping molecular and metabolic characteristics, the degree to which these properties are realized will undoubtedly depend on the specific niches in which these cells reside.

Gross anatomy of (**A**) anterior subcutaneous; (**B**) brown; (**C**) epididymal (b, bladder); (**D**) ovarian (u, uterine horns); (**E**) posterior subcutaneous (dorsolumbar (d), inguinal (i), gluteal (g)); (**F**) inguinal; (**G**) mesenteric; (**H**) perirenal (k, kidney); (**I**) retroperitoneal (k, kidney); (**J**) pericardial (h, heart); and (**K**) popliteal adipose depots in C57BL/6J adult mice. Arrows point to specific depots if multiple depots are depicted. Relevant organs are designated by appropriate letters.

Histology of (**A**) anterior subcutaneous; (**B**) dorsolumbar; (**C**) inguinal; (**D**) gluteal; (**E**) brown; (**F**) gonadal; (**G**) perirenal; (**H**) retroperitoneal; (**I**) omental; (**J**) mesenteric; (**K**) pericardial; (**L**) popliteal; (**M**) constitutive and (**N**) regulated bone marrow adipose; (**O**) intermuscular; and (**P**) infrapatellar depots in C57BL/6J adult mice. Tissues were isolated according to the presented protocol, fixed overnight in 10% neutral buffered formalin, processed, and embedded in paraffin. 5 μm sections were stained with H&E. Most images were taken at 100x magnification; scale bar = 100 μm.

# Chapter III: Wntless Regulates Lipogenic Gene Expression in Adipocytes and Protects Against Diet-Induced Metabolic Dysfunction

(**A-B**) MSCs isolated from C57BL/6J mice were cultured under standard conditions and induced to differentiate. Wntless gene (n = 6) and protein expression at indicated days of differentiation. (**C**) Wntless gene expression in the stromal-vascular (SVF) and adipocyte (Ads) fractions isolated from epididymal (eWAT) and inguinal (iWAT) white adipose tissues of C57BL/6J mice (males; n = 6). (**D**) Representative immunoblot of Wntless protein expression across C57BL/6J mouse tissues (asWAT, anterior subcutaneous WAT; pWAT, perirenal WAT; BAT, brown adipose tissue); Adiponectin, Laminin, and Ponceau S included as controls. (**E-F**) Regulation of Wntless gene and protein expression in eWAT and iWAT of 16-week-old wildtype mice fed with normal chow diet (NCD) or 8 weeks of high fat diet (HFD) (males; n = 6). (**G**) Wnt-related genes found to be significantly up- or down-regulated by RNA-seq analyses of day 12 adipocytes vs. day 0 cultured MSCs (n = 4). (**H**) Select Wnt-related genes found to be significantly up- or down-regulated by RNA-seq analyses of day 12 cultured adipocytes treated with recombinant Wnt3a (20 ng/ml) for 4 h (n = 4). RNA expression normalized to PPIA. Data presented as mean $\pm$ S.D. * indicates significance at $p < 0.05$.

(**A**) Wntless gene expression in the stromal-vascular (SVF) and adipocyte (Ads) fractions isolated from eWAT and inguinal iWAT of 16-week-old wildtype mice fed with NCD or 8 weeks of HFD (males; n = 6). (**B**) Regulation of Wntless gene expression in eWAT and iWAT by nutritional and environmental conditions: fast: 18 h; refed: 6 h after 18 h fast; 30% calorie restriction (CR): 6 wks; 4°C cold exposure: 6 hr. Each condition normalized to its individual experimental control set to 1 (indicated by grey dashed line). (**C**) Wntless gene expression in male and female human tissues; data obtained from the GTEx-RNA-Seq dataset. (**D**) Wnt-related genes found by RNA-seq analyses to be expressed at similar levels in both day 12 adipocytes and confluent day 0 cultured MSCs (n = 4). RNA expression normalized to PPIA. Data presented as mean $\pm$ S.D. * indicates significance at $p < 0.05$.

(**A**) Schematic model for deletion of Wntless in cultured adipocytes using adenoviral Cre recombinase. (**B**) Genetic recombination of Wntless in $Wls^{fl/fl}$ and $Wls^{-/-}$ adipocytes using a 3-primer PCR system (n = 3). (**C-D**) Wntless RNA and protein expression in adipocytes following adenoviral GFP or Cre infection (n = 3). (**E**) Representative brightfield and Oil Red-O images, and (**F**) triacylglycerol accumulation in $Wls^{fl/fl}$ and $Wls^{-/-}$ adipocytes (n = 4). (**G**) Expression of known Wnt target genes in $Wls^{fl/fl}$ and $Wls^{-/-}$ adipocytes (n = 3). (**H**) Free cytosolic and membrane $\beta$-catenin protein expression in $Wls^{fl/fl}$ and $Wls^{-/-}$ confluent MSCs under basal conditions (n = 6) and after 20 ng/ml Wnt3a treatment for 4 h (n = 2); GAPDH and laminin shown as cytosolic and membrane loading controls, respectively. RNA expression normalized to PPIA. Data presented as mean $\pm$ S.D. * indicates significance at $p < 0.05$.

(**A**) Densitometry quantification of cytosolic and membranous $\beta$-catenin protein expression in $Wls^{fl/fl}$ and $Wls^{-/-}$ day 0 MSCs under basal conditions and after 20 ng/ml Wnt3a treatment for 4 h (n = 6). (**B**) Proteins found by untargeted proteomics analysis to be increased over 200% or decreased more than 50% in cultured day 12 $Wls^{-/-}$ adipocytes (n = 1). Data presented as mean $\pm$ S.D. * indicates significance at $p < 0.05$.

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(**A**) Lipogenic proteins quantified by densitometry (n = 3). (**B**) *De novo* lipogenesis was evaluated in cultured *Wls*<sup>fl/fl</sup> and *Wls*<sup>-/-</sup> adipocytes using [$^{14}$C]-acetate for 1, 2, 4 and 8 h. Radiolabel incorporation into PL and DAG fractions extracted from *Wls*<sup>fl/fl</sup> and *Wls*<sup>-/-</sup> adipocytes was quantified by scintillation counting (n = 3). (**C**) Basal and induced glycerol release from *Wls*<sup>fl/fl</sup> and *Wls*<sup>-/-</sup> adipocytes using 1 μM isoproterenol (Iso), 5 μM forskolin (Forsk), or 1 μM CL-316,243 (CL-316) for 3 h (n = 3). (**D**) β-oxidation of [$^{3}$H]-palmitic acid; 100 μM etomoxir used as negative control (n = 3). (**E**) Expression of proteins related to lipolysis or β-oxidation (n = 3). Data presented as mean ± S.D. * indicates significance at $p < 0.05$.

(**A-B**) Gene and protein expression of indicated transcription factors (n = 3); I: immature, insoluble form of SREBP1c at relative mobility of 100 kDa; M: mature, soluble form of SREBP1c at 50 kDa. (**C**) Mature versus immature forms of SREBP1c quantified by densitometry. (**D**) Basal and insulin-stimulated glucose uptake into *Wls*<sup>fl/fl</sup> and *Wls*<sup>-/-</sup> adipocytes (n = 3). (**E**) Representative immunoblot of AKT phosphorylation in *Wls*<sup>fl/fl</sup> and *Wls*<sup>-/-</sup> adipocytes following treatment with indicated insulin concentrations for 10 min. (**F**) Expression of *Srebf1* or *Mlxipl* mRNAs in *Wls*<sup>fl/fl</sup> and *Wls*<sup>-/-</sup> adipocytes following treatment with indicated insulin concentrations for 24 h (n = 3). (**G**) Expression of *Scap* mRNA in *Wls*<sup>fl/fl</sup> and *Wls*<sup>-/-</sup> adipocytes following treatment with indicated insulin concentrations for 24 h (n = 3). (**H**) Representative immunoblot of SCAP protein expression in *Wls*<sup>fl/fl</sup> and *Wls*<sup>-/-</sup> adipocytes following treatment with indicated insulin concentrations for 10 min; pAKT(thr308) shown as a control for insulin response. RNA expression normalized to PPIA. Data presented as mean ± S.D. * indicates significance at $p < 0.05$.

(**A**) Growth curve of 20-week-old female *Wls*<sup>fl/fl</sup> and *Wls*<sup>-/-</sup> mice on NCD. (**B**) Glucose tolerance test in 16-week-old *Wls*<sup>fl/fl</sup> and *Wls*<sup>-/-</sup> mice. (**C**) Insulin tolerance test in 18-week-old mice. (**D**) Body composition of 16-week-old *Wls*<sup>fl/fl</sup> and *Wls*<sup>-/-</sup> mice. (**E**) Blood glucose concentrations in random-fed and 16 h fasted mice. Serum concentrations of (**F**) insulin, (**G**) adiponectin, and (**H**) triacylglycerols in 20-week-old mice. (**I**) Tissue weights at time of sacrifice. *Wls*<sup>fl/fl</sup>: n = 7; *Wls*<sup>-/-</sup>: n = 9. Data presented as mean ± S.D. * indicates significance at $p < 0.05$.

(**A**) Genetic recombination of Wntless in tissues isolated from *Wls*^{-/-} mice. (**B-C**) Wntless mRNA and protein expression in iWAT and eWAT of *Wls*^{fl/fl} and *Wls*^{-/-} mice. (**D**) Wntless mRNA expression in SVF and adipocytes isolated from iWAT and eWAT of *Wls*^{fl/fl} and *Wls*^{-/-} mice (n = 3). (**E**) Body composition of 16-week-old *Wls*^{fl/fl} and *Wls*^{-/-} mice on NCD. (**F**) Growth curve of 20-week-old *Wls*^{fl/fl} and *Wls*^{-/-} mice. (**G**) Glucose tolerance test in 16-week-old *Wls*^{fl/fl} and *Wls*^{-/-} mice. (**H**) Insulin tolerance test in 18-week-old mice. (I) Blood glucose concentrations in random-fed and 16 h fasted mice. Serum concentrations of (**J**) insulin, (**K**) adiponectin, and (**L**) triacylglycerols in 20-week-old mice. (**M**) Tissue weights at time of sacrifice. RNA expression normalized to PPIA. Data shown in **E-M** from male mice, n = 5 per group. Data presented as mean $\pm$ S.D. * indicates significance at $p < 0.05$.

(**A**) Representative histological images of H&E-stained distal and proximal tibia of male and female *Wls*^{fl/fl} and *Wls*^{-/-} mice fed NCD for 20 weeks; 100x magnification; scale bar, 50 µm. µCT analyses of (**B**) tibial length measured by distance from growth plate (GP) to tibia/fibula junction (T/F J), (**C**) mid-tibia cortical bone area (Ct. BA/TA), (**D**) bone mineral density (Ct. BMD), (**E**) tibial trabecular bone volume fraction (Tb. BV/TV), (**F**) trabecular number (Tb. N), (**G**) trabecular bone mineral density (Tb. BMD), (**H**) trabecular separation (Tb. Sp) and (**I**) trabecular thickness (Tb. Th). Data presented as mean $\pm$ S.D. * indicates significance at $p < 0.05$.

(**A**) Representative histological images of H&E-stained tissues from *Wls*^{fl/fl} and *Wls*^{-/-} mice fed normal chow diet for 20 weeks; 200x magnification; scale bar, 100 µm. (**B**) Lipogenic gene expression in eWAT and iWAT isolated from *Wls*^{fl/fl} and *Wls*^{-/-} mice. (**C**) Lipogenic gene expression in isolated eWAT adipocytes (n = 7). (**D**) Wnt target gene expression in isolated eWAT adipocytes and SVF of *Wls*^{fl/fl} and *Wls*^{-/-} mice (n = 5). (**E**) Expression of Wnt mRNAs in SVF isolated from eWAT of *Wls*^{fl/fl} and *Wls*^{-/-} mice (n = 6). Data shown from male mice. RNA expression normalized to PPIA. Data presented as mean $\pm$ S.D. * indicates significance at $p < 0.05$.

(**A**) Lipogenic gene expression in livers isolated from male $Wls^{fl/fl}$ and $Wls^{-/-}$ mice on NCD. (**B**) Representative plots of flow cytometric analyses of eWAT SVF isolated from male $Wls^{fl/fl}$ and $Wls^{-/-}$ mice, stained with CD31-APC (endothelial cells) and CD45-PE (immune cells). (**C**) Quantification of SVF cell populations based on flow cytometric analyses (n = 6). RNA expression normalized to PPIA. Data presented as mean ± S.D. * indicates significance at p < 0.05.

(**A**) Growth curve over time of 32-week-old $Wls^{fl/fl}$ and $Wls^{-/-}$ mice fed 60% HFD for 24 weeks. (**B**) Body composition analysis of 28-week-old mice. (**C**) Glucose tolerance test in 28-week-old mice. (**D**) Serum insulin concentrations in fed and 16 h fasted mice. (**E**) Serum insulin concentrations in 16 h fasted mice at indicated times after intraperitoneal glucose injection (1 mg/kg body weight). (**F**) Insulin tolerance test in 30-week-old mice. (**G**) Blood glucose concentrations in random-fed and 16 h fasted mice. Circulating concentrations of (**H**) leptin, (**I**) adiponectin, (**J**) triacylglycerols, and (**K**) total and free cholesterol in 32-week-old mice. (**L**) Tissue weights of mice at time of sacrifice. Data shown from male mice; $Wls^{fl/fl}$: n = 8; $Wls^{-/-}$: n = 7. Data presented as mean ± S.D. * indicates significance at p < 0.05.

(**A**) Daily food intake of male $Wls^{fl/fl}$ and $Wls^{-/-}$ mice fed HFD for 24 weeks. (**B**) Adipocyte size quantification of iWAT (400-500 adipocytes/mouse; n = 8, 7). (**C**) Representative H&E-stained images and UCP1 protein expression in BAT of male $Wls^{fl/fl}$ and $Wls^{-/-}$ mice fed HFD for 24 weeks; 200x magnification; scale bar, 100 μm. (**D**) Expression of Wnt mRNAs in SVF isolated from eWAT of HFD-fed male $Wls^{fl/fl}$ and $Wls^{-/-}$ mice (n = 10). (**E**) Lipogenic gene expression in livers isolated from male $Wls^{fl/fl}$ and $Wls^{-/-}$ mice on HFD. RNA expression normalized to PPIA. Data presented as mean ± S.D. * indicates significance at p < 0.05.

(**A**) Representative histological images of H&E-stained tissues from *Wls*<sup>fl/fl</sup> and *Wls*<sup>-/-</sup> mice fed 60% HFD for 24 weeks; 200x magnification; scale bar, 100 μm. (**B**) Adipocyte size quantification of eWAT (400-500 adipocytes/mouse, n = 8, 7). (**C**) Wnt target gene expression in adipocytes and SVF isolated from eWAT. (**D-E**) Lipogenic mRNA and protein expression in isolated eWAT adipocytes. (**F**) Lipogenic gene expression in eWAT and iWAT from *Wls*<sup>fl/fl</sup> and *Wls*<sup>-/-</sup> mice. Data shown from male mice. RNA expression normalized to PPIA. Data presented as mean ± S.D. * indicates significance at $p < 0.05$.

Adipocyte-derived Wnts are required for expression of a network of lipogenic genes and loss of secreted Wnts in *Wls*<sup>-/-</sup> adipocytes is sensed and compensated for by surrounding stromal-vascular cells to maintain tissue-wide Wnt signaling homeostasis.

**CHAPTER IV: Wnt/β-catenin Signaling Regulates Adipose Tissue Lipogenesis and Adipocyte-Specific Loss is Rigorously Defended by Surrounding Stromal-Vascular Cells.**

(**A-B**) Mesenchymal stem cells (MSC) isolated from C57BL/6J mice were cultured under standard conditions and induced to differentiate. Ctnnb1 gene (n = 6) and protein (n = 2) expression at indicated days of adipogenesis. (**C**) Ctnnb1 gene expression in stromal-vascular (SVF) and adipocyte (Ads) fractions isolated from epididymal (eWAT) and inguinal (iWAT) white adipose tissues (WAT) of C57BL/6J mice (males; n = 5). (**D**) Expression of Ctnnb1 in eWAT and iWAT of mice fed normal chow diet (NCD) or high fat diet (HFD) for 10 weeks. (**E**) Ctnnb1 expression in SVF and Ads of eWAT and iWAT isolated from NCD- and HFD-fed mice (males; n = 6). (**F**) Ctnnb1 allele structure and genetic recombination in β-catfl/fl and β-cat-/- adipocytes using a 3-primer PCR system (n = 3). (**G-H**) Ctnnb1 RNA (n = 6) and protein (n = 3) expression in adipocytes following adenoviral GFP or Cre infection. (**I**) Representative brightfield and Oil Red-O images, and (**J**) triacylglycerol (TAG) accumulation in β-catfl/fl and β-cat-/- adipocytes (n = 6). (**K**) Expression of Ctnnb1 and downstream Wnt target genes in β-catfl/fl and β-cat-/- adipocytes treated with vehicle or 3

µM CHIR99021 for 4 h (n = 6). RNA expression normalized to PPIA. Data presented as mean ± S.D. * indicates significance at p < 0.05.

(**A**) Representative immunoblot of β-catenin protein expression across C57BL/6J mouse tissues (BAT, brown adipose tissue; pWAT, perirenal WAT); adiponectin and laminin included as controls. (**B**) *Dlk1* and *Adipoq* gene expression in SVF and adipocyte fractions isolated from eWAT and iWAT of C57BL/6J mice (males; n = 5). (**C-D**) *Lep* and *Nkd1* mRNA expression in SVF and adipocytes isolated from eWAT and iWAT of mice fed NCD or 10 weeks of HFD (males; n = 5). (**E**) Regulation of *Ctnnb1* gene expression in eWAT and iWAT by nutritional and environmental conditions: fast: 18 h; refed: 6 h after 18 h fast; 30% calorie restriction (CR): 6 weeks; 4°C cold exposure: 6 h. Each condition normalized to its individual experimental control set to 1 (indicated by dashed line; n = 6). (**F**) *Ctnnb1* gene expression in male and female human tissues; data obtained from the GTEx-RNA-Seq dataset. (**G**) Schematic model for deletion of β-catenin in cultured preadipocytes or adipocytes using adenoviral Cre recombinase. (**H**) *Ctnnb1* gene recombination (n = 2) and β-catenin protein expression (n = 4) in preadipocytes treated with adenoviral GFP or Cre. (**I**) Expression of downstream Wnt target genes in *β-cat*$^{fl/fl}$ and *β-cat*$^{-/-}$ adipocytes treated with vehicle or 3 µM CHIR99021 for 4 h (n = 6). RNA expression normalized to PPIA. Data presented as mean ± S.D. * indicates significance at p < 0.05.

RNA-seq analyses were performed on *β-cat*$^{fl/fl}$ and *β-cat*$^{-/-}$ adipocytes under basal conditions or after 4 h treatment with recombinant Wnt3a (20 ng/ml; n = 4 per group). (**A**) Heat maps of differential gene expression changes in *β-cat*$^{fl/fl}$ and *β-cat*$^{-/-}$ adipocytes under basal conditions (left panel) and *β-cat*$^{fl/fl}$ cells treated with vehicle or Wnt3a (right panel). (**B**) Gene Set Enrichment Analyses (GSEA) of genes expressed in *β-cat*$^{fl/fl}$ and *β-cat*$^{-/-}$ adipocytes under basal conditions (top panel) and *β-cat*$^{fl/fl}$ cells treated with vehicle or Wnt3a (bottom panel). (**C-D**) MA plots of gene expression changes following Wnt3a treatment of *β-cat*$^{fl/fl}$ or *β-cat*$^{-/-}$ adipocytes. (**E**) Venn diagram depicting meta-analysis of gene expression changes in *β-cat*$^{fl/fl}$ or *β-cat*$^{-/-}$ adipocytes treated with vehicle or Wnt3a for 4 h. (**F**) Expression of *Ctnnb1* and downstream Wnt target genes in *β-cat*$^{fl/fl}$ and *β-cat*$^{-/-}$ adipocytes treated with vehicle or 20 ng/ml recombinant Wnt3a for 4, 12 or 24 h (n = 6). RNA expression normalized to PPIA. Data presented as mean ± S.D. * indicates significance at p < 0.05.

RNA-seq analyses were performed on *β-cat*<sup>fl/fl</sup> and *β-cat*<sup>-/-</sup> preadipocytes under basal conditions or after 4 h treatment with recombinant Wnt3a (20 ng/ml; n = 4 per group). (**A**) Heat maps of differential gene expression changes in *β-cat*<sup>fl/fl</sup> and *β-cat*<sup>-/-</sup> preadipocytes under basal conditions (left panel) and *β-cat*<sup>fl/fl</sup> cells treated with vehicle or Wnt3a (right panel). (**B**) Gene Set Enrichment Analyses (GSEA) of genes in *β-cat*<sup>fl/fl</sup> and *β-cat*<sup>-/-</sup> preadipocytes under basal conditions (top panel) and *β-cat*<sup>fl/fl</sup> cells treated with vehicle or Wnt3a (bottom panel). (**C-D**) MA plots of gene expression changes following Wnt3a treatment of *β-cat*<sup>fl/fl</sup> or *β-cat*<sup>-/-</sup> preadipocytes. (**E**) Venn diagram depicting meta-analysis of gene expression changes in *β-cat*<sup>fl/fl</sup> or *β-cat*<sup>-/-</sup> preadipocytes treated with vehicle or Wnt3a for 4 h. (**F**) Expression of *Ctnnb1* and downstream Wnt target genes in *β-cat*<sup>fl/fl</sup> and *β-cat*<sup>-/-</sup> preadipocytes treated with vehicle or 20 ng/ml recombinant Wnt3a for 4, 12 or 24 h (n = 6). RNA expression normalized to PPIA. Data presented as mean ± S.D. * indicates significance at p < 0.05.

(**A-B**) Heat map and MA plot showing differentially expressed genes related to fatty acid, cholesterol, and bile acid metabolism in cultured *β-cat*<sup>fl/fl</sup> and *β-cat*<sup>-/-</sup> adipocytes (n = 4). (**C-D**) Lipogenic gene (n = 6) and protein (n = 3) expression in *β-cat*<sup>fl/fl</sup> and *β-cat*<sup>-/-</sup> adipocytes. (**E**) Proportion of total saturated versus unsaturated fatty acids in lipids extracted from *β-cat*<sup>fl/fl</sup> and *β-cat*<sup>-/-</sup> adipocytes (n = 3). (**F**) Relative proportions of myristic (C14:0) and palmitic (C16:0) versus myristoleic (C14:1, n-5) and palmitoleic (C16:1, n-7) acids (n = 3). (**G**) *De novo* lipogenesis (DNL) was evaluated in cultured *β-cat*<sup>fl/fl</sup> and *β-cat*<sup>-/-</sup> adipocytes using [$^{14}$C]-acetate for 2, 4 and 8 h. Incorporation of [$^{14}$C]-radiolabel into TAG fractions extracted from *β-cat*<sup>fl/fl</sup> and *β-cat*<sup>-/-</sup> adipocytes was quantified by scintillation counting (n = 6). (**H-I**) Gene (n = 6) and protein (n = 3) expression of indicated transcription factors in *β-cat*<sup>fl/fl</sup> and *β-cat*<sup>-/-</sup> adipocytes. (**J**) Protein expression in *β-cat*<sup>fl/fl</sup> and *β-cat*<sup>-/-</sup> adipocytes treated for 72 h with adenovirus expressing GFP, ChREBP, or SREBP1c (1 x 10$^5$ viral particles/ml). (**K**) Integrative Genomics Viewer capture showing Tcf7l2 peaks (indicating binding occupancy) in the regions ± 3 kb from transcription start sites (black arrows) of indicated genes in cultured *Tcf7l2*<sup>fl/fl</sup> and *Tcf7l2*<sup>-/-</sup> adipocytes. RNA expression normalized to PPIA. Data presented as mean ± S.D. * indicates significance at p < 0.05.

(**A**) Lipogenic proteins quantified by densitometry (n = 3). (**B**) Relative proportions of stearic (C18:0) versus vaccenic (C18:1, n-7) and oleic (C18:1, n-9) acids in $\beta$-cat$^{fl/fl}$ and $\beta$-cat$^{-/-}$ adipocytes (n = 3). [$^{14}$C]-radiolabel in (**C**) conditioned media versus (**D**) whole cell lysates after indicated incubation times measured by scintillation counting (n = 6). (**E**) Expression of genes related to acetate uptake and activation (n = 6). (**F-G**) Radiolabel incorporation into DAG and PL fractions extracted from $\beta$-cat$^{fl/fl}$ and $\beta$-cat$^{-/-}$ adipocytes quantified by scintillation counting (n = 6). (**H**) Linear regression analyses comparing slopes of acetate uptake over time from conditioned media into $\beta$-cat$^{fl/fl}$ and $\beta$-cat$^{-/-}$ adipocytes. (**I**) Linear regression analyses comparing slopes of radiolabel incorporation over time into TAG fractions of $\beta$-cat$^{fl/fl}$ and $\beta$-cat$^{-/-}$ adipocytes. (**J**) Gene expression in $\beta$-cat$^{fl/fl}$ and $\beta$-cat$^{-/-}$ adipocytes treated with adenovirus expressing GFP, ChREBP, or SREBP1c for 72 h (1 x 10$^5$ viral particles/ml; n = 4). RNA expression normalized to PPIA. Data presented as mean $\pm$ S.D. * indicates significance at $p < 0.05$.

(**A**) Genetic recombination in tissues isolated from $\beta$-cat$^{-/-}$ mice. (**B**) Growth curve of 28-week-old $\beta$-cat$^{fl/fl}$ and $\beta$-cat$^{-/-}$ mice. (**C**) Body composition of 16-week-old $\beta$-cat$^{fl/fl}$ and $\beta$-cat$^{-/-}$ mice on NCD. (**D**) Glucose tolerance tests in 16-week-old $\beta$-cat$^{fl/fl}$ and $\beta$-cat$^{-/-}$ mice. (**E**) Insulin tolerance test in 19-week-old mice. (**F**) Blood glucose concentrations in random-fed and 16 h fasted mice. Serum concentrations of (**G**) random-fed and fasted insulin and (**H**) adiponectin levels in 28-week-old mice. (**I**) Basal and stimulated lipolysis in 22-week-old mice (iso, isoproterenol: 10 mg/kg body weight). (**J**) Serum TAG in 28-week-old mice. (**K**) Tissue weights at time of sacrifice. (**L**) Representative histological images of H&E-stained tissues from $\beta$-cat$^{fl/fl}$ and $\beta$-cat$^{-/-}$ mice fed NCD for 28 weeks; 200x magnification; scale bar, 100 $\mu$m. Data in **B-L** from male mice, n = 8 per group. Data presented as mean $\pm$ S.D. * indicates significance at $p < 0.05$.

(**A**) Representative traces and quantification of daily running distance in chow-fed male 23-week-old $\beta$-cat$^{fl/fl}$ and $\beta$-cat$^{-/-}$ mice given access to voluntary exercise wheels for six weeks. (**B**) Growth curve of 28-week-old female $\beta$-cat$^{fl/fl}$ and $\beta$-cat$^{-/-}$ mice. (**C**) Body composition of 16-week-old $\beta$-cat$^{fl/fl}$ and $\beta$-cat$^{-/-}$ mice on NCD. (**D**) Glucose and insulin tolerance tests in 16- and 19-week-old $\beta$-cat$^{fl/fl}$ and $\beta$-cat$^{-/-}$ mice, respectively. (**E**) Blood

glucose concentrations in random-fed and 16 h fasted mice. Serum concentrations of (**F**) insulin, (**G**) adiponectin, and (**H**) TAG in 28-week-old mice. (**I**) Tissue weights at time of sacrifice. (**J**) Representative histological images of H&E-stained tissues from female $\beta$-cat$^{fl/fl}$ and $\beta$-cat$^{-/-}$ mice fed NCD for 28 weeks; 200x magnification; scale bar, 100 $\mu$m. Data in **B-J** from female mice, n = 6 per group. Data presented as mean $\pm$ S.D. * indicates significance at p < 0.05.

(**A**) Genetic recombination in tissues isolated from $\beta$-cat$^{fl/fl}$ and $\beta$-cat$^{-/-}$ mice (n = 3). (**B-C**) *Ctnnb1* mRNA (n = 3) and protein (n = 6) expression in eWAT and iWAT of $\beta$-cat$^{fl/fl}$ and $\beta$-cat$^{-/-}$ mice. (**D**) Genomic recombination of β-catenin in adipocytes and SVF isolated from eWAT and iWAT of $\beta$-cat$^{fl/fl}$ and $\beta$-cat$^{-/-}$ mice. (**E**) *Ctnnb1* mRNA expression in isolated eWAT adipocytes and SVF of $\beta$-cat$^{fl/fl}$ and $\beta$-cat$^{-/-}$ mice (n = 6). (**F**) β-catenin protein expression in isolated eWAT adipocytes and SVF of $\beta$-cat$^{fl/fl}$ and $\beta$-cat$^{-/-}$ mice; adiponectin and laminin shown as protein loading controls. (**G-H**) Wnt target gene expression in adipocytes and SVF isolated from eWAT of $\beta$-cat$^{fl/fl}$ and $\beta$-cat$^{-/-}$ mice (n = 8). (**I**) Representative plots showing flow cytometry analysis of SVF isolated from $\beta$-cat$^{fl/fl}$ and $\beta$-cat$^{-/-}$ mice (3 mice per sample; n = 3 samples). (**J**) Quantification of SVF cell proportions evaluated by flow cytometry analysis (3 mice per sample; n = 3 samples). (**K**) *Ctnnb1* mRNA expression normalized to PPIA in cellular fractions isolated by FACS analysis (3 mice per sample; n = 3 samples). Data presented as mean $\pm$ S.D. * indicates significance at p < 0.05.

(**A**) Representative histological images of H&E-stained proximal tibia (regulated bone marrow adipose tissue) of male and female $\beta$-cat$^{fl/fl}$ and $\beta$-cat$^{-/-}$ mice fed NCD for 28 weeks (male: n = 8; female: n = 6). (**B**) $\mu$CT analyses of tibial trabecular bone volume fraction (Tb. BV/TV) and trabecular bone mineral density (Tb. BMD). (**C**) Representative histological images of H&E-stained distal tibia (constitutive bone marrow adipose tissue) of male and female $\beta$-cat$^{fl/fl}$ and $\beta$-cat$^{-/-}$ mice. (**D**) $\mu$CT analyses of mid-tibial cortical bone area (Ct. BA/TA) and cortical bone mineral density (Ct. BMD). Histological images shown at 100x magnification; scale bar, 50 $\mu$m. Data presented as mean $\pm$ S.D. * indicates significance at p < 0.05.

(**A**) Growth curve over time of 32-week-old *β-cat*^{fl/fl} and *β-cat*[/-] mice fed 60% HFD for 24 weeks. (**B**) Body composition analysis of 28-week-old mice. (**C-D**) Glucose tolerance test and area under the curve analysis in 28-week-old mice. (**E**) Blood glucose concentrations in random-fed and 16 h fasted mice. (**F**) Insulin tolerance test in 30-week-old mice. (**G**) Serum insulin concentrations in random-fed mice or in 16 h fasted mice at indicated times after intraperitoneal glucose injection (1 mg/kg body weight). Serum (**H**) TAG, (**I**) total cholesterol, and (**J**) adiponectin in 32-week-old mice. (**K**) Tissue weights at time of sacrifice. (**L**) Representative histological images of H&E-stained tissues from *β-cat*^{fl/fl} and *β-cat*[/-] mice fed HFD for 24 weeks; 200x magnification; scale bar, 100 μm. (**M**) Quantification of liver TAG in 32-week-old mice. Data shown from male mice; *β-cat*^{fl/fl}: n = 5, *β-cat*[/-]: n = 11. Data presented as mean ± S.D. * indicates significance at p < 0.05.

(**A-D**) Lipogenic gene expression in whole eWAT, iWAT, liver, or isolated eWAT adipocytes of *β-cat*^{fl/fl} and *β-cat*[/-] mice (n = 8). (**E**) Expression of immune, endothelial, and stromal cell markers in SVF isolated from eWAT of *β-cat*^{fl/fl} and *β-cat*[/-] mice (n = 6). (**F**) Expression of inflammatory markers in whole eWAT of *β-cat*^{fl/fl} and *β-cat*[/-] mice (n = 6; n = 10). (**G-H**) Expression of cell markers and Wnt target genes in SVF sub-populations isolated by FACS analysis (3 mice per sample; n = 3 samples). RNA expression normalized to PPIA. Data presented as mean ± S.D. * indicates significance at p < 0.05.

(**A**) *Ctnnb1* mRNA expression in eWAT and iWAT of *β-cat*^{fl/fl} and *β-cat*[/-] mice fed HFD for 28 weeks (n = 5). (**B**) *Ctnnb1* mRNA expression in isolated eWAT adipocytes and SVF of HFD-fed *β-cat*^{fl/fl} and *β-cat*[/-] mice (n = 5). (**C**) β-catenin protein expression in isolated eWAT adipocytes and SVF of *β-cat*^{fl/fl} and *β-cat*[/-] mice fed HFD; adiponectin and laminin shown as controls. (**D-E**) Wnt target gene expression in SVF and adipocytes isolated from eWAT of obese *β-cat*^{fl/fl} and *β-cat*[/-] mice (n = 5). (**F**) Lipogenic gene expression in eWAT adipocytes isolated from HFD-fed *β-cat*^{fl/fl} and *β-cat*[/-] mice (n = 8). (**G**) Expression of immune, endothelial, and stromal cell markers in SVF isolated from eWAT of obese *β-cat*^{fl/fl} and *β-cat*[/-] mice (n = 8). (**H-I**) Expression of inflammatory markers in whole eWAT and iWAT of *β-cat*^{fl/fl} and *β-cat*[/-] mice fed HFD (n = 8). RNA

expression normalized to PPIA. Data presented as mean ± S.D. * indicates significance at $p < 0.05$.

(**A**) Daily food intake of male $\beta\text{-}cat^{fl/fl}$ and $\beta\text{-}cat^{-/-}$ mice fed HFD for 24 weeks. (**B**) ITT area under the curve analysis of male $\beta\text{-}cat^{fl/fl}$ and $\beta\text{-}cat^{-/-}$ HFD-fed mice. (**C**) Pancreas weights at time of sacrifice of HFD-fed mice ($n = 5$, $n = 11$). (**D-E**) mRNA expression of adipokines in eWAT and iWAT of HFD-fed mice ($n = 8$). (**F**) Immunoblot and densitometry quantification of UCP1 and β-catenin protein expression in BAT isolated from HFD-fed $\beta\text{-}cat^{fl/fl}$ and $\beta\text{-}cat^{-/-}$ mice ($n = 4$). Data presented as mean ± S.D. * indicates significance at $p < 0.05$.

Canonical Wnt/β-catenin signaling is required for coordinate regulation of adipocyte lipogenesis and fatty acid desaturation. In chow-fed mice, loss of adipocyte β-catenin is sensed and compensated for by neighboring stromal cells to defend tissue-wide canonical Wnt signaling homeostasis. Chronic overnutrition overrides this compensatory mechanism, revealing that adipocyte-specific β-catenin knockout mice are protected from diet-induced obesity and metabolic dysfunction.

# LIST OF ABBREVIATIONS

*β-cat*$^{-/-}$: adipocyte-specific β-catenin knockout

BAT: brown adipose tissue

DNL: *de novo* lipogenesis

ER: endoplasmic reticulum

eWAT: epididymal white adipose tissue

Fzd: frizzled

HFD: high fat diet

iWAT: inguinal white adipose tissue

LRP: low-density lipoprotein receptor-related protein

MSC: mesenchymal stem cell

NCD: normal chow diet

pWAT: perirenal white adipose tissue

sEV: small extracellular vesicle

SVC: stromal-vascular cell

SVF: stromal-vascular fraction

TAG: triacylglycerol

TCF/LEF: T-cell factor/lymphoid enhancer-binding factor

WAT: white adipose tissue

*Wls*$^{-/-}$: adipocyte-specific Wntless knockout

# ABSTRACT

Obesity is a key risk factor for many secondary chronic illnesses, including type 2 diabetes and cardiovascular disease. Canonical Wnt/$\beta$-catenin signaling is well-established as an important regulator of mesenchymal cell fate determination and differentiation, inhibiting adipogenesis and promoting osteoblastogenesis. Emerging genetic evidence in humans has linked various Wnt pathway members to body fat distribution, obesity, and metabolic dysfunction, suggesting that this pathway is also operative in terminally-differentiated adipocytes. Indeed, recent studies in mice have uncovered compelling evidence suggesting that the Wnt pathway plays important roles in adipocyte metabolism, particularly under obesogenic conditions. However, the exact functional roles of the Wnt pathway and its underlying molecular mechanisms in this context remain unclear due to complexities of Wnt signaling and differences in experimental models, approaches, and results.

My dissertation work endeavored to unravel the unique contributions of Wnt pathway members to adipocyte metabolism. To this end, I generated novel cultured cell and mouse models to functionally characterize the differential roles of two key pathway members, Wntless and $\beta$-catenin, in terminally-differentiated adipocytes. Deletion of Wntless, a dedicated intracellular chaperone for Wnt proteins, allowed me to investigate the functional roles of both canonical and non-canonical Wnts secreted from adipocytes, whereas loss of $\beta$-catenin allowed me to specifically interrogate the contribution of canonical Wnt signaling to adipocyte function.

These studies revealed for the first time that loss of adipocyte-derived Wnts or canonical Wnt/$\beta$-catenin signaling in adipocytes coordinately down-regulates a wide network of lipogenic genes, resulting in impaired *de novo* lipogenesis and fatty acid desaturation. Further, these effects on lipid metabolism are mediated by repression of

*Srebf1* and *Mlxipl*, known master transcriptional regulators of lipogenic enzyme expression. At first glance, Wntless and β-catenin do not appear to influence global metabolism in mice maintained on chow diet. However, my studies revealed a striking phenomenon by which adipose tissues defend adipocyte-specific loss of Wntless or β-catenin through compensatory up-regulation of Wnt signaling in neighboring stromal-vascular cells. Finally, long-term overnutrition overrides this compensatory mechanism, revealing that both adipocyte-specific Wntless and β-catenin knockout mice are resistant to diet-induced obesity, adipocyte hypertrophy, and metabolic dysfunction.

Taken together, my doctoral findings demonstrate that Wnt signaling in adipocytes is required for fatty acid and glucose metabolism. In addition, adipose tissues rigorously defend Wnt signaling homeostasis under standard nutritional conditions, underscoring the critical importance of this pathway in mature adipocyte metabolism and adipose tissue function. Finally, given the evolutionary conservation and ubiquitous nature of this pathway, it is likely that the findings herein, including compelling evidence of Wnt-mediated cross-talk between diverse cell types, will be widely applicable to the biology of other tissues.

## CHAPTER I

## Introduction

**Adipose tissues have important roles in metabolic health and disease.**

Historically, adipose tissue was considered to be an inert organ primarily responsible for storage of excess energy. The ability to store nutrients that can be mobilized during periods when energy demands exceed caloric intake is an evolutionarily preserved function; indeed, the ability to store energy in the form of cytoplasmic triacylglycerol is conserved from *Saccharomyces cerevisiae* to *Drosophila melanogaster* to *Homo sapiens*[1-3]. Over the past two decades, however, tremendous strides in understanding the molecular underpinnings of adipocyte development and physiology have replaced this simplistic view of adipose tissue function. We now know that adipose tissues have fundamental and complex roles not only in whole-body homeostasis, including storage and release of energy in response to local and global needs, but also in thermoregulation, mechanical support, and secretion of adipokines to regulate energy balance, metabolism and immune responses[4,5].

In 1949, researchers at Jackson Laboratory discovered a strain of mice harboring an autosomal recessive allele that caused them to weigh three times as much as normal mice[6]. These mice were fittingly designated *obese* (*ob*) and their profound phenotype suggested for the first time the existence of a single gene critical for feeding behavior, body weight, and global metabolism. Over 40 years later, the mouse *ob* allele and its corresponding human homolog were successfully positionally cloned in 1994[7]. The *ob* gene was found to encode a novel adipocyte hormone that was named leptin, after the Greek root *leptos* meaning "thin", based on the notion that leptin functions to keep mice from becoming obese[8]. Similar to *ob/ob* mice, humans lacking leptin due to genetic mutations are characterized by profound hyperphagic obesity, decreased energy expenditure and insulin insensitivity; these effects can be rescued in both humans and mice with recombinant leptin therapy[9,10]. Thus, the break-through

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discovery of leptin revealed the central role of adipocytes in global energy homeostasis and initiated a decades-long explosion of interest in adipose tissue biology.

Of course, interest has also surged in recent years due to the dramatic increase in global prevalence of obesity[11-13]. Indeed, nearly a third of the world's population is now classified as overweight or obese, and the epidemic has significantly impacted all age groups and both sexes, irrespective of ethnicity, geography, and socioeconomic status[12]. Obesity, defined as excess accumulation of white adipose tissue (WAT), has far-reaching consequences: it is correlated with elevated risk for many secondary chronic illnesses, including type 2 diabetes, dyslipidemia, and cardiovascular disease, reduced quality of life, increased medical care costs, and premature mortality[13-15].

Given the association between obesity and metabolic dysfunction, it is easy to assign the role of villain to WAT and overlook its beneficial functions. However, adipocytes provide a safe location for lipid storage, and expansion of WAT with obesity is the result, rather than the cause, of energy imbalance[16-18]. When circulating energy exceeds the storage capacity of WAT, lipids accumulate in ectopic locations, including muscle and liver; this ectopic storage is believed to cause metabolic dysfunction, including insulin resistance and hepatosteatosis[17,18]. Interestingly, lipodystrophy, a disorder characterized by WAT loss and redistribution, is associated with many of the same metabolic complications observed with obesity[19,20], providing further support for the protective role of adipocytes (**Figure 1.1**).

**Adipocytes located in discrete depots and niches have specialized characteristics and functions.**

Adipocytes are distributed throughout the body in discrete depots, and in some cases serve specialized roles within their microenvironments[21-25] (**Figure 1.2**). Most unilocular white adipocytes are distributed throughout the body in subcutaneous and visceral WAT depots[26,27] and the characteristics of these depots are important for differential susceptibility to metabolic diseases[3,18,26,28]. Indeed, subcutaneous white adipocytes, located beneath the skin, have been associated with protective metabolic effects[18,25,26], whereas visceral white adipocytes, which surround the vital organs and are contained within the gonadal, perirenal, retroperitoneal, omental and pericardial depots, are

commonly linked to metabolic disorders, including type 2 diabetes and cardiovascular disease[5,18,26].

First identified as thermogenic cells in 1961, classical multilocular brown adipocytes are contained in the interscapular brown adipose tissue (BAT) depot[21,29,30]. Additionally, clusters of brown adipocytes are also found in other locations throughout the body, including in supraclavicular, infra/subscapular, cervical, paravertebral and periaortic depots[30-32]. Brown and brown-like adipocytes are rich in mitochondria, express uncoupling protein 1 (UCP1), and play important roles in adaptive thermogenesis and glucose homeostasis[33-35]. When activated, UCP1 uncouples the electrochemical gradient driving ATP synthesis, thereby stimulating the respiratory chain to generate heat[36,37]. In response to various stimuli, including cold exposure, clusters of UCP1-expressing adipocytes, termed beige or "brite" (brown in white), also develop within WAT[38-41]. Although brown and beige/brite adipocytes share key features, like the ability to undergo thermogenesis, emerging evidence suggests that these cell populations are distinct in their development, molecular characteristics, and regulation[39,42-44].

Since fluorodeoxyglucose positron emission tomography (FDG-PET) studies identified the presence and functionality of BAT in adult humans ten years ago[45-49], this tissue has been considered an attractive target for weight loss and has thus enjoyed the spotlight in AT research over the last decade. Considerable efforts have been put forth to harness BAT biology as a therapeutic target for the treatment of obesity, including recruitment of new brown adipocytes or stimulation of thermogenesis in existing brown adipocytes to increase energy expenditure[41,50,51]. Although the question of whether BAT can feasibly be targeted in humans remains unanswered, the wider field of adipose biology has undoubtedly benefitted from a deeper understanding of brown adipocyte development and function.

In addition to their location in major WAT and BAT depots, adipocytes also exist in specialized niches throughout the body[22,23]. These depots are typically small in size, closely associated with other anatomic structures, and are thought to perform unique functions within their microenvironments[52]. Although recognition of their importance is growing, the underlying biology of these distinct groups of adipocytes remains, for the

most part, incompletely understood. For example, bone marrow adipose tissue (BMAT) serves as a lipid reservoir, is a major source of circulating adiponectin, and closely interacts with osteoblasts, osteoclasts, and hematopoietic cells[53-55]. Dermal adipocytes contribute to widespread processes, including wound healing, immune response, thermoregulation, and hair follicle growth[56-59]. Further, epicardial adipocytes may produce several adipokines and chemokines that exert local and systemic effects on the development and progression of coronary artery disease[60,61]. Expansion of inter/intramuscular WAT has been positively correlated with increased adiposity, systemic insulin resistance, and decreased muscular strength and mobility[62]. In addition, popliteal adipocytes serve as a lipid reservoir for lymphatic expansion during infection[63,64]. While the specific roles of different articular depots are generally unknown, the Hoffa (infrapatellar) depot within the knee is now thought to contribute to pathologies, including anterior knee pain and osteoarthritis[65].

Thus, although adipocytes throughout the body have overlapping molecular and metabolic characteristics, the degree to which these properties are realized may depend on the specific niches in which these cells reside. The developmental and functional differences between depots are currently under intense study. Investigation of discrete adipose depots will undoubtedly allow further definition of critical differences in their development, gene expression, and function.

**Wnts play fundamental and diverse roles in tissue development and maintenance.** The first mammalian *Wnt* gene, originally named *Integration 1* (*Int1*) and now known as *Wnt1*, was initially discovered in 1982 as a gene activated by integration of mouse mammary tumor virus (MMTV) pro-viral DNA in virally-induced mammary carcinomas[66]. During this time, independent studies in *Drosophila* larval development identified the fly *Wingless* (*Wg*) gene as a pivotal regulator of segment polarity and wing formation[67]. In 1987, *Int1* and *Wg* were found to be homologous genes[68]. Thus, the *Wnt* nomenclature was derived from the combination of both gene names to yield a consistent classification system across species; today, most homologs of *Int1* and *Wg*, from *Drosophila* to humans, share the *Wnt* name.

Since the initial discovery of *Wnt1*, interest in Wnt signaling and its fundamental functions in organismal biology has grown steadily. Wnt signaling is an ancient pathway used by all multicellular animals, from the earliest metazoans to mice to humans[69-72]. The mammalian Wnt signaling pathway is complex: the 19 different *Wnt* genes, 10 *Frizzled* genes and two *Lrp* co-receptor genes are accompanied by an intricate network of intracellular components, giving rise to promiscuous interactions on the cell surface and innumerable downstream molecular responses[71,73-78].

In 1991, mutation in the *Apc* gene, which encodes adenomatous polypopsis coli (APC), was identified as the genetic cause of familial adenomatous polypopsis, a hereditary cancer syndrome[79-81]. Shortly thereafter, APC was found to interact directly with central Wnt pathway protein β-catenin[82,83], providing the first direct connection between Wnt signaling and human disease. Since then, aberrant Wnt signaling has been implicated in diverse pathologic processes, including developmental disorders, cancers, and degenerative diseases[70,72,74,84,85].

Wnt ligands are evolutionarily conserved secreted, lipid-modified glycoproteins with well-established and diverse roles in cell proliferation, fate determination, and differentiation[70,72,75,86]. Analyses of Wnt primary amino acid sequences reveal that Wnt proteins share a conserved pattern of 23 cysteine residues and an N-terminal signal sequence for endoplasmic reticulum (ER) targeting and subsequent secretion[71,87,88]. During synthesis, Wnt proteins, which are ~30-40 kDa in size, undergo extensive post-translational processing to ensure proper secretion and function.

In addition to variable N-glycosylation by the oligosaccharyl transferase (OST) complex[88,89], all 19 Wnts are predicted to be lipid-modified by the O-acyltransferase enzyme Porcupine (Porcn)[90-93]: Wnt proteins undergo palmitoylation (addition of palmitic acid; C16:0) at conserved Cys77[94] and palmitoleoylation (addition of palmitoleic acid; C16:1) at conserved Ser209[95,96] (**Figure 1.3 A**). Porcn is a dedicated and highly conserved component of Wnt signaling; indeed, it is only active in Wnt-producing cells. Mutations in the *Porcn* gene lead to impaired Wnt lipidation, retention at the ER, and defective release from cells[92,96-98]. Whereas the functional necessity of N-glycosylation remains unclear[99,100], lipid modifications are critically coupled to the secretion and function of Wnts: Ser209 acylation is required for intracellular targeting and secretion[95],

whereas Cys77 acylation is required for proper extracellular signaling[94,101,102]. Indeed, mutation of Ser209 to Ala disrupts Wnt3a secretion and promotes accumulation at the ER; in contrast, mutation of Cys77 to Ala does not impact secretion but impairs downstream signaling activity.

**Wntless is a dedicated chaperone protein required for Wnt secretion.**

Wntless (Wls; also known as GPR177, Sprinter, or Evenness interrupted) is an evolutionarily conserved, multipass, transmembrane protein dedicated to trafficking of Wnts to the plasma membrane for secretion[103-105]. First discovered in *Drosophila* in 2006, the structure of Wls provides important clues for its intracellular function[103]. Analysis of its primary amino acid sequence predicts that Wls contains a long N-terminal region, seven to eight transmembrane domains, and an intracellular C-terminus (**Figure 1.3 B**). Similar to most membrane-associated proteins, Wls is modified with N-linked oligosaccharides; indeed, SDS-PAGE analysis of N-glycosidase-treated Wls protein yields a collapsed fast-migrating band[106,107]. Further, a lipocalin-like domain in the N-terminal region is essential for the association between Wls and Wnt1, Wnt3, and Wnt5a[108]. Thus, this lipocalin-like domain affords Wls the ability to bind to hydrophobic regions of Wnt proteins, such as the post-translational lipid modifications. Finally, Wls contains a conserved endocytosis signal motif that is recognized by clathrin adapter protein 2 and is critical for internalization and recycling of Wls from the plasma membrane; mutations in this motif result in accumulation of Wls at the cell surface[109-111].

After Wnts are produced, the covalent addition of acyl moieties at the ER renders Wnt proteins hydrophobic and limits their mobility through the secretory pathway[71]. Wls is localized throughout the Wnt secretory route, including the ER, Golgi apparatus, intracellular vesicles, and plasma membrane[112]. Thus, Wls binds modified Wnts at the ER and Golgi apparatus and facilitates their movement throughout the cell to the plasma membrane for extracellular release[103-105,113] (**Figure 1.4**). Importantly, palmitoleoylation at conserved Ser209 on Wnt proteins is thought to be required for binding to Wls; mutants lacking this lipid modification are unable to interact with Wls and are thus retained at the ER[95,114,115]. In contrast, Wnt proteins with defective N-glycosylation bind appropriately to Wls and are secreted normally. Following secretion

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of Wnts, Wls undergoes clathrin-mediated endocytosis[112,116]. Wls then has two distinct fates: it is either transported back to the ER and Golgi for reuse or is targeted for lysosomal degradation[116-118].

Two *Wls* knockout mouse models generated by distinct gene targeting strategies demonstrated that global homozygous *Wls* deletion is embryonic lethal[108,119]. Both models exhibit phenotypes observed with global deletion of *Wnt1*, *Wnt3*, *Wnt1* and *Wnt3* together, or *Ctnnb1* (β-catenin)[120-124], providing *in vivo* support for the role of Wls in Wnt secretion and function. Generation of a mouse harboring a floxed *Wls* allele has allowed the study of conditional tissue-specific Wls deletion[119]. Thus, ablation of Wls in various vertebrate and invertebrate animal models and tissues results in phenotypes consistent with loss of Wnt function, supporting the notion that Wls regulates signaling at the level of Wnt transport and secretion in signal-producing cells[103,119,125-130].

**Signal transduction through the canonical Wnt pathway**

Several pathways can be activated by binding of Wnt proteins to their receptors on receiving cells, including the canonical, non-canonical Wnt/calcium, and non-canonical planar cell polarity pathways[131-133]. Of these, the best studied is the canonical pathway, which regulates activation of Wnt target genes through stabilization of the central protein β-catenin[72,84,134].

In the late 1980s, two independent groups simultaneously identified β-catenin, based on its different functions in cell structure and signaling[135,136]. Studies of proteins associated with E-cadherin, a pivotal player in cell adhesion, identified α-catenin, β-catenin, and γ-catenin; collectively, these proteins were called catenins, based on the Latin word *catena* meaning chain, to reflect their roles in anchoring E-cadherin to the cytoskeleton[135,137]. Studies of its *Drosophila* ortholog Armadillo first uncovered the potential function of β-catenin in Wnt signaling: *armadillo* mutant flies exhibit developmental abnormalities in segment polarity, similar to *wg* mutants[136,138]. Subsequent studies demonstrated that *Drosophila* segmentation dysfunction in *armadillo* mutants is due to impaired *Wg* signaling[139]. These initial studies were pivotal for the ensuing characterization of the Wnt/β-catenin (Wg/Armadillo) signaling cascade.

The protein structure of β-catenin makes it uniquely suited for roles in both cell mechanics and signaling (**Figure 1.5 A**). β-catenin is comprised of a central region containing 12 Armadillo repeats and flanked on either side by distinct N- and C-terminal domains[140,141]. The Armadillo repeats form a superhelix featuring a positively charged groove that serves as a platform for interactions with various β-catenin binding partners in the adherens junctions, cytosol, and nucleus. Structural analyses have revealed that many β-catenin binding partners, including E-cadherin, APC, and T-cell factor/lymphoid enhancer-binding factor (TCF/LEF) proteins, share overlapping binding sites in this groove; thus, partners typically cannot bind simultaneously (**Figure 1.5 B**). All of these β-catenin interactors bind to a core binding site comprised of Armadillo repeats 3 through 9, forming salt bridges with two key amino acid residues, Lys 312 and Lys 435[140-143].

The majority of endogenous intracellular β-catenin is bound to E-cadherin and α-catenin at adherens junctions, whereas a small pool exists as free cytosolic β-catenin[134,144,145]. Association of β-catenin with E-cadherin protects it from binding to a multi-unit destruction complex comprised of Axin, APC, protein phosphatase 2A (PP2A), protein kinases casein kinase 1 α (CK1α) and glycogen synthase 3 β (GSK3β), and the E3 ligase β-TrCP[72,134,141,146]. In the absence of Wnt signaling, this destruction complex triggers rapid CK1α and GSK3β-mediated phosphorylation of N-terminal serine and threonine residues on free cytosolic β-catenin[147,148], thereby promoting its binding to and ubiquitination by β-TrCP. Phosphorylated and ubiquitinated β-catenin is subsequently targeted for proteasomal degradation to prevent activation of Wnt target genes[84,148-150] (**Figure 1.6**). The half-life of free cytosolic β-catenin is on the order of minutes, whereas the pool associated with adherens junctions is stable; thus, free cytosolic β-catenin is the Wnt-responsive signaling pool[134,151-153].

Once secreted, hydrophobic Wnt proteins have limited abilities to diffuse in the aqueous extracellular environment and thus act in an autocrine/paracrine manner. When Wnts bind to their membrane-spanning frizzled (Fzd) receptors and low-density lipoprotein receptor-related protein (LRP) co-receptors, they trigger an intracellular signaling cascade that disrupts the destruction complex and prevents phosphorylation

of free cytosolic β-catenin, thereby promoting its stabilization[72,84,134,146,154]. Accumulated β-catenin then translocates to the nucleus, where it associates with DNA-binding TCF/LEF proteins, which act as transcriptional switches. In the absence of Wnt signals, TCF/LEF factors form complexes with Groucho/transducin-like enhancer of split (TLE) to act as transcriptional repressors. Binding of β-catenin physically displaces Groucho/TLE, forming bipartite TCF/β-catenin co-activators that then mediate transcription of downstream Wnt target genes[134,153,155-159] (**Figure 1.6**).

**Canonical Wnt signaling is an important endogenous inhibitor of adipogenesis.**

Mesenchymal stem cells (MSC) arising from the mesoderm are multipotent and have the potential to differentiate into adipocytes, osteocytes, chondrocytes, or myocytes[160]; adipogenesis is the process by which MSCs differentiate into mature lipid-laden adipocytes. The transcriptional programming of adipogenesis has been studied extensively and is comprised of two phases: determination and terminal differentiation[161-164]. During determination, MSCs are committed to the adipogenic lineage and are converted to preadipocytes. Terminal differentiation is the process whereby preadipocytes then acquire characteristics of the mature adipocyte, including expression of cellular machinery required for lipid synthesis and storage, insulin sensitivity, and adipokine secretion. Studies have shown that induction of differentiation in precursors results in rapid activation of transcription factors, leading to dramatic changes in the epigenome[165,166]. These events culminate in the temporal induction of the master regulator of adipogenesis, peroxisome proliferator activated receptor γ (PPARγ), and other key adipogenic transcription factors, including CCAAT-enhancer binding protein α (C/EBPα) and C/EBPβ, which together drive the adipocyte gene program[162,167-169].

Canonical Wnt/β-catenin signaling has well-established roles in MSC fate determination and terminal differentiation. Although studies have explored Wnt-mediated regulation of myogenesis and chondrogenesis[170-172], the differential roles of this pathway in osteogenesis versus adipogenesis have been particularly well-characterized (**Figure 1.7**). Indeed, enforced expression of canonical Wnt3a, Wnt6, Wnt10a, Wnt10b, or a β-catenin stable mutant, in multipotent progenitor cells stabilizes

9

Wnt/β-catenin signaling, which promotes osteoblastogenesis and inhibits inhibits adipogenesis[173-179].

Further, canonical Wnt signaling activation in committed preadipocytes through pharmacological inhibition of GSK3 with lithium or CHIR99021, or enforced expression of Wnt1, Wnt6, Wnt10a, Wnt10b, or a dominant-stable form of β-catenin, suppresses induction of PPARγ and C/EBPα and thus blocks adipogenesis[151,152,180-182]. Similarly, overexpression of Pygo2, a nuclear promoter of β-catenin/TCF/LEF transcription, in preadipocytes mediates β-catenin signaling to inhibit differentiation, whereas Pygo2 deletion induces spontaneous differentiation[183]. Although β-catenin is often considered to function exclusively as a canonical Wnt effector, it can also signal through unrelated pathways in certain contexts. In response to testosterone, for example, β-catenin binds to the androgen receptor and is subsequently shuttled to the nucleus to coactivate TCF/LEFs to inhibit adipocyte differentiation[184]. Additionally, tumor necrosis factor α (TNFα) treatment blocks differentiation of preadipocytes by stabilizing β-catenin and enhancing TCF7L2-dependent transcription, suggesting that β-catenin mediates effects of inflammatory cytokines on adipogenesis[185].

Conversely, inhibition of Wnt signaling in precursor cells promotes adipogenic differentiation. Indeed, treatment of preadipocytes with soluble Wnt inhibitors such as secreted frizzled-related protein 1 (SFRP1) or SFRP2, or overexpression of negative pathway regulators, such as Axin or dominant-negative TCF4, results in spontaneous differentiation into adipocytes[151,152]. Enforced expression of nuclear β-catenin antagonist Chibby induces spontaneous adipogenesis by binding to β-catenin and blocking activation of TCF/LEF, thus repressing β-catenin-mediated transcription[186,187]. In contrast, loss of Chibby up-regulates β-catenin transcriptional activity and blocks differentiation[186,187].

In mice, conditional deletion of β-catenin in uterine mesenchymal cells impairs smooth muscle myogenesis and stimulates conversion of these progenitors into adipocytes[188]. This report provided the first *in vivo* evidence linking Wnt signaling and MSC fate determination. Since then, studies have shown that AT-specific overexpression of canonical Wnt10b results in dramatically reduced WAT mass and

resistance to genetic and diet-induced obesity, absence of BAT development, and increased trabecular bone thickness and strength, whereas loss of Wnt10b causes reduction of trabecular bone mass[189-191]. Additionally, deletion of Wnt signaling activator Pygo2 in progenitor cells promotes accumulation of visceral WAT, glucose intolerance, and insulin insensitivity in diet-induced obese mice[183].

A robust literature has also emerged to highlight the role of canonical Wnt/β-catenin signaling in bone development and homeostasis *in vivo*. Interest in Wnt signaling within the bone was first sparked by identification of *LRP5* loss-of-function mutations in human patients diagnosed with osteoporosis pseudoglioma[192]. Since then, mutations in many members of the Wnt signaling pathway, including *LRP4*, *LRP5*, *LRP6*, *LGR4*, *WNT1*, *WNT16*, and *SOST* have been associated with altered bone mass in humans[193-200]. Of particular interest, genome-wide studies across various human populations have also identified single-nucleotide polymorphism (SNP) in *CTNNB1* and *WLS* that are correlated with reduced bone mineral density and osteoporosis[201,202].

Thus, various mouse models manipulating Wnt components in bone development and function have been generated and characterized to date[200]. Deletion of either *Wls* or *Ctnnb1* in progenitor cells blocks osteoblast commitment and differentiation, resulting in severe osteopenia[203,204], whereas overexpression of β-catenin promotes bone formation[205]. Osteoblast-specific *Wls* knockout mice are characterized by severe impairment in bone formation, dramatic reductions in both cortical and trabecular bone mass, increased fracture risk, and premature lethality[128]. Further, *Ctnnb1* deletion in osteoblasts results in severe osteopenia due to increased osteoclastogenesis; in contrast, constitutive activation of β-catenin in osteoblasts impairs formation of osteoclasts[206,207]. Indeed, FABP4- and OCN-Wnt10b mice exhibit increased osteoblastogenesis and extensive trabeculation throughout the entire endocortical compartment, whereas *Wnt10b^{-/-}* mice are characterized by decreased trabecular bone volume and bone mineral density[191,208].

Finally, investigations into the role of sclerostin, a glycoprotein secreted predominantly by osteocytes, have opened a new avenue of inquiry into endocrine-mediated communication between WAT and bone. Sclerostin, encoded by *Sost*, interacts with LRP5 and LRP6 co-receptors, thereby preventing downstream

intracellular propagation of Wnt signaling[209-211]. In humans, elevated serum sclerostin levels are associated with increased body mass index, adiposity, fasting glucose concentrations, and insulin resistance[212,213]. Global deletion of *Sost* in mice profoundly influences both WAT and bone: *Sost*^{-/-} mice exhibit dramatically increased bone formation, decreased adiposity, and improved insulin sensitivity, whereas overproduction of sclerostin causes adipocyte hypertrophy and insulin insensitivity[214]. Taken together, these studies and many others illustrate the fundamental roles of Wnt signaling in directing MSC fate determination and the development of adipose tissue and bone.

**Recent genetic studies in humans and mice suggest fundamental roles for Wnt signaling in mature adipocyte metabolism.**

Whereas the critical role of Wnt signaling in regulation of adipogenesis has been well-studied, its function in terminally-differentiated adipocytes remains unclear. However, increasing genetic evidence is linking various Wnt signaling pathway members to body fat distribution, obesity, and metabolic dysfunction in humans. Indeed, missense *WNT10B* variants and certain *WNT5B* SNPs are associated with increased risk of obesity and type 2 diabetes, respectively[215,216], whereas polymorphisms in the Wnt inhibitor *SFRP5* locus have been linked to decreased adiposity in men[217]. Further, common variants in Wnt signaling inhibitor *ZNRF3* and Wnt signaling activator *RSPO3* are associated with increased waist-to-hip ratio[218-220]. Patients harboring loss-of-function mutations in Wnt co-receptors *LRP5* and *LRP6* are predisposed to develop impaired glucose homeostasis, coronary disease, and osteoporosis[221,222], whereas gain-of-function *LRP5* mutations are correlated with altered body fat distribution, increased adiposity, and osteosclerosis[223]. Nonsense mutations in *LGR4*, which encodes a protein that stabilizes Wnt receptors, are associated with reduced adiposity and impaired bone formation and remodeling[225]; in contrast, gain-of-function mutations in *LGR4* are correlated with increased visceral adiposity[224]. Strikingly, genome-wide association studies across a broad range of human populations have found that polymorphisms in the canonical Wnt effector *TCF7L2* are perhaps the strongest genetic risk factors associated with development of type 2 diabetes risk[226-228]. Finally, rare gain-of-function

mutations in *CTNNB1* (β-catenin) have recently been linked to altered body fat distribution and increased obesity risk[229]. Together, these studies provide compelling genetic evidence for critical roles of canonical Wnt/β-catenin signaling in regulation of adiposity, body composition, and metabolic health.

Recent studies in mice have also found that Wnt signaling is operative and important in adipocyte metabolism, but its exact functional roles in this context remain unclear[189,190,229-231]. For example, stabilization of Wnt signaling through global deletion of secreted frizzled-related protein 5 (SFRP5) – an adipocyte protein highly induced by obesity that binds to and sequesters Wnts – provides protection from diet-induced obesity[230]. Whereas total adipocyte numbers are unaffected, adipocytes in SFRP5-deficient mice have increased mitochondrial number and are smaller in size compared to those of control mice, resulting in reduced WAT and improved glucose tolerance. Adipocyte-specific deletion of *Ctnnb1* has recently been reported to cause decreased subcutaneous WAT mass and improved glycemic control in diet-induced obese mice[229]. In contrast, adipocyte-specific deletion of the Wnt target *Tcf7l2* results in adipocyte hypertrophy and impaired glucose homeostasis with high fat diet feeding[231].

These reports provide the first evidence that canonical Wnt signaling plays an important role in the ability of existing adipocytes to accommodate excess energy, but additional studies are required to unravel the complex mechanisms underlying this regulation. Thus, my doctoral work described in the following chapters endeavors to provide novel insights into the specific and differential contributions of various Wnt pathway members, including Wls and β-catenin, to mature adipocyte metabolism.

**Figure 1.1**



**OBESITY**                  **LIPODYSTROPHY**

Excess circulating lipids exceed ability of adipocytes to expand

Circulating lipids exceed ability of few existing adipocytes to expand

**ECTOPIC LIPID DEPOSITION**

| LIVER | MUSCLE | PANCREAS | SERUM |
|---|---|---|---|
| ↑ Hepatic steatosis | ↑ Insulin resistance | ↑ β-cell failure | ↓ Adiponectin |
| ↑ Insulin resistance | ↓ Fatty acid oxidation | ↓ Insulin secretion | ↓ Leptin signaling |
| | | | ↑ Glucose and TAG |

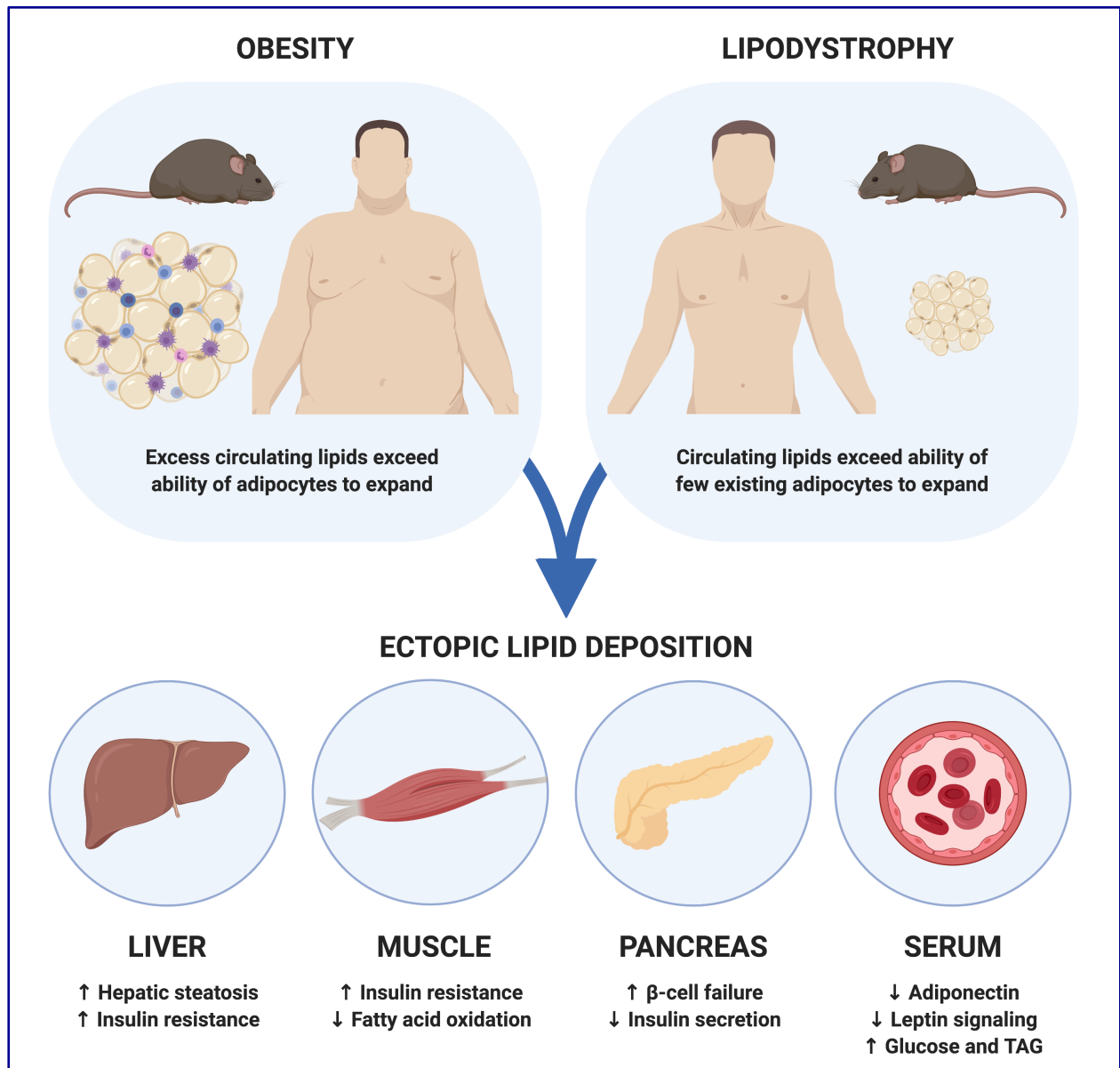**Figure 1.1. Adipocytes serve protective roles and safely store circulating lipids.** When circulating energy exceeds the storage capacity of WAT, such as in obesity or lipodystrophy, lipids accumulate in ectopic locations, including liver and muscle. Ectopic storage of lipids causes metabolic dysfunction, including insulin resistance and hepatosteatosis. *Adapted from Savage, 2009[232]; Rochford, 2014[233].*

# Figure 1.2

| | Subcutaneous | Visceral | Brown | Marrow |
|---|---|---|---|---|
| **ANATOMY** | | | | |
| Murine depots | Anterior subcutaneous: interscapular, subscapular, axillary, cervical  Posterior subcutaneous: dorsolumbar, inguinal, gluteal | Perigonadal (epididyma/parametrial), perirenal, mesenteric, omental, retroperitoneal | Interscapular (characteristics below have been studied in interscapular brown fat; other brown and brown-like depots are not addressed here) | Constitutive: distal tibia, tail vertebrae  Regulated: mid-to-proximal tibia, femur, lumbar vertebrae |
| Developmental origin | Lateral mesoderm  Mesodermal precursor | Lateral mesoderm  Mesodermal precursor | Paraxial mesoderm  Dermomyotomal precursor | ?  Mesodermal precursor |
| Metabolic risk with expansion | + | +++ | – | ? |
| **CELLULAR CHARACTERISTICS** | | | | |
| Adipocyte size (diameter) | +++  70 μm | ++++  100 μm | +  20 μm | ++  cMAT: 40 μm \| rMAT: 30 μm |
| Expansion with obesity | Hypertrophy  Hyperplasia | Hypertrophy | – | cMAT: none  rMAT: hyperplasia |
| Mitochondrial respiration | + | + | ++ | ? |
| Adiponectin secretion | ++ | +++ | + | ++++ (rabbit) |
| Leptin secretion | +++ | ++ | + | ? |
| Pro-inflammatory cytokine secretion | ++ | +++ | ? | ? |
| **METABOLIC CHARACTERISTICS** | | | | |
| Insulin-stimulated glucose uptake | + | + | +++ | ? |
| Cold-stimulated glucose uptake | + | + | +++ | ? |
| $\beta_3$AR-mediated lipolysis | ++ | +++ | +++ | ? |
| Triacylglycerol turnover rate | + | ++ | ? | ? |

Note: unless noted, all characteristics above have been defined in rodents housed in standard conditions on normal chow.

| Diet-induced obesity | Calorie restriction | Cold exposure |
|---|---|---|
| Subcutaneous: ↑ depot size, ↑ inflammation, ↑ metabolic dysfunction  Visceral: ↑ depot size, ↑ inflammation, ↑ metabolic dysfunction  Regulated marrow: ↑ depot size, ↓ bone mass | Subcutaneous: ↓ depot size, ↑ browning, ↑ metabolic function  Visceral: ↓ depot size, ↑ metabolic function  Brown: ↑ thermogenic activity  Regulated marrow: ↑ depot size, ↓ bone mass | Subcutaneous: ↓ depot size, ↑ browning, ↑ metabolic function  Visceral: ↓ depot size, ↑ browning, ↑ metabolic function  Brown: ↑ thermogenic activity  Regulated marrow: ↑ depot size |

**Figure 1.2. Adipocytes located in discrete depots and niches have specialized characteristics and functions.** The intrinsic cellular and metabolic properties of different adipocytes are shaped by the specific niches in which they reside. Important regional differences in the developmental, molecular, and functional profiles of four major types of adipocytes (subcutaneous, visceral, brown, marrow) are depicted. *Adapted from Bagchi et al., 2018[23].*

**Figure 1.3**
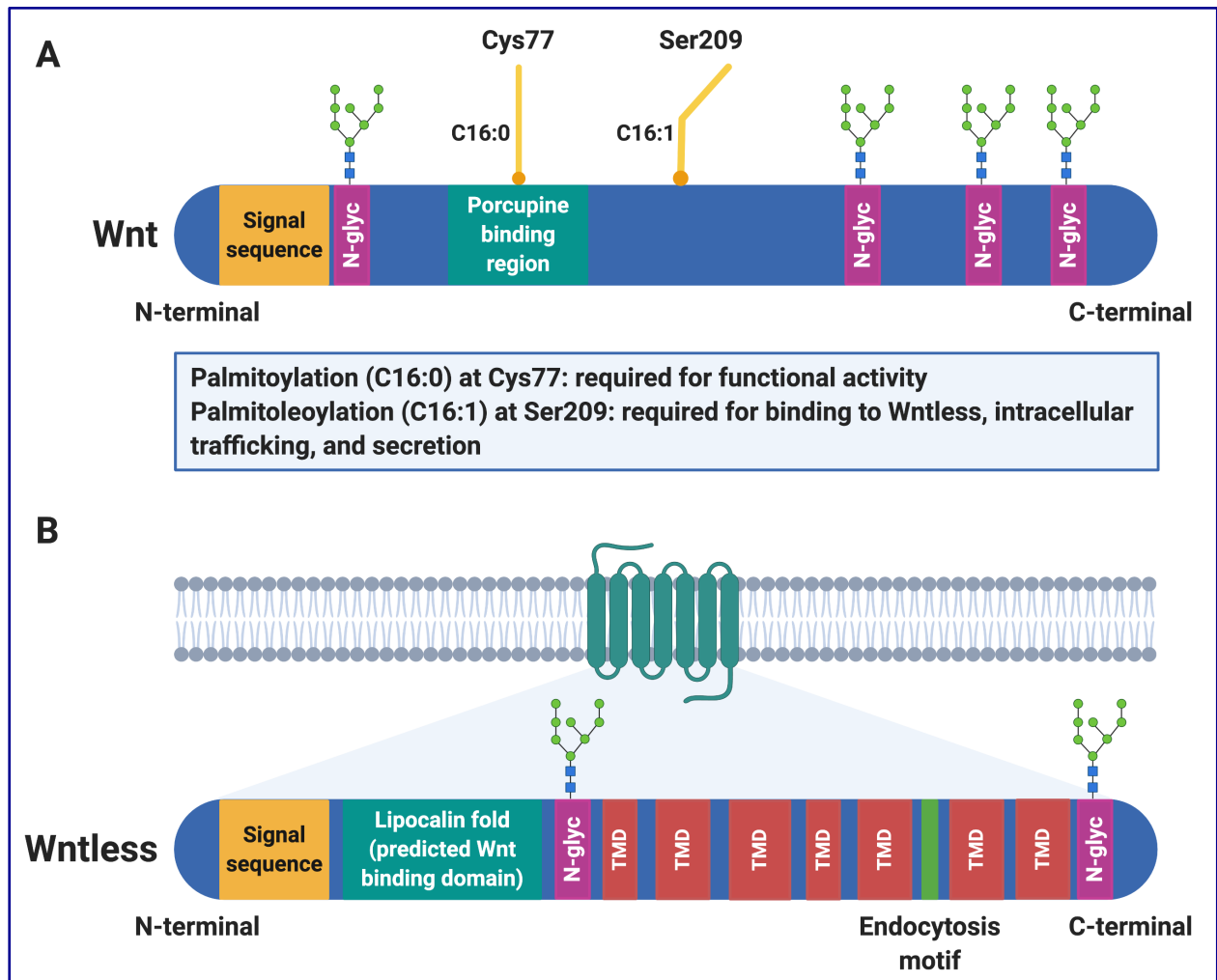


**Figure 1.3. Lipidation of Wnt ligands are required for binding to Wntless and subsequent intracellular trafficking, secretion, and functional activity.** (**A**) Wnts are lipid-modified secreted glycoproteins (~30-40 kDa) characterized by a signal peptide sequence (yellow), varying numbers of N-glycosylation sites (N-glyc; purple), and two conserved lipid modifications: palmitic acid (C16:0) at Cys77 and palmitoleic acid (C16:1) at Ser209. Palmitoleoylation (C16:1) at Ser209 is required for the interaction between Wnts and their dedicated chaperone protein, Wntless; palmitoylation (C16:0) at Cys77 is required for functionality of secreted Wnts. (**B**) Wntless is an evolutionarily conserved transmembrane protein required for intracellular trafficking and secretion of lipidated Wnts. Wntless (~62 kDa) is predicted to have seven transmembrane domains (TMD; red), a signal peptide sequence (yellow), an endocytosis motif (green), and a hydrophobic lipocalin domain (teal) thought to be the site of interaction with Wnts. *Adapted from Das et al., 2012[234]; Herr et al., 2012[115].*

**Figure 1.4**
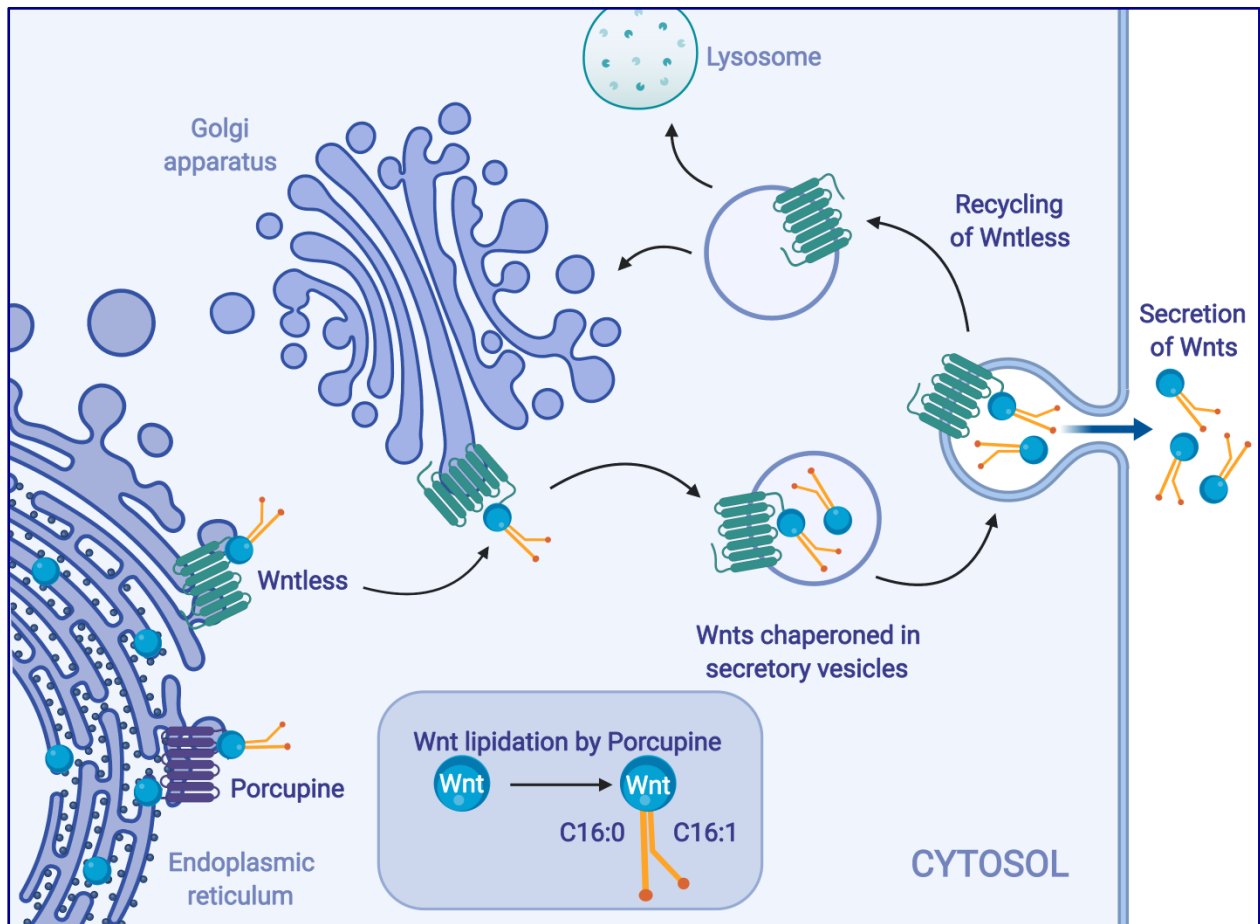


**Figure 1.4. Wntless is an evolutionarily conserved chaperone protein dedicated to trafficking lipidated Wnts through the intracellular secretory compartment.** After Wnt proteins are synthesized in the endoplasmic reticulum (ER), they undergo significant post-translational modifications, including N-glycosylation and lipidation. The ER acyltransferase Porcupine catalyzes addition of palmitic (C16:0) and palmitoleic (C16:1) acid moieties at conserved Cys77 and Ser209 residues, respectively. Palmitoleoylation (addition of C16:1) is required for interaction of Wnts with their chaperone protein, Wntless. Wntless binds to and chaperones Wnt proteins from the ER through the trans-Golgi network, and to the plasma membrane in secretory vesicles. Once Wnts are secreted, Wntless is transported back via the retromer complex to the Golgi for reuse or to lysosomes for degradation. *Adapted from Herr et al., 2012[115].*

**Figure 1.5**
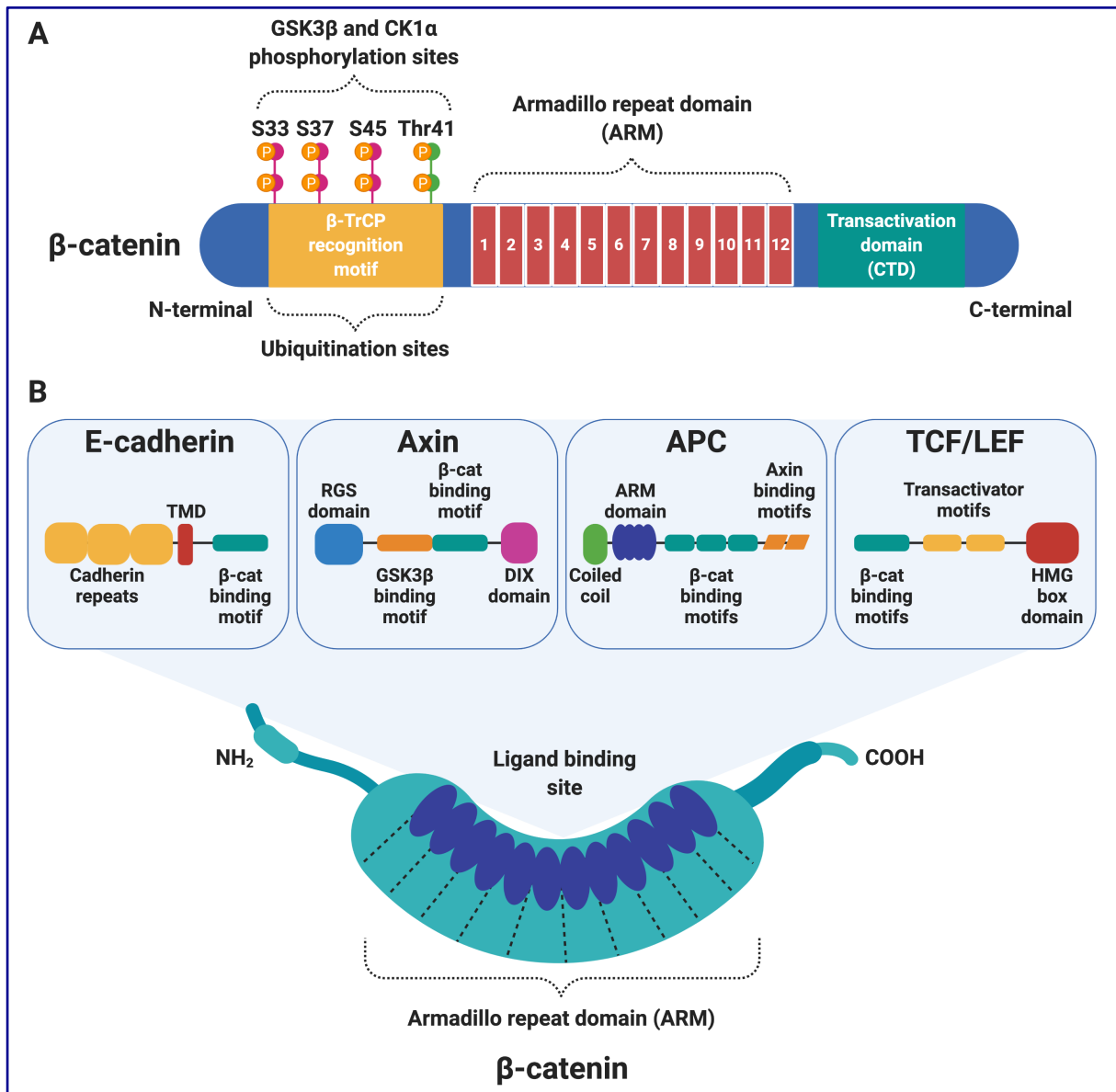


**Figure 1.5. Structural composition of β-catenin provides insights into its dual roles in cell mechanics and Wnt signaling.** (**A**) β-catenin is comprised of a central 12-unit Armadillo repeat domain (ARM) flanked on either side by distinct N- and C-terminal domains. The N-terminal region consists of conserved serine and threonine residues that are sequentially phosphorylated by CK1α and GSK3β; phosphorylation of these sites promotes binding to β-TrCP, subsequent ubiquitination, and proteasomal degradation. (**B**) The ARM region forms a superhelix featuring a positively charged groove that serves as a platform for interactions with various β-catenin binding partners, including E-cadherin, Axin, APC, and TCF/LEF proteins. These partners share overlapping binding sites in the ARM groove, and thus typically cannot bind simultaneously. *Adapted from Xu et al., 2007[235]; Graham et al., 2000[140].*

**Figure 1.6**
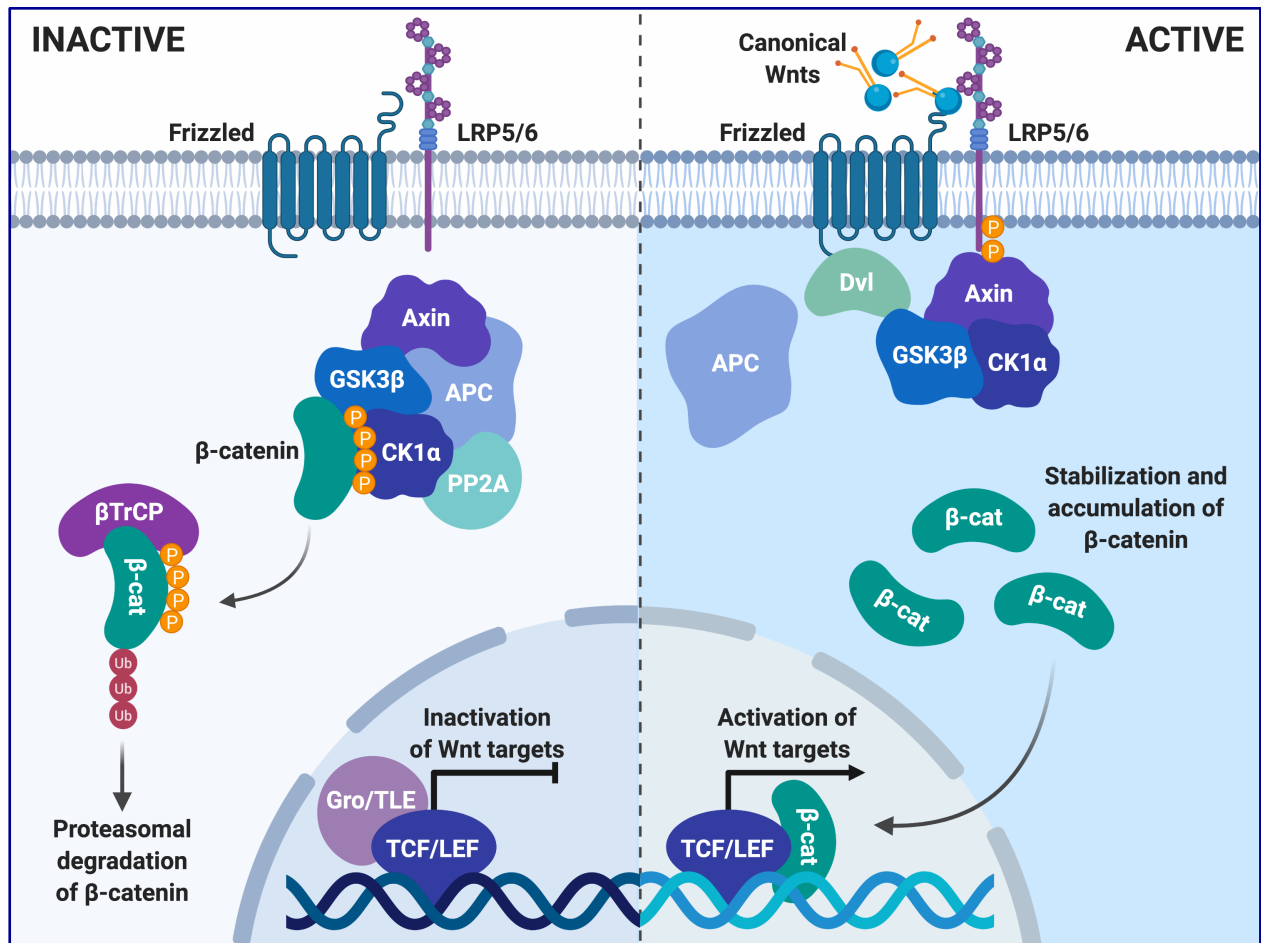


**Figure 1.6. Activation of the canonical Wnt signaling pathway promotes β-catenin-mediated transcription of Wnt target genes.** In the absence of extracellular Wnts (inactive; left panel), β-catenin is bound by a destruction complex comprised of Axin, APC, GSK3β, CK1α, and PP2A. CK1α and GSK3β sequentially phosphorylate free cytosolic β-catenin, targeting it for ubiquitination by βTrCP and subsequent proteasomal degradation. When canonical Wnts bind to their frizzled receptors and LRP co-receptors (active; right panel), they trigger an intracellular signaling cascade that leads to hypo-phosphorylation, stabilization, and accumulation of cytosolic β-catenin. β-catenin can then translocate to the nucleus, displace Groucho/TLE repressors, and coactivate TCF/LEF transcription factors to mediate Wnt target gene expression. *Adapted from Palomer et al., 2019[236].*

**Figure 1.7**
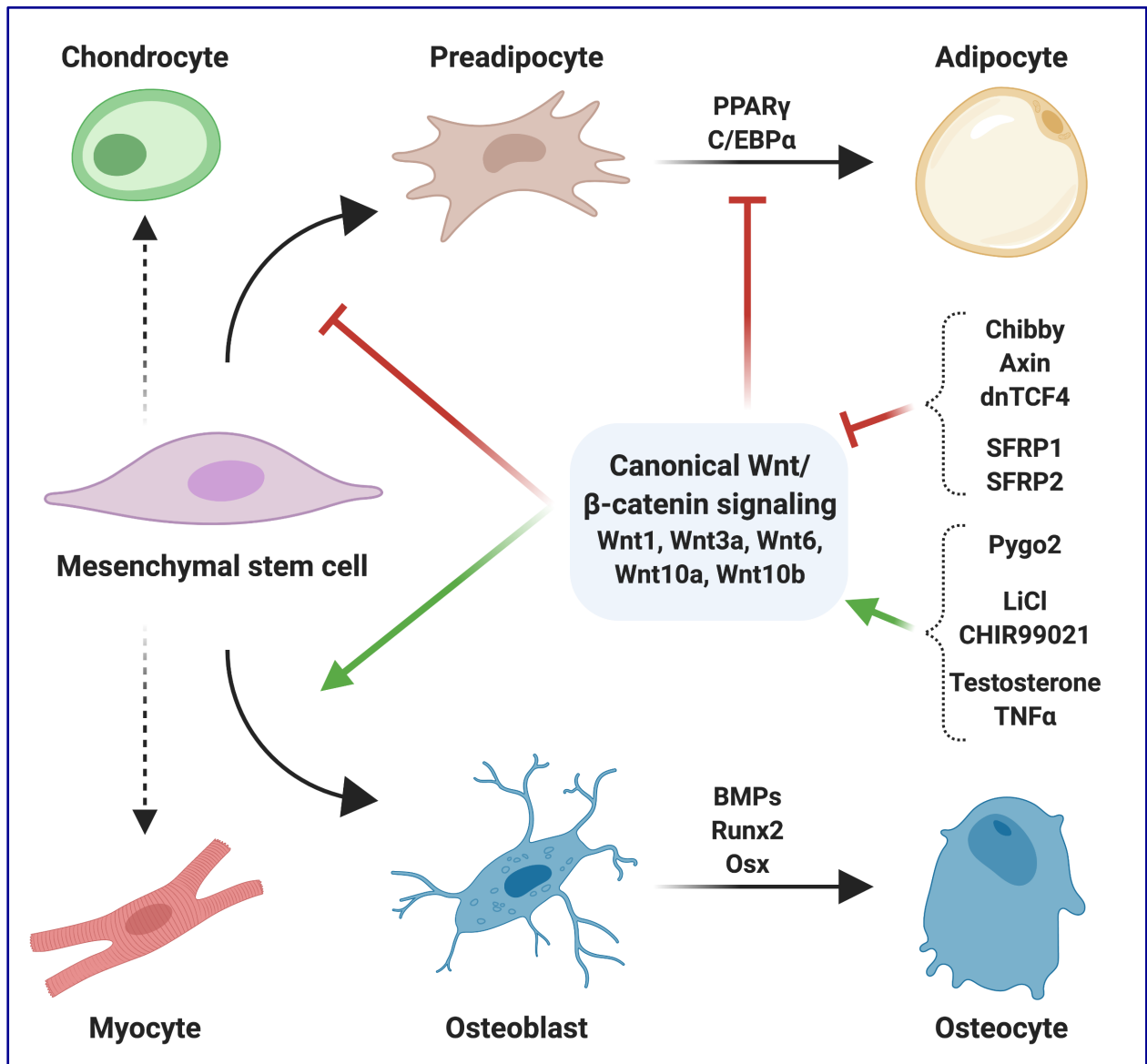


**Figure 1.7. The canonical Wnt signaling pathway is an important endogenous regulator of cell fate determination.** Activation of canonical Wnt/β-catenin signaling in mesenchymal stem cells suppresses adipogenesis and promotes osteoblastogenesis. Wnt signaling suppresses adipocyte differentiation by inhibiting expression of PPARγ and C/EBPα, the central regulators of adipogenesis.

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# CHAPTER II

## Methodology for Dissection of Diverse Murine Adipose Depots

## Abstract

Adipose tissues are complex organs with a wide array of functions, including storage and mobilization of energy in response to local and global needs, uncoupling of metabolism to generate heat, and secretion of adipokines to regulate whole-body homeostasis and immune responses. Emerging research is identifying important regional differences in the developmental, molecular, and functional profiles of adipocytes located in discrete depots throughout the body. Different properties of the depots are medically relevant since metabolic diseases often demonstrate depot-specific effects. This protocol will provide investigators with a detailed anatomic atlas and dissection guide for the reproducible and accurate identification and excision of diverse mouse adipose tissues. Standardized dissection of discrete adipose depots will allow detailed comparisons of their molecular and metabolic characteristics and contributions to local and systemic pathologic states under various nutritional and environmental conditions.

**Introduction**

Adipose tissues play critical roles in whole-body homeostasis, including storage and release of energy in response to local and global needs, thermoregulation, and secretion of adipokines to regulate energy balance, metabolism, and immune responses[1,2]. Adipocytes are located throughout the body in discrete depots[3-5], as well as singly and in small clusters associated with vascular and lymphatic structures, and in some cases serve specialized roles within their microenvironments[6,7]. Although adipocytes throughout the body have overlapping molecular and metabolic characteristics, the degree to which these properties are realized may depend on the specific niches in which these cells reside.

Most adipocytes are distributed in subcutaneous and visceral white adipose tissues (WAT)[8,9]. Subcutaneous adipocytes are contained within the anterior and posterior subcutaneous depots. The interscapular depot contains multilocular brown adipocytes, which are rich in mitochondria[10]. Brown and brown-like adipocytes express uncoupling protein 1 (UCP1), which when activated can uncouple mitochondrial respiration, leading to non-shivering thermogenesis[11]. This increased mitochondrial respiration also promotes glucose and fatty acid uptake and oxidation in brown adipocytes[12,13]. Some WAT depots are prone to browning; although the posterior subcutaneous depot appears to be continuous, centrally located inguinal adipocytes become brown-like more readily than those in adjacent depots. However, at very low environmental temperatures, even epididymal adipose tissue is recruited to form brown-like thermogenic adipocytes[14].

The intra-abdominal depots, such as the mesenteric, perirenal, retroperitoneal, and gonadal fat pads, are contained within the peritoneal cavity. Relative to adipocytes in subcutaneous depots, intra-abdominal adipocytes are typically larger and secrete less leptin. In addition, expansion of the intra-abdominal depots is highly associated with development of systemic insulin resistance. With positive energy balance, subcutaneous adipose tissue expands by adipocyte hypertrophy and hyperplasia[15]. In contrast, intra-abdominal depots expand during obesity predominantly by adipocyte hypertrophy[15], which is associated with metabolic dysfunction because larger adipocytes have increased lipolysis, attract inflammatory macrophages, and are insulin

resistant. Thus, the different characteristics of these depots are important for differential susceptibility to metabolic diseases[2,7,9,16-18].

Marrow adipose tissue accounts for ~10% of total fat mass in healthy humans, and in rodents can be classified as constitutive or regulated. Studies have demonstrated region-specific differences in marrow adipocyte properties, including development, regulation, gene expression, and lipid composition[19]. In addition to serving as a lipid reservoir, marrow adipose tissue is a disproportionate source of circulating adiponectin, and because of its enclosure within bone, is integral to our understanding of osteoblasts, osteoclasts, and hematopoietic cells[20,21].

In addition to the adipose depots discussed above, there are several other understudied depots that are likely to have important local roles yet to be discovered. These include intramuscular, periarticular, paracardial, epicardial, and retro-orbital adipocytes[22-25]. The popliteal depot provides lipids for expansion of lymphatic tissue following infection[26]. The dermal depot has roles in thermal insulation, wound healing, and hair follicle growth, and serves as a barrier to infection[27-30].

Although regional differences in adipocyte character and function are under intense study, the field is currently limited by the lack of a standardized protocol for the identification and dissection of diverse mouse depots. Previously published methods have typically described the isolation of one or two specific depots and lack the level of detail required for uniform excision[31,32]. The protocol described herein provides a comprehensive guide for the specific anatomic locations and isolation steps of many different mouse adipose depots. Although WAT depots are the primary focus, the excision of interscapular BAT is also described in detail. Adipose tissues excised using this protocol can be used for a wide variety of experimental endpoints, including explant studies, histology, and gene expression analyses.

The goal of this protocol is to provide investigators with a detailed protocol to clearly and precisely identify and isolate both prominent and less-studied murine adipose depots (**Figure 2.1**). This resource will facilitate a more complete investigation of the developmental, molecular, and functional characteristics of adipocytes within diverse niches.

**Protocol**

*All animal procedures are performed with the approval of the Institutional Animal Care and Use Committee (IACUC) of the University of Michigan.*

**Euthanization**

1. Place the mouse in an isoflurane vaporizer chamber and adjust the isoflurane flow rate to 5% or greater. Continue isoflurane exposure until one minute after breathing stops. Then remove the mouse from the vaporizer chamber and confirm euthanasia using an approved secondary measure.
2. Place the euthanized mouse on the dissection pan. Sterilize the dorsal and ventral external surfaces of the mouse using 70% ethanol. Ensure that the external surface of the mouse is sufficiently wet to minimize contamination from fur during dissection.

**Identification and isolation of major subcutaneous WAT depots (anterior subcutaneous, dorsolumbar, inguinal, gluteal) and interscapular BAT**

**Anterior subcutaneous WAT**

*This method isolates both anterior subcutaneous WAT and interscapular BAT. Anterior subcutaneous WAT is located between the scapulae, descending from the nape of the neck to the axillae of the mouse[11]. This depot has alternatively been described as suprascapular[10] or interscapular[33] WAT and lies directly on top of the interscapular BAT depot.*

1. To isolate the anterior subcutaneous depot, lay the mouse on its stomach in a prone position. Secure the upper and lower limbs to the dissection pan with dissection pins.
2. Use forceps to lift the dorsal skin at the nape of the neck. Use iris scissors to make a small (1 mm) cut in the skin.
3. Insert one blade of the iris scissors into the initial cut and make a midline vertical incision (2–3 cm) through the skin, beginning at the nape of the neck and descending along the spine to mid-back.

4. Make two horizontal incisions (1 cm each) using the iris scissors, extending laterally from midline, at the top and bottom of the initial vertical incision.
5. Use forceps to carefully peel back the skin and expose the anterior subcutaneous depot.
6. Use iris scissors to remove the depot following the natural borders of the tissue.
7. Place the dissected depot on the dissection pan and carefully remove the contaminating BAT using iris scissors.

**Classical BAT**

*Classical BAT is located beneath the anterior subcutaneous WAT depot.*

1. To isolate BAT, use iris scissors to cut horizontally along the bottom edge of the anterior subcutaneous tissue, following the natural border of the depot.
2. Then, use iris scissors to make two vertical incisions along the lateral edges of the depot, following the natural borders of the tissue.
3. Use forceps to carefully flip the depot up and reveal the butterfly-shaped interscapular BAT embedded within the WAT. Carefully dissect the BAT out from the surrounding WAT.

**Posterior subcutaneous WAT**
1. Lay the mouse on its back in a supine position.
2. After securing the upper and lower limbs with dissection pins, use forceps to lift up the skin at the base of the sternum and make a small (1 mm) cut in the skin.
3. Insert one blade of the iris scissors into the initial cut and make a midline incision (4–5 cm) through the skin, beginning at the base of the sternum and descending to the base of the tail. Exercise caution when making this incision because the ventral skin is very thin and is closely associated with the underlying peritoneal cavity wall.
4. Make two horizontal incisions (1 cm each) using iris scissors, extending laterally from midline, at the top of the initial vertical incision.
5. Use forceps to carefully peel back the skin from the peritoneal cavity and the leg to find posterior subcutaneous WAT, which should remain associated with the skin.

Secure the outstretched skin with dissection pins to ease complete excision of the WAT.

*Although posterior subcutaneous WAT appears to be continuous, it is actually comprised of three discrete depots: dorsolumbar, inguinal, and gluteal[1]. The dorsolumbar depot extends from the lumbar spine to the base of the hindlimb. The triangular inguinal depot extends from the base of the hindlimb ventrally across the groin and contains a prominent lymph node. The gluteal depot extends inferiorly from the base of the groin and wraps around the leg to the base of the tail.*

**Identification and isolation of visceral WAT depots (gonadal, perirenal, retroperitoneal, omental, pericardial)**

*WAT depots, which are largely contained within the thoracic and peritoneal cavities, keep the mouse in a supine position. Secure the upper and lower limbs to the dissection pan with dissection pins.*

1. Use forceps to lift up the thin peritoneal cavity wall at the base of the sternum and make a small cut (1 mm) using iris scissors.
2. Insert one blade of the iris scissors into the cut and make a descending vertical incision (4–5 cm) from the top of the peritoneal cavity (base of the sternum) to the rectum.
3. Make two horizontal incisions (1 cm each) with iris scissors, extending laterally from midline, at the top and bottom of the vertical incision.
4. Use forceps to peel back the peritoneum and expose the abdominal cavity contents. Pin the outstretched peritoneum to the dissection pan.

**Gonadal WAT**

*Gonadal WAT surrounds the uterus and ovaries in females (ovarian or parametrial) and the epididymis and testes in males (epididymal). Ovarian WAT surrounds the ovaries, uterus, and bladder. In obese animals, gonadal and perirenal WAT can appear to be continuous – in this case, separate the depots at the uterine horn and ovaries.*

*Epididymal WAT is found bound to the epididymis, testes, and the prominent epididymal blood vessel.*

1. Locate the gonads (testes or ovaries) and use forceps to lift up the associated gonadal WAT.
2. Use iris scissors to carefully excise the WAT from the gonads.

**Perirenal WAT**

*Perirenal WAT surrounds the kidneys bilaterally. In obese animals, this depot can appear to extend inferiorly to the top of the uterine horn and ovaries. Although the perirenal WAT has traditionally been classified as a visceral depot[3], several groups have identified it as a brown depot based on lineage tracing and radiolabeled glucose uptake studies[10,33,34]. Histologically it is comprised of a mixture of white and brown adipocytes.*

1. To excise the perirenal depot, locate the kidney and use forceps to lift it up and pull it midline to see a clear division between the perirenal and retroperitoneal depots.
2. Excise the WAT directly associated with the kidneys. Ensure that the adrenal glands, located above the kidneys, are removed from the WAT.

**Retroperitoneal WAT**

*Retroperitoneal WAT is located in a paravertebral position, along the border between the posterior abdominal wall and the spinal cord.*

1. To excise this depot, use forceps to lift the kidney up and towards midline to clearly see the border between the perirenal and retroperitoneal depots.
   *Note: In obese animals, identifying this border can be challenging.*
2. Then, use iris scissors to carefully dissect the retroperitoneal WAT from the posterior peritoneal wall.

**Omental WAT**

*Omental WAT is located along the greater curvature of the stomach. Although omental adipose is an important depot in humans, it is generally only readily visible in morbidly obese mice.*

1. To identify visceral omental WAT, use forceps to lift the stomach up. Use iris scissors to remove the associated adipose tissue along the inferior border of the stomach. Do not confuse omental WAT with the pancreas.

**Mesenteric WAT**

*Mesenteric WAT is a web-like structure surrounding and associated with the small and large intestines.*

1. To excise this depot, remove the intestines from the rest of the digestive tract by cutting at the base of the stomach and the rectum. Use forceps to lift the intestines out of the visceral cavity and unravel them.
2. Use iris scissors to carefully dissect the mesenteric WAT away from the intestines, beginning at the duodenum and continuing to the end of the colon. Carefully remove lymph nodes that are closely associated with the mesenteric depot.

**Pericardial WAT**

*Pericardial WAT is located outside the visceral pericardium and on the external surface of the parietal pericardium, often along the inferior aspect of the heart[24].*

1. To gain access to the thoracic cavity, keep the mouse in a supine position. Secure the upper and lower limbs to the dissection pan with dissection pins.
2. Use forceps to lift the xyphoid process (cartilage at the base of the sternum) and make a small (1 mm) cut in the thoracic cavity wall.
3. Insert one blade of the iris scissors into the cut and make two horizontal incisions (1 cm each) extending laterally from the base of the sternum. This will expose the diaphragm and inferior border of the thoracic cavity.

4. Use dissecting scissors to make two ascending vertical incisions (3–4 cm) through the ribcage, extending superiorly from the lateral borders of the thoracic cavity to the clavicle.

5. Use forceps to lift up the ventral half of the ribcage. Use dissecting scissors to make a final cut (2 cm) along the inferior length of the clavicle to remove the ribcage and expose the thoracic cavity contents.

6. If present, carefully dissect the pericardial adipose from the outer surface of the pericardium.

**Identification and isolation of other adipose depots**

**Epicardial WAT**

*Epicardial WAT is contained within the visceral pericardium and is directly associated with the surface of the myocardium.*

1. If present, epicardial adipocytes can be observed histologically after isolation and fixation of the perfused heart in 10% neutral buffered formalin for 24 h.

**Popliteal WAT**

*Popliteal WAT is located in the popliteal fossa in the posterior knee and contains a large lymph node. This depot is not typically visible in young animals.*

1. To isolate popliteal WAT, use iris scissors to carefully remove the skin from the base of the hind limb to the foot.

2. Place the patella against the dissection pan and secure the outstretched leg with a pin in the foot. Ensure that the popliteal fossa at the back of the knee is facing upward.

3. Use iris scissors to make a cut at the inferior border of the medial and lateral heads of the gastrocnemius muscle. Use forceps to lift up the muscle and reveal the triangular popliteal depot.

4. Use iris scissors to excise the depot along the natural tissue borders.

**Dermal WAT**

*Dermal WAT is a thin layer of adipocytes located between the reticular dermis and the panniculus carnosus muscle layer.*

1. To identify dermal adipocytes by histological methods, place the mouse on its stomach in a prone position. After securing the upper and lower limbs with dissection pins, spray the external surface of the mouse with 70% ethanol to wet the skin.
2. Use a scalpel to remove the wetted fur from a square portion of skin on the back of the mouse.
3. Use iris scissors to carefully excise the shaved portion of skin, which will include the reticular dermis, dermal WAT, panniculus carnosus, and some subcutaneous WAT.
4. Use a scalpel to cut the excised skin into thin vertical strips.
5. Position the thin vertical strips with the sticky subcutaneous WAT layer facing down.
6. Starting at one end, roll the strip onto itself to form a spiral. The sticky subcutaneous WAT layer on the outside of the spiral will allow the roll to maintain its shape during fixation.
7. Place the spiral in a well of a 24-well plate containing 10% neutral buffered formalin for 24 h prior to histological processing[35].

**Intermuscular WAT**

*Intermuscular WAT is broadly defined as adipocytes located beneath the deep fascia of muscles. This term includes adipocytes interspersed between muscle fibers of skeletal muscle, also known as intramuscular WAT, and adipocytes located within muscle bundles themselves. Wildtype mice generally do not have large numbers of intermuscular adipocytes. However, it is possible under certain conditions to identify intermuscular WAT by histological methods. Under some conditions, intermuscular adipocytes can also be found in smooth muscles, such as the diaphragm.*

1. To isolate the tibialis anterior (TA) muscle, for example, place the mouse on its back in a supine position and secure the lower limbs to the dissection pan using dissection pins. Wet the skin with 70% ethanol to minimize contamination with fur.

2. Use iris scissors to carefully remove the skin from the leg and expose the quadriceps muscle group, located above the knee on the femur shaft, and the TA located below the knee on the ventral surface of the tibia.

   *Note: The TA is thick near the proximal end of the tibia and more tendinous near the distal end.*

3. Use iris scissors to excise a piece of the muscle and fix in 10% neutral buffered formalin for 24 h prior to histological processing[35].

**Intra-articular WAT depots**

*Intra-articular WAT depots are located within synovial joints.*

1. To identify infrapatellar WAT, for example, by histological methods, harvest leg bones as detailed in the BMAT section.

2. After the removing the femur-tibial complex, use iris scissors and gauze pads to remove as much muscle and connective tissue as possible. Do not break the tibiofemoral joint.

3. Fix the femur-tibial complex in 10% neutral buffered formalin for 24 h prior to decalcification and histological processing (described below).

**Bone marrow adipose tissues**

*Bone marrow adipose tissue (BMAT) is contained within bones, interspersed with hematopoietic cells. Anatomically, BMAT can be classified as constitutive (distal tibia and caudal vertebrae) or regulated (mid-to-proximal tibia, femur, and lumbar vertebrae)[21,36]. Clean isolation of bone marrow adipocytes for RNA and protein analyses is challenging in mice. However, mouse bones can readily be harvested, fixed, decalcified and paraffin-embedded for histological analyses.*

1. To harvest leg bones, for example, first remove the legs from the mouse. Use dissection scissors to cut the acetabulofemoral joint, keeping the femoral head intact.

2. Use iris scissors to carefully remove the skin from the leg, revealing the leg muscles.

3. Carefully dissect away the main muscles from the femur and tibia using iris scissors and gauze pads.

4. When the femur is exposed, follow the edge of the bone to the articulation of the pelvis and femur and carefully release the femoral head from the acetabulofemoral joint. Use gauze to clean any remaining tissue from the femur.

5. Follow the border of the tibia to the ankle joint. Carefully release the medial malleolus, located at the tip of the tibia, from the ankle joint.

6. Once the femur-tibial complex has been isolated, remove as much muscle and connective tissue as possible using iris scissors and/or gauze.

7. Separate the tibia from the femur by inserting one blade of the iris scissors into the tibiofemoral joint and gently cut through the medial and lateral collateral ligaments and the anterior and posterior cruciate ligaments. Do not remove the capsule of the knee joint to ensure that the tibia remains intact. Use gauze to clean any remaining tissue from the tibia.

8. Fix bones in 10% neutral buffered formalin for 24 h at room temperature and then wash and store according to future needs.

   1. To analyze bone parameters using microcomputed tomography (µCT), store bones in Sorensen's phosphate buffer, pH 7.4[19].

   2. For BMAT quantification and histological analyses, decalcify bones in 14% EDTA, pH 7.4, for 10-14 days.

   3. Following decalcification, use osmium tetroxide staining and µCT analysis to quantify BMAT[37]. Otherwise, process and paraffin-embed bones for histology[19].

**Discussion**

As the importance of the diverse molecular and functional characteristics of discrete adipocyte clusters is increasingly recognized, it is crucial that investigators within the field uniformly identify and excise adipose depots for further analyses. To date, few protocols exist for standardized localization and isolation of the wide range of mouse adipose depots. Previously published methods are focused primarily on one or two depots and lack the details necessary for uniform identification and excision by different investigators[31,32]. This protocol is a novel and important contribution to the field of adipose biology since it provides an in-depth guide to the anatomic location and precise dissection of commonly studied and lesser-known depots throughout the mouse. Gross anatomical location (**Figure 2.2**) and histological analyses (**Figure 2.3**) are provided to demonstrate the diversity of adipocyte niches.

Successful isolation of discrete adipose depots using this protocol is dependent on several critical steps. Maintenance of a clean dissection environment is crucial; 70% ethanol can be used as necessary to remove contaminating hairs or blood from the dissection tray and tools. When isolating posterior subcutaneous WAT, it is imperative that the initial incision in the skin does not penetrate the closely associated peritoneal cavity so that the two layers can be efficiently separated to expose the WAT. Similarly, when cutting through the peritoneal wall to expose the abdominal cavity contents, incisions must not perforate the underlying intestines to prevent contamination with digestive elements. Interscapular BAT must be carefully excised from the surrounding tissue to prevent WAT contamination, which will skew future analyses. Consistent identification of the posterior subcutaneous dorsolumbar, inguinal, and gluteal depots depends on the utilization of precise anatomic landmarks described in the protocol. The distinction between these depots is particularly important; although the depots can appear to be continuous, centrally located inguinal adipocytes acquire brown-like characteristics more readily than their surrounding counterparts.

The adipose tissues isolated using this protocol can be analyzed using a variety of techniques. In addition to histological evaluation (i.e. immunohistochemistry, H&E staining), adipose tissues can be used for a wide array of molecular analyses, from regulation of transcription through to posttranslational protein modifications. Adipocytes

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and stromal vascular cells within individual depots can be isolated by collagenase digestion and differential centrifugation. These fractions can then be used for molecular, metabolic, and cell culture studies. Depot explants can also be used for *ex vivo* metabolic and enzymatic assays.

Several challenges and limitations arise during the isolation and excision of mouse adipose tissues. First, some depots that are physiologically important in humans are not commonly present in lean, adult mice. For example, omental WAT, which is the major visceral depot in humans, can only be seen in genetically- or diet-induced obese mice. The expansion of some depots can, however, be induced by nutritional challenges or drug treatments. For example, high fat diet (HFD) feeding can cause the expansion of omental and pericardial WAT. Intermuscular WAT can be induced by skeletal muscle injury[38,39] or HFD[40]. Regulated BMAT also expands in response to various challenges, including HFD, calorie restriction and thiazolidinedione treatment[36,41].

Second, due to the small size of mice, obtaining enough sample for molecular or metabolic analyses can be difficult. For example, isolation of enough pure bone marrow adipocytes for subsequent analyses is challenging, even after pooling bones from multiple mice. Third, accurately defining borders of certain depots can be difficult. For example, posterior subcutaneous WAT appears to be one continuous depot but is actually comprised of discrete dorsolumbar, inguinal, and gluteal depots. Additionally, the perirenal can appear to be fused to both the gonadal and retroperitoneal depots in very obese animals. The lack of distinct borders between adjacent depots can make clean isolation of these tissues challenging. However, this dissection protocol provides investigators with a detailed atlas and step-by-step guidance for accurate and reproducible dissection of a wide range of mouse adipose depots.

Field-wide standardization of the identification and isolation of discrete mouse adipose depots described above will undoubtedly help further elucidate differences in the development, gene expression, and local and systemic functions of previously under-studied adipocyte niches.

**Figure 2.1**
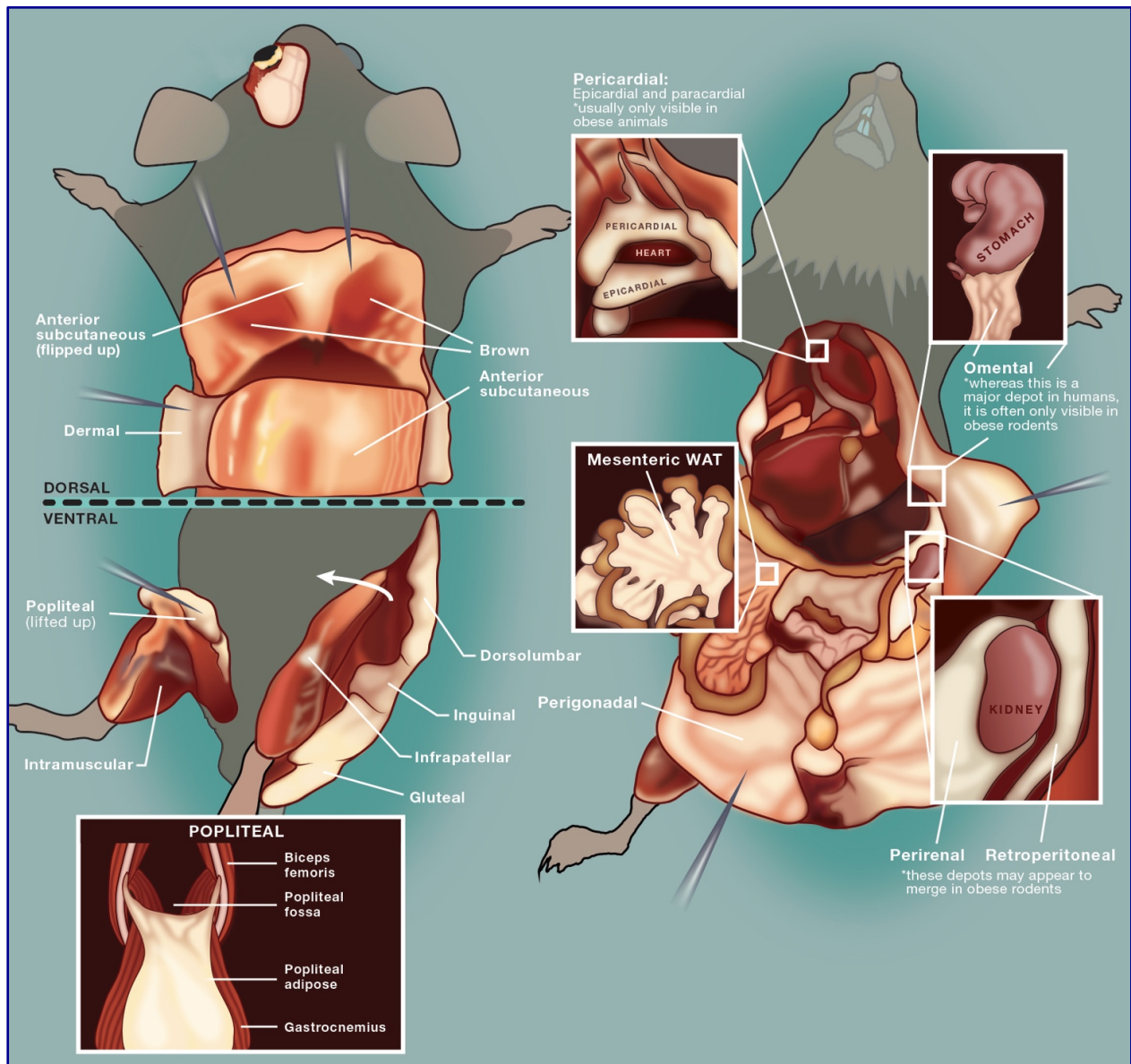


**Figure 2.1. Schematic depiction of dissected mouse adipose depots.** Adipocytes are located throughout the body in discrete depots, as well as singly and in small clusters associated with vascular and lymphatic structures. Although adipocytes throughout the body have overlapping molecular and metabolic characteristics, the degree to which these properties are realized will undoubtedly depend on the specific niches in which these cells reside.

**Figure 2.2**
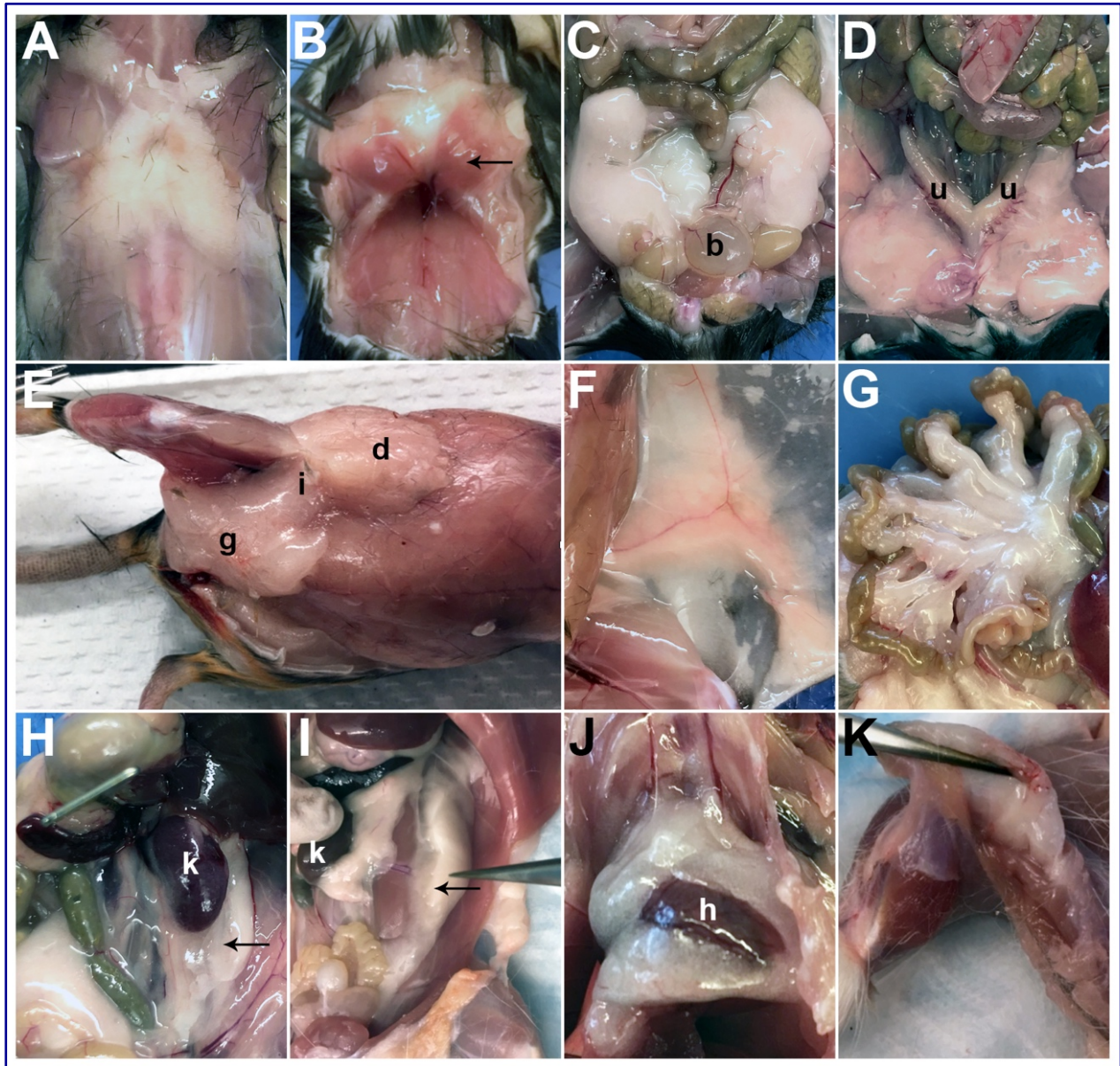


**Figure 2.2. Gross anatomical locations of mouse adipose depots.** Gross anatomy of (**A**) anterior subcutaneous; (**B**) brown; (**C**) epididymal (b, bladder); (**D**) ovarian (u, uterine horns); (**E**) posterior subcutaneous (dorsolumbar (d), inguinal (i), gluteal (g)); (**F**) inguinal; (**G**) mesenteric; (**H**) perirenal (k, kidney); (**I**) retroperitoneal (k, kidney); (**J**) pericardial (h, heart); and (**K**) popliteal adipose depots in C57BL/6J adult mice. Arrows point to specific depots if multiple depots are depicted. Relevant organs are designated by appropriate letters.

**Figure 2.3**
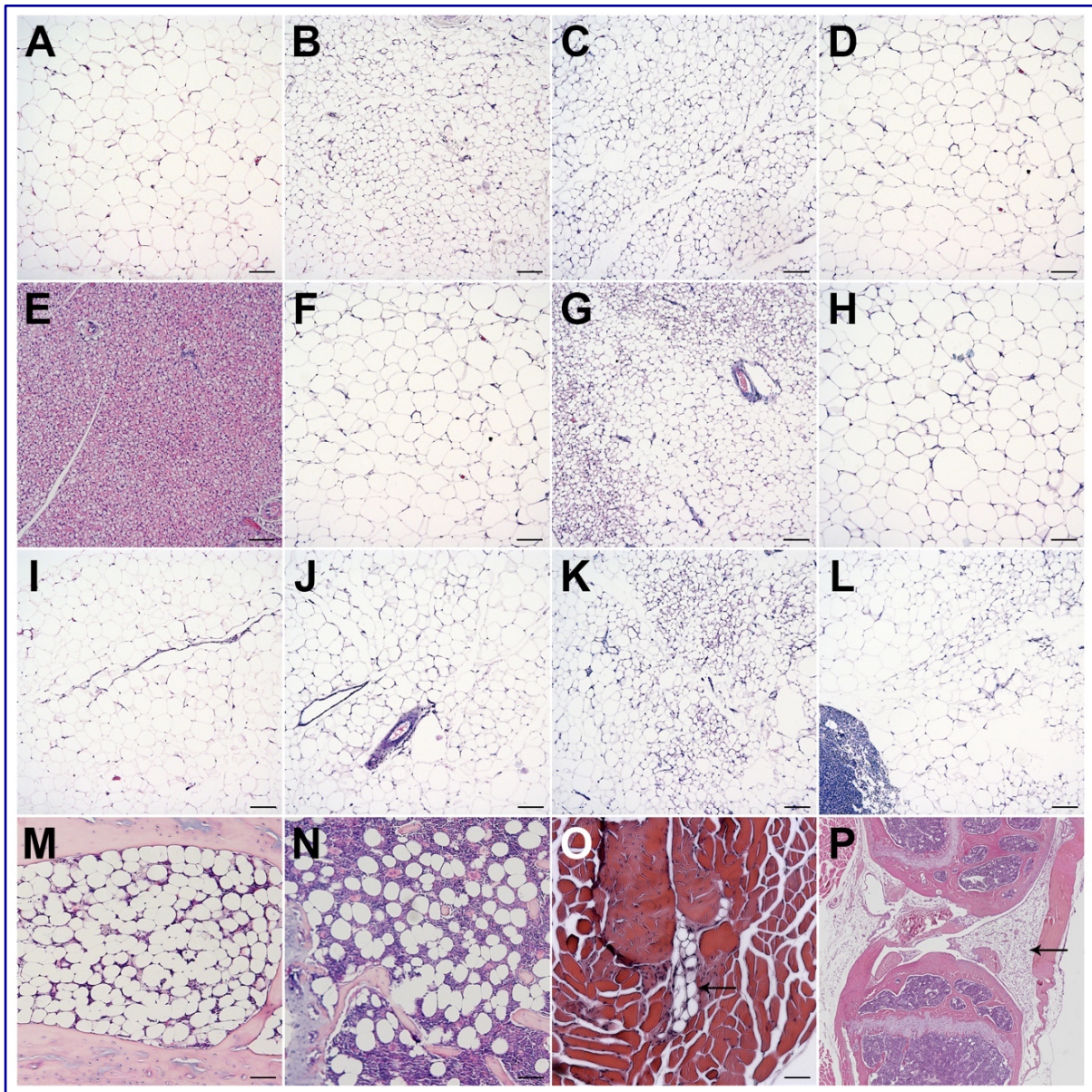


**Figure 2.3. Histological evaluation of discrete mouse adipose depots.** Histology of (**A**) anterior subcutaneous; (**B**) dorsolumbar; (**C**) inguinal; (**D**) gluteal; (**E**) brown; (**F**) gonadal; (**G**) perirenal; (**H**) retroperitoneal; (**I**) omental; (**J**) mesenteric; (**K**) pericardial; (**L**) popliteal; (**M**) constitutive and (**N**) regulated bone marrow adipose; (**O**) intermuscular; and (**P**) infrapatellar depots in C57BL/6J adult mice. Tissues were isolated according to the presented protocol, fixed overnight in 10% neutral buffered formalin, processed, and embedded in paraffin. 5 μm sections were stained with H&E. Most images were taken at 100x magnification; scale bar = 100 μm.

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# CHAPTER III

# Wntless Regulates Lipogenic Gene Expression in Adipocytes and Protects Against Diet-Induced Metabolic Dysfunction

## Abstract

*Objective:* Obesity is a key risk factor for many secondary chronic illnesses, including type 2 diabetes and cardiovascular disease. Canonical Wnt/ β-catenin signaling is well-established as an important endogenous inhibitor of adipogenesis. This pathway is operative in mature adipocytes; however, its roles in this context remain unclear due to complexities of Wnt signaling and differences in experimental models. Herein, we use novel cultured cell and mouse models to investigate functional roles of Wnts secreted from adipocytes. *Methods:* We generated adipocyte-specific Wntless (*Wls*) knockout mice and cultured cell models to investigate molecular and metabolic consequences of disrupting Wnt secretion from mature adipocytes. To characterize *Wls*-deficient cultured adipocytes, we evaluated expression of Wnt target and lipogenic genes, and downstream functional effects on carbohydrate and lipid metabolism. In addition, we investigated the impact of adipocyte-specific *Wls* deletion on adipose tissues and global glucose metabolism in mice fed normal chow or high fat diets. *Results:* We report that many aspects of the Wnt signaling apparatus are expressed and operative in mature adipocytes, including the Wnt chaperone Wntless. Deletion of Wntless in cultured adipocytes results in inhibition of *de novo* lipogenesis and lipid monounsaturation, likely through repression of *Srebf1* (SREBP1c) and *Mlxipl* (ChREBP), and impaired cleavage

of immature SREBP1c into its active form. Adipocyte-specific *Wls* knockout mice (*Wls*^{-/-}) have lipogenic gene expression in adipose tissues and isolated adipocytes similar to that of controls when fed a normal chow diet. However, closer investigation reveals that a subset of Wnts and downstream signaling targets are up-regulated within stromal-vascular cells of *Wls*^{-/-} mice, suggesting that adipose tissues defend loss of Wnt secretion from adipocytes. Interestingly, this compensation is lost with long-term high fat diet challenge. Thus, after six months of high fat diet, *Wls*^{-/-} mice are characterized by decreased adipocyte lipogenic gene expression, reduced visceral adiposity, and improved glucose homeostasis. *Conclusion:* Taken together, these studies demonstrate that adipocyte-derived Wnts regulate *de novo* lipogenesis and lipid desaturation, and coordinate expression of lipogenic genes in adipose tissues. In addition, we report that Wnt signaling within adipose tissues is defended, such that a loss of Wnt secretion from adipocytes is sensed and compensated for by neighboring stromal-vascular cells. With chronic overnutrition, this compensatory mechanism is lost, revealing that *Wls*^{-/-} mice are resistant to diet-induced obesity, adipocyte hypertrophy, and metabolic dysfunction.

**Introduction**

Wnts are members of an evolutionarily conserved family of secreted glycoproteins with well-established roles in cell proliferation and differentiation during tissue development[1,2]. When canonical Wnts bind to their membrane-spanning frizzled (Fzd) receptors and low-density lipoprotein receptor-related protein (LRP) co-receptors, they trigger an intracellular signaling cascade that stabilizes free cytosolic β-catenin, which then translocates to the nucleus and coactivates DNA-binding T-cell factor/lymphoid enhancer-binding factor (TCF/LEF) proteins to mediate downstream gene transcription[1-3]. The endogenous Wnt pathway is a critical regulator of mesenchymal cell fate where it inhibits adipogenesis and promotes osteogenesis[4-18].

Genetic studies in humans have also identified associations between Wnt pathway members and body fat distribution, obesity and metabolic function. For example, genome-wide association studies across diverse human populations have found strong correlations between polymorphisms in the canonical Wnt transcriptional effector *TCF7L2* and susceptibility to type 2 diabetes[19-21]; indeed, *TCF7L2* is one of the strongest risk loci for development of type 2 diabetes. In addition, loss-of-function mutations in Wnt co-receptors *LRP5* and *LRP6* are associated with impaired glucose tolerance, osteoporosis, and cardiovascular disease[22,23], whereas gain-of-function *LRP5* mutations are associated with increased adiposity and altered fat distribution[24]. Common variants in Wnt signaling inhibitor *ZNRF3* and Wnt signaling activator *RSPO3* are associated with increased waist-to-hip ratio[25-27]. Further, gain-of-function mutations in *LGR4*, a protein that stabilizes Wnt receptors, are correlated with increased visceral adiposity[28]. Polymorphisms in the *SFRP5* locus have been associated with decreased adiposity in men[29], whereas missense variants in *WNT10B* and certain *WNT5B* single nucleotide polymorphisms are correlated with higher risk of obesity and type 2 diabetes, respectively[30,31]. Finally, variants in *CTNNB1* (β-catenin) have been associated with increased body mass index and risk of obesity[32]. Taken together, these studies provide strong genetic evidence for the influence of Wnt/β-catenin signaling on white adipose tissue (WAT) function, body composition, and metabolic health.

Although recent studies have demonstrated that Wnt signaling is active in mature adipocytes, its functional roles in this context remain unclear due to complexity of the

Wnt pathway, and differences in experimental models, approaches, and results[13,14,32-34]. For example, stabilization of Wnt signaling through global deletion of secreted frizzled-related protein 5 (SFRP5) - an adipocyte protein highly induced by obesity that binds to and sequesters Wnts - causes resistance to diet-induced obesity in mice[33]. Whereas total adipocyte numbers are unaffected, adipocytes in *Sfrp5* mutant mice have increased mitochondrial number and are smaller in size compared to control mice, resulting in reduced WAT and improved glucose tolerance. Adipocyte-specific deletion of β-catenin has also been reported to cause decreased subcutaneous WAT mass and improved glycemic control in diet-induced obese mice[32]. In contrast, adipocyte-specific deletion of the transcription factor *Tcf7l2* leads to adipocyte hypertrophy and impaired glucose homeostasis with diet-induced obesity[34]. These reports provide the first evidence that canonical Wnt signaling regulates ability of existing adipocytes to accommodate excess energy. Additional studies targeting the Wnt pathway in adipocytes are required to further understand how various components of this pathway differentially contribute to adipocyte metabolism.

Although it is clear that Wnt signaling is important within adipose tissues, one significant gap in our knowledge is the cellular source of physiologically relevant Wnts. To address this shortfall, we have targeted Wntless (*Wls*), an evolutionarily conserved chaperone protein required for transport of Wnts from the endoplasmic reticulum (ER) and Golgi apparatus to the cell membrane for secretion[35,36]. Wnt proteins are modified with palmitoleic acid (C16:1) at conserved serine residues, and the lipocalin-like structure of Wntless may facilitate binding to these hydrophobic modifications[37-41]. In fact, palmitoleoylation at Ser209 of Wnt3a, which is required for Wnt activity, is required for its physical binding to Wntless and subsequent release from the ER[37,42,43]. Deletion of Wntless in vertebrate and invertebrate animal models results in phenotypes consistent with loss of Wnt function, suggesting that Wntless regulates signaling at the level of Wnt transport and secretion in signal-producing cells[36,44-50].

Here, we report that Wntless is expressed in mature adipocytes and that adipocyte-specific *Wls* deletion (hereby designated as *Wls*^{-/-}) leads to reduced *de novo* lipogenesis (DNL) and lipid monounsaturation. Further, inhibition of a network of lipogenic genes is correlated with repression of *Srebf1* and *Mlxipl*, which encode

SREBP1c and ChREBP, known transcriptional regulators of this gene set[51-53]. Of particular interest, in mice fed a normal chow diet (NCD), loss of adipocyte-specific Wnt secretion is defended by increased expression of Wnts in surrounding stromal-vascular cells (SVC); however, this compensation is lost in mice fed a high fat diet (HFD). Thus, *Wls*^{-/-} mice challenged with long-term HFD demonstrate decreased adipocyte lipogenic gene expression, reduced size of epididymal adipocytes and WAT, and resistance to HFD-induced metabolic dysfunction. Studies from the cancer field have firmly established that inhibition of stearoyl CoA desaturase 1 (SCD1), which catalyzes the synthesis of palmitoleic acid, results in suppression of Wnt/β-catenin signaling[41,54-61]. Our studies reveal a reciprocal relationship in which Wnts are required for expression of lipogenic genes and DNL, including expression of SCD1 and formation of palmitoleic acid. Thus, these results highlight the novel and important roles of adipocyte-derived Wnts in critical mature adipocyte functions, including accumulation of lipid in WAT with HFD.

**Results**

**Wntless is expressed in mature adipocytes and is up-regulated by diet-induced obesity.**

Canonical Wnt signaling has previously been well-established as a significant endogenous regulator of adipogenesis[4]. Although recent studies have provided compelling evidence that this pathway also plays a role in the metabolism of adipocytes[32-34], particularly under obesogenic conditions, the contribution of adipocyte-derived Wnt proteins has not been directly studied in this context. To this end, we first evaluated whether Wntless, the chaperone protein required for secretion of Wnts, is expressed and regulated in cultured adipocytes and WAT. In cultured MSCs derived from wildtype C57BL/6J mice, expression of *Wls* mRNA and protein was high in precursors, transiently repressed during differentiation, and elevated again in mature adipocytes (**Figure 3.1 A-B**). As expected, mRNA and protein expression of the adipocyte marker *Adipoq* (Adiponectin) increased during adipogenesis, whereas expression of the preadipocyte marker *Dlk1* (Pref1) decreased (**Figure 3.1 A-B**).

In addition to adipocytes, WAT contain myriad cell types, including preadipocytes, stromal cells, endothelial cells, and immune cells. These cells comprise the SVF of WAT and many have been shown to have active Wnt signaling in other contexts[5,6,62-64]. We measured *Wls* mRNA in fractionated eWAT and iWAT of C57BL/6J mice and found that it is expressed at higher levels in isolated adipocytes than in the SVF (**Figure 3.1 C**). We then performed immunoblot analyses on various mouse tissues and observed that Wntless is highly expressed in diverse adipose depots (**Figure 3.1 D**), with relatively low abundance in lung and pancreas. Once we established that *Wls* is expressed in mouse adipose tissues, we explored whether its expression in WAT is regulated by nutritional or environmental challenges. We found that *Wls* gene expression in eWAT and iWAT is up-regulated by diet-induced obesity (**Figure 3.1 E**); notably, this increase in *Wls* expression occurs specifically in the adipocyte fraction and not in the SVF of eWAT and iWAT (**Supplemental Figure 3.1 A**). Further, *Wls* is specifically regulated by HFD feeding as its expression is not altered by acute fasting, fasting/refeeding, calorie restriction or acute cold exposure (**Supplemental Figure 3.1**

**B**). Wntless protein expression is also up-regulated by diet-induced obesity in eWAT and iWAT (**Figure 3.1 F**). We also queried the publicly available GTEx dataset to determine the distribution of *Wls* gene expression across male and female human tissues and found that *Wls* is expressed at higher levels in WAT depots when compared to liver or skeletal muscle (**Supplemental Figure 3.1 C**). These data suggest that Wntless, which is expressed in adipocytes and induced by obesity, likely plays a metabolic role in these cells, particularly under obesogenic conditions.

**Wnt/β-catenin signaling is operative in cultured adipocytes.**

Although previous studies have characterized in detail the mechanisms by which Wnt signaling represses adipogenesis of mesenchymal precursors and promotes alternative cell fates[4,5,15,17,18], the expression pattern and roles of Wnt-related proteins in mature adipocytes is less clear. RNA-seq analyses of cultured MSCs and differentiated adipocytes reveals that many Wnts are induced during adipogenesis, including *Wnt2b*, *Wnt4*, *Wnt5b*, *Wnt8b*, *Wnt9a*, and *Wnt9b* (**Figure 3.1 G**). We also observed induction of *Fzd4* and *Fzd9* receptors and constitutive expression of other pathway members, such as *Ctnnb1* (β-catenin), *Lrp5, Lrp6, Sfrp1, Dvl1, Dvl2, Dvl3, Wnt16, Wnt2*, and *Wnt7b* (**Supplemental Figure 3.1 D**). Consistent with all major components of the signaling pathway being either induced or expressed constitutively, a number of downstream Wnt targets were elevated in mature adipocytes, including *Wisp1*, *Wisp2*, *Axin2*, *Tcf7l2* and *Tcf7l1*, amongst others. Further, treatment of mature adipocytes with recombinant Wnt3a regulates many Wnt pathway genes involved in negative feedback, as well as many adipocyte genes (**Figure 3.1 H**; data not shown). Taken together, these data indicate that the canonical Wnt signaling apparatus is operative in mature adipocytes.

**Wntless deletion in cultured adipocytes inhibits DNL and lipid monounsaturation.**

To study the molecular functions of Wntless in adipocytes, we isolated and cultured MSCs from *Wls*^{fl/fl} mice. This model allowed us to perform metabolic and mechanistic studies in cells derived directly from our mouse model. *Wls*^{fl/fl} MSCs were differentiated into adipocytes using a standard adipogenic cocktail. At day four of differentiation, adipocytes were infected in serum-free medium with adenoviral GFP as a control or

adenoviral Cre recombinase to induce *Wls* gene deletion. Adipocytes were allowed to recover following infection and were analyzed at day 12 to 14 of differentiation (**Figure 3.2 A**). Recombination of the floxed allele was confirmed by a 3-primer PCR system (**Figure 3.2 B**). Both qPCR and immunoblot analyses of Cre-infected adipocytes showed efficient deletion of *Wls* at the mRNA (**Figure 3.2 C**) and protein (**Figure 3.2 D**) levels. Deletion of Wntless does not affect differentiation and lipid accumulation, as assessed by adiponectin protein expression (**Figure 3.2 D**), phase-contrast microscopy, Oil Red-O staining (**Figure 3.2 E**), and measurement of TAG (**Figure 3.2 F**). To investigate whether Wnt signaling is altered by *Wls* deletion, we measured expression of known targets and found that Wnt-induced genes, including *Axin2*, *Tcf7l2*, *Cmyc*, and *Ppard* are down-regulated, whereas Wnt-suppressed gene *Id2* is up-regulated in *Wls*^{-/-} adipocytes (**Figure 3.2 G**). The downregulation of Wnt target genes in *Wls*^{-/-} adipocytes is relatively mild, suggesting that other pathways contribute to basal expression of these genes.

Of note, *Ctnnb1* mRNA is not altered, which is consistent with its primary regulation through protein turnover[6,65]. Free cytosolic β-catenin, which is not bound to E-cadherins and comprises a very small and highly regulated proportion of total cellular β-catenin in preadipocytes, is the pool that mediates Wnt signaling[5,6,66,67]. Thus, we measured free cytosolic β-catenin as a surrogate for Wnt signaling downstream of adipocyte-derived Wnts. Compared to *Wls*^{fl/fl} cells, we found that free cytosolic β-catenin is significantly lower (~33%) in *Wls*^{-/-} MSCs at baseline (**Figure 3.2 H**; quantified in **Supplemental Figure 3.2 A**). Interestingly, free cytosolic β-catenin also appears to be lower in *Wls*^{-/-} MSCs upon stimulation with Wnt3a, which may be secondary to desensitization of the Wnt signaling pathway in the absence of Wntless and secreted Wnts. In contrast, similar levels of membrane β-catenin are observed in control and *Wls*^{-/-} MSCs (**Figure 3.2 H**; quantified in **Supplemental Figure 3.2 A**). These data suggest that Wnt signaling is active in mature adipocytes and impaired secretion of adipocyte-derived Wnts influences downstream signaling in an autocrine/paracrine manner.

Since Wntless functions to chaperone Wnt proteins from the ER and Golgi apparatus to the plasma membrane for secretion, we hypothesized that its deletion might influence the adipocyte proteome. We therefore performed untargeted proteomic

analyses of *Wls*^{fl/fl} and *Wls*^{-/-} cultured adipocytes and observed substantial up- and down-regulation of adipocyte proteins (**Supplemental Figure 3.2 B**). Of these potential Wnt targets, we focused our investigations on the down-regulation of SCD1, an important adipocyte enzyme in the ER that catalyzes the desaturation of palmitic (C16:0) and stearic (C18:0) acids into palmitoleic (C16:1, n-7) and oleic (C18:1, n-9) acids, respectively[68,69]. In addition to its role as the rate-limiting enzyme in fatty acid desaturation, SCD1 is also a key member of the DNL pathway (**Figure 3.3 A**), which converts excess dietary carbohydrates and amino acids into fatty acids. A subset of these fatty acids are esterified to form TAG, which can later be hydrolyzed and fatty acids either secreted or metabolized by β-oxidation to provide energy for the adipocyte[70,71]. Consistent with proteomic data (**Supplemental Figure 3.2 B**), we found that Wntless deficiency in adipocytes down-regulates expression of *Scd1* mRNA (**Figure 3.3 A**) and protein (**Figure 3.3 B**). In addition, we also observed coordinate regulation of several enzymes involved in DNL and lipid metabolism (**Figure 3.3 A-B**; quantified in **Supplemental Figure 3.3 A**). Given the ~75% inhibition of SCD1 following *Wls* deletion, we hypothesized that *Wls*^{-/-} adipocytes would have impaired fatty acid monounsaturation and therefore a larger proportion of saturated fatty acids. Thus, we next used GC to analyze the composition of esterified fatty acids in total lipids extracted from *Wls*^{fl/fl} and *Wls*^{-/-} adipocytes. Consistent with our hypothesis, we found that *Wls*^{-/-} adipocytes contain a smaller proportion of monounsaturated fatty acids compared to *Wls*^{fl/fl} adipocytes (**Figure 3.3 C**). More specifically, when compared to control adipocytes, differentiated *Wls*^{-/-} cells contain higher levels of palmitic (C16:0) and stearic (C18:0) acids and lower amounts of myristoleic (C14:1, n-5), palmitoleic (C16:1, n-7) and vaccenic acids (C18:1, n-7) (**Figure 3.3 D-F**). Of note, *Wls*^{-/-} adipocytes contain mildly elevated levels of oleic acid (C18:1, n-9), consistent with previous reports of SCD1 inhibition in 3T3-L1 adipocytes[72].

In addition to SCD1, *Wls* deletion also regulates expression of genes involved in lipid metabolism, including repression of ATP citrate lyase (ACLY), acetyl-CoA carboxylase (*Acaca*/ACC1) and fatty acid synthase (*Fasn*/FASN) (**Figure 3.3 A-B**). We therefore performed DNL assays using [14C]-acetate to determine whether this pathway is less active in *Wls*^{-/-} adipocytes. Cultured adipocytes were incubated in medium

containing [$^{14}$C]-acetate for 1, 2, 4 or 8 hours. Cells were then collected, and lipids were extracted to measure radiolabel incorporation into TAG, diacylglycerol (DAG), and phospholipid (PL) fractions. Analyses of conditioned media (**Figure 3.3 G**) and whole cell lysates (**Figure 3.3 H**) demonstrated that *Wls*$^{-/-}$ adipocytes take up less labeled acetate over time. Importantly, *Wls*$^{-/-}$ adipocytes incorporate significantly less radiolabel over time into the TAG fraction of cellular lipid (**Figure 3.3 I-J**) but not the PL or DAG fractions (**Figure 3.3 I**; quantified in **Supplementary Figure 3.3 B**). Thus, down-regulation of the DNL pathway secondary to Wntless deficiency is rate-limiting in adipocytes. Taken together, these data demonstrate that deletion of Wntless coordinately represses the network of lipogenic genes, thus altering the rate of lipogenesis and steady-state lipid composition in cultured adipocytes.

**Wntless regulation of lipogenic genes is likely mediated by SREBP1c and ChREBP, and is independent of insulin responsivity.**

We next sought to understand the mechanism by which Wntless regulates the expression of *Scd1* and other lipogenic genes. Sterol regulatory element-binding protein 1c (SREBP1c) and carbohydrate-responsive element-binding protein (ChREBP) are known key upstream transcriptional regulators in WAT and liver of many genes involved in DNL, including *Acaca*, *Fasn*, and *Scd1*[51-53]. Thus, we were interested in exploring whether these proteins are regulated in *Wls*$^{-/-}$ adipocytes. We found that expression of *Srebf1* and *Mlxipl*, which encode SREBP1c and ChREBP respectively, are both down-regulated following loss of Wntless, whereas other transcription factors involved in adipogenesis and adipocyte metabolism, including *Pparg*, *Lxra*, and *Lxrb,* are not influenced (**Figure 3.4 A**). Although PPARγ protein was unchanged, *Cebpa* mRNA and protein were both slightly repressed in *Wls*$^{-/-}$ adipocytes, suggesting that this adipogenic transcription factor may also regulate lipogenic genes in mature adipocytes (**Figure 3.4 A-B**). Importantly, immunoblot analyses revealed that SREBP1c and ChREBP proteins were both repressed in *Wls*$^{-/-}$ adipocytes (**Figure 3.4 B**), which is of interest since the literature to date has not reported regulation by Wnt signaling of *Srebf1* or *Mlxipl* in mature adipocytes. In addition, SREBP1c is post-translationally cleaved at the ER and only mature, soluble SREBP1c translocates to the nucleus to mediate gene

transcription. Of note, we observed that whereas the mature form of SREBP1c (~50 kDa relative mobility) is significantly decreased in $Wls^{-/-}$ adipocytes, the larger immature form (~100 kDa) is reciprocally increased (**Figure 3.4 B-C**). Taken together, these data suggest that Wntless regulates lipogenic gene expression through expression of ChREBP, C/EBPα, and SREBP1c mRNA and proteins, with SREBP1c also potentially regulated at the post-translational level.

Previous studies have established insulin as an important regulator of *Srebf1* gene expression in the liver[73-76]. We therefore next investigated whether decreased responsivity to insulin mediates the effects of *Wls* deletion on SREBP1c and ChREBP in adipocytes. We first measured basal and insulin-stimulated glucose uptake and did not observe differences between $Wls^{fl/fl}$ and $Wls^{-/-}$ adipocytes (**Figure 3.4 D**). We next assessed signaling downstream of insulin by immunoblot analyses, and found that phosphorylation on specific serine or threonine amino acids in AKT was not impaired in $Wls^{-/-}$ adipocytes (**Figure 3.4 E**). Although insulin has been shown to promote *Srebf1* mRNA expression in the liver, insulin treatment did not induce *Srebf1* or *Mlxipl* gene expression in either $Wls^{fl/fl}$ or $Wls^{-/-}$ adipocytes (**Figure 3.4 F**). After translation, SREBP1 is anchored to the ER by a protein complex that includes SREBP cleavage-activating protein (SCAP) and insulin-induced gene 1 or 2 (Insig1 or Insig2)[51,73,77-79]. The Insigs function to prevent the maturation of SREBP1. In response to stimuli, including insulin, Insig activity is decreased and SCAP escorts SREBP1 from the ER to the Golgi apparatus, where it is cleaved into a soluble and transcriptionally active form[79,80]. We therefore explored whether SCAP mRNA or protein expression is altered by *Wls* deletion but found both to be unchanged in $Wls^{-/-}$ adipocytes (**Figure 3.4 G-H**). Taken together, these data support the conclusion that in adipocytes, Wntless regulates SREBP1c and ChREBP through mechanisms independent of insulin responsivity.

**Adipose-specific Wntless deletion does not influence body composition or whole-body metabolism in mice on NCD.**

Our studies thus far demonstrate that Wntless expression is promoted by diet-induced obesity, and Wntless deficiency suppresses a network of lipogenic genes and DNL, and reduces fatty acid monounsaturation of adipocyte lipids. Thus, we hypothesized that

Wntless plays an important role *in vivo* in the appropriate storage of circulating nutrients, particularly under obesogenic conditions. To test this hypothesis, we generated adipose-specific *Wls* knockout mice by crossing *Wls*^{fl/fl} mice with adiponectin-Cre mice. Adiponectin-Cre has been well-established as a model to delete floxed genes specifically and efficiently in adipocytes[81]. In our model, genetic recombination of *Wls* is only observed in adipose depots, and not in other tissues, such as liver, pancreas, and muscle (**Figure 3.5 A**). As expected, we found that Wntless mRNA (**Figure 3.5 B**) and protein (**Figure 3.5 C**) were decreased in iWAT and eWAT of *Wls*^{-/-} mice on NCD, when compared to *Wls*^{fl/fl} counterparts. Analyses of mRNA from fractionated WAT demonstrated that *Wls* deletion occurs specifically in the adipocyte fraction and not in the SVF of iWAT and eWAT depots (**Figure 3.5 D**). We then explored possible metabolic phenotypes in the *Wls*^{-/-} mice and did not detect differences in body composition (**Figure 3.5 E**), growth over time (**Figure 3.5 F**), glucose tolerance (**Figure 3.5 G**), or insulin sensitivity (**Figure 3.5 H**). Consistent with these data, *Wls*^{-/-} mice did not exhibit altered fed or fasted concentrations of blood glucose (**Figure 3.5 I**), or serum insulin (**Figure 3.5 J**), adiponectin (**Figure 3.5 K**), or TAG (**Figure 3.5 L**). Finally, tissue weights such as iWAT, eWAT, brown adipose tissue (BAT) or liver were not different between *Wls*^{fl/fl} and *Wls*^{-/-} mice (**Figure 3.5 M**). Similar results were observed in female mice fed NCD (**Supplemental Figure 3.4**). Histological analyses of tissues did not yield substantial differences in adipocyte size or number within iWAT, eWAT, perirenal WAT, or BAT (**Figure 3.6 A**; data not shown). In addition, liver morphology was unchanged (**Figure 3.6 A**).

Wnt signaling plays an important role in bone, where it increases bone mass and impairs marrow adiposity. For example, FABP4- or OCN-Wnt10b mice have extensive trabeculation throughout the entire endocortical compartment[15,16]. We therefore evaluated whether marrow cells traced by adiponectin-Cre are a critical source of Wnts in the bone marrow niche[82,83]. Histological analyses of tibia from both male and female cohorts suggest that Wntless deficiency does not influence size or number of marrow adipocytes (**Supplemental Figure 3.5 A**). In addition, micro-computed tomography ($\mu$CT) analyses indicate that cortical and trabecular bone mass variables were not influenced by *Wls* deletion (**Supplemental Figure 3.5 B-I**).

Since Wntless was found to regulate lipogenic gene expression *in vitro*, we next measured expression of a subset of key DNL genes in eWAT and iWAT of *Wls*<sup>fl/fl</sup> and *Wls*<sup>-/-</sup> mice. As expected, *Wls* gene expression was significantly decreased in both eWAT and iWAT of *Wls*<sup>-/-</sup> mice (**Figure 3.6 B**). Surprisingly, expression of most DNL pathway genes was not changed, with the exception of *Mlxipl* (**Figure 3.6 B**). We also did not observe changes in DNL gene expression within livers of *Wls*<sup>-/-</sup> mice (**Supplemental Figure 3.6 A**). Analyses of isolated adipocytes from *Wls*<sup>-/-</sup> mice showed that, despite efficient knockout of *Wls*, expression of lipogenic genes was not altered (**Figure 3.6 C**). This result was puzzling, so we next evaluated whether Wnt signaling is active within adipose tissues *in vivo.* To this end, we measured expression of downstream Wnt target genes in isolated adipocytes and SVF of *Wls*<sup>-/-</sup> mice and their *Wls*<sup>fl/fl</sup> counterparts, and found that expression of *Axin2* and *Id2* were dysregulated as expected in adipocytes with Wntless deficiency. Interestingly, for those adipocyte genes that were not suppressed as expected (i.e. *Cmyc, Tcf7l2,* and *Ppard*), compensatory up-regulation was observed in the SVF (**Figure 3.6 D**). Flow cytometric analyses of SVF isolated from *Wls*<sup>fl/fl</sup> and *Wls*<sup>-/-</sup> mice did not reveal differences in subpopulation proportions (**Supplemental Figure 3.6 B-C**); thus, we considered the possibility that SVCs of *Wls*<sup>-/-</sup> mice sense the adipocyte-specific loss of Wnt secretion and respond by increasing their own production of Wnts to compensate for the deficiency. To this end, we measured the mRNA expression of many Wnts in SVF isolated from *Wls*<sup>fl/fl</sup> or *Wls*<sup>-/-</sup> mice fed NCD and found significantly increased expression of several Wnts, including *Wnt6*, *Wnt9b*, *Wnt10a*, *Wnt10b*, and *Wnt16* (**Figure 3.6 E**). We also observed decreased expression of *Wnt1*, *Wnt4*, and *Wnt8b*, whereas expression of *Wnt2a*, *Wnt2b*, *Wnt3*, *Wnt5a*, *Wnt5b*, and *Wnt11* was unchanged. These data provide strong evidence for altered Wnt production by neighboring SVCs in response to impaired secretion from adipocytes. Dramatic induction of *Wnt6*, *Wnt9b*, *Wnt10a*, *Wnt10b*, and *Wnt16*, along with induction of Wnt target genes, suggests that WATs rigorously defend adipocyte-specific loss of Wnts by increased signaling within SVCs.

***Wls^{-/-}* mice on HFD have decreased adipocyte DNL gene expression, reduced size of epididymal adipocytes and WAT, and are protected from metabolic dysfunction.**

Previous studies have suggested that Wnt signaling plays an important role in the metabolism of adipocytes under conditions of nutrient excess[32-34]. We have also found that *Wls* expression is up-regulated within both subcutaneous and visceral WAT with diet-induced obesity (**Figure 3.1 E-F**; **Supplemental Figure 3.1 A**). We therefore challenged *Wls^{fl/fl}* and *Wls^{-/-}* mice with 60% HFD for a minimum of 20 weeks and a striking metabolic phenotype emerged. Although HFD feeding did not cause a difference in body weight gain (**Figure 3.7 A**), *Wls^{-/-}* mice had decreased fat mass and increased lean mass (**Figure 3.7 B**), which was not due to altered daily food intake (**Supplemental Figure 3.7 A**). *Wls^{-/-}* mice had significantly improved glucose tolerance (**Figure 3.7 C**) and consistent with this, demonstrated higher random-fed (**Figure 3.7 D**) and glucose-induced circulating insulin concentrations (**Figure 3.7 E**) when compared to controls. Insulin sensitivity was not different (**Figure 3.7 F**), nor were random-fed or fasting blood glucose concentrations (**Figure 3.7 G**) or serum leptin (**Figure 3.7 H**), adiponectin (**Figure 3.7 I**), TAG (**Figure 3.7 J**), or free or total cholesterol (**Figure 3.7 K**). Consistent with leaner body composition, eWAT weights of *Wls^{-/-}* mice were decreased, whereas liver weights were increased (**Figure 3.7 L**).

Histological analyses of *Wls^{-/-}* tissues demonstrated significantly decreased eWAT adipocyte hypertrophy (**Figure 3.8 A-B**) with a trend toward smaller cells observed in iWAT (**Supplemental Figure 3.7 B**). Livers of *Wls^{-/-}* mice showed improved hepatosteatosis (**Figure 3.8 A**), likely contributing to the observed protection from glucose intolerance. Of note, we did not observe differences in histology of BAT or expression of UCP1 protein in *Wls^{-/-}* mice (**Supplemental Figure 3.7 C**), suggesting that metabolic effects of Wntless deletion are not secondary to altered BAT thermogenesis. We next isolated SVF and adipocytes from *Wls^{-/-}* mice fed HFD and measured Wnt target gene expression. We were intrigued to find that with HFD, SVF of *Wls^{-/-}* mice no longer had elevated Wnt expression or downstream signaling (**Figure 3.8 C**; **Supplemental Figure 3.7 D**). Analyses of the adipocyte fraction showed that Wnt target gene expression was significantly decreased (**Figure 3.8 C**), suggesting that HFD

challenge diminishes the ability of WAT to maintain Wnt signaling homeostasis. Consistent with this result, expression of several lipogenic genes, including *Mlxipl*, *Acaca*, *Fasn*, *Scd1* and *Elovl7*, was coordinately regulated in isolated epididymal adipocytes (**Figure 3.8 D-E**) and whole eWAT of *Wls*$^{-/-}$ mice (**Figure 3.8 F**), remarkably consistent with our studies in cultured adipocytes (**Figures 3.3**, **3.4**). A subset of these genes was also repressed in iWAT of *Wls*$^{-/-}$ mice (**Figure 3.8 F**). Finally, to determine whether reduced WAT DNL was compensated for elsewhere, we isolated livers from *Wls*$^{-/-}$ mice and found that they did not have altered DNL gene expression (**Supplemental Figure 3.7 E**). In summary, our studies support the conclusion that with chronic overnutrition, adipose tissues of *Wls*$^{-/-}$ mice no longer defend Wnt signaling homeostasis, which leads to down-regulation of DNL enzyme expression, reduced adipocyte hypertrophy and adiposity, and overall improved metabolic health.

**Discussion**

An extensive literature has characterized mechanisms by which Wnt signaling in MSCs represses adipogenesis and promotes alternative cell fates, such as osteoblastogenesis[5,12,17,18,84]. The Wnts expressed in MSCs that function cell-autonomously include Wnt10b, Wnt10a, and Wnt6, and other family members may conceivably participate as paracrine regulators[5,6,12]. The preponderance of evidence indicates that canonical Wnt/β-catenin signaling dominates control of MSC fate, with lesser contributions from the non-canonical pathway[5,84]. In this regard, the expression of β-catenin is suppressed during adipogenesis in both mouse and human models, and its activity is further inhibited by induced expression of Wnt antagonist Chibby[85]. Much of the early data within this field focused on the inhibition of adipogenesis by Wnt signaling, and coordinate suppression of many genes involved in Wnt/β-catenin signaling during the early stages of differentiation. However, our work herein and other recently published reports demonstrate that repression of the canonical Wnt pathway during differentiation is transient, and that proteins including β-catenin, Wntless and Tcf7l2, play important functional roles in mature adipocytes[32,34].

Here, we report that essentially all aspects of the canonical Wnt signaling apparatus are expressed in mature adipocytes, including Wntless, Wnts, Fzd receptors, LRP co-receptors, Dishevelleds, β-catenin, TCF/LEFs, and modulatory proteins like Axin2, SFRP4 and SFRP5. Indeed, Wnt signaling in cultured adipocytes is likely to have contributions from many Wnt proteins, including Wnt16, Wnt2, and Wnt7b, which are expressed at similar levels in MSCs and adipocytes, and Wnt2b, Wnt4, Wnt5b, Wnt8b, Wnt9a, and Wnt9b, which are induced during adipogenesis. These Wnts are proposed to act through LRP5 and LRP6[9,10], in conjunction with Fzd4 and Fzd9, which are also increased during differentiation. We also observed induction of previously reported downstream Wnt targets WISP1 and WISP2, secreted adipokines that are increased with obesity and proposed to regulate whole-body insulin sensitivity[86,87]. In some cases, there may also be interspecies differences that will be important for our long-term understanding of these complex processes[88]. We have shown that the core Wnt/β-catenin signaling molecules are expressed and operative within mature adipocytes, and expect that the unique combination of Wnts, receptors, and transcription factors

provides distinct functional specificity; however, the cellular processes in adipocytes controlled by this pathway were previously poorly understood.

To evaluate the cell-autonomous functions of Wnt signaling in adipocytes, we chose to block secretion of adipocyte-derived Wnts through deletion of the Wnt chaperone protein, Wntless. As expected, Wntless deletion and presumed impairment of Wnt secretion from cultured adipocytes suppressed accumulation of free cytosolic β-catenin and subsequent expression of several classic Wnt target genes such as *Axin2*, *Tcf7l2*, and *Cmyc*, responses that have been observed in many other cell types. In the context of adipocytes, we observed that Wnt secretion is also required for coordinate expression of lipogenic genes, including *Acly*, *Acaca*, *Fasn* and *Scd1*. Consistent with repression of these genes, *Wls*^{-/-} adipocytes exhibit functional impairments in DNL and lipid monounsaturation. These effects on adipocyte metabolism are highly specific: Wntless deletion does not influence insulin-stimulated glucose uptake, adrenergic stimulation of lipolysis, or β-oxidation (**Supplemental Figure 3.3 C-E**). These data are supported by that of Geoghegan *et al.*, who reported that Tcf7l2 deletion in mesenchymal progenitors promotes adipogenesis and elevated expression of gene clusters involved in fatty acid and TAG metabolism[34], consistent with the repressive role of β-catenin and TCF/LEF signaling in this context[5]. Tcf7l2 binding sites were reported within 1 kb of the transcriptional start site of most lipogenic genes, including *Acly*, *Fasn*, *and Scd1*, consistent with Wnt/β-catenin signaling directly coordinating adipocyte lipid metabolism[34].

A common feature of Wnt signaling in a wide range of cell types and animal models is inhibitory feedback, which provides an intricate restraint on Wnt-dependent gene activation[89,90]. Our lab previously observed Wnt1-induced feedback on members of the Wnt pathway in preadipocytes[91]; here, we uncovered a similar response to recombinant Wnt3a treatment of fully differentiated adipocytes. Thus, exogenous Wnt3a stimulates many of the classic inhibitory responses to limit the effects of Wnt signaling. These include induction of known feedback genes like *Nkd1*, *Nkd2*, *Axin2*, *Notum*, *Lgr5*, and *Tcf7*, coincident with repression of *Tcf7l2*, *Fzd8*, and *Lrp5*. Because Wnt secretion and functionality is largely dependent upon lipid modification by SCD1-derived palmitoleic acid[41], inhibition of SCD1 results in reduced Wnt/β-catenin signaling, an

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observation extensively investigated within the cancer field[54-61]. Herein, we have determined that the reciprocal relationship also holds true: endogenous Wnt signaling in adipocytes is required for lipogenesis and monounsaturation of lipids, including the formation of palmitoleic acid. Although signaling downstream of adipocyte Wnts is necessary for maintenance of lipogenic genes and production of palmitoleic acid, stimulation of adipocytes with exogenous Wnt3a does not further promote expression of these genes. These results may indicate that endogenous Wnt signaling plays a permissive role in expression of lipogenic genes, or perhaps that stimulation with a Wnt ligand other than Wnt3a may be necessary to observe dynamic regulation of this gene network.

In mice maintained on NCD, adipocyte-specific *Wls* deletion does not overtly influence body composition or whole-body metabolism. Consistent with our results, recent investigations into the role of Wnt signaling in mature adipocytes, including global deletion of *Sfrp5* or adipocyte-specific deletion of *Ctnnb1* or *Tcf7l2*, also did not yield metabolic phenotypes on a chow diet[32-34]. Although these previous studies did not probe further into a lack of detectable phenotype, our experiments uncovered a novel compensatory mechanism by which adipose tissues defend the loss of adipocyte-derived Wnt signaling in mice fed NCD. We found that despite efficient Wntless ablation at the DNA, RNA and protein levels, adipocytes isolated from *Wls*^{-/-} mice do not have altered Wnt target or DNL gene expression. However, upon further investigation, we observed that SVF cells isolated from *Wls*^{-/-} mice up-regulate Wnt mRNAs, including *Wnt6*, *Wnt9b*, *Wnt10a*, *Wnt10b*, and *Wnt16*. Consistent with these data, we also found elevated Wnt target gene expression in *Wls*^{-/-} SVCs, as evidenced by increased expression of *Cmyc, Tcf7l2*, and *Ppard*. These data suggest the intriguing possibility that under standard nutritional conditions, SVF cell populations sense the loss of adipocyte-derived Wnts and respond by up-regulating their own Wnt production and signaling to maintain downstream target gene expression within adipocytes.

Although mechanisms by which SVCs compensate for loss of adipocyte Wnts is not yet clear, a number of possibilities exist. Wntless deficiency may alter the SVF cellular composition, enriching for subpopulations that have highly active Wnt signaling. However, flow cytometry analysis of immune, endothelial, and stromal cell proportions

within the SVF did not yield observable differences between $Wls^{fl/fl}$ and $Wls^{-/-}$ mice, making this possibility less likely. Alternatively, a particular cell type within the SVF may detect loss of Wnt signaling from adipocytes and compensate by increasing its own Wnt production and secretion. It is also possible that the intricate intracellular feedback mechanisms described above may involve other families of secreted signaling molecules, including BMPs and FGFs, both of which are regulated in adipocytes by Wnt3a, and which have been shown in other contexts to interact with Wnt/β-catenin signaling[92-95]. Wnts are low-abundance, lipid-modified proteins that act locally in an autocrine/paracrine manner; these characteristics create major technical challenges for isolating and quantifying Wnts from tissue or serum of $Wls^{fl/fl}$ and $Wls^{-/-}$ mice. Nevertheless, further studies will be necessary to fully elucidate the compensatory mechanisms by which specific SVF cells sense and respond to the loss of adipocyte-derived Wnt secretion.

Adipose tissues are critical for maintenance of insulin sensitivity and glucose homeostasis through storage of excess energy and secretion of adipokines[96-100]. To date, inhibition of adipose-specific Wnt signaling has been shown to influence whole-body glucose homeostasis under obesogenic conditions; however, discordant results have been reported between animal models that should, ostensibly, all be loss-of-function for Wnt signaling. For example, our work in $Wls^{-/-}$ mice found that more than 20 weeks of HFD overrides the compensatory Wnt signaling in adipose SVCs, revealing a striking phenotype. $Wls^{-/-}$ mice fed HFD are characterized by decreased fat and increased lean mass, improved glucose homeostasis, increased circulating insulin, and decreased lipogenic gene expression within WAT depots. Interestingly, these mice exhibit increased liver weight but improved hepatosteatosis by histological analysis. Although highly speculative, one possible explanation underlying this finding might be the existence of a Wnt-regulated adipocyte factor that signals to the pancreas to suppress insulin secretion. When this signal is removed by impaired Wnt secretion from adipose tissue, elevated circulating insulin may act upon the liver to suppress gluconeogenesis, stimulate glycogen storage and increase liver weight. Future quantitative measurements of regulated insulin secretion, glucose homeostasis, glycogen and TAG in whole livers, as well as examination of hepatic cell size and

number, may uncover mechanisms by which Wntless deficiency improves glucose tolerance in obese mice.

Consistent with our studies, adipocyte-specific β-catenin-deficient mice also exhibit decreased fat mass and improved glucose tolerance when fed HFD long-term[32]. In contrast, diet-induced obese *Tcf7l2*^{-/-} mice demonstrate increased adipose tissue mass, impaired glucose tolerance and greater insulin resistance[34]. Expression of a subset of lipogenic genes, including *Acly* and *Scd1*, was elevated in WAT of these mice, in line with observed adipocyte hypertrophy, but counter to our results in cultured adipocytes and WAT from *Wls*^{-/-} mice. The bases for these apparently conflicting results may include reported discrepancies between their RNAseq and qPCR results, generation of alternative splice variants with differential cis-element binding or transcriptional activity, or compensatory binding of other TCF/LEF transcription factors to cis-elements[101-104]. Alternatively, it is conceivable that there are distinctions between knockout of Wnt secretion and deletion of a downstream transcriptional effector of β-catenin; to this end, Tcf7l2 has also been shown to have transcriptional activity independent of β-catenin in certain contexts[105] and its deletion may therefore impact distinct pathways. Indeed, Geogheghan *et al.* report that *Tcf7l2* deletion repressed expression of lipolytic genes, possibly contributing to adipocyte hypertrophy and subsequent metabolic dysfunction on HFD. In any case, results from both groups point to regulation of lipid metabolism by Wnt signaling in adipocytes.

In a gain-of-function Wnt signaling model, global *Sfrp5*[Q27Stop] mice fed HFD were characterized by decreased adipocyte size and WAT mass, elevated mitochondrial variables including oxygen consumption, and a mild improvement in glucose homeostasis[33]. These effects of SFRP5 on glucose homeostasis are largely supported by injection of SFRP5 neutralizing antibody[106]. Consistent with these data, transgenic mice with enforced expression of Wnt10b from the FABP4 promoter have less WAT when maintained on NCD, are resistant to HFD-induced or genetic obesity, and are more glucose tolerant and insulin sensitive than controls[13,14]. Taken together, these gain-of-function models support the notion that increased adipose Wnt signaling influences whole-body glucose homeostasis; however, further research is required to establish mechanisms and to resolve differences in experimental outcomes.

In summary, we have demonstrated that Wnt/β-catenin signaling is active in adipocytes and adipocyte-derived Wnts are required for expression of a network of lipogenic genes. Further, adipocytes compensate for Wnt deficiency through cell-autonomous mechanisms, and WAT defends loss of adipocyte-derived Wnts by up-regulating Wnt signaling in SVCs (**Figure 3.9**). Finally, this compensatory mechanism for maintaining homeostasis is overridden by long-term HFD, revealing that *Wls*$^{-/-}$ mice are resistant to diet-induced obesity, adipocyte hypertrophy, and metabolic dysfunction.

**Materials and methods**

*Animals*

$Wls^{fl/fl}$ mice (#012888, Jackson Lab, Ellsworth, ME), which harbor loxP sites flanking exon 1, were crossed with Adiponectin (Adipoq)-Cre mice (#028020, Jackson Lab, Ellsworth, ME) to generate $Wls^{fl/fl}$ or $Wls^{-/-}$ mice. Animals were housed in a 12 h light/12 h dark cycle with free access to water and food. For HFD studies, mice were fed rodent diet with 60 kcal% from fat (#12492, Research Diets, New Brunswick, NJ). All animal studies were approved by and performed in compliance with policies of the University of Michigan Institutional Animal Care and Use Committee. Daily care of mice was overseen by the Unit for Laboratory Animal Medicine at the University of Michigan.

*Body composition*

Fat, lean, and free fluid masses were estimated with a Bruker Minispec LF90II NMR (Bruker, Billerica, MA) within the University of Michigan Mouse Metabolic Phenotyping Center.

*Glucose and insulin tolerance tests*

For glucose tolerance tests, mice were fasted for 16 h and then administered an intraperitoneal injection of glucose (1 mg/kg body weight). For insulin tolerance tests, mice were fasted for 6 h and then given an intraperitoneal injection of insulin (Eli Lilly, Indianapolis, IN). Mice on NCD received 0.5 U insulin/kg body weight whereas mice on HFD were administered 1.0 U insulin/kg body weight. Blood was collected from the tail vein, and glucose concentrations were monitored at 0, 15, 30, 60, and 120 minutes after intraperitoneal injection using a glucometer and Contour Next blood glucose strips (Bayer AG, Leverkusen, Germany).

*Serum measurements*

Blood was collected from the tail vein, or by cardiac puncture at time of harvest, and allowed to coagulate on ice for 2 h. After centrifugation at 2,000 x g for 20 min at 4°C, serum was transferred to a new tube and stored at -80°C. ELISA was used to estimate

concentrations of serum insulin (Crystal Chem USA, Elk Grove, IL), adiponectin (R&D Systems Inc., Minneapolis, MN), and leptin (PeproTech Inc., Rocky Hill, NJ). Triacylglycerols (TAG), and total and free cholesterol were measured by colorimetric assays (Cayman Chemical, Ann Arbor, MI; Abcam, Cambridge, UK, respectively).

*Adipocyte and stromal-vascular cell fractionation*

Inguinal WAT (iWAT) and epididymal WAT (eWAT) were excised from mice[99,107], minced with scissors and digested for 1 h at 37°C with shaking (600 rpm) in 2 mg/ml collagenase type I (Worthington Biochemical, Lakewood, NJ) in Krebs-Ringer-HEPES (KRH; pH 7.4) buffer containing 3% fatty acid-free bovine serum albumin (BSA; Gold Biotechnology, St. Louis, NJ), 1 g/L glucose, and 500 nM adenosine. The resulting cell suspensions were filtered through 100 μm cell strainers and centrifuged at 100 x g for 8 min to separate the stromal-vascular fraction (SVF) and the buoyant adipocytes. Fractions were washed 2x with KRH buffer containing 3% fatty acid-free BSA, 1 g/L glucose, and 500 nM adenosine. For immunoblot analyses, the fractions were subsequently washed 1x with KRH buffer containing 0.5% fatty acid-free BSA, 1 g/L glucose, and 500 nM adenosine.

*Histology*

Soft tissues were harvested and fixed in 10% neutral buffered formalin overnight at 4°C. Bones were fixed in 10% neutral buffered formalin for 24 h, rinsed with water and decalcified in 14% EDTA, pH 7.4 for 14 days. Paraffin-embedded tissues were sectioned at 5 μm thickness and stained with hematoxylin and eosin (H&E) as previously described[108]. Stained sections were imaged using a Zeiss inverted microscope at 100x or 200x magnification.

*Adipocyte histomorphometry*

Adipocyte size and number were quantified from WAT sections stained with hematoxylin and eosin. After digital processing, individual adipocyte cross-sectional areas were quantified using MetaMorph Microscopy Automation & Image Analysis Software 2.0 (Molecular Devices, Sunnyvale, CA) as previously described[108]. To

compare adipocyte sizes between genotypes, frequency distribution curves were generated with bin values as follows: bin range: 0 to 18,000 $\mu m^2$; bin width: 500 $\mu m^2$. Nonlinear curves were fit to the data to visualize frequency distribution patterns.

*Micro-computed tomography ($\mu$CT) analysis of bone*

Harvested tibiae were fixed in 10% neutral buffered formalin for 24 h, washed with water, placed in Sorensen's phosphate buffer, pH 7.4, and stored at 4°C prior to $\mu$CT analysis. After placement in a 19-mm diameter tube, the length of each bone was scanned using a $\mu$CT system ($\mu$CT100 Scanco Medical, Bassersdorf, Switzerland). Scan settings were as follows: voxel size 12 $\mu m$, 70 kVp, 114 $\mu A$, 0.5 mm AL filter, integration time 500 ms. Density measurements were calibrated to the manufacturer's hydroxyapatite phantom. Analysis was performed with the manufacturer's evaluation software, using a threshold of 180 for trabecular bone and 280 for cortical bone.

*Cell culture*

Primary mesenchymal stem cells (MSC) were isolated from the ears of wildtype C57BL/6J (Jackson Labs, Bar Harbor, Maine) or *Wls*^{fl/fl} mice as previously described[33,109,110] and cultured at 5% $CO_2$. Subconfluent MSCs were maintained in DMEM:F12 medium (Thermo Fisher Scientific, Waltham, MA) containing 10% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO) and supplemented with 10 ng/ml recombinant basic fibroblast growth factor (PeproTech Inc., Rocky Hill, NJ). At two days post-confluence, adipogenesis was induced with 0.5 mM methylisobutylxanthine, 1 $\mu$M dexamethasone, 5 $\mu$g/ml insulin and 5 $\mu$M rosiglitazone in DMEM:F12 containing 10% FBS. From day 2 to 4 of differentiation, cells were fed with fresh DMEM:F12 medium containing 10% FBS, 5 $\mu$g/ml insulin and 5 $\mu$M rosiglitazone. Thereafter, cells were maintained in DMEM:F12 containing 10% FBS. Accumulation of neutral lipids in adipocytes was visualized with Oil Red-O staining[111]. Briefly, cells were washed with phosphate-buffered saline (PBS), fixed in 0.5% glutaraldehyde in PBS for 5 min, incubated in Oil Red-O working solution for 10 min at room temperature with gentle agitation, and then washed 2x with PBS. Total TAG accumulation was quantified by colorimetric assay (Cayman Chemical, Ann Arbor, MI). Cells cultured for analyses of

lipid composition were differentiated from day 6 to 12 in DMEM:F12 containing 10% charcoal-stripped FBS (Sigma-Aldrich, St. Louis, MO). Where indicated, confluent MSCs or day 12 adipocytes were treated with 20 ng/ml recombinant Wnt3a (R&D Systems Inc., Minneapolis, MN) for 4 h prior to collection.

*Genetic recombination in cultured cells*

To induce gene deletion, *Wls*^{fl/fl} adipocytes were treated with adenoviral GFP or adenoviral Cre recombinase ($1x10^{10}$ viral particles/ml) in serum-free maintenance medium from day 4 to 6 of differentiation. Adenoviruses were obtained from the University of Michigan Vector Core. Genetic recombination was confirmed by PCR with a three-primer system. Primer sequences as follows: P1, CTTCCCTGCTTCTTTAAGCGTC; P2, AGGCTTCGAACGTAACTGACC; P3, CTCAGAACTCCCTTCTTGAAGC; floxed band: 556 bp; recombined band: 410 bp.

*Cellular fraction for isolation of free cytosolic and membrane β-catenin*

To evaluate levels of free cytosolic β-catenin, confluent MSCs were treated for 4 h with 20 ng/ml recombinant Wnt3a (R&D Systems Inc., Minneapolis, MN) and cellular fractionation was performed as previously described. Briefly, cells were washed twice with cold PBS and homogenized using a BioVortexer mixer (Chemglass, Vineland, NJ) in ice-cold buffer containing 10 mM Tris pH 7.4, 140 mM NaCl, 5 mM EDTA, 2mM DTT, 1:100 protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). Lysates were then centrifuged for 10 min at 500 x g at 4°C to remove cellular debris. Lysates were subsequently ultracentrifuged at 100,000 x g for 90 min at 4°C to obtain the cytosolic (supernatant) and membrane (pellet) fractions. Protein concentrations of cell fractions were measured by BCA protein assay (Thermo Scientific, Waltham, MA) and β-catenin expression was measured by immunoblotting[6,66].

*De novo lipogenesis (DNL) assay*

Cultured adipocytes were incubated overnight in fresh serum-free DMEM:F12 medium prior to evaluation of DNL. Cells were then incubated in fresh DMEM:F12 medium (containing 0.5 mM sodium pyruvate, 0.5 mM L-glutamine, 2.5 mM glucose)

supplemented with 1% fatty acid-free BSA, and containing 0.5 $\mu$Ci [$^{14}$C]-acetate (PerkinElmer, Waltham, MA) and 5 $\mu$M sodium acetate for 1, 2, 4, or 8 h at 37°C. At the end of the indicated incubation times, adipocytes were harvested, and lipids extracted for analyses by thin-layer chromatography and scintillation counting.

*Insulin-stimulated glucose uptake*

Cultured adipocytes were fasted for 6 h in Dulbecco's PBS (dPBS; without calcium, magnesium, phenol red; Thermo Fisher Scientific, Waltham, MA) with 0.7% fatty acid-free BSA at 37°C. Cells were washed 1x with dPBS. Adipocytes were then stimulated with 0, 2, 10, 20 or 100 nM insulin in dPBS containing 0.7% fatty acid-free BSA. After 30 min, 0.1 $\mu$Ci/ml [$^{14}$C]-2-deoxy-glucose and 1 mM 2-deoxy-glucose was added per well and incubated for an additional 10 min. To measure background glucose uptake, 50 $\mu$M cytochalasin B (glucose transport inhibitor) was added to a subset of wells during the last 10 min of insulin stimulation. Glucose uptake was terminated with addition of 2-deoxy-glucose to a final concentration of 20 mM. Medium was aspirated and cells were washed 2x with ice-cold PBS. Adipocytes were lysed in 0.1% sodium dodecyl sulfate, lysates centrifuged for 10 min at 13,600 x g, and supernatants transferred to a new tube. Radioactivity in the supernatant was measured using a scintillation counter, background glucose uptake subtracted, and labeled glucose uptake normalized to protein concentration of lysed cells.

*Lipolysis assay*

Cultured adipocytes were washed 1x with PBS and then incubated in Hank's Balanced Salt Solution (HBSS; Thermo Fisher Scientific, Waltham, MA) containing 2% BSA for 1 h at 37°C. Cells were then stimulated with 1 $\mu$M isoproterenol , 5 $\mu$M forskolin, or 1 $\mu$M CL-316,243 in fresh HBSS containing 2% BSA for 3 h at 37°C; untreated cells were used to assess basal lipolysis. Conditioned medium was collected at the end of the incubation, released glycerol measured by colorimetric assay (Sigma-Aldrich, St. Louis, MO), and normalized to protein concentration of lysed cells.

*β-oxidation assay*

Cultured adipocytes were washed 1x with PBS and then incubated with 5 mCi/ml [$^3$H]-palmitic acid and 22 μM palmitic acid in β-oxidation buffer (following manufacturer's guidelines; catalog #D5030; Sigma-Aldrich, St. Louis, MO) containing 10 mg/ml BSA and 200 μM L-carnitine for 3 h at 37°C. A subset of wells was pre-treated with 100 μM etomoxir (CPT1 inhibitor; Cayman Chemical, Ann Arbor, MI; Abcam, Cambridge, UK) to block mitochondrial β-oxidation. Fresh etomoxir was added to these wells for the duration of the assay. After 3 h, conditioned media from cells was passed through columns containing anion exchange resin and collected in scintillation vials. Columns were subsequently washed 2x with deionized water. Radioactivity in the supernatant was measured using a scintillation counter and normalized to protein concentration of lysed cells.

*Lipid extraction for analyses by gas chromatography*

Cultured adipocytes were washed 2x with PBS and then suspended in 500 μl of a 1:2.5 methanol/water mixture. The cell suspension was transferred to a borosilicate glass tube. Wells were rinsed with 500 μl of the 1:2.5 methanol/water mixture and the volume was transferred to the glass tube. Then 375 μl chloroform and 375 μl 0.9% NaCl were added to each tube, which were then vortexed vigorously and centrifuged at 2500 rpm for 20 min at 4°C. The lower organic chloroform layer containing total lipids was transferred to a new tube and stored at -20°C until analyzed.

*Fatty acid composition by gas chromatography*

Fatty acids within extracted lipids were derivatized into their methyl esters by trans-esterification with boron trifluoride-methanol as previously described [112]. Derivatized methyl esters were re-dissolved in a small volume of hexane and purified by thin-layer chromatography using n-hexane-diethyl ether-acetic acid (50:50:2, v/v/v) as the developing solvents. After development, plates were dried and sprayed with Premulin. The products were identified under ultraviolet light by comparison to the retention flow of the methyl heptadecanoate (C17:0) standard (retention flow, 0.67) applied side-by-side on the same plate. Methyl esters were extracted from the thin-layer chromatography

powder with diethyl ether, concentrated under nitrogen, and re-dissolved in 100 µl hexane. Fatty acid compositions of the lipids were analyzed by gas chromatography (GC) as follows. Analysis of FAMEs was performed with 1 µl sample injection on an Agilent GC machine, model 6890N equipped with flame ionization detector, an auto sampler and a Chemstation software for data analysis. An Agilent HP 88, 30 m GC column with a 0.25 mm inner diameter and 0.20 mm film thickness was used. Hydrogen was used as a carrier gas and nitrogen was used as a makeup gas. Analyses were carried out with a temperature programming of 125-220°C. Fatty acid components within unknown samples were identified with respect to the retention times of authentic standard methyl ester mixtures run side-by-side. Fatty acid components were quantified with respect to the known amount of internal standard added and the calibration ratio derived from each fatty acid of a standard methyl esters mixture and the methyl heptadecanoate internal standard. The coefficient of variation for GC analyses was found to be within 2.3-3.7%.

*Quantitative RT-PCR*

Total RNA was purified from frozen tissue or cultured cells using RNA STAT-60 (Tel Test, Alvin, TX) according to the manufacturer's instructions. One µg of total RNA was reverse-transcribed to cDNA using M-MLV Reverse Transcriptase (Invitrogen, Carlsbad, CA). qRT-PCR was performed on the StepOnePlus System (Applied Biosystems, Foster City, CA) with qPCRBIO Sygreen Mix (Innovative Solutions, Beverly Hill, MI). Prior to use, all primers were validated with a cDNA titration curve and product specificity was confirmed by melting curve analyses and gel electrophoresis of qPCR products. Expression of each gene was calculated using a cDNA titration curve within each plate and was then normalized to the expression of peptidylprolyl isomerase A (PPIA) mRNA.

*RNA sequencing (RNA-seq) analyses*

Confluent MSCs or day 12 adipocytes were treated with 20 ng/ml recombinant Wnt3a (R&D Systems Inc., Minneapolis, MN) or vehicle for 4 h prior to lysis and RNA isolation.

After DNase treatment, purified RNA samples (n = 4 per condition) were submitted to the University of Michigan Advanced Genomics Core for quality control, library preparation, and sequencing on an Illumina Hi-Seq platform. Read files were downloaded and collated into a single .fastq file for each sample. Quality of raw read data was checked using FastQC (version v0.11.30) to identify features of the data that may indicate quality problems (i.e. low-quality scores, over-represented sequences, inappropriate GC content). The Tuxedo Suite software package was used for alignment, differential expression analysis, and post-analysis diagnostics. Briefly, reads were aligned to the UCSC reference genome using TopHat (version 2.0.13) and Bowtie2 (version 2.2.1). FastQC was used for a second round of quality control post-alignment to ensure that only high-quality data was input to expression quantitation and differential expression analyses. Differential expression analysis was done using two distinct methods: Cufflinks/CuffDiff and HTSeq/DESeq2, using UCSC build mm10 as the reference genome sequence. To generate lists of Wnt-related genes significantly regulated by adipogenesis or recombinant Wnt3a treatment, data were analyzed using GOrilla (Gene Ontology Enrichment Analysis and Visualization Tool) and the PANTHER Classification System. The Wnt Homepage (http://web.stanford.edu/group/nusselab/cgi-bin/wnt/) was also used to generate a list of established Wnt pathway genes. The complete RNAseq dataset was then mined to identify Wnt-related genes that are up- or down-regulated during adipogenesis, or in response to Wnt3a.

*Untargeted proteomics analysis*

Cultured day 12 *Wls*fl/fl and *Wls*-/- adipocytes were washed three times with PBS to remove serum. Cells were homogenized using a BioVortexer mixer (Chemglass, Vineland, NJ) in ice-cold lysis buffer (1% SDS, 12.7 mM EDTA, 60 mM Tris-HCl; pH 6.8) containing 1:100 protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). Lysates were then centrifuged at 13,600 x g for 10 min at 4°C and the supernatant was submitted to the University of Michigan Proteomics and Peptide Synthesis Core for untargeted proteomics analysis. In short, 12 μl of each sample was processed by SDS-PAGE using a 10% Bis-Tris NuPAGE mini-gel (Invitrogen, Carlsbad, CA) with MES buffer. The gel was excised into 10 equally sized bands and processed by in-gel

digestion with trypsin using a Digilab ProGest robot (Accela, Prague, Czech Republic). Half of each digest was analyzed by nano LC-MS/MS with a Waters NanoAcquity HPLC system interfaced to a ThermoFisher Q Exactive. Peptides were loaded on a trapping column and eluted over a 75 μm analytic column at 350 nL/min; both columns were packed with Luna C18 resin. The mass spectrometer was operated in data-dependent mode, with the Orbitrap operating at 70,000 FWHM and 17,500 FWHM for MS and MS/MS, respectively. The 15 most abundant ions were selected for MS/MS. Data was queried using Mascot with the following parameters: enzyme: Trypsin/P; database: SwissProt Mouse; fixed modification: carbamidomethyl (C); variable modifications: oxidation (M), acetyl (N-term), pyro-Glu (N-term Q); deamidation (N, Q); mass value: monoisotropic; peptide mass tolerance: 10 ppm; fragment mass tolerance: 0.02 Da; max missed cleavages: 2. Mascot DAT files were parsed into Scaffold (Proteome Software, Portland, OR) for validation, filtering and to create a non-redundant list per sample. Data were filtered using a 1% protein and peptide FDR and requiring at least 2 unique peptides per protein.

*Immunoblot analyses*

Tissues were collected from mice and homogenized using a BioVortexer mixer (Chemglass, Vineland, NJ) in ice-cold lysis buffer (1% SDS, 12.7 mM EDTA, 60 mM Tris-HCl; pH 6.8) containing 1:100 protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). Lysates were then centrifuged at 13,600 x g for 10 min at 4°C. For WAT lysates, the top lipid layer was removed, and extracts were centrifuged again. Cultured cells were washed once with PBS and homogenized in lysis buffer containing 1:100 protease inhibitor cocktail. Lysates were then centrifuged at 13,600 x g for 10 min at 4°C. Protein concentrations of cell or tissue lysates were measured by BCA protein assay (Thermo Scientific, Waltham, MA). Lysates were diluted to equal protein concentrations in lysis buffer and Laemmli sample buffer, vortexed vigorously, and heated at 95°C for 5 min. Cell or tissue extracts (20 μg) were separated by SDS-PAGE on 4-12% gradient polyacrylamide gels (Invitrogen, Carlsbad, CA) and transferred to Immobilon PVDF membranes (Millipore, Billerica, MA). Membranes were blocked in 5% non-fat dried milk in Tris-buffered saline, pH 7.4, containing 0.05% Tween-20 (TTBS) for 1 h at room

temperature and then immunoblotted with indicated primary antibodies (1:1000) in 5% BSA in TTBS overnight at 4°C. Blots were probed with horseradish peroxidase-conjugated secondary antibodies (1:5000) diluted in 5% non-fat dried milk in TTBS for 1.5 h at room temperature and visualized with Clarity Western ECL Substrate (Bio-Rad, Hercules, CA) or SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific, Waltham, MA).

*Human gene expression data*

Human *Wls* gene expression data were acquired from the publicly available Genotype-Tissue Expression (GTEx) database.

*Statistics*

All data are presented as mean ± S.D. When comparing two groups, significance was determined using two-tailed Student's t-test. When comparing multiple experimental groups, an analysis of variance (ANOVA) was followed by post hoc analyses with Dunnett's or Sidak's test, as appropriate. Differences were considered significant at $p < 0.05$ and are indicated with asterisks.
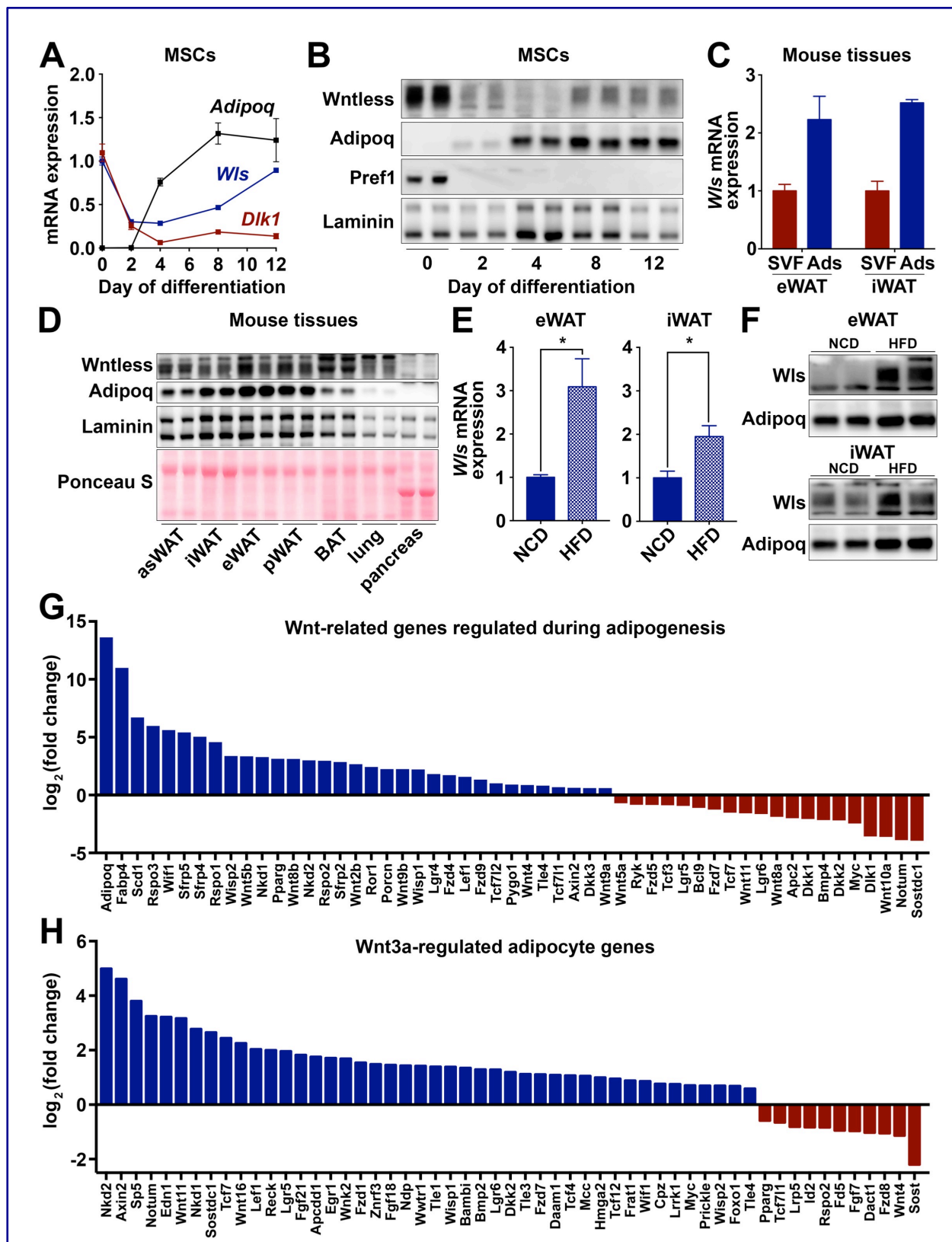
# Figure 3.1

**Figure 3.1. Canonical Wnt signaling is active in cultured adipocytes and Wntless is up-regulated by diet-induced obesity.** (**A-B**) MSCs isolated from C57BL/6J mice were cultured under standard conditions and induced to differentiate. Wntless gene (n = 6) and protein expression at indicated days of differentiation. (**C**) Wntless gene expression in the stromal-vascular (SVF) and adipocyte (Ads) fractions isolated from epididymal (eWAT) and inguinal (iWAT) white adipose tissues of C57BL/6J mice (males; n = 6). (**D**) Representative immunoblot of Wntless protein expression across C57BL/6J mouse tissues (asWAT, anterior subcutaneous WAT; pWAT, perirenal WAT; BAT, brown adipose tissue); Adiponectin, Laminin, and Ponceau S included as controls. (**E-F**) Regulation of Wntless gene and protein expression in eWAT and iWAT of 16-week-old wildtype mice fed with normal chow diet (NCD) or 8 weeks of high fat diet (HFD) (males; n = 6). (**G**) Wnt-related genes found to be significantly up- or down-regulated by RNA-seq analyses of day 12 adipocytes vs. day 0 cultured MSCs (n = 4). (**H**) Select Wnt-related genes found to be significantly up- or down-regulated by RNA-seq analyses of day 12 cultured adipocytes treated with recombinant Wnt3a (20 ng/ml) for 4 h (n = 4). RNA expression normalized to PPIA. Data presented as mean ± S.D. * indicates significance at $p < 0.05$.

**Supplemental Figure 3.1**



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**Supplemental Figure 3.1**. (**A**) Wntless gene expression in the stromal-vascular (SVF) and adipocyte (Ads) fractions isolated from eWAT and inguinal iWAT of 16-week-old wildtype mice fed with NCD or 8 weeks of HFD (males; n = 6). (**B**) Regulation of Wntless gene expression in eWAT and iWAT by nutritional and environmental conditions: fast: 18 h; refed: 6 h after 18 h fast; 30% calorie restriction (CR): 6 wks; 4°C cold exposure: 6 hr. Each condition normalized to its individual experimental control set to 1 (indicated by grey dashed line). (**C**) Wntless gene expression in male and female human tissues; data obtained from the GTEx-RNA-Seq dataset. (**D**) Wnt-related genes found by RNA-seq analyses to be expressed at similar levels in both day 12 adipocytes and confluent day 0 cultured MSCs (n = 4). RNA expression normalized to PPIA. Data presented as mean $\pm$ S.D. * indicates significance at $p < 0.05$.

**Figure 3.2**



**Figure 3.2. Wntless is required for canonical Wnt signaling in differentiated primary adipocytes.** (**A**) Schematic model for deletion of Wntless in cultured adipocytes using adenoviral Cre recombinase. (**B**) Genetic recombination of Wntless in $Wls^{fl/fl}$ and $Wls^{-/-}$ adipocytes using a 3-primer PCR system (n = 3). (**C-D**) Wntless RNA and protein expression in adipocytes following adenoviral GFP or Cre infection (n = 3). (**E**) Representative brightfield and Oil Red-O images, and (**F**) triacylglycerol accumulation in $Wls^{fl/fl}$ and $Wls^{-/-}$ adipocytes (n = 4). (**G**) Expression of known Wnt target genes in $Wls^{fl/fl}$ and $Wls^{-/-}$ adipocytes (n = 3). (**H**) Free cytosolic and membrane β-catenin protein expression in $Wls^{fl/fl}$ and $Wls^{-/-}$ confluent MSCs under basal conditions (n = 6) and after 20 ng/ml Wnt3a treatment for 4 h (n = 2); GAPDH and laminin shown as cytosolic and membrane loading controls, respectively. RNA expression normalized to PPIA. Data presented as mean ± S.D. * indicates significance at p < 0.05.

**Supplemental Figure 3.2**



**Supplemental Figure 3.2.** (**A**) Densitometry quantification of cytosolic and membranous β-catenin protein expression in *Wls*^fl/fl^ and *Wls*^-/-^ day 0 MSCs under basal conditions and after 20 ng/ml Wnt3a treatment for 4 h (n = 6). (**B**) Proteins found by untargeted proteomics analysis to be increased over 200% or decreased more than 50% in cultured day 12 *Wls*^-/-^ adipocytes (n = 1). Data presented as mean ± S.D. * indicates significance at p < 0.05.

**Figure 3.3**
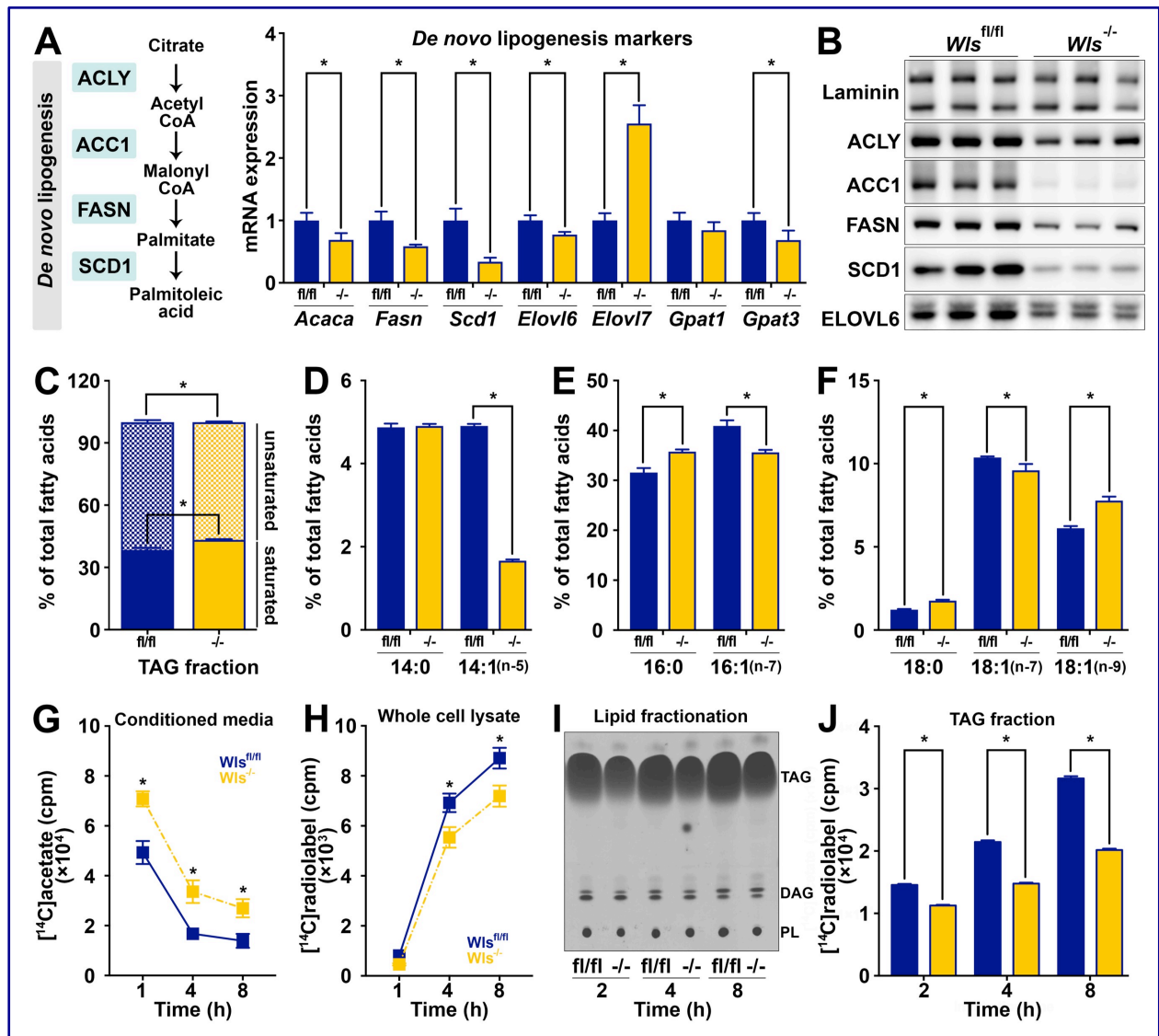


**Figure 3.3. Adipocyte Wntless regulates expression of a network of lipogenic genes and influences triacylglycerol fatty acid composition and** *de novo* **lipogenesis.** (**A-B**) Lipogenic gene and protein expression in *Wls*^{fl/fl} and *Wls*^{-/-} adipocytes (n = 3). RNA expression normalized to PPIA. (**C**) Proportion of total saturated (solid) versus unsaturated fatty acids (hatched) in lipids extracted from *Wls*^{fl/fl} and *Wls*^{-/-} adipocytes (n = 6). Relative proportions of (**D**) myristic (C14:0) versus myristoleic acid (C14:1), (**E**) palmitic (C16:0) versus palmitoleic acid (C16:1), and (**F**) stearic (C18:0) versus vaccenic (C18:1, n-7) or oleic acid (C18:1, n-9). *De novo* lipogenesis was evaluated in cultured *Wls*^{fl/fl} and *Wls*^{-/-} adipocytes using [$^{14}$C]-acetate for 1, 2, 4 and 8 h. [$^{14}$C]-radiolabel in (**G**) conditioned media versus (**H**) whole cell lysates after indicated incubation times was measured by scintillation counting (n = 3). (**I**) Representative thin-layer chromatography analysis of [$^{14}$C]-acetate incorporation into lipid species over indicated incubation times; TAG, triacylglycerol; DAG, diacylglycerol;

PL, phospholipid. (**J**) Radiolabel incorporation into TAG fractions extracted from *Wls*^{fl/fl} and *Wls*^{-/-} adipocytes was quantified by scintillation counting (n = 3). Data presented as mean ± S.D. * indicates significance at $p < 0.05$.

**Supplemental Figure 3.3**



**Supplemental Figure 3.3.** (**A**) Lipogenic proteins quantified by densitometry (n = 3). (**B**) *De novo* lipogenesis was evaluated in cultured *Wls*$^{fl/fl}$ and *Wls*$^{-/-}$ adipocytes using [$^{14}$C]-acetate for 1, 2, 4 and 8 h. Radiolabel incorporation into PL and DAG fractions extracted from *Wls*$^{fl/fl}$ and *Wls*$^{-/-}$ adipocytes was quantified by scintillation counting (n = 3). (**C**) Basal and induced glycerol release from *Wls*$^{fl/fl}$ and *Wls*$^{-/-}$ adipocytes using 1 μM isoproterenol (Iso), 5 μM forskolin (Forsk), or 1 μM CL-316,243 (CL-316) for 3 h (n = 3). (**D**) β-oxidation of [$^{3}$H]-palmitic acid; 100 μM etomoxir used as negative control (n = 3). (**E**) Expression of proteins related to lipolysis or β-oxidation (n = 3). Data presented as mean ± S.D. * indicates significance at p < 0.05.

**Figure 3.4**



Figure 3.4. **Wntless is required for expression of *Srebf1* and *Mlxipl*, transcriptional regulators of lipogenesis.** (**A-B**) Gene and protein expression of indicated transcription factors (n = 3); I: immature, insoluble form of SREBP1c at relative mobility of 100 kDa; M: mature, soluble form of SREBP1c at 50 kDa. (**C**) Mature versus immature forms of SREBP1c quantified by densitometry. (**D**) Basal and insulin-stimulated glucose uptake into *Wls*fl/fl and *Wls*-/- adipocytes (n = 3). (**E**) Representative immunoblot of AKT phosphorylation in *Wls*fl/fl and *Wls*-/- adipocytes following treatment with indicated insulin concentrations for 10 min. (**F**) Expression of *Srebf1* or *Mlxipl* mRNAs in *Wls*fl/fl and *Wls*-/- adipocytes following treatment with indicated insulin concentrations for 24 h (n = 3). (**G**) Expression of *Scap* mRNA in *Wls*fl/fl and *Wls*-/- adipocytes following treatment with indicated insulin concentrations for 24 h (n = 3). (**H**) Representative immunoblot of SCAP protein expression in *Wls*fl/fl and *Wls*-/- adipocytes following treatment with indicated insulin concentrations for 10 min; pAKT(thr308) shown as a control for insulin response. RNA expression normalized to PPIA. Data presented as mean ± S.D. * indicates significance at p < 0.05.

**Supplemental Figure 3.4**



**Supplemental Figure 3.4.** (**A**) Growth curve of 20-week-old female $Wls^{fl/fl}$ and $Wls^{-/-}$ mice on NCD. (**B**) Glucose tolerance test in 16-week-old $Wls^{fl/fl}$ and $Wls^{-/-}$ mice. (**C**) Insulin tolerance test in 18-week-old mice. (**D**) Body composition of 16-week-old $Wls^{fl/fl}$ and $Wls^{-/-}$ mice. (**E**) Blood glucose concentrations in random-fed and 16 h fasted mice. Serum concentrations of (**F**) insulin, (**G**) adiponectin, and (**H**) triacylglycerols in 20-week-old mice. (**I**) Tissue weights at time of sacrifice. $Wls^{fl/fl}$: n = 7; $Wls^{-/-}$: n = 9. Data presented as mean ± S.D. * indicates significance at $p < 0.05$.

**Figure 3.5**
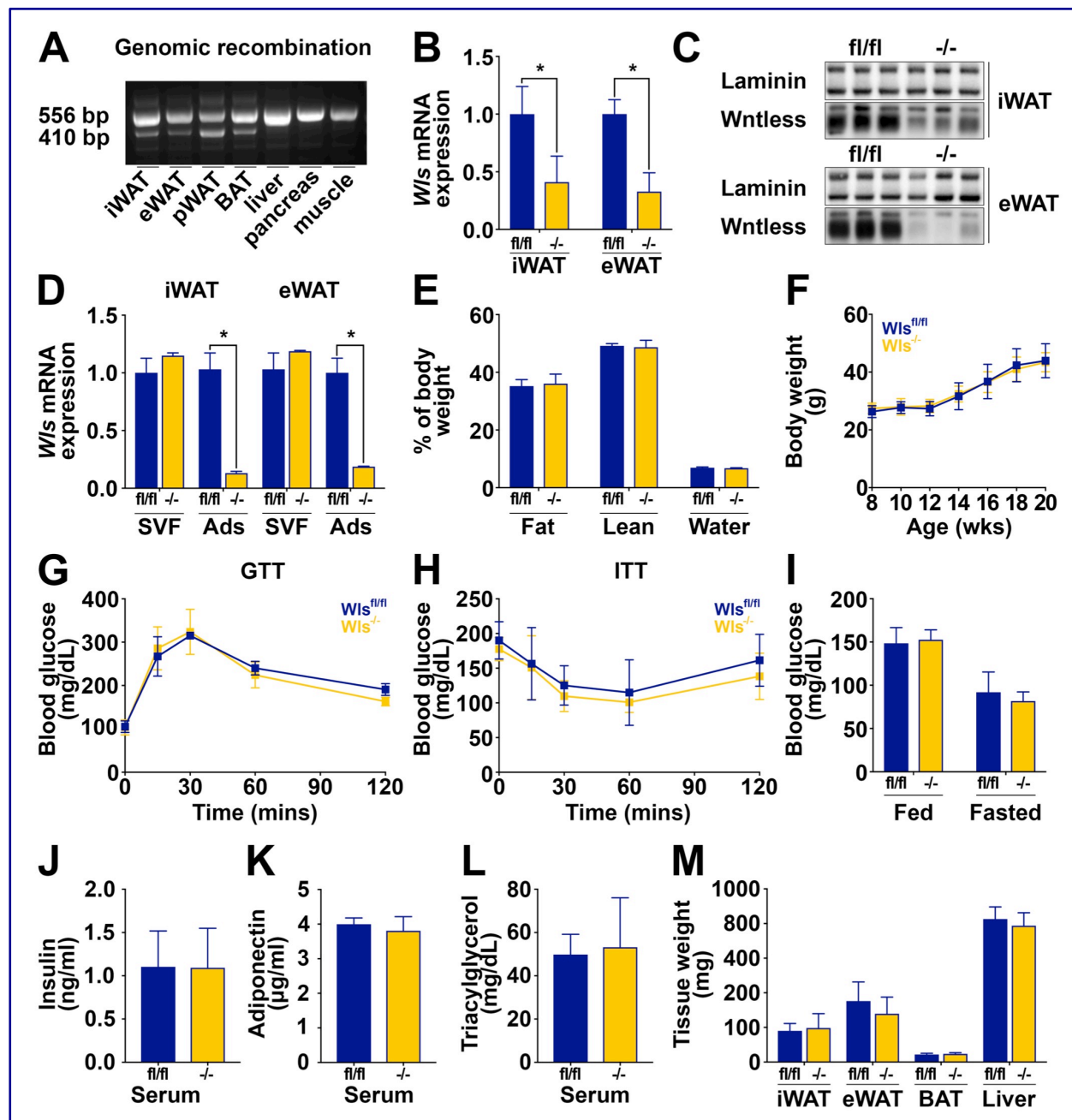


Figure 3.5. Adipose-specific Wntless deletion does not influence whole-body metabolism on a normal chow diet. (**A**) Genetic recombination of Wntless in tissues isolated from *Wls*^{-/-} mice. (**B-C**) Wntless mRNA and protein expression in iWAT and eWAT of *Wls*^{fl/fl} and *Wls*^{-/-} mice. (**D**) Wntless mRNA expression in SVF and adipocytes isolated from iWAT and eWAT of *Wls*^{fl/fl} and *Wls*^{-/-} mice (n = 3). (**E**) Body composition of 16-week-old *Wls*^{fl/fl} and *Wls*^{-/-} mice on NCD. (**F**) Growth curve of 20-week-old *Wls*^{fl/fl} and *Wls*^{-/-} mice. (**G**) Glucose tolerance test in 16-week-old *Wls*^{fl/fl} and *Wls*^{-/-} mice. (**H**) Insulin tolerance test in 18-week-old mice. (I) Blood glucose concentrations in random-fed and

16 h fasted mice. Serum concentrations of (**J**) insulin, (**K**) adiponectin, and (**L**) triacylglycerols in 20-week-old mice. (**M**) Tissue weights at time of sacrifice. RNA expression normalized to PPIA. Data shown in **E**-**M** from male mice, n = 5 per group. Data presented as mean $\pm$ S.D. * indicates significance at $p < 0.05$.

**Supplemental Figure 3.5**



**Supplemental Figure 3.5.** (**A**) Representative histological images of H&E-stained distal and proximal tibia of male and female $Wls^{fl/fl}$ and $Wls^{-/-}$ mice fed NCD for 20 weeks; 100x magnification; scale bar, 50 μm. μCT analyses of (**B**) tibial length measured by distance from growth plate (GP) to tibia/fibula junction (T/F J), (**C**) mid-tibia cortical bone area (Ct. BA/TA), (**D**) bone mineral density (Ct. BMD), (**E**) tibial trabecular bone volume fraction (Tb. BV/TV), (**F**) trabecular number (Tb. N), (**G**) trabecular bone mineral density (Tb. BMD), (**H**) trabecular separation (Tb. Sp) and (**I**) trabecular thickness (Tb. Th). Data presented as mean ± S.D. * indicates significance at $p < 0.05$.

**Figure 3.6**

**Figure 3.6. Adipose tissues compensate for adipocyte-specific Wntless deficiency by increasing Wnt signaling in stromal-vascular cells.** (**A**) Representative histological images of H&E-stained tissues from *Wls*fl/fl and *Wls*-/- mice fed normal chow diet for 20 weeks; 200x magnification; scale bar, 100 μm. (**B**) Lipogenic gene expression in eWAT and iWAT isolated from *Wls*fl/fl and *Wls*-/- mice. (**C**) Lipogenic gene expression in isolated eWAT adipocytes (n = 7). (**D**) Wnt target gene expression in isolated eWAT adipocytes and SVF of *Wls*fl/fl and *Wls*-/- mice (n = 5). (**E**) Expression of Wnt mRNAs in SVF isolated from eWAT of *Wls*fl/fl and *Wls*-/- mice (n = 6). Data shown from male mice. RNA expression normalized to PPIA. Data presented as mean ± S.D. * indicates significance at $p < 0.05$.

**Supplemental Figure 3.6**



**Supplemental Figure 3.6.** (**A**) Lipogenic gene expression in livers isolated from male *Wls*fl/fl and *Wls*-/- mice on NCD. (**B**) Representative plots of flow cytometric analyses of eWAT SVF isolated from male *Wls*fl/fl and *Wls*-/- mice, stained with CD31-APC (endothelial cells) and CD45-PE (immune cells). (**C**) Quantification of SVF cell populations based on flow cytometric analyses (n = 6). RNA expression normalized to PPIA. Data presented as mean ± S.D. * indicates significance at $p < 0.05$.

**Figure 3.7**
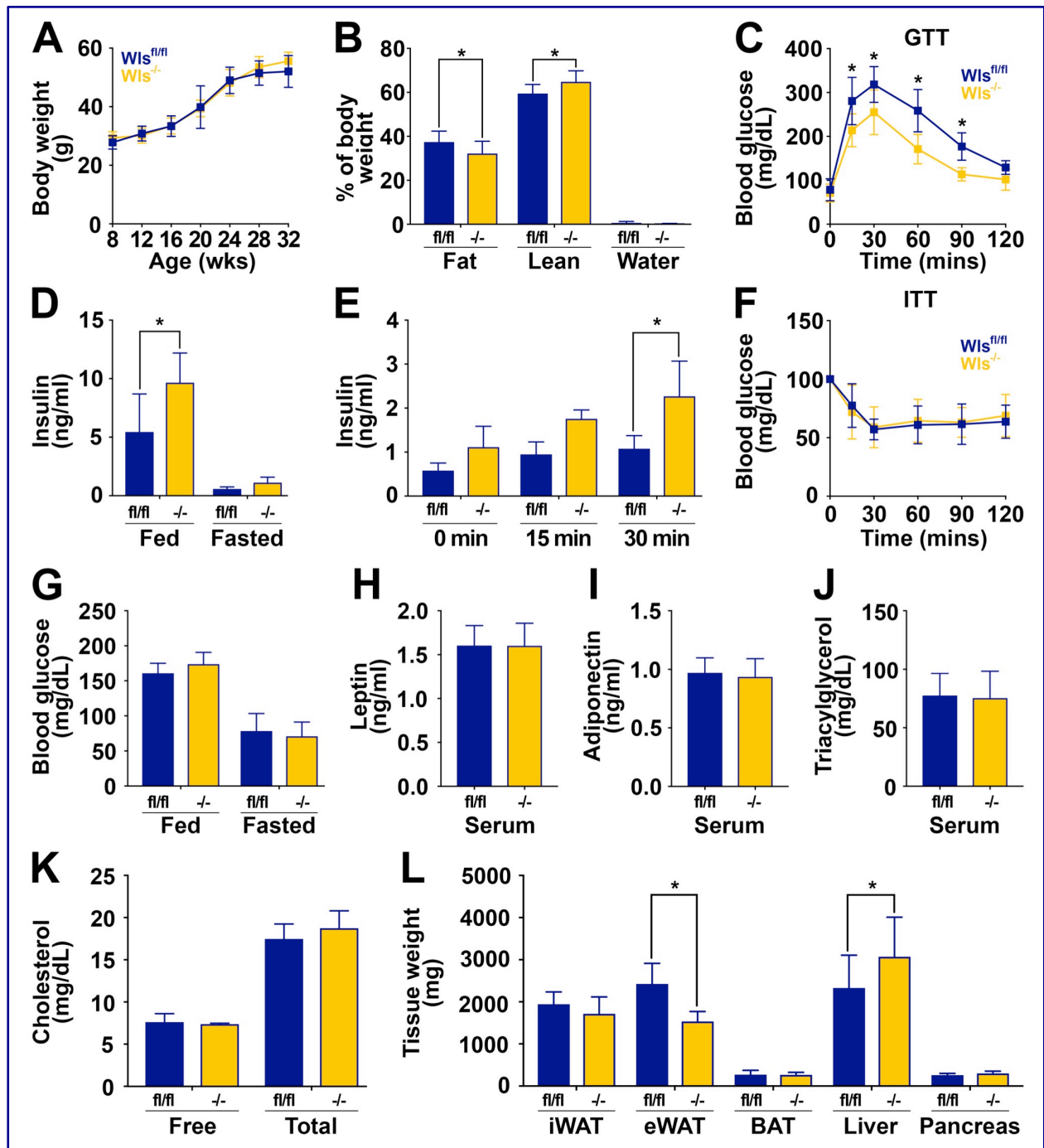


Figure 3.7. *Wls*^{-/-} **mice on HFD have decreased eWAT and improved glucose tolerance.** (**A**) Growth curve over time of 32-week-old *Wls*^{fl/fl} and *Wls*^{-/-} mice fed 60% HFD for 24 weeks. (**B**) Body composition analysis of 28-week-old mice. (**C**) Glucose tolerance test in 28-week-old mice. (**D**) Serum insulin concentrations in fed and 16 h fasted mice. (**E**) Serum insulin concentrations in 16 h fasted mice at indicated times after intraperitoneal glucose injection (1 mg/kg body weight). (**F**) Insulin tolerance test in

30-week-old mice. (**G**) Blood glucose concentrations in random-fed and 16 h fasted mice. Circulating concentrations of (**H**) leptin, (**I**) adiponectin, (**J**) triacylglycerols, and (**K**) total and free cholesterol in 32-week-old mice. (**L**) Tissue weights of mice at time of sacrifice. Data shown from male mice; *Wls*$^{fl/fl}$: n = 8; *Wls*$^{-/-}$: n = 7. Data presented as mean $\pm$ S.D. * indicates significance at $p < 0.05$.

**Supplemental Figure 3.7**



**Supplemental Figure 3.7.** (**A**) Daily food intake of male $Wls^{fl/fl}$ and $Wls^{-/-}$ mice fed HFD for 24 weeks. (**B**) Adipocyte size quantification of iWAT (400-500 adipocytes/mouse; n = 8, 7). (**C**) Representative H&E-stained images and UCP1 protein expression in BAT of male $Wls^{fl/fl}$ and $Wls^{-/-}$ mice fed HFD for 24 weeks; 200x magnification; scale bar, 100 µm. (**D**) Expression of Wnt mRNAs in SVF isolated from eWAT of HFD-fed male $Wls^{fl/fl}$ and $Wls^{-/-}$ mice (n = 10). (**E**) Lipogenic gene expression in livers isolated from male $Wls^{fl/fl}$ and $Wls^{-/-}$ mice on HFD. RNA expression normalized to PPIA. Data presented as mean $\pm$ S.D. * indicates significance at $p < 0.05$.

**Figure 3.8**



Figure 3.8. *Wls*-/- mice on HFD have reduced epididymal adipocyte size and decreased DNL gene expression. (**A**) Representative histological images of H&E-stained tissues from *Wls*fl/fl and *Wls*-/- mice fed 60% HFD for 24 weeks; 200x magnification; scale bar, 100 μm. (**B**) Adipocyte size quantification of eWAT (400-500 adipocytes/mouse, n = 8, 7). (**C**) Wnt target gene expression in adipocytes and SVF isolated from eWAT. (**D-E**) Lipogenic mRNA and protein expression in isolated eWAT adipocytes. (**F**) Lipogenic gene expression in eWAT and iWAT from *Wls*fl/fl and *Wls*-/- mice. Data shown from male mice. RNA expression normalized to PPIA. Data presented as mean ± S.D. * indicates significance at $p < 0.05$.

**Figure 3.9**
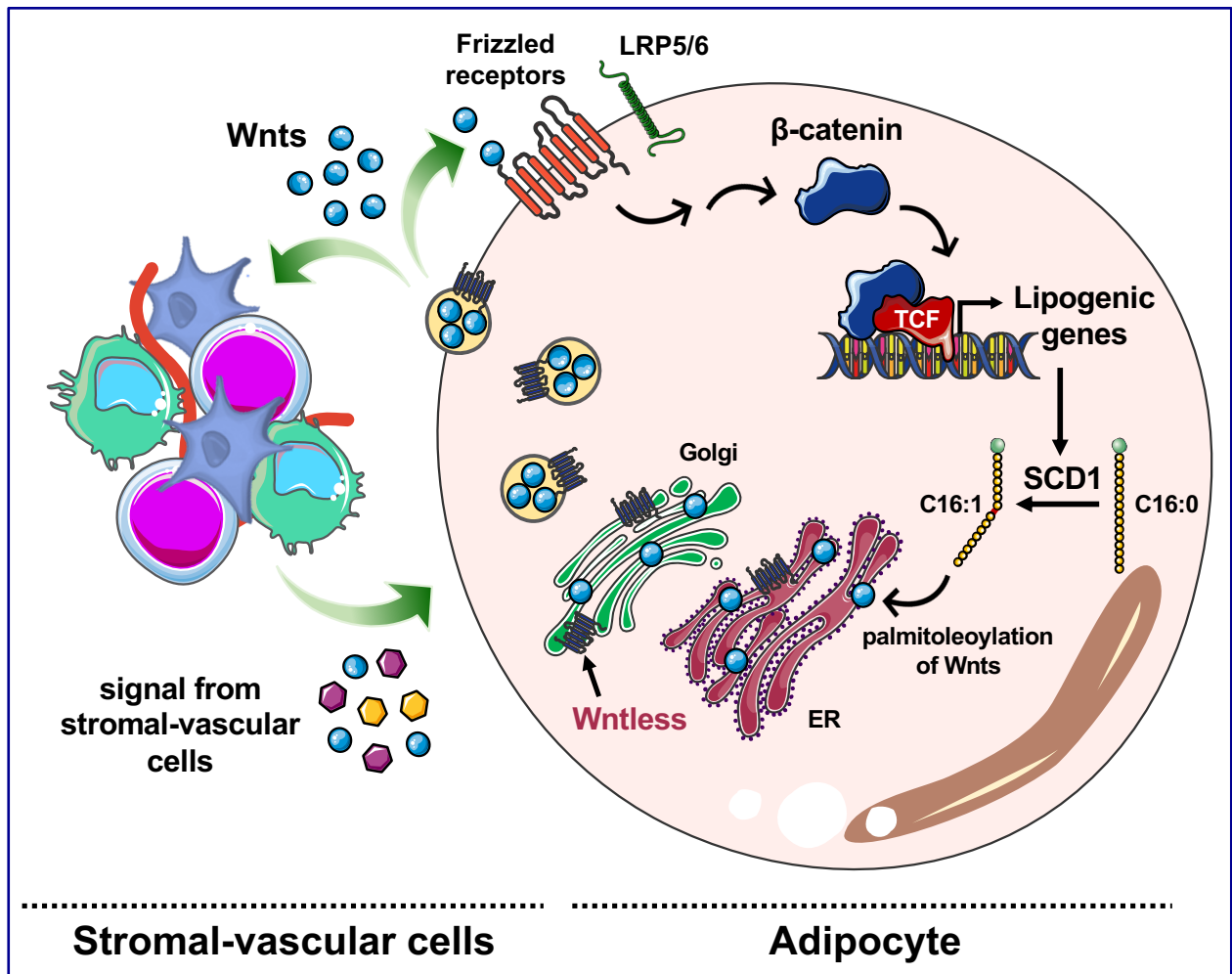


**Figure 3.9. Schematic depicting the consequences of adipocyte-specific deletion of Wntless.** Adipocyte-derived Wnts are required for expression of a network of lipogenic genes and loss of secreted Wnts in *Wls*^{-/-} adipocytes is sensed and compensated for by surrounding stromal-vascular cells to maintain tissue-wide Wnt signaling homeostasis.

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# CHAPTER IV

## Wnt/β-catenin Signaling Regulates Adipose Tissue Lipogenesis and Adipocyte-Specific Loss is Rigorously Defended by Surrounding Stromal-Vascular Cells

## Abstract

*Objective:* Canonical Wnt/β-catenin signaling is a well-studied endogenous regulator of mesenchymal cell fate determination, promoting osteoblastogenesis and inhibiting adipogenesis. However, emerging genetic evidence in humans links a number of Wnt pathway members to body fat distribution, obesity, and metabolic dysfunction, suggesting that this pathway also functions in adipocytes. Recent studies in mice have uncovered compelling evidence that the Wnt signaling pathway plays important roles in adipocyte metabolism, particularly under obesogenic conditions. However, complexities in Wnt signaling and differences in experimental models and approaches have thus far limited our understanding of its specific roles in this context. *Methods:* To investigate the roles of the canonical Wnt pathway in regulation of adipocyte metabolism, we generated adipocyte-specific β-catenin (*β-cat*) knockout mouse and culture cell models. We used RNA sequencing, ChIP sequencing, and molecular approaches to assess expression of Wnt targets and lipogenic genes. We then used functional assays to evaluate effects of β-catenin deficiency on adipocyte metabolism, including lipid and carbohydrate handling. In mice maintained on normal chow and high fat diets, we interrogated the cellular and functional consequences of adipocyte-specific β-catenin deletion on adipose tissues and systemic metabolism. *Results:* We report that in adipocytes, canonical Wnt/β-catenin signaling regulates *de novo* lipogenesis (DNL) and fatty acid

monounsaturation. Further, β-catenin mediates effects of Wnt signaling on lipid metabolism in part by transcriptional regulation of *Mlxipl* and *Srebf1*. Intriguingly, adipocyte-specific loss of β-catenin is sensed and defended by CD45$^-$/CD31$^-$ stromal cells to maintain tissue-wide Wnt signaling homeostasis in chow-fed mice. With long-term high fat diet, this compensatory mechanism is overridden, revealing that β-catenin deletion promotes resistance to diet-induced obesity and adipocyte hypertrophy and subsequent protection from metabolic dysfunction. *Conclusions:* Taken together, these studies demonstrate that Wnt signaling in adipocytes is required for lipogenic gene expression, *de novo* lipogenesis, and lipid desaturation. In addition, adipose tissues rigorously defend Wnt signaling homeostasis under standard nutritional conditions, such that stromal-vascular cells sense and compensate for the adipocyte-specific loss. These findings underscore the critical importance of this pathway in adipocyte lipid metabolism and adipose tissue function.

**Introduction**

Canonical Wnt/β-catenin signaling is an evolutionarily conserved pathway with well-established and diverse roles in cell proliferation and differentiation[1,2]. Binding of canonical Wnts to their membrane-spanning frizzled receptors and low-density lipoprotein receptor-related protein (LRP) co-receptors activates a complex intracellular signaling cascade that results in the stabilization and accumulation of free cytosolic β-catenin[3,4]. β-catenin can then translocate to the nucleus to bind to and coactivate DNA-binding T-cell factor/lymphoid enhancer-binding factor (TCF/LEF) proteins, thereby regulating Wnt target gene transcription[2,5,6].

Wnt signaling through the β-catenin-dependent pathway has been shown to have profound effects on mesenchymal cell fate determination and differentiation. Although this pathway is also involved in myogenesis and chondrogenesis[7-9], its differential regulatory roles in adipogenesis and osteogenesis have been particularly well-studied. Indeed, stabilization of canonical signaling by enforced expression of Wnt3a, Wnt6, Wnt10a or Wnt10b, or a β-catenin stable mutant, in multipotent progenitor cells inhibits adipogenesis and promotes osteoblastogenesis[10-14]. Further, activation of canonical Wnt signaling in preadipocytes by enforced expression of Wnt1, Wnt10b, or a dominant-stable form of β-catenin, or pharmacological inhibition of glycogen synthase kinase 3, blocks adipogenesis by suppressing induction of the master adipogenic transcription factors PPARγ and C/EBPα[15-17]. In contrast, knockdown experiments in preadipocytes demonstrate that of the 19 Wnts, endogenous expression of Wnt6, Wnt10a, and Wnt10b contributes significantly to inhibition of adipogenesis[10]. Further, treatment of preadipocytes with soluble Wnt inhibitors sFRP1 or sFRP2, or overexpression of negative regulators of the canonical pathway, such as Axin or dominant-negative TCF4, results in spontaneous adipogenic differentiation[18,19]. Enforced expression of β-catenin antagonist Chibby induces adipogenesis, whereas loss of Chibby up-regulates β-catenin transcriptional activity and inhibits adipogenic differentiation[20,21]. Taken together, this significant body of work demonstrates that endogenous Wnt/β-catenin signaling is a critical repressor of adipocyte differentiation.

Although Wnt signaling is an important regulator of adipose tissue development[22,23], increasing genetic evidence also links this pathway to adiposity, body fat distribution, and metabolic dysfunction in humans. For example, missense variations in canonical *WNT10B* are correlated with increased risk of type 2 diabetes[24], whereas polymorphisms in the Wnt inhibitor *SFRP5* locus are associated with decreased adiposity in men[25]. In addition, common variants in *RSPO3* and *ZNRF3* have been implicated in increased waist-to-hip ratio[26-28]. Patients harboring loss-of-function mutations in Wnt co-receptors *LRP5* and *LRP6* are more likely to suffer from impaired glucose homeostasis, coronary disease, and osteoporosis[29,30]; in contrast, gain-of-function mutations in *LRP5* are associated with increased adiposity and osteosclerosis[31]. Nonsense mutations in *LGR4*, a protein involved in stability of the Wnt co-receptor complex, are correlated with reduced adiposity and impaired bone formation and remodeling[32], whereas patients with gain-of-function *LGR4* mutations are predisposed to increased visceral adiposity[33]. Perhaps most striking, genome-wide association studies across a broad range of ethnic populations have strongly linked polymorphisms in the canonical Wnt transcriptional activator *TCF7L2* to increased susceptibility to type 2 diabetes[34-36]. Finally, during the course of our work, Chen *et al.* reported novel gain-of-function mutations in *CTNNB1* ($\beta$-catenin) that are associated with altered body fat distribution and predisposition to obesity[37]. These studies provide compelling genetic evidence for distinct roles of canonical Wnt/$\beta$-catenin signaling in body fat distribution and systemic metabolism.

A flurry of recent studies in mice have begun to investigate Wnt signaling in mature adipocytes, but its functional roles in this context remain unclear. For example, enforced expression of Wnt10b from the FABP4 promoter or stabilization of Wnt signaling through global deletion of the Wnt inhibitor secreted frizzled-related protein 5 (SFRP5) provides protection from diet-induced obesity[22,23,38]. In contrast, adipocyte-specific *Tcf7l2* deletion in obese mice results in increased adipocyte hypertrophy and impaired glucose homeostasis[39]. Work from our lab has demonstrated that loss of Wntless, and thus signaling by adipocyte-derived Wnts, protects mice from diet-induced obesity and metabolic dysfunction[40]. Consistent with these findings, Chen *et al.* recently reported that $\beta$-catenin knockout mice are resistant to diet-induced obesity and exhibit

improved glucose homeostasis and insulin sensitivity compared to control counterparts[37]. Taken together, these reports demonstrate that Wnt signaling plays an important role in mature adipocytes, particularly under obesogenic conditions. However, additional studies are required to unravel the detailed mechanisms by which different Wnt pathway members contribute to metabolic function of terminally-differentiated cells. To this end, we have chosen to investigate roles of $\beta$-catenin, the central protein in the canonical Wnt pathway, specifically in adipocytes.

Herein, we report for the first time that $\beta$-catenin is a key regulator of adipocyte *de novo* lipogenesis (DNL) and fatty acid desaturation. Although chow-fed adipocyte-specific $\beta$-catenin knockout mice do not exhibit an overt phenotype, deeper investigation reveals a striking phenomenon in which surrounding stromal-vascular cells (SVC) dramatically up-regulate $\beta$-catenin production and return mRNA and/or protein back to deficient adipocytes, such that knockout cells have sustained expression of $\beta$-catenin despite complete genomic deletion. Thus, Wnt signaling within and between cell types is monitored in white adipose tissue (WAT), and loss of $\beta$-catenin signaling in adipocytes is compensated for by $CD45^-/CD31^-$ stromal cells to maintain whole-tissue Wnt signaling homeostasis under standard nutritional conditions. This compensatory mechanism is overridden by diet-induced obesity such that high fat diet (HFD)-fed adipocyte-specific $\beta$-catenin knockout mice exhibit decreased adiposity and improved glucose homeostasis and hepatosteatosis. These findings underscore the critical importance of the canonical Wnt/$\beta$-catenin pathway in adipocyte lipid metabolism and adipose tissue function.

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**Results**

**β-catenin is highly expressed in terminally differentiated adipocytes and up-regulated by obesity.**

The canonical Wnt/β-catenin pathway is a critical regulator of mesenchymal cell fate determination and an endogenous inhibitor of adipogenesis[15,16]. Although Wnt signaling is commonly believed to be most important in precursors, recent studies have begun to shed light on important contributions of this pathway to functions within terminally-differentiated adipocytes[37-40]; however, specific roles of β-catenin in this context require further investigation. We thus first confirmed that β-catenin is expressed in mature adipocytes. *Ctnnb1* (β-catenin) mRNA and protein expression is high in cultured mesenchymal stem cells (MSC) derived from wildtype C57BL/6J mice, transiently suppressed during differentiation, and subsequently increased in terminally-differentiated adipocytes (**Figure 4.1 A-B**). Expression of the adipocyte marker *Adipoq* (adiponectin) increases during adipogenesis, whereas expression of the preadipocyte marker *Dlk1* (Pref1) decreases as expected (**Figure 4.1 A-B**).

To determine β-catenin levels in adipose tissues *in vivo*, we performed immunoblot analyses in various tissues isolated from wildtype mice and found that β-catenin is widely expressed, including in diverse adipose depots, pancreas and lung (**Supplemental Figure 4.1 A**). In addition to adipocytes, WAT is comprised of many other cell types, including endothelial, immune, and stromal cells and preadipocytes. These non-adipocyte cells are known as the stromal-vascular fraction (SVF) of WAT, and previous studies have demonstrated that β-catenin is widely expressed in many of these populations[15,16,41-44]. To assess whether adipocyte-specific expression contributes significantly to β-catenin levels within whole adipose tissues, we measured *Ctnnb1* mRNA expression in fractionated epididymal (eWAT) and inguinal (iWAT) WAT of wildtype mice. We found that *Ctnnb1* is expressed highly in isolated adipocytes compared to the SVF (**Figure 4.1 C**), consistent with recently reported data[37]. As expected, *Adipoq* expression is found exclusively in the adipocyte fractions, whereas *Dlk1* expression is limited to the SVF (**Supplemental Figure 4.1 B**), providing evidence of clean separation.

Recent studies have demonstrated that Wnt signaling plays a role in adipocyte metabolism under obesogenic conditions[37-40]. Thus, we examined the regulation of β-catenin in WAT after a number of nutritional and environmental treatments and found that its expression is up-regulated in both eWAT and iWAT with diet-induced obesity (**Figure 1D**), parallel with induction of *Lep* (leptin) (**Supplemental Figure 4.1 C**). Consistent with *Ctnnb1* expression patterns, downstream Wnt target *Nkd1* is increased in eWAT with HFD treatment; effects in iWAT are not statistically different (**Supplemental Figure 4.1 D**). Further, analyses of fractionated WAT demonstrate that up-regulation of *Ctnnb1* largely occurs in adipocytes, rather than in the SVF (**Figure 4.1 E**). This regulation is specific; *Ctnnb1* expression is not altered by other conditions, including acute fasting, fasting/refeeding, calorie restriction, or acute cold exposure (**Supplemental Figure 4.1 E**). Finally, we interrogated the GTEx database to determine distribution of *Ctnnb1* gene expression across male and female human tissues and found that β-catenin is highly expressed in subcutaneous and visceral WAT depots, with relatively low expression in liver, skeletal muscle, and pancreas (**Supplemental Figure 4.1 F**). Together, these data demonstrate that β-catenin is expressed in mature adipocytes and up-regulated by diet-induced obesity, and thus conceivably plays an important metabolic role in these cells.

**Canonical Wnt signaling is operative in cultured adipocytes.**

To interrogate the molecular functions of β-catenin in adipocytes, we utilized a cultured cell model using MSCs isolated directly from *β-cat*^{fl/fl} mice. To efficiently ablate β-catenin in either preadipocytes or adipocytes, we established a protocol for gene deletion using adenoviral Cre recombinase (**Supplemental Figure 4.1 G**). To induce gene deletion in preadipocytes, *β-cat*^{fl/fl} MSCs at 30-40% confluence were infected in serum-free medium with adenoviral GFP as a control, or adenoviral Cre to stimulate recombination. Preadipocytes were allowed to recover following infection and were analyzed at confluence. To induce gene deletion in adipocytes, *β-cat*^{fl/fl} MSCs were differentiated using a standard adipogenic cocktail and at day four of differentiation, adipocytes were infected with adenoviral GFP or Cre in serum-free medium. Cells were then allowed to recover and were subsequently analyzed at day 12 of differentiation. A 3-primer PCR

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system was used to confirm recombination of the floxed allele (**Figure 4.1 F,** preadipocytes shown in **Supplemental Figure 4.1 H**). Efficient deletion of *Ctnnb1* mRNA (**Figure 4.1 G**) and protein (**Figure 4.1 H,** preadipocytes shown in **Supplemental Figure 4.1 H**) was observed in Cre-infected adipocytes. Adipocyte-specific β-catenin deletion does not affect differentiation status or lipid accumulation, as evidenced by adiponectin and perilipin protein expression (**Figure 4.1 H**), phase-contrast microscopy and Oil Red-O staining (**Figure 4.1 I**), and quantification of triacylglycerol (TAG) content per well (**Figure 4.1 J**). Importantly, protein levels of AXIN2 and WISP2, two known Wnt targets, are decreased in *β-cat$^{-/-}$* adipocytes (**Figure 4.1 H,** preadipocytes shown in **Supplemental Figure 4.1 H**).

Thus, we next investigated in more detail whether Wnt target genes are altered in adipocytes lacking β-catenin. To this end, we measured expression of well-characterized downstream genes under basal conditions and after stimulation with the small molecule CHIR99021, which stabilizes β-catenin through inhibition of GSK3 activity[15]. We found that Wnt-induced genes, including *Axin2*, *Nkd1*, and *Tcf7l2*, are down-regulated in *β-cat$^{-/-}$* adipocytes, whereas Wnt-repressed genes *Id2* and *Wif1* are up-regulated (**Figure 4.1 K, Supplemental Figure 4.1 I**). Importantly, Wnt pathway stimulation with CHIR99021 induces expression of most target genes only in *β-cat$^{fl/fl}$* adipocytes and not in *β-cat$^{-/-}$* cells, suggesting that expression of these genes is β-catenin-dependent in terminally differentiated cells (**Figure 4.1 K, Supplemental Figure 4.1 I**). Of note, *Id2* expression is down-regulated in both control and knockout adipocytes with CHIR99021 treatment, albeit to different degrees, suggesting that it is also regulated by other cellular pathways affected by GSK3 inhibition (**Figure 4.1 K**). Taken together, these data indicate that the canonical Wnt signaling pathway is operative in mature adipocytes, and likely regulates functions that are specialized to these cells.

## β-catenin regulates metabolic pathways in adipocytes and exclusively mediates effects of canonical Wnt3a signaling.

Although β-catenin-dependent canonical Wnt signaling is a critical regulator of mesenchymal cell fate, the role of this pathway in mature adipocytes is less clear. To

identify the transcriptional pathways directly regulated by β-catenin in terminally-differentiated cells, we performed RNA-sequencing (RNA-seq) analyses of *β-cat*^{fl/fl} and *β-cat*^{-/-} adipocytes at baseline and following 4-hour treatment with recombinant Wnt3a (**Figure 4.2 A, E**). Of the 18,931 identified genes, β-catenin deletion in adipocytes resulted in significant up-regulation of 1,582 genes and down-regulation of 484 genes. We then used Gene Set Enrichment Analyses (GSEA) to identify significantly up- and down-regulated pathways in knockout cells. Strikingly, the majority of up-regulated pathways are related to inflammatory response, including TNFα, IL6, interferon γ, and interferon α signaling; in contrast, loss of β-catenin leads to suppression of several metabolic pathways, including those involved in glycolysis, oxidative phosphorylation, and fatty acid, cholesterol, and bile acid metabolism (**Figure 4.2 B**). Of note, mTORC1 signaling is also significantly down-regulated in *β-cat*^{-/-} adipocytes, which is of interest since this pathway has been shown to regulate lipid metabolism in adipocytes[45-48].

Wnt3a treatment of *β-cat*^{fl/fl} adipocytes resulted in significant up-regulation of 535 genes and down-regulation of 417 genes, including dramatic induction of known Wnt targets *Axin2*, *Nkd1*, *Nkd2*, and *Wnt11* (**Figure 4.2 A, C**). Consistent with canonical Wnt/β-catenin signaling predominating in adipocytes, Wnt3a treatment of *β-cat*^{-/-} adipocytes only altered expression of one gene, *Gpr161,* which has no known function (**Figure 4.2 D**). As expected, treatment of control adipocytes with Wnt3a stimulates the expression of genes known to be related to Wnt/β-catenin signaling (**Figure 4.2 B**). Hedgehog and TGFβ signaling, which have broad effects on differentiation and mature cell functions, are also up-regulated (**Figure 4.2 B**); cross-talk between these pathways and Wnt signaling have been reported in other contexts[49,50]. Differential regulation of a subset of Wnt target genes was confirmed by qPCR analyses of control and knockout adipocytes treated with Wnt3a for different lengths of time (**Figure 4.2 F**).

Consistent with an extensive literature describing the role of Wnt signaling in precursors, RNA-seq analyses of *β-cat*^{fl/fl} and *β-cat*^{-/-} preadipocytes identified 866 up-regulated genes and 936 down-regulated genes (**Supplemental Figure 4.2 A, E)**. Deletion of β-catenin in precursors suppresses many pathways critical for cell function, including DNA repair, proliferation, stress response, and glycolysis (**Supplemental**

**Figure 4.2 B**), consistent with known roles of Wnt signaling in cell proliferation and cancer metabolism[51]. Similar to observations in control adipocytes, Wnt3a treatment of *β-cat*^fl/fl preadipocytes promoted expression of many genes related to TGFβ, Hedgehog and Notch signaling (**Supplemental Figure 4.2 A-C**). Unlike in *β-cat*^-/- adipocytes, Wnt3a treatment of *β-cat*^-/- preadipocytes resulted in up-regulation of 146 genes and down-regulation of 171 genes, including induction of Wnt target genes like *Axin2* and *Nkd1* (**Supplemental Figure 4.2 D-F**). These data indicate that in preadipocytes, Wnt target genes are also regulated by non-canonical Wnt signaling pathways, whereas in adipocytes, Wnt3a acts exclusively through a β-catenin-dependent mechanism.

**β-catenin-dependent signaling regulates lipogenesis and fatty acid desaturation in adipocytes.**

Lipogenesis and lipid accumulation are two specialized functions of adipocytes. Thus, we were intrigued to find that the RNA-seq dataset identified many genes related to lipid metabolism, including *Srebf1 and Scd1*, as significantly down-regulated in *β-cat*^-/- adipocytes (**Figure 4.3 A-B**). Consistent with the RNA-seq data, we found that β-catenin deficiency in adipocytes down-regulates mRNA (**Figure 4.3 C**) and protein (**Figure 4.3 D,** quantification in **Supplemental Figure 4.3 A**) expression of many genes in the *de novo* lipogenesis (DNL) pathway, including ATP citrate lyase (*Acly*, ACLY), acetyl-CoA carboxylase (*Acaca*, ACC1), fatty acid synthase (*Fasn*, FASN), and stearoyl CoA desaturase 1 (*Scd1*, SCD1). DNL converts excess dietary amino acids and carbohydrates into fatty acids, a subset of which can be esterified into TAG and later mobilized to provide energy to adipocytes and other cells throughout the body[52,53]. SCD1, a member of the DNL pathway, is the rate-limiting adipocyte enzyme localized to the endoplasmic reticulum that catalyzes desaturation of palmitic (C16:0) and stearic (18:0) acids into palmitoleic (C16:1, n-7) and oleic (C18:1, n-9) acids, respectively[54,55]. Since SCD1 protein expression is inhibited by ~55% in *β-cat*^-/- adipocytes (**Figure 4.3 D, Supplemental Figure 4.3 A**), we hypothesized that TAG isolated from these cells would have decreased unsaturated fatty acids and increased saturated fatty acids. Indeed, we found that *β-cat*^-/- adipocytes contain a smaller proportion of monounsaturated fatty acid species compared to control adipocytes (**Figure 4.3 E**).

Closer examination revealed that compared to $\beta$-cat$^{fl/fl}$ adipocytes, lipids isolated from terminally-differentiated $\beta$-cat$^{-/-}$ cells contain significantly higher proportions of palmitic (16:0) and stearic (18:0) acids and less myristoleic (14:1, n-5) and palmitoleic (16:1, n-7) acids (**Figure 4.3 F, Supplemental Figure 4.3 B**). In addition, $\beta$-cat$^{-/-}$ adipocytes also contain slightly elevated proportions of oleic (C18:1, n-9), vaccenic (C18:1, n-7), and arachidonic (C20:4) acids (**Supplemental Figure 4.3 B**, data not shown), consistent with previously published studies of SCD1 inhibition in 3T3-L1 adipocytes[56]. Although it is surprising that ELOVL6 did not follow expression of other ChREBP- and SREBP1-regulated lipogenic genes, up-regulation of ELOVL7 has been observed in a variety of contexts in which Wnt signaling is repressed[40,57-59]. The functional significance of increased ELOVL7 in adipocytes remains unclear since the proportion of very long chain saturated fatty acids was not altered in $\beta$-cat$^{-/-}$ adipocytes (data not shown).

Since $\beta$-catenin deletion down-regulates a network of key DNL pathway members (**Figure 4.3 A-D**), we performed DNL assays using [$^{14}$C]-acetate to evaluate whether lipogenesis is functionally impaired in $\beta$-cat$^{-/-}$ adipocytes. Differentiated adipocytes were incubated in medium containing [$^{14}$C]-acetate for 2, 4 or 8 hours. Cells were then lysed, and lipids extracted to measure the incorporation of radiolabel into TAG, diacylglycerol (DAG), and phospholipid (PL) fractions. Conditioned media (**Supplemental Figure 4.3 C**) and whole cell lysate (**Supplemental Figure 4.3 D**) analyses indicated that $\beta$-cat$^{-/-}$ adipocytes take up less labeled acetate over time. Consistent with this, *Slc16a1* (*Mct1),* an acetate transporter, and *Acss2*, the cytosolic enzyme that catalyzes activation of acetate for use in lipid synthesis, are suppressed in $\beta$-cat$^{-/-}$ adipocytes (**Supplemental Figure 4.3 B, E**). $\beta$-cat$^{-/-}$ adipocytes incorporate significantly less radiolabel over time into the TAG and DAG fractions of cellular lipid (**Figure 4.3 G, Supplemental Figure 4.3 F**), but not the PL fraction (**Supplemental Figure 4.3 G**). This is important, as it suggests that acetate uptake is not the rate-limiting factor for radiolabel incorporation into TAG; indeed, decreased acetate incorporation in lipid synthesis may actually feedback to suppress transporter expression. Further, linear regression analyses demonstrate that decreased incorporation into TAG is not proportional to decreased acetate uptake (**Supplemental Figure 4.3 H-I**), strongly suggesting that suppressed expression of DNL enzymes, not

genes related to acetate transport or activation, is rate-limiting for lipogenesis in *β-cat*^{-/-} adipocytes. Thus, impaired DNL is secondary to loss of β-catenin-dependent Wnt signaling. These data suggest that adipocyte β-catenin signaling is required for lipogenic gene expression, DNL and steady-state lipid composition.

**β-catenin exerts effects on lipogenesis through transcriptional regulation of Srebf1 and Mlxipl.**

Extensive studies in liver have identified SREBP1c and ChREBP as key upstream transcriptional regulators of many genes involved in DNL, including *Acaca*, *Fasn*, and *Scd1*[60,61]. We recently demonstrated for the first time that Wnt signaling mediates adipocyte lipogenic gene expression through regulation of sterol regulatory element-binding protein 1c (SREBP1c) and carbohydrate-responsive element-binding protein (ChREBP)[40]. Thus, we next investigated whether these genes are specifically regulated by β-catenin-dependent Wnt signaling. Indeed, we found that expression of *Srebf1* and *Mlxipl*, which encode SREBP1c and ChREBP respectively, are both repressed in *β-cat*^{-/-} adipocytes; other transcription factors involved in adipogenesis and mature adipocyte function, including *Pparg* and *Cebpa*, are not altered (**Figure 4.3 A-B, H**). Consistent with expression of their respective mRNAs, SREBP1c and ChREBP protein levels are also decreased, whereas PPARγ and C/EBPα levels are unaffected (**Figure 4.3 I**).

To establish whether repression of *Mlxipl* or *Srebf1* directly mediates impaired lipogenesis and fatty acid monounsaturation following β-catenin deletion, we evaluated the effects of increasing ChREBP or SREBP1c expression in β-catenin knockout adipocytes slightly above that observed in control adipocytes. Thus, we treated *β-cat*^{fl/fl} and *β-cat*^{-/-} adipocytes with adenoviral GFP as a control or adenoviruses encoding ChREBP or SREBP1c. Ectopic expression of ChREBP or SREBP1c was sufficient to induce SCD1 expression in control adipocytes, and partially rescue SCD1 mRNA and protein levels in *β-cat*^{-/-} adipocytes (**Figure 4.3 J, Supplemental Figure 4.3 J**). Expression of *Fasn* was also partially rescued by overexpression of these transcription factors (**Supplemental Figure 4.3 J**).

Since β-catenin coactivates TCF/LEF proteins like Tcf7l2 (Tcf4) to mediate transcription of downstream targets, we analyzed the promoter regions of *Mlxipl*, *Srebf1*, *Acaca*, *Fasn*, and *Scd1* and found multiple predicted Tcf7l2 binding sites on each gene (data not shown). Thus, we interrogated a recently published chromatin immunoprecipitation sequencing (ChIP-seq) dataset of Tcf7l2 binding in cultured *Tcf7l2*^{fl/fl} and *Tcf7l2*^{-/-} adipocytes[39] and found enrichment of Tcf7l2 binding sites in the promoter, exon and/or first intronic regions of *Mlxipl*, *Srebf1*, *Fasn*, and *Scd1*; binding occupancy of Tcf7l2 was subsequently lost in *Tcf7l2*^{-/-} cells (**Figure 4.3 K**). Taken together, these results support a model in which repressive effects of β-catenin deletion on lipogenic gene expression are mediated, in part, through down-regulation of ChREBP and SREBP1c. However, identification of Tcf7l2 binding sites in transcriptional regulatory regions of several lipogenic genes, including *Acaca*, *Fasn*, and *Scd1,* suggests that β-catenin likely regulates expression of these genes through a combination of direct (TCF/LEF) and indirect (ChREBP and SREBP1c) mechanisms.

**Loss of β-catenin signaling within adipocytes does not influence global metabolism in chow-fed mice.**

Our studies in cultured adipocytes revealed that canonical Wnt signaling through β-catenin is an important regulator of adipocyte lipogenesis and lipid desaturation (**Figure 4.3, Supplemental Figure 4.3**). We next hypothesized that β-catenin has effects on these pathways *in vivo* and thus generated adipocyte-specific β-catenin knockout mice by crossing *β-cat*^{fl/fl} mice with adiponectin-Cre mice. Genetic recombination is specifically observed in various adipose depots, but not in other tissues, including liver, muscle, pancreas, lung or heart (**Figure 4.4 A**). We evaluated possible metabolic phenotypes in *β-cat*^{-/-} mice maintained on normal chow diet (NCD) but did not observe differences in growth over time (**Figure 4.4 B**), body composition (**Figure 4.4 C**), or glucose and insulin tolerances (**Figure 4.4 D-E**). *β-cat*^{-/-} mice do not exhibit altered fed or fasted blood glucose (**Figure 4.4 F**), serum insulin concentrations (**4.4 G**), or circulating adiponectin levels (**Figure 4.4 H**). We did not detect differences in voluntary exercise capacity (**Supplemental Figure 4.4 A**), basal or induced lipolysis (**Figure 4.4**

I), or serum TAG levels (**Figure 4.4 J**). Upon harvest, weights of iWAT, eWAT, brown adipose tissue (BAT), perirenal WAT (pWAT), and liver were not different between *β-cat*^fl/fl^ and *β-cat*^-/-^ mice (**Figure 4.4 K**). Finally, histological analyses of tissues did not yield substantial differences in adipocyte size or number within BAT, iWAT, eWAT, or pWAT (**Figure 4.4 L**, data not shown), and liver morphology was not influenced (**Figure 4.4 L**). Similar results were found in female mice (**Supplemental Figure 4.4 B-J**).

Wnt signaling through β-catenin has been shown to have profound effects on determination of MSC fate and differentiation into adipocytes, osteocytes, chondrocytes or myocytes. The role of this pathway in fate selection between adipogenesis and osteogenesis has been particularly well-studied. Indeed, studies have demonstrated that Wnt signaling increases bone mass and trabeculation, and impairs accumulation of bone marrow adipocytes[62,63]. We therefore examined whether marrow adiposity and bone characteristics are influenced by adipocyte-specific β-catenin deficiency. Histological analyses of tibia from both male and female mice suggest that β-catenin deletion does not alter size or number of regulated or constitutive marrow adipocytes (**Supplemental Figure 4.5 A, C**). Further, micro-computed tomography (μCT) analyses did not yield differences in either trabecular or cortical bone mass variables in *β-cat*^-/-^ mice (**Supplemental Figure 4.5 B, D**).

**β-catenin is up-regulated in the stromal-vascular fraction of adipose tissues from knockout mice.**

Although we did not observe an overt metabolic phenotype in *β-cat*^-/-^ mice maintained on chow diet, we expected to find decreased DNL gene expression in eWAT and iWAT isolated from knockout mice. However, lipogenic gene expression is not influenced in whole eWAT or iWAT of *β-cat*^-/-^ mice (**Supplemental Figure 4.6 A-B**). We also did not observe changes in DNL gene expression within livers of *β-cat*^-/-^ mice (**Supplemental Figure 4.6 C**). Perhaps most perplexing, analyses of isolated adipocytes from *β-cat*^-/-^ mice did not demonstrate altered DNL gene expression, except for mild suppression of *Scd1* (**Supplemental Figure 4.6 D**). These data were concerning and suggested that perhaps β-catenin is not efficiently deleted *in vivo*. Indeed, we were surprised to find

that although β-catenin appears to be recombined at the genomic level in whole WAT of *β-cat*^{-/-} mice (**Figure 4.5 A**), suppression of *Ctnnb1* mRNA is much less substantial than expected (**Figure 4.5 B**). Further, β-catenin protein levels are virtually unaltered in eWAT and iWAT of *β-cat*^{-/-} mice (**Figure 4.5 C**).

Since SVF cell populations are known to express β-catenin, we considered the possibility that adipocyte-specific loss was being masked by the relatively high expression of β-catenin in SVCs. Thus, we fractionated WAT to determine whether efficient deletion occurs within adipocytes. Indeed, we found that β-catenin gene recombination occurs specifically in adipocytes and not SVCs of *β-cat*^{-/-} mice (**Figure 4.5 D**). However, we were surprised to find that *Ctnnb1* mRNA is only reduced by ~50% in the adipocyte fraction of *β-cat*^{-/-} mice, and its expression is highly induced in the SVF of knockout mice (**Figure 4.5 E**). We also observed sustained levels of β-catenin protein in knockout adipocytes, and significant induction of β-catenin protein within the SVF of *β-cat*^{-/-} mice (**Figure 4.5 F**). We next measured Wnt target genes in isolated adipocytes and SVCs of *β-cat*^{fl/fl} and *β-cat*^{-/-} mice. Consistent with β-catenin expression patterns, we found that most Wnt targets are not altered in the adipocyte fraction of *β-cat*^{-/-} mice (**Figure 4.5 G**), whereas these genes are significantly up-regulated in the SVF with β-catenin deficiency (**Figure 4.5 H**).

These striking findings suggest that adipose tissues are able to sense depletion of β-catenin and subsequently maintain canonical Wnt signaling across the tissue by up-regulating the pathway in SVCs. Thus, we next evaluated whether a specific cellular sub-population is enriched within the SVF of *β-cat*^{-/-} mice and found that several macrophage markers, including *Adgre1* (*F4/80*), *Cd68*, and *Cd11c*, are elevated, whereas markers for endothelial and stromal cells are not changed (**Supplemental Figure 4.6 E**). These data may suggest that macrophage number is increased in the SVF of *β-cat*^{-/-} mice; these macrophages may in turn contribute *Ctnnb1* mRNA or β-catenin protein back to deficient adipocytes, either directly via small extracellular vesicles (sEVs) or indirectly by stimulating production in neighboring cells. Although macrophage markers are increased, expression of classical inflammatory markers are decreased or unchanged in whole WAT of *β-cat*^{-/-} mice (**Supplemental Figure 4.6 F**).

We next performed flow cytometry analysis of SVF isolated from chow-fed *β-cat*^{fl/fl} and *β-cat*^{-/-} mice. We used CD45 and CD31 as markers for immune and endothelial cells, respectively; CD45^{-}/CD31^{-} cells were designated as the stromal cell population. Flow cytometry analysis did not yield differences in proportions of CD45^{+}, CD31^{+}, or CD45^{-}/CD31^{-} populations (**Figure 4.5 I-J**). Since proportions of different SVF cell types were not influenced by adipocyte-specific β-catenin deletion, we next hypothesized that a specific cell type might be up-regulating its own β-catenin expression. Thus, we used fluorescence-activated cell sorting (FACS) to separate CD45^{+}, CD31^{+}, and CD45^{-}/CD31^{-} cell fractions. Expression of *F4/80*, *Pecam1*, and *Pdgfra* were evaluated by qPCR to confirm that we had specifically separated immune, endothelial, and stromal cell populations (**Supplemental Figure 4.6 G**). We next measured *Ctnnb1* mRNA expression in the three cell fractions and were intrigued to find that *Ctnnb1* is up-regulated in the CD45^{-}/CD31^{-} population of chow-fed *β-cat*^{-/-} mice (**Figure 4.5 K**). Consistent with *Ctnnb1* expression patterns, downstream Wnt targets *Axin2* and *Nkd1* are also increased in CD45^{-}/CD31^{-} cells isolated from *β-cat*^{-/-} mice (**Supplemental Figure 4.6 H**). These data suggest that a sub-population of CD45^{-}/CD31^{-} stromal cells is able to sense the loss of adipocyte β-catenin, either directly or indirectly, and subsequently up-regulate its own expression to maintain Wnt signaling homeostasis within WAT of chow-fed mice. Further studies will be required to identify the specific cells within this population that mediate the observed compensatory effects.

**β-cat^{-/-} mice are protected from diet-induced obesity and metabolic dysfunction.**
Previous investigations into the role of Wnt signaling in WAT have found that this pathway is important in adipocyte metabolism under obesogenic conditions[37-40]. Consistent with these studies, we report that *Ctnnb1* expression is up-regulated within both eWAT and iWAT with diet-induced obesity (**Figure 4.1 D-E**). We thus challenged *β-cat*^{fl/fl} and *β-cat*^{-/-} mice with HFD and found that beginning at 20 weeks of feeding, knockout mice demonstrate decreased weight gain (**Figure 4.6 A**) and fat mass (**Figure 4.6 B**) compared to control mice, whereas lean mass is not affected. Of note, *β-cat*^{-/-} mice do not have decreased food intake (**Supplemental Figure 4.7 A**). Consistent with

decreased adiposity, $\beta$-cat$^{-/-}$ mice exhibit significantly improved glucose tolerance (**Figure 4.6 C-D**). Although fasting and random-fed blood glucose concentrations are not different (**Figure 4.6 E**) and insulin sensitivity only trends toward improvement in knockout mice (**Figure 4.6 F, Supplemental Figure 4.7 B**), glucose-induced circulating insulin concentrations are decreased in $\beta$-cat$^{-/-}$ mice compared to controls (**Figure 4.6 G**). Circulating TAG levels are decreased in knockout mice (**Figure 4.6 H**), whereas serum cholesterol (**Figure 4.6 I**) and adiponectin (**Figure 4.6 J**) are not changed. Upon harvest, weights of iWAT, eWAT and pWAT were significantly decreased in knockout mice, consistent with leaner body composition, whereas BAT, liver, and pancreas weights were unchanged (**Figure 4.6 K, Supplemental Figure 4.7 C**). Histological analyses of $\beta$-cat$^{-/-}$ tissues suggest that adipocyte sizes within iWAT and eWAT are mildly decreased (**Figure 4.6 L**). In addition, livers of knockout mice have less hepatosteatosis compared to control counterparts (**Figure 4.6 L**), in line with recently reported findings[37]. Consistent with the observed protection from glucose intolerance and the histological findings, livers of $\beta$-cat$^{-/-}$ mice have decreased TAG content (**Figure 4.6 M**). *Cdf* (adipsin) mRNA levels are increased in eWAT of $\beta$-cat$^{-/-}$ mice, whereas *Lep* expression is decreased and *Adipoq and Retn* (resistin) remain unchanged; similar trends were observed in iWAT of knockout mice (**Supplemental Figure 4.7 D-E**). Finally, expression of UCP1 protein in BAT of $\beta$-cat$^{-/-}$ mice was unchanged despite a ~50% decrease in *Ctnnb1* expression (**Supplemental Figure 4.7 F**); these data, along with comparable BAT morphology (**Figure 4.6 L**), suggest that altered BAT thermogenesis is likely not responsible for improved metabolic function observed in obese $\beta$-catenin knockout mice.

We next measured *Ctnnb1* expression in eWAT and iWAT of $\beta$-cat$^{-/-}$ mice fed HFD and found much lower mRNA levels in tissues of knockout mice (**Figure 4.7 A**). Further investigation revealed that *Ctnnb1* mRNA expression in isolated adipocytes is largely ablated in knockout mice, whereas expression in SVF is comparable to those of control mice (**Figure 4.7 B**). Consistent with these data, $\beta$-catenin protein is effectively deleted in the adipocyte fraction isolated from $\beta$-cat$^{-/-}$ mice, and protein levels are no longer elevated in the SVF of these animals (**Figure 4.7 C**). These data are compelling

and suggest that with HFD, the compensatory increase in canonical Wnt signaling within stromal cells of knockout mice is lost. Indeed, the SVF of *β-cat*[*-/-*] mice no longer exhibit elevated Wnt target gene expression (**Figure 4.7 D**). Further, analyses of the adipocyte fraction demonstrated decreased expression of Wnt targets, including *Axin2*, *Nkd1*, *Wnt10b, and Wnt16* (**Figure 4.7 E**).

Consistent with our *in vitro* studies showing the effects of β-catenin deletion on DNL, adipocytes of HFD-fed knockout mice are also characterized by decreased expression of key lipogenic genes, including *Srebf1*, *Mlxipl*, *Acly*, *Acaca*, *Fasn*, and *Scd1* (**Figure 4.7 F**). We also found that macrophage markers are decreased in the SVF of HFD-fed knockout mice (**Figure 4.7 G**), corroborating results reported by Chen *et al.*[37] and suggesting that these cells mediate the compensatory mechanism observed in chow-fed mice. Consistent with decreased immune cell markers, whole WAT of *β-cat*[*-/-*] mice have suppressed expression of some inflammatory markers, including *Tnfa* and *Inos2* (**Figure 4.7 H-I**). Expression of *Saa3*, which encodes a secreted protein that activates macrophages, is also decreased in WAT of HFD-fed knockout mice (**Figure 4.7 H-I**). Thus, taken together, our findings demonstrate that diet-induced obesity overrides stromal compensation for adipocyte-specific β-catenin deletion, leading to impaired lipogenic gene expression, decreased adipose accumulation and adipocyte hypertrophy, and protection from metabolic dysfunction.

## Discussion

Canonical β-catenin-dependent Wnt signaling is well-established as a key player in MSC fate determination, acting as a potent endogenous repressor of adipogenesis and promoter of osteoblastogenesis[10,12,13,15]. The preponderance of data within adipocyte biology to date has focused on the inhibition of adipogenesis by Wnt signaling, undoubtedly because many genes involved in this pathway are suppressed during the early stages of differentiation[64]. However, our work herein and recently published studies from our laboratory and others have demonstrated that canonical Wnt pathway members are present and operative in terminally-differentiated adipocytes, and thus have distinct functional roles in this context[37-40].

To evaluate specific roles of canonical Wnt signaling in adipocyte function, we chose to ablate β-catenin, the central protein in the pathway. We report here that β-catenin deletion in cultured adipocytes suppresses expression of known downstream target genes, including *Axin2*, *Nkd1*, and *Tcf7l2*. Further, stimulation of Wnt signaling using either a GSK3 inhibitor or recombinant Wnt3a is largely blocked in adipocytes lacking β-catenin, suggesting that effects of canonical Wnt signaling are mediated exclusively by β-catenin in terminally-differentiated cells. Global RNA-seq analyses of *β-cat*^{fl/fl} and *β-cat*^{-/-} adipocytes identified several metabolic pathways as down-regulated by inhibition of Wnt signaling, including oxidative phosphorylation, glycolysis, and fatty acid, cholesterol, and bile acid metabolism. Of particular interest to adipocyte biology, we found that signaling through β-catenin is required for coordinate expression of many lipogenic genes, including *Acly*, *Acaca*, *Fasn*, and *Scd1.* Of note, RNA-seq analyses of livers from *β-cat*^{-/-} mice have also demonstrated decreased expression of lipogenic genes, including *Acly* and *Scd1*[65].

Consistent with repressed expression of DNL enzymes, we report that *β-cat*^{-/-} adipocytes are characterized by impaired lipogenesis and fatty acid monounsaturation. Effects of β-catenin deletion on adipocyte metabolism are specific, as knockout adipocytes do not exhibit altered insulin-stimulated glucose uptake, adrenergic stimulation of lipolysis, or β-oxidation of fatty acids (data not shown). These data are strongly supported by recently published work from our group showing that blocking

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secretion and downstream signaling of adipocyte-derived Wnts also inhibits DNL and lipid unsaturation[40]. In addition, Geoghegan *et al.* recently reported that *Tcf7l2* deletion in precursor cells stimulates adipogenesis and increased expression of genes related to lipid metabolism, providing further support for the presumptive role of β-catenin-dependent signaling in regulation of this process in adipocytes[39].

Extensive studies in the liver have identified *Srebf1* and *Mlxipl*, encoding SREBP1c and ChREBP respectively, as key upstream transcriptional regulators of DNL enzymes[53]. Here, we report that expression of *Srebf1* and *Mlxipl* is significantly decreased in *β-cat^{-/-}* adipocytes, and that ectopic expression of ChREBP or SREBP1c partially rescues expression of DNL genes in adipocytes lacking β-catenin. These data are consistent with genome-wide ChIP-seq analysis of ChREBP binding sites in WAT, which identified binding sites on many genes related to metabolism, including *Acaca*, *Fasn*, and *Scd1*[66]. Thus, β-catenin mediates effects on DNL genes in part by regulating expression of key transcription factors *Srebf1* and *Mlxipl*. Indeed, ChIP-seq analyses of Tcf7l2 binding sites in cultured adipocytes, used as a surrogate for β-catenin/TCF/LEF activity, identified specific Tcf7l2 occupancies in the regions surrounding the transcriptional start sites of *Mlxipl* and *Srebf1*, but also downstream lipogenic genes such as *Fasn* and *Scd1*. Although these data suggest that β-catenin regulates transcription of lipogenic genes through a combination of indirect (via ChREBP and SREBP1c) and direct mechanisms, ChIP-seq analyses of β-catenin binding sites in adipocytes will be required to further interrogate the direct versus indirect effects of β-catenin on transcription of lipogenic genes.

Adipocyte-specific β-catenin deletion does not appear to influence global metabolism in chow-fed mice. Indeed, consistent with our studies, recent investigations into roles of Wnt signaling in mature adipocytes, including global *Sfrp5* deletion or adipocyte-specific deletion of *Tcf7l2*, *Wntless*, or *Ctnnb1,* also did not reveal an overt metabolic phenotype in chow-fed mice[37-40]. However, we recently published a study showing that surrounding SVCs compensate for loss of adipocyte-derived Wnts secondary to *Wntless* deletion[40]. Thus, we probed further into the lack of a detectable phenotype in *β-cat^{-/-}* mice and were surprised to find that although β-catenin is efficiently

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ablated at the genomic level in $\beta$-cat$^{-/-}$ adipocytes, the mRNA and protein are still detectable at levels much higher than expected. These data are consistent with those recently published by Chen *et al.*, who reported a ~50% reduction in $\beta$-catenin mRNA expression in adipocytes isolated from iWAT or eWAT of knockout mice[37]. Perhaps unsurprisingly, given the substantial β-catenin expression remaining, we found that Wnt targets and lipogenic genes are not altered in isolated knockout adipocytes. However, further investigation revealed that SVCs isolated from $\beta$-cat$^{-/-}$ mice exhibit significantly up-regulated expression of *Ctnnb1* and downstream Wnt targets, including *Axin2*, *Nkd1*, *Tcf7l2*, *Wnt10b*, and *Wnt16*. Although Chen *et al.* did not report elevated *Ctnnb1* expression in SVF of knockout mice, their data does suggest a trend toward increased expression in SVF isolated from eWAT[37]. These data support the compelling conclusion that Wnt signaling is critical for autocrine and paracrine communication within WAT, such that loss of Wnt/β-catenin signaling in adipocytes is sensed and compensated for by SVCs to maintain whole-tissue Wnt signaling homeostasis.

An important question raised by our studies is the underlying mechanism by which β-catenin expression is up-regulated in the SVF of knockout animals. Gene expression analysis of CD45$^+$, CD31$^+$ and CD45$^-$/CD31$^-$ populations isolated by FACS demonstrated that *Ctnnb1* and downstream targets *Axin2* and *Nkd1* are significantly up-regulated in CD45$^-$/CD31$^-$ stromal cells of knockout mice. These data suggest that a sub-population of CD45$^-$/CD31$^-$ cells, which include adipose stem cells, committed preadipocytes, and pericytes[67], is able to directly or indirectly sense the loss of adipocyte β-catenin and subsequently up-regulates its own expression to maintain Wnt signaling homeostasis within WAT of chow-fed mice. This compensatory mechanism may be the result of a dynamic network of intercellular Wnt signals; alternatively, it may conceivably arise from complex interactions between canonical Wnt signaling and other pathways, including Hedgehog, BMP and FGF signaling[49,68,69].

Gene expression analysis of whole SVF indicated that several macrophage markers, including *F4/80*, *Cd68*, and *Cd11c*, were increased in chow-fed $\beta$-cat$^{-/-}$ mice. It is important to note that various isoforms of CD45 are present on almost all differentiated hematopoietic cells, and although *Cd68* and *Cd11c* are commonly used as macrophage markers, they are also expressed by non-hematopoietic cell types,

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including endothelial and stromal cells[70,71]. Thus, it is possible that β-catenin indeed causes a mild increase in macrophage number, but that this difference is not observed when using a broad marker like CD45, which stains virtually all hematopoietic cells. Further studies using single-cell RNA sequencing analysis of SVF from *β-cat*^{fl/fl} and *β-cat*^{-/-} mice may help clarify specific sub-population changes following adipocyte-specific loss of β-catenin.

The relatively high levels of *Ctnnb1* mRNA and β-catenin protein in knockout adipocytes also suggests the intriguing possibility that β-catenin is being delivered back to deficient cells, perhaps via SVF-derived sEVs. Many different cell types are known to secrete sEVs containing proteins, lipids, and genetic material; these sEVs serve as a unique mechanism for intercellular communication and have varied and intricate effects on receiving cells[72,73]. This is of interest in the context of adipose biology, as WAT-derived sEVs have widespread effects[74,75], from regulation of hepatic FGF21 expression and glucose handling by the liver[76] to promotion of fatty acid oxidation within melanoma cells, contributing to aggressive tumor cell migration and invasion[77]. sEVs are also secreted from non-adipocyte cell types within WAT, including macrophages[78], endothelial cells[79], and stromal cells[80] and can have profound effects on glucose homeostasis, insulin sensitivity, and inflammation. Recently, Crewe *et al.* found that endothelial-derived sEVs mediate cross-talk between adipocyte and SVF cell populations[79]. Indeed, they reported a phenomenon strikingly similar to the one we observe in chow-fed *β-cat*^{-/-} mice: despite efficient genetic ablation of *Cav1* (caveolin-1), cav1 protein is readily detectable in deficient adipocytes. Ultimately, this was found to be the result of sEV-mediated trafficking of cav1 protein from surrounding endothelial cells back to *Cav1*^{-/-} adipocytes. In addition to cav1, proteomic analysis of isolated endothelial sEVs identified members of the Wnt signaling pathway, including β-catenin[79]. Thus, a similar mechanism may explain the sustained expression of β-catenin protein in knockout adipocytes. In addition to identification of the cell population contributing elevated β-catenin expression, further studies will be required to determine whether β-catenin protein is being trafficked back to deficient adipocytes.

To date, studies of Wnt signaling in mature adipocytes have consistently reported that metabolic phenotypes are revealed with long-term HFD. However, conflicting results have emerged: our work in $\beta$-cat$^{-/-}$ mice fed HFD demonstrate decreased weight gain and fat mass, significantly improved glucose homeostasis, decreased circulating TAG and glucose-stimulated insulin release, and protection from hepatosteatosis. Of note, the metabolic effects observed in our mice are remarkably consistent with data recently reported by Chen *et al.*; in their study deleting β-catenin from adipocytes, obese $\beta$-cat$^{-/-}$ mice also exhibited decreased body weight and adiposity, accompanied by improved glucose tolerance, insulin sensitivity, and hepatosteatosis[37]. Additionally, adipocyte-specific *Wntless*$^{-/-}$ mice fed HFD are also characterized by decreased fat mass and protection from glucose intolerance and hepatosteatosis[40]. In contrast, HFD-fed *Tcf7l2*$^{-/-}$ mice demonstrated increased WAT mass, impaired glucose tolerance, and insulin insensitivity[39]. Further, WAT isolated from Tcf7l2 knockout mice had elevated lipogenic gene expression, including *Scd1*. There are many potential reasons for these discordant results, including the generation of alternative Tcf7l2 splice variants[81], compensatory activity of other TCF/LEF transcription factors[18,19], or downstream effects on signaling pathways independent of β-catenin[82]. Nevertheless, it is clear from these studies that Wnt signaling within adipocytes, while complex, plays a critical role in regulation of lipid metabolism.

One final point of interest is the functional protection from diet-induced obesity and subsequent metabolic dysfunction that loss of adipocyte-specific β-catenin signaling appears to afford mice. Chen *et al.* found that WAT of HFD-fed $\beta$-cat$^{-/-}$ mice contained fewer PDGFR$\alpha^+$ preadipocytes, indicating that reduced fat mass is caused by decreased hyperplasia[37]. Further, they observed decreased expression of Saa3, a secreted protein that is elevated with obesity and type 2 diabetes, and which functions to activate macrophages to promote local inflammatory responses[83-85]. Thus, Chen *et al.* put forth the hypothesis that reduced Saa3 in HFD-fed $\beta$-cat$^{-/-}$ mice leads to less macrophage recruitment and activation, and subsequently less PDGFR$\alpha^+$ cell proliferation. Indeed, global Saa3-deficient mice are resistant to diet-induced obesity, adipose tissue inflammation and dyslipidemia[86]. Our own studies demonstrate that consistent with macrophage marker expression patterns, *Saa3* is mildly higher in $\beta$-cat$^{-/-}$

mice maintained on chow diet and is subsequently suppressed in knockout mice with HFD feeding. Thus, it is possible that β-catenin mediates cross-talk between mature adipocytes and surrounding cells through Saa3, but future studies will be required to directly answer this question.

In summary, we report that β-catenin-dependent canonical Wnt signaling regulates various metabolic pathways in mature adipocytes, including lipid metabolism (**Figure 4.8**). Indeed, β-catenin is required for coordinate regulation of DNL and fatty acid desaturation, partly mediated through the key transcription factors *Srebf1* and *Mlxipl*. Perhaps most interestingly, in chow-fed mice, CD45$^-$/CD31$^-$ stromal cells respond to adipocyte-specific β-catenin depletion by up-regulating β-catenin and downstream target gene expression to defend canonical Wnt signaling homeostasis within WAT. We contend that this compensatory mechanism may explain the lack of observable phenotypes under standard nutritional conditions in virtually all mouse models that have been developed to interrogate the function of Wnt signaling in adipocytes. Finally, HFD feeding obesity overrides this compensatory mechanism, revealing that *β-cat$^{-/-}$* mice are protected from diet-induced obesity and metabolic dysfunction. Together, these novel findings underscore the critical importance of Wnt signaling in regulation of glucose and lipid metabolism in mature adipocytes.

**Materials and methods**

*Animals*

*Ctnnb1*$^{fl/fl}$ mice (hereby referred to as *β-cat*$^{fl/fl}$) (#004152, Jackson Lab, Ellsworth, ME), which harbor loxP sites flanking exons 2 to 6, were crossed with Adiponectin (Adipoq)-Cre mice (#028020, Jackson Lab, Ellsworth, ME) to generate *β-cat*$^{fl/fl}$ or *β-cat*$^{-/-}$ mice. Animals were housed in a 12 h light/12 h dark cycle with free access to water and food. For high fat diet (HFD) studies, mice were fed rodent diet with 60 kcal% from fat (#12492, Research Diets, New Brunswick, NJ). All animal studies were approved by and performed in compliance with policies of the University of Michigan Institutional Animal Care and Use Committee. Daily care of mice was overseen by the Unit for Laboratory Animal Medicine at the University of Michigan.

*Body composition*

Lean, fat, and free fluid masses were measured by a Bruker Minispec LF90II NMR (Bruker, Billerica, MA) at the University of Michigan Mouse Metabolic Phenotyping Center.

*Glucose and insulin tolerance tests*

For glucose tolerance tests, mice were fasted for 16 h and then given glucose (1 mg/kg body weight) via intraperitoneal injection. For insulin tolerance tests, mice were fasted for 6 h and then administered insulin (Eli Lilly, Indianapolis, IN) via intraperitoneal injection. Chow-fed mice received 0.5 U insulin/kg body weight, whereas HFD-fed mice were given 1.0 U insulin/kg body weight. Glucose concentrations were monitored in blood collected from the tail vein at 0, 15, 30, 60, and 120 minutes after injection using a glucometer and Contour Next blood glucose strips (Bayer AG, Leverkusen, Germany).

*In vivo lipolysis*

Mice were administered an intraperitoneal injection of saline as control or isoproterenol (10 mg/kg body weight) to stimulate lipolysis. Blood was collected from the tail vein immediately prior to and 15, 30, 60, and 120 minutes after injection. Blood was allowed

to coagulate on ice for 2 h. After centrifugation at 2,000 x g for 20 min at 4°C, serum was transferred to a new tube. Serum glycerol concentrations were measured by colorimetric assay (Sigma-Aldrich, St. Louis, MO).

*Voluntary running wheel exercise*

Mice were single-housed for one week prior to beginning exercise studies. Mice were then given free access to running wheels (Columbus Instruments, Columbus, OH) for voluntary exercise. Daily running totals were calculated from wheel revolutions collected at 1 min intervals for a total of six weeks.

*Serum measurements*

Blood was collected from the tail vein or by cardiac puncture at time of sacrifice. After coagulation on ice for 2 h and centrifugation at 2,000 x g for 20 min at 4°C, separated serum was transferred to a new tube and stored at -80°C until use. Serum insulin and adiponectin concentrations were measured by ELISA (Crystal Chem USA, Elk Grove, IL; R&D Systems Inc., Minneapolis, MN. respectively). Colorimetric assays were used to estimate triacylglycerols (TAG) and total cholesterol levels (Cayman Chemical, Ann Arbor, MI; Abcam, Cambridge, UK, respectively).

*Adipocyte and stromal-vascular cell fractionation*

Epididymal WAT (eWAT) and inguinal WAT (iWAT) depots were excised from mice as previously described[87,88]. WAT depots were minced with scissors and digested in 2 mg/ml collagenase type I (Worthington Biochemical, Lakewood, NJ) in Krebs-Ringer-HEPES (KRH; pH 7.4) buffer containing 3% fatty acid-free bovine serum albumin (BSA; Gold Biotechnology, St. Louis, NJ), 1 g/L glucose, and 500 nM adenosine for 1 h at 37°C with shaking (600 rpm). Buoyant adipocytes were separated from the stromal-vascular fraction (SVF) by filtering cell suspensions through 100 $\mu$m cell strainers and then centrifuging at 100 x g for 8 min. Fractions were washed 2x with KRH buffer containing 3% fatty acid-free BSA, 1 g/L glucose, and 500 nM adenosine. For immunoblot samples, fractions were then washed 1x with KRH buffer containing 0.5% fatty acid-free BSA, 1 g/L glucose, and 500 nM adenosine.

*Fluorescence-activated cell sorting of stromal-vascular cell sub-populations*

Excised eWAT was digested at 37°C in RPMI medium (Thermo Fisher Scientific, Waltham, MA) containing 0.5% BSA and 1 mg/ml type II collagenase for 30 mins with shaking. In order to ensure sufficient cell numbers, three mice were combined per sample and three samples were analyzed per genotype. SVF was separated from buoyant adipocytes by differential centrifugation following filtration through 100 $\mu$m cell strainers. Cells were stained with anti-CD45-PE (clone 30-F11; eBioscience, San Diego, CA) and anti-CD31-APC (clone 390; eBioscience, San Diego, CA) antibodies prior to flow cytometry analysis. Fluorescence-activated cell sorting was performed on a BD FACSAria III cell sorter (BD Biosciences, San Jose, CA) and data were processed and analyzed using FlowJo software (BD Biosciences, San Jose, CA). Sorted cells were pelleted by 500 x g for 5 mins, and RNA was subsequently isolated for evaluation of gene expression.

*Histology*

After harvest, soft tissues were fixed overnight in 10% neutral buffered formalin at 4°C. Bones were fixed for 24 h in 10% neutral buffered formalin, rinsed with water and then decalcified for 14 days in 14% EDTA, pH 7.4. Tissues were then processed, paraffin-embedded, and sectioned at 5 $\mu$m thickness. Sections were stained with hematoxylin and eosin as previously described[89] and subsequently imaged using a Zeiss inverted microscope at 100x or 200x magnification, as indicated.

*Micro-computed tomography (μCT) analysis of bone*

Tibia were harvested and fixed in 10% neutral buffered formalin for 24 h, rinsed with water, and stored at 4°C in Sorensen's phosphate buffer, pH 7.4, prior to μCT analysis. For analysis, each bone was placed in a 19-mm diameter tube and its length was scanned using a μCT system as previously described[40] (μCT100 Scanco Medical, Bassersdorf, Switzerland). Analyses of bone parameters were performed with the manufacturer's evaluation software, using a threshold of 280 for cortical bone and 180 for trabecular bone.

*Cell culture*

Primary mesenchymal stem cells (MSC) were isolated from outer ears of wildtype C57BL/6J (Jackson Labs, Bar Harbor, Maine) or *β-cat*^fl/fl mice as previously described[38,90] and cultured at 37°C in 5% $CO_2$. Subconfluent MSCs were maintained in DMEM:F12 medium (Thermo Fisher Scientific, Waltham, MA) containing 10% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO) and supplemented with 10 ng/ml recombinant basic fibroblast growth factor (PeproTech Inc., Rocky Hill, NJ). Adipogenesis was induced at two days post-confluence with 5 μg/ml insulin, 5 μM rosiglitazone, 1 μM dexamethasone, and 0.5 mM methylisobutylxanthine in DMEM:F12 containing 10% FBS. Cells were fed with fresh DMEM:F12 medium containing 10% FBS, 5 μg/ml insulin and 5 μM rosiglitazone from days 2 to 4 of differentiation. For the remainder of differentiation, cells were maintained in DMEM:F12 containing 10% FBS. Oil Red-O staining was used to visualize accumulation of neutral lipids, as previously described[91]. Colorimetric assay (Cayman Chemical, Ann Arbor, MI) was used to quantify total TAG accumulation per well. For lipid composition analyses, cells were differentiated in DMEM:F12 containing 10% charcoal-stripped FBS (Sigma-Aldrich, St. Louis, MO) from days 6 to 12. As indicated, confluent MSCs or mature adipocytes were treated with 3 μM CHIR99021 (Cayman Chemical, Ann Arbor, MI) or 20 ng/ml recombinant Wnt3a (R&D Systems Inc., Minneapolis, MN) for 4 h prior to lysis and RNA isolation.

*Genetic recombination in cultured cells*

To induce gene deletion in preadipocytes, *β-cat*^fl/fl precursors were treated at ~30% confluence with adenoviral GFP or adenoviral Cre recombinase (3 x $10^9$ viral particles/ml) in serum-free DMEM:F12 supplemented with 10 ng/ml recombinant basic fibroblast growth factor for 24 h. Preadipocytes were analyzed once they reached confluence. To induce gene recombination in adipocytes, *β-cat*^fl/fl cells were treated with adenoviral GFP or adenoviral Cre recombinase (1 x $10^{10}$ viral particles/ml) in serum-free DMEM:F12 from days 4 to 6 of differentiation. Adipocytes were then analyzed at day 12 of differentiation. Adenoviruses were obtained from the University of Michigan Vector Core. PCR with a three-primer system was used to confirm genetic recombination.

Primer sequences are as follows: P1, AAGGTAGAGTGATGAAAGTTGTT; P2, CACCATGTCCTCTGTCTATTC; P3, TACACTATTGAATCACAGGGACTT; floxed band: 324 bp, recombined band: 500 bp.


*ChREBP or SREBP1c overexpression in cultured cells*

*β-cat*^{fl/fl} and *β-cat*^{-/-} adipocytes were treated with adenoviral GFP, adenovirus overexpressing ChREBP, or adenovirus overexpressing SREBP1c (1 x $10^5$ viral particles/ml) in DMEM:F12 containing 10% FBS from days 9 to 12 of differentiation. Adipocytes were then analyzed at day 12 for rescue of DNL enzyme expression. Adenoviruses were obtained from Vector Biolabs (Vector Biolabs, Malvern, PA).


*De novo lipogenesis (DNL) assay*

Prior to evaluation of DNL, cultured adipocytes were incubated in fresh serum-free DMEM:F12 medium overnight. To measure DNL, cells were then incubated in fresh DMEM:F12 medium (with 0.5 mM sodium pyruvate, 0.5 mM L-glutamine, 2.5 mM glucose, 1% fatty acid-free BSA) containing 5 $\mu$M sodium acetate and 0.5 $\mu$Ci [$^{14}$C]-acetate (PerkinElmer, Waltham, MA) for 2, 4, or 8 h at 37°C. Following indicated incubation times, cell were harvested and lipids extracted for analyses by scintillation counting and thin-layer chromatography.


*Lipid extraction for analyses by gas chromatography*

Lipids were extracted from cultured adipocytes as previously described[40]. Briefly, cells were washed 2x with PBS and then collected in 500 $\mu$l of a 1:2.5 methanol/water mixture. Cell suspensions were transferred to clean borosilicate glass tubes. Wells were rinsed with 500 $\mu$l of the 1:2.5 methanol/water mixture and volumes were transferred to the glass tubes. After addition of 750 $\mu$l chloroform and 375 $\mu$l 0.9% NaCl, tubes were vortexed vigorously and centrifuged at 2,500 rpm for 20 min at 4°C. Lower organic chloroform layers containing total lipids were transferred to clean glass tubes and stored at -20°C until use.

*Fatty acid composition by gas chromatography*

Fatty acids within extracted lipids were derivatized into their methyl esters by trans-esterification with boron trifluoride-methanol, as previously described[92]. Derivatized methyl esters were re-dissolved in a small volume of hexane and purified by thin-layer chromatography using n-hexane-diethyl ether-acetic acid (50:50:2, v/v/v) as the developing solvent. Plates were dried and sprayed with Premulin after development. Products were identified under ultraviolet light by comparison to retention flow of the methyl heptadecanoate (C17:0) standard (retention flow, 0.67) applied side-by-side on the same plate. Methyl esters were extracted from thin-layer chromatography powder with diethyl ether, concentrated under nitrogen, and re-dissolved in 100 µl hexane. Fatty acid compositions of lipids were analyzed by gas chromatography (GC) as follows: FAMEs analysis was performed with 1 µl sample injection on an Agilent GC machine, model 6890N equipped with flame ionization detector, an auto sampler and a Chemstation software for data analysis. An Agilent HP 88, 30 m GC column with a 0.25 mm inner diameter and 0.20 mm film thickness was used, with hydrogen as a carrier gas and nitrogen as a makeup gas. Analyses were carried out with a temperature programming of 125-220°C. Fatty acid components within unknown samples were identified with respect to retention times of authentic standard methyl ester mixtures run side-by-side. Fatty acid components were quantified with respect to a known amount of internal standard added and the calibration ratio derived from each fatty acid of a standard methyl esters mixture and the methyl heptadecanoate internal standard. Coefficient of variation for GC analyses was found to be 2.3-3.7%.

*Quantitative RT-PCR*

Total RNA was isolated and purified from cultured cells or frozen tissue using RNA STAT-60 (Tel Test, Alvin, TX) according to the manufacturer's instructions. M-MLV Reverse Transcriptase (Invitrogen, Carlsbad, CA) was used to reverse-transcribe 1 µg RNA to cDNA. qRT-PCR was performed using qPCRBIO Sygreen Mix (Innovative Solutions, Beverly Hill, MI) on a StepOnePlus System (Applied Biosystems, Foster City, CA). All primers were validated with cDNA titration curves prior to use; qPCR product specificities were confirmed by melting curve analyses and gel electrophoresis. Gene

expression was calculated using a cDNA titration curve within each plate and was subsequently normalized to peptidylprolyl isomerase A (PPIA) mRNA expression.

*RNA sequencing (RNA-seq) analyses*

Confluent *β-cat*<sup>fl/fl</sup> and *β-cat*<sup>-/-</sup> preadipocytes and terminally-differentiated adipocytes were treated with 20 ng/ml recombinant Wnt3a (R&D Systems Inc., Minneapolis, MN) or vehicle for 4 h prior to lysis and RNA isolation (n = 4 per group). After DNase treatment, samples were submitted to the University of Michigan Advanced Genomics Core for quality control, library preparation, and sequencing on an Illumina Hi-Seq platform. Read files for each sample were subsequently downloaded and collated into a single .fastq file. FastQC (version v0.11.30) was used to evaluate the quality of raw read data and to identify problematic features (inappropriate GC content, over-represented sequences, low-quality scores). Alignment, differential expression analyses, and post-analysis diagnostics were performed using the Tuxedo Suite software package. Briefly, TopHat (version 2.0.13) and Bowtie2 (version 2.2.1) were used to align reads to the UCSC reference genome. A second round of quality control was performed post-alignment using FastQC to ensure that only high-quality data was used for gene expression quantitation and differential expression analyses. Differential expression analyses were performed using two independent methods: Cufflinks/CuffDiff and HTSeq/DESeq2, using UCSC build mm10 as the reference genome sequence. Pathway analyses were conducted on ranked lists of log2 fold change using Gene Set Enrichment Analysis (GSEA) software v4.0.3 and Molecular Signature Database (MSigDB) by the Broad Institute[93]. Prior to running analyses, mouse gene symbols were remapped to human ortholog symbols using chip annotation files. Mouse genes that did not have equivalent human orthologs were excluded from analyses. Output from DESeq2 analyses were used to generate lists of genes ranked by the metric -log10 FDR*log2 fold change. The resulting lists were run through pre-ranked GSEA using the Molecular Signatures Database v7.1 (H, hallmark gene sets). Enriched pathways were defined by an FDR < 0.05. Normalized enrichment score (NES) is the primary metric from GSEA for evaluating the magnitude of differentially expressed pathways. Pathway impact analyses were conducted on AdvaitaBio's iPathwayGuide[94] to identify enriched

KEGG pathways. The ggplot2 package in R was used for further visualization of enriched pathways.


*Chromatin immunoprecipitation (ChIP-seq) analyses*

ChIP-seq data of Tcf7l2 binding sites in *Tcf7l2*^{fl/fl} and *Tcf7l2*^{-/-} cultured adipocytes was previously generated[39] and is publicly available. Data was obtained from Gene Expression Omnibus (GEO accession: GSE129403). The Integrative Genomics Viewer (IGV) was used for visualization of Tcf7l2 peaks[95].


*Human gene expression data*

Human *Ctnnb1* gene expression data were obtained from the publicly available Genotype-Tissue Expression (GTEx) database.


*Immunoblot analyses*

Tissue samples were homogenized with a BioVortexer mixer (Chemglass, Vineland, NJ) in ice-cold lysis buffer (1% SDS, 12.7 mM EDTA, 60 mM Tris-HCl; pH 6.8) containing 1:100 protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). Lysates were centrifuged at 13,600 x g for 10 min at 4°C, the top lipid layer removed, and extracts were centrifuged again. Cultured cells were washed 2x with PBS, lysed in ice-cold lysis buffer containing 1:100 protease inhibitor cocktail, and homogenized. Lysates were then centrifuged at 13,600 x g for 10 min at 4°C. Protein concentrations of tissue or cell lysates were measured by BCA protein assay (Thermo Scientific, Waltham, MA). Lysates were diluted to equal protein concentrations in Laemmli sample buffer and lysis buffer, vigorously vortexed, and denatured at 95°C for 5 min. Tissue or cell extracts (20 $\mu$g) were separated by SDS-PAGE on 4-12% gradient polyacrylamide gels (Invitrogen, Carlsbad, CA) and transferred to Immobilon PVDF membranes (Millipore, Billerica, MA). Prior to immunoblotting with primary antibodies, membranes were blocked in 5% non-fat dried milk in Tris-buffered saline, pH 7.4, containing 0.05% Tween-20 (TTBS) for 1 h at room temperature. All primary antibodies were used at a concentration of 1:1000 in TTBS containing 5% BSA overnight at 4°C. Membranes were probed with horseradish peroxidase-conjugated secondary antibodies (1:5000) diluted in 5% non-fat dried milk in

TTBS for 1.5 h at room temperature and subsequently visualized with Clarity Western ECL Substrate (Bio-Rad, Hercules, CA) or SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific, Waltham, MA).

*Statistics*

All data are presented as mean ± S.D. Significance was determined using two-tailed Student's t-test when comparing two groups. An analysis of variance (ANOVA) was followed by post hoc analyses with Dunnett's or Sidak's test, as appropriate, when comparing multiple experimental groups. Observed differences were considered to be significant at $p < 0.05$ and are indicated with asterisks.
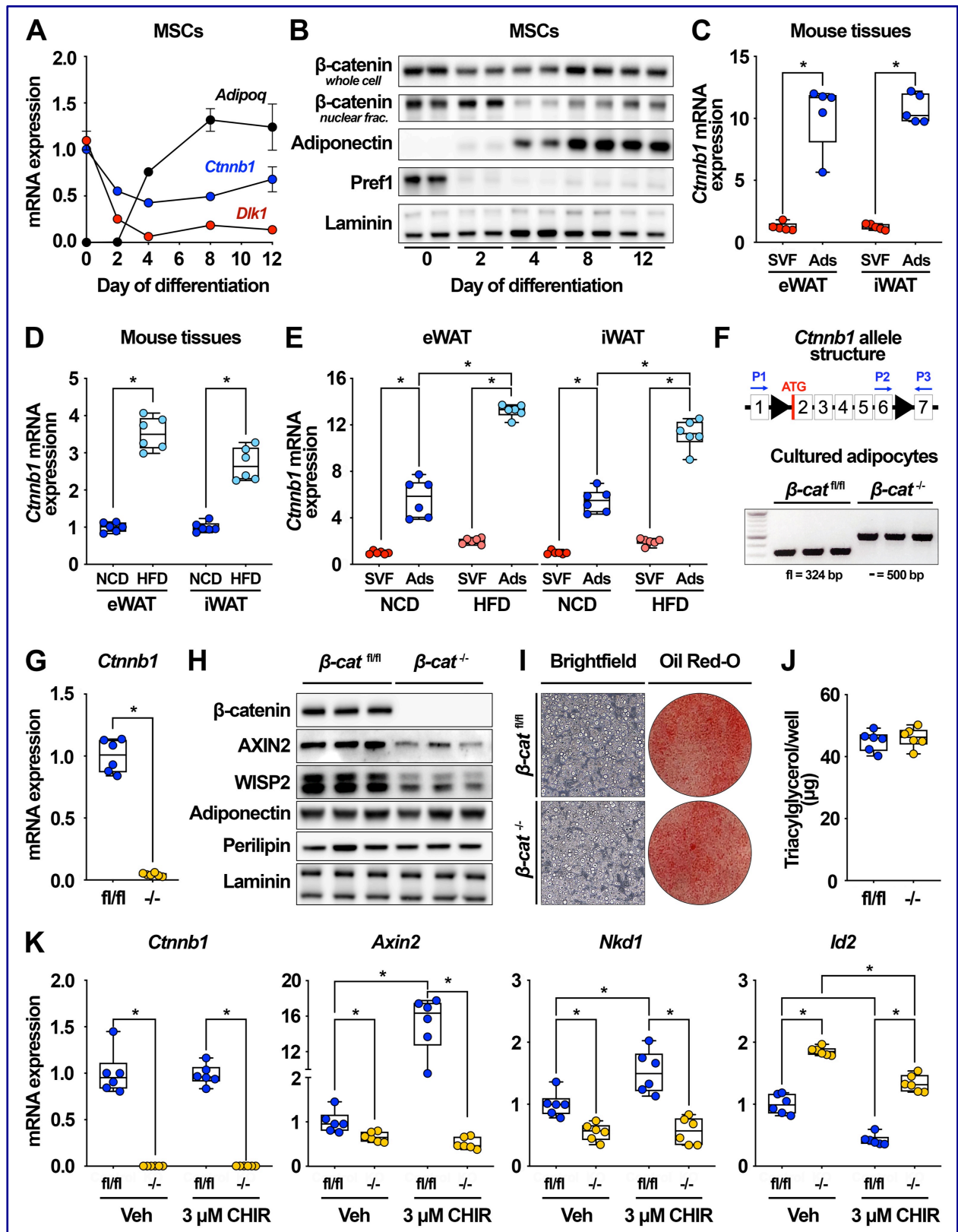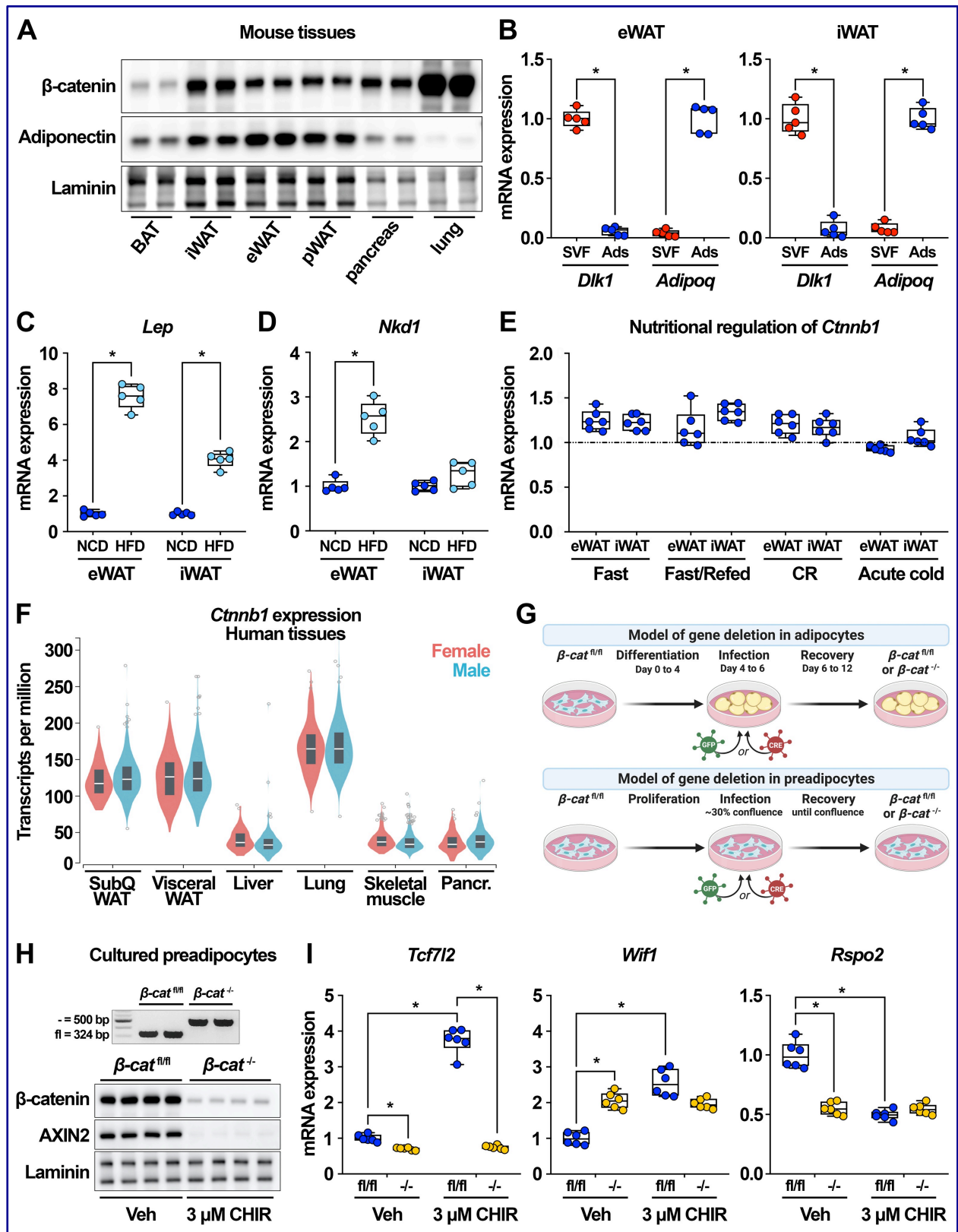
**Figure 4.1**

**Figure 4.1. β-catenin is expressed in cultured and primary adipocytes and up-regulated by diet-induced obesity.** (**A-B**) Mesenchymal stem cells (MSC) isolated from C57BL/6J mice were cultured under standard conditions and induced to differentiate. *Ctnnb1* gene (n = 6) and protein (n = 2) expression at indicated days of adipogenesis. (**C**) *Ctnnb1* gene expression in stromal-vascular (SVF) and adipocyte (Ads) fractions isolated from epididymal (eWAT) and inguinal (iWAT) white adipose tissues (WAT) of C57BL/6J mice (males; n = 5). (**D**) Expression of *Ctnnb1* in eWAT and iWAT of mice fed normal chow diet (NCD) or high fat diet (HFD) for 10 weeks. (**E**) *Ctnnb1* expression in SVF and Ads of eWAT and iWAT isolated from NCD- and HFD-fed mice (males; n = 5). (**F**) *Ctnnb1* allele structure and genetic recombination in *β-cat*$^{fl/fl}$ and *β-cat*$^{-/-}$ adipocytes using a 3-primer PCR system (n = 3). (**G-H**) *Ctnnb1* RNA (n = 6) and protein (n = 3) expression in adipocytes following adenoviral GFP or Cre infection. (**I**) Representative brightfield and Oil Red-O images, and (**J**) triacylglycerol (TAG) accumulation in *β-cat*$^{fl/fl}$ and *β-cat*$^{-/-}$ adipocytes (n = 6). (**K**) Expression of *Ctnnb1* and downstream Wnt target genes in *β-cat*$^{fl/fl}$ and *β-cat*$^{-/-}$ adipocytes treated with vehicle or 3 μM CHIR99021 for 4 h (n = 6). RNA expression normalized to PPIA. Data presented as mean ± S.D. * indicates significance at $p < 0.05$.

# Supplemental Figure 4.1



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**Supplemental Figure 4.1.** (**A**) Representative immunoblot of β-catenin protein expression across C57BL/6J mouse tissues (BAT, brown adipose tissue; pWAT, perirenal WAT); adiponectin and laminin included as controls. (**B**) *Dlk1* and *Adipoq* gene expression in SVF and adipocyte fractions isolated from eWAT and iWAT of C57BL/6J mice (males; n = 5). (**C-D**) *Lep* and *Nkd1* mRNA expression in SVF and adipocytes isolated from eWAT and iWAT of mice fed NCD or 10 weeks of HFD (males; n = 5). (**E**) Regulation of *Ctnnb1* gene expression in eWAT and iWAT by nutritional and environmental conditions: fast: 18 h; refed: 6 h after 18 h fast; 30% calorie restriction (CR): 6 weeks; 4°C cold exposure: 6 h. Each condition normalized to its individual experimental control set to 1 (indicated by dashed line; n = 6). (**F**) *Ctnnb1* gene expression in male and female human tissues; data obtained from the GTEx-RNA-Seq dataset. (**G**) Schematic model for deletion of β-catenin in cultured preadipocytes or adipocytes using adenoviral Cre recombinase. (**H**) *Ctnnb1* gene recombination (n = 2) and β-catenin protein expression (n = 4) in preadipocytes treated with adenoviral GFP or Cre. (**I**) Expression of downstream Wnt target genes in *β-cat*^fl/fl and *β-cat*^-/- adipocytes treated with vehicle or 3 μM CHIR99021 for 4 h (n = 6). RNA expression normalized to PPIA. Data presented as mean ± S.D. * indicates significance at p < 0.05.
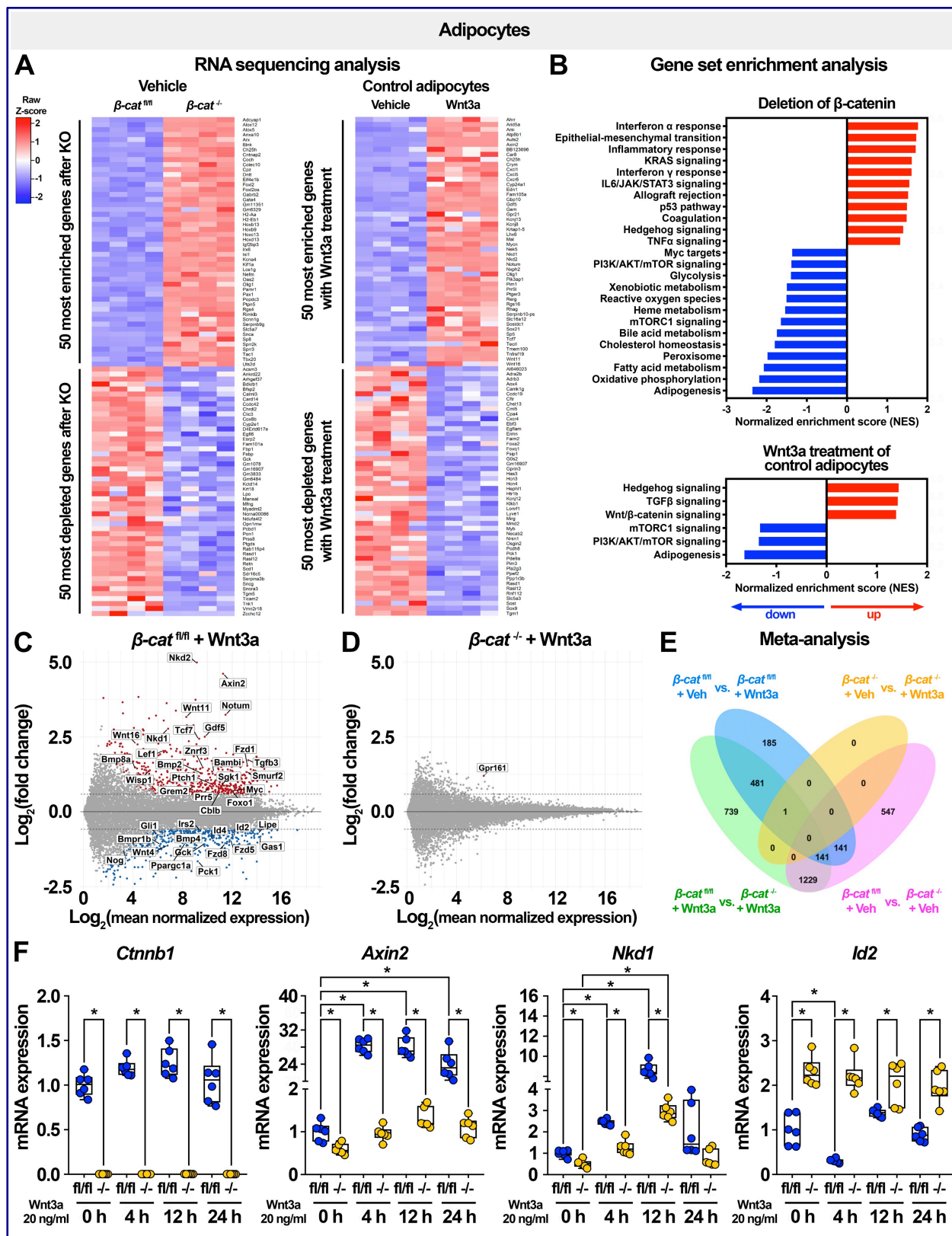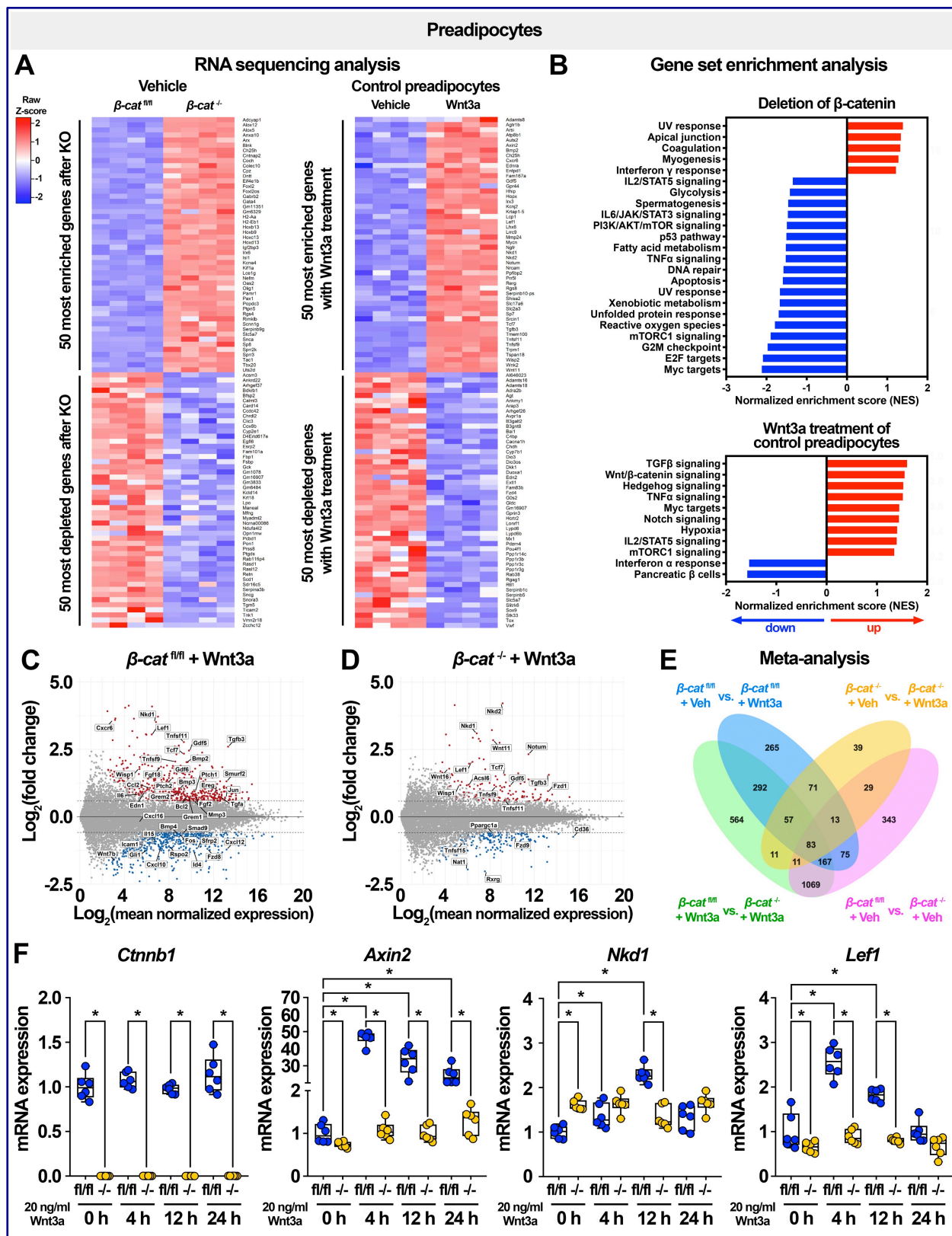
**Figure 4.2**

**Figure 4.2. β-catenin regulates metabolic pathways in adipocytes and exclusively mediates effects of canonical Wnt3a signaling.** RNA-seq analyses were performed on *β-cat*^fl/fl^ and *β-cat*^-/-^ adipocytes under basal conditions or after 4 h treatment with recombinant Wnt3a (20 ng/ml; n = 4 per group). (**A**) Heat maps of differential gene expression changes in *β-cat*^fl/fl^ and *β-cat*^-/-^ adipocytes under basal conditions (left panel) and *β-cat*^fl/fl^ cells treated with vehicle or Wnt3a (right panel). (**B**) Gene Set Enrichment Analyses (GSEA) of genes expressed in *β-cat*^fl/fl^ and *β-cat*^-/-^ adipocytes under basal conditions (top panel) and *β-cat*^fl/fl^ cells treated with vehicle or Wnt3a (bottom panel). (**C-D**) MA plots of gene expression changes following Wnt3a treatment of *β-cat*^fl/fl^ or *β-cat*^-/-^ adipocytes. (**E**) Venn diagram depicting meta-analysis of gene expression changes in *β-cat*^fl/fl^ or *β-cat*^-/-^ adipocytes treated with vehicle or Wnt3a for 4 h. (**F**) Expression of *Ctnnb1* and downstream Wnt target genes in *β-cat*^fl/fl^ and *β-cat*^-/-^ adipocytes treated with vehicle or 20 ng/ml recombinant Wnt3a for 4, 12 or 24 h (n = 6). RNA expression normalized to PPIA. Data presented as mean ± S.D. * indicates significance at $p < 0.05$.

# Supplemental Figure 4.2

**Supplemental Figure 4.2.** RNA-seq analyses were performed on $\beta$-*cat*$^{fl/fl}$ and $\beta$-*cat*$^{-/-}$ preadipocytes under basal conditions or after 4 h treatment with recombinant Wnt3a (20 ng/ml; n = 4 per group). (**A**) Heat maps of differential gene expression changes in $\beta$-*cat*$^{fl/fl}$ and $\beta$-*cat*$^{-/-}$ preadipocytes under basal conditions (left panel) and $\beta$-*cat*$^{fl/fl}$ cells treated with vehicle or Wnt3a (right panel). (**B**) Gene Set Enrichment Analyses (GSEA) of genes in $\beta$-*cat*$^{fl/fl}$ and $\beta$-*cat*$^{-/-}$ preadipocytes under basal conditions (top panel) and $\beta$-*cat*$^{fl/fl}$ cells treated with vehicle or Wnt3a (bottom panel). (**C-D**) MA plots of gene expression changes following Wnt3a treatment of $\beta$-*cat*$^{fl/fl}$ or $\beta$-*cat*$^{-/-}$ preadipocytes. (**E**) Venn diagram depicting meta-analysis of gene expression changes in $\beta$-*cat*$^{fl/fl}$ or $\beta$-*cat*$^{-/-}$ preadipocytes treated with vehicle or Wnt3a for 4 h. (**F**) Expression of *Ctnnb1* and downstream Wnt target genes in $\beta$-*cat*$^{fl/fl}$ and $\beta$-*cat*$^{-/-}$ preadipocytes treated with vehicle or 20 ng/ml recombinant Wnt3a for 4, 12 or 24 h (n = 6). RNA expression normalized to PPIA. Data presented as mean $\pm$ S.D. * indicates significance at $p < 0.05$.
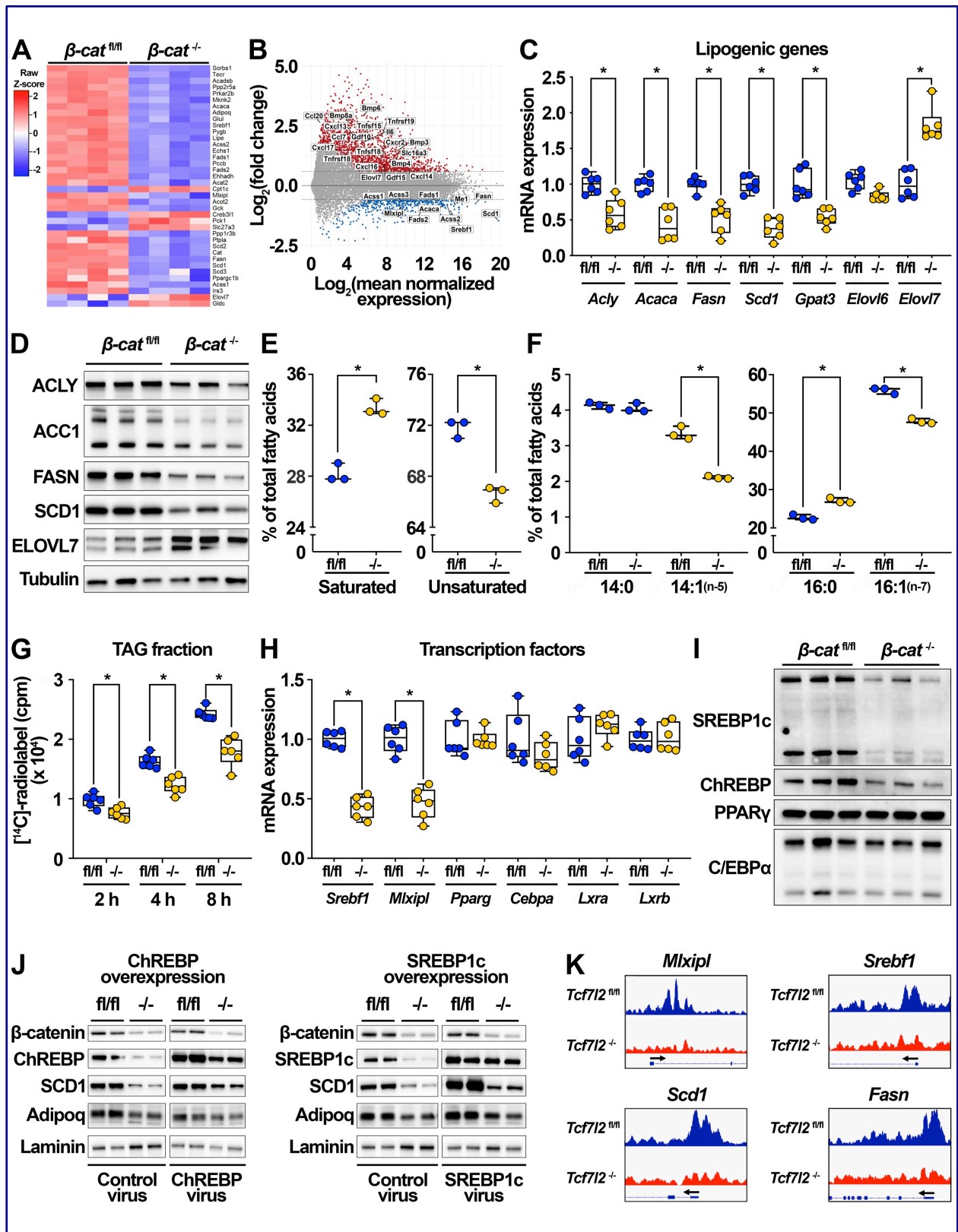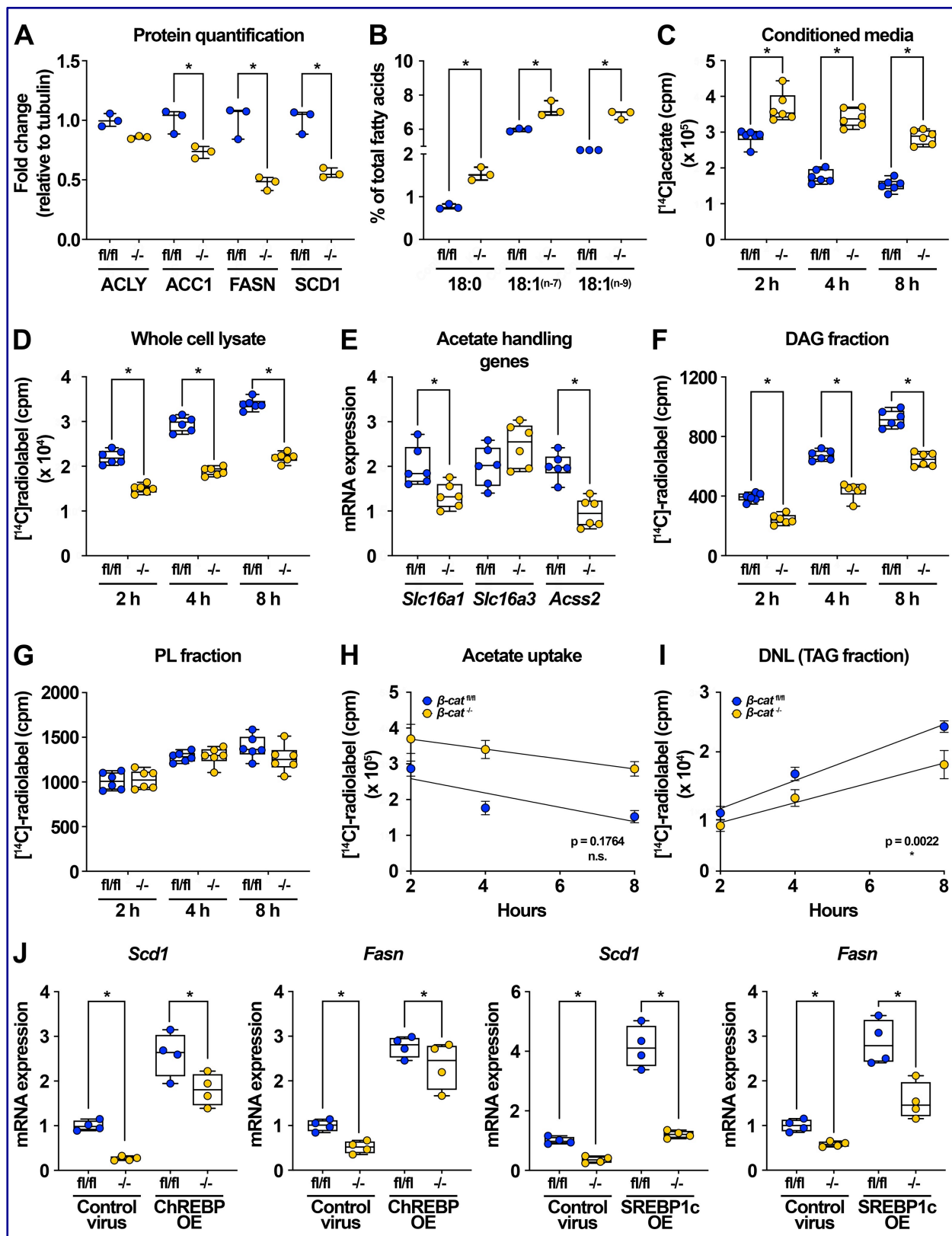
**Figure 4.3**

**Figure 4.3. β-catenin-dependent Wnt signaling regulates lipogenesis and fatty acid desaturation in adipocytes.** (**A-B**) Heat map and MA plot showing differentially expressed genes related to fatty acid, cholesterol, and bile acid metabolism in cultured *β-cat*$^{fl/fl}$ and *β-cat*$^{-/-}$ adipocytes (n = 4). (**C-D**) Lipogenic gene (n = 6) and protein (n = 3) expression in *β-cat*$^{fl/fl}$ and *β-cat*$^{-/-}$ adipocytes. (**E**) Proportion of total saturated versus unsaturated fatty acids in lipids extracted from *β-cat*$^{fl/fl}$ and *β-cat*$^{-/-}$ adipocytes (n = 3). (**F**) Relative proportions of myristic (C14:0) and palmitic (C16:0) versus myristoleic (C14:1, n-5) and palmitoleic (C16:1, n-7) acids (n = 3). (**G**) *De novo* lipogenesis (DNL) was evaluated in cultured *β-cat*$^{fl/fl}$ and *β-cat*$^{-/-}$ adipocytes using [$^{14}$C]-acetate for 2, 4 and 8 h. Incorporation of [$^{14}$C]-radiolabel into TAG fractions extracted from *β-cat*$^{fl/fl}$ and *β-cat*$^{-/-}$ adipocytes was quantified by scintillation counting (n = 6). (**H-I**) Gene (n = 6) and protein (n = 3) expression of indicated transcription factors in *β-cat*$^{fl/fl}$ and *β-cat*$^{-/-}$ adipocytes. (**J**) Protein expression in *β-cat*$^{fl/fl}$ and *β-cat*$^{-/-}$ adipocytes treated for 72 h with adenovirus expressing GFP, ChREBP, or SREBP1c (1 x 10$^5$ viral particles/ml). (**K**) Integrative Genomics Viewer capture showing Tcf7l2 peaks (indicating binding occupancy) in the regions ± 3 kb from transcription start sites (black arrows) of indicated genes in cultured *Tcf7l2*$^{fl/fl}$ and *Tcf7l2*$^{-/-}$ adipocytes. RNA expression normalized to PPIA. Data presented as mean ± S.D. * indicates significance at $p < 0.05$.

**Supplemental Figure 4.3**

**Supplemental Figure 4.3.** (**A**) Lipogenic proteins quantified by densitometry (n = 3). (**B**) Relative proportions of stearic (C18:0) versus vaccenic (C18:1, n-7) and oleic (C18:1, n-9) acids in $\beta$-cat$^{fl/fl}$ and $\beta$-cat$^{-/-}$ adipocytes (n = 3). [$^{14}$C]-radiolabel in (**C**) conditioned media versus (**D**) whole cell lysates after indicated incubation times measured by scintillation counting (n = 6). (**E**) Expression of genes related to acetate uptake and activation (n = 6). (**F-G**) Radiolabel incorporation into DAG and PL fractions extracted from $\beta$-cat$^{fl/fl}$ and $\beta$-cat$^{-/-}$ adipocytes quantified by scintillation counting (n = 6). (**H**) Linear regression analyses comparing slopes of acetate uptake over time from conditioned media into $\beta$-cat$^{fl/fl}$ and $\beta$-cat$^{-/-}$ adipocytes. (**I**) Linear regression analyses comparing slopes of radiolabel incorporation over time into TAG fractions of $\beta$-cat$^{fl/fl}$ and $\beta$-cat$^{-/-}$ adipocytes. (**J**) Gene expression in $\beta$-cat$^{fl/fl}$ and $\beta$-cat$^{-/-}$ adipocytes treated with adenovirus expressing GFP, ChREBP, or SREBP1c for 72 h (1 x $10^5$ viral particles/ml; n = 4). RNA expression normalized to PPIA. Data presented as mean $\pm$ S.D. * indicates significance at $p < 0.05$.
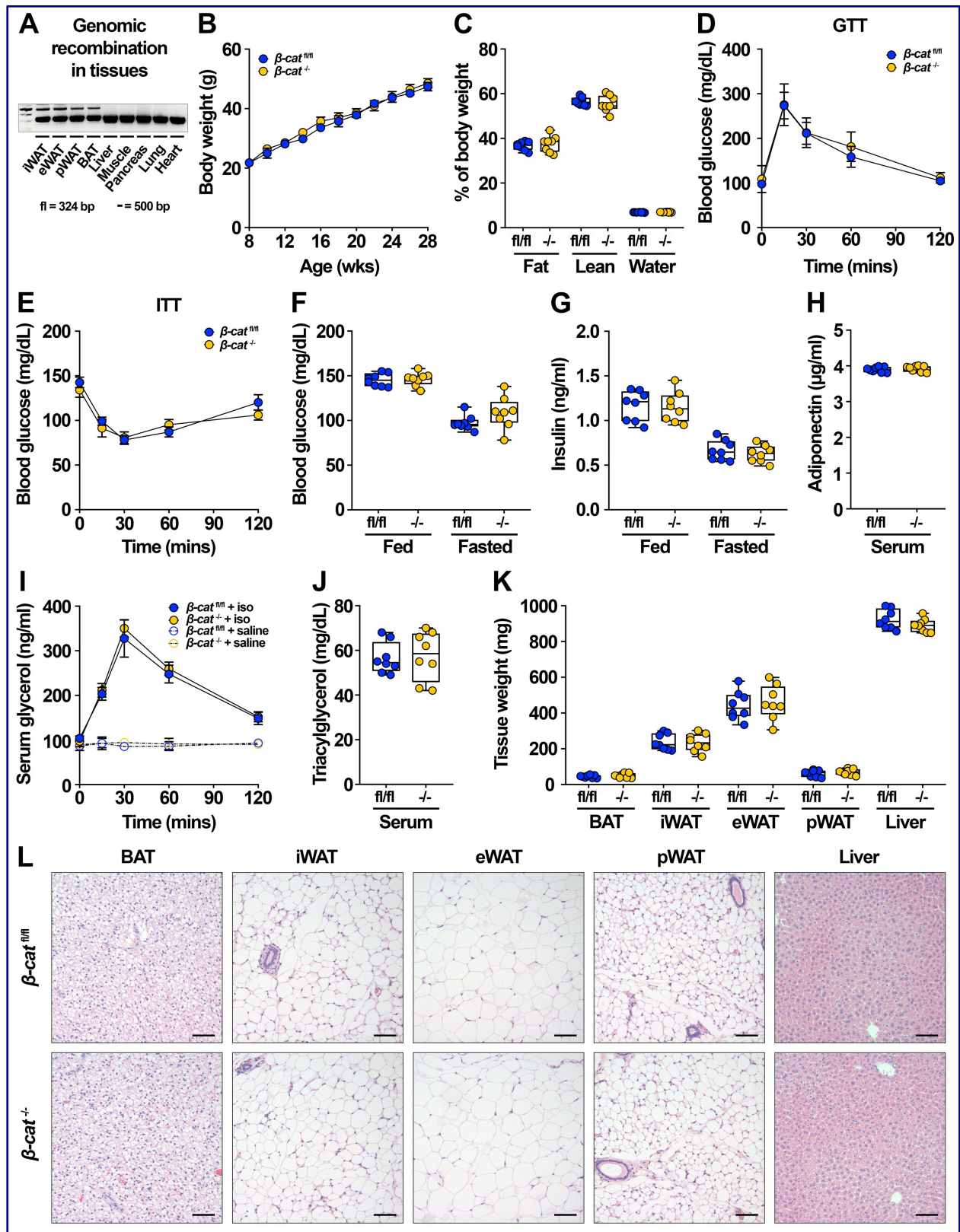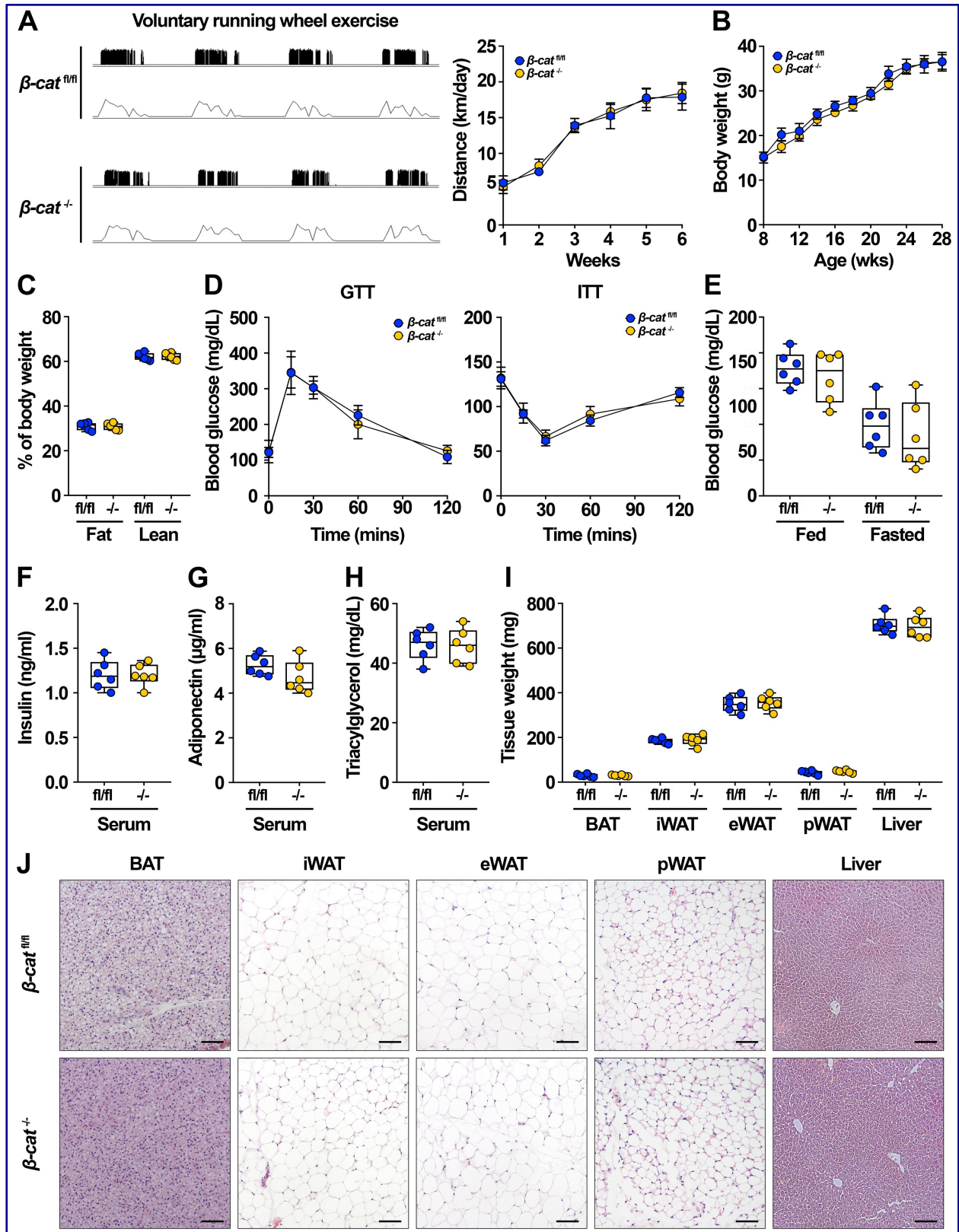
**Figure 4.4**

**Figure 4.4. Adipocyte-specific β-catenin deletion does not influence global metabolism on a normal chow diet.** (**A**) Genetic recombination in tissues isolated from *β-cat*$^{-/-}$ mice. (**B**) Growth curve of 28-week-old *β-cat*$^{fl/fl}$ and *β-cat*$^{-/-}$ mice. (**C**) Body composition of 16-week-old *β-cat*$^{fl/fl}$ and *β-cat*$^{-/-}$ mice on NCD. (**D**) Glucose tolerance tests in 16-week-old *β-cat*$^{fl/fl}$ and *β-cat*$^{-/-}$ mice. (**E**) Insulin tolerance test in 19-week-old mice. (**F**) Blood glucose concentrations in random-fed and 16 h fasted mice. Serum concentrations of (**G**) random-fed and fasted insulin and (**H**) adiponectin levels in 28-week-old mice. (**I**) Basal and stimulated lipolysis in 22-week-old mice (iso, isoproterenol: 10 mg/kg body weight). (**J**) Serum TAG in 28-week-old mice. (**K**) Tissue weights at time of sacrifice. (**L**) Representative histological images of H&E-stained tissues from *β-cat*$^{fl/fl}$ and *β-cat*$^{-/-}$ mice fed NCD for 28 weeks; 200x magnification; scale bar, 100 μm. Data in **B-L** from male mice, n = 8 per group. Data presented as mean ± S.D. * indicates significance at $p < 0.05$.

## Supplemental Figure 4.4

**Supplemental Figure 4.4.** (**A**) Representative traces and quantification of daily running distance in chow-fed male 23-week-old *β-cat*^fl/fl^ and *β-cat*^-/-^ mice given access to voluntary exercise wheels for six weeks. (**B**) Growth curve of 28-week-old female *β-cat*^fl/fl^ and *β-cat*^-/-^ mice. (**C**) Body composition of 16-week-old *β-cat*^fl/fl^ and *β-cat*^-/-^ mice on NCD. (**D**) Glucose and insulin tolerance tests in 16- and 19-week-old *β-cat*^fl/fl^ and *β-cat*^-/-^ mice, respectively. (**E**) Blood glucose concentrations in random-fed and 16 h fasted mice. Serum concentrations of (**F**) insulin, (**G**) adiponectin, and (**H**) TAG in 28-week-old mice. (**I**) Tissue weights at time of sacrifice. (**J**) Representative histological images of H&E-stained tissues from female *β-cat*^fl/fl^ and *β-cat*^-/-^ mice fed NCD for 28 weeks; 200x magnification; scale bar, 100 μm. Data in **B-J** from female mice, n = 6 per group. Data presented as mean ± S.D. * indicates significance at $p < 0.05$.
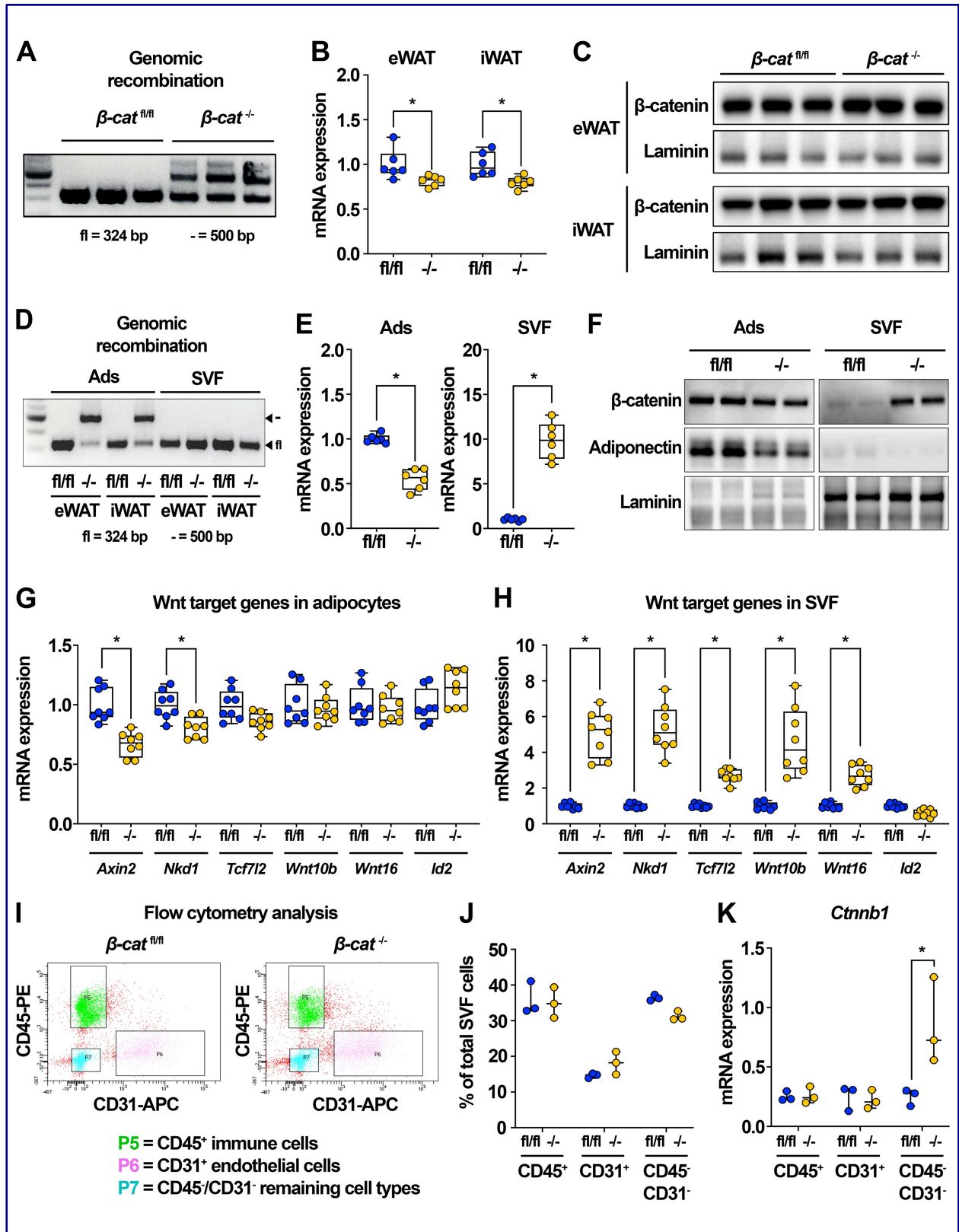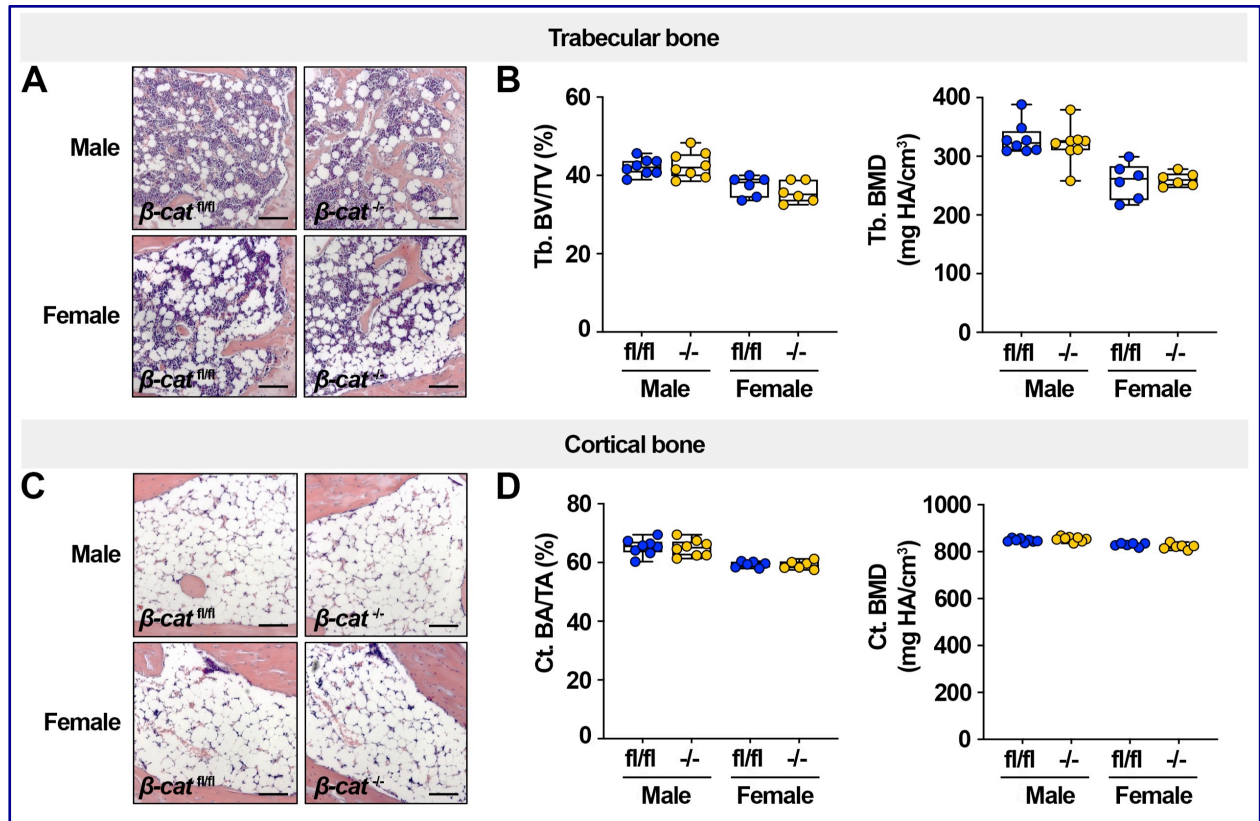
# Figure 4.5



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**Figure 4.5. β-catenin is up-regulated in the stromal-vascular fraction of adipose tissues from knockout mice.** (**A**) Genetic recombination in tissues isolated from *β-cat*$^{fl/fl}$ and *β-cat*$^{-/-}$ mice (n = 3). (**B-C**) *Ctnnb1* mRNA (n = 3) and protein (n = 6) expression in eWAT and iWAT of *β-cat*$^{fl/fl}$ and *β-cat*$^{-/-}$ mice. (**D**) Genomic recombination of β-catenin in adipocytes and SVF isolated from eWAT and iWAT of *β-cat*$^{fl/fl}$ and *β-cat*$^{-/-}$ mice. (**E**) *Ctnnb1* mRNA expression in isolated eWAT adipocytes and SVF of *β-cat*$^{fl/fl}$ and *β-cat*$^{-/-}$ mice (n = 6). (**F**) β-catenin protein expression in isolated eWAT adipocytes and SVF of *β-cat*$^{fl/fl}$ and *β-cat*$^{-/-}$ mice; adiponectin and laminin shown as protein loading controls. (**G-H**) Wnt target gene expression in adipocytes and SVF isolated from eWAT of *β-cat*$^{fl/fl}$ and *β-cat*$^{-/-}$ mice (n = 8). (**I**) Representative plots showing flow cytometry analysis of SVF isolated from *β-cat*$^{fl/fl}$ and *β-cat*$^{-/-}$ mice (3 mice per sample; n = 3 samples). (**J**) Quantification of SVF cell proportions evaluated by flow cytometry analysis (3 mice per sample; n = 3 samples). (**K**) *Ctnnb1* mRNA expression normalized to PPIA in cellular fractions isolated by FACS analysis (3 mice per sample; n = 3 samples). Data presented as mean ± S.D. * indicates significance at p < 0.05.

**Supplemental Figure 4.5**



**Supplemental Figure 4.5.** (**A**) Representative histological images of H&E-stained proximal tibia (regulated bone marrow adipose tissue) of male and female *β-cat*$^{fl/fl}$ and *β-cat*$^{-/-}$ mice fed NCD for 28 weeks (male: n = 8; female: n = 6). (**B**) μCT analyses of tibial trabecular bone volume fraction (Tb. BV/TV) and trabecular bone mineral density (Tb. BMD). (**C**) Representative histological images of H&E-stained distal tibia (constitutive bone marrow adipose tissue) of male and female *β-cat*$^{fl/fl}$ and *β-cat*$^{-/-}$ mice. (**D**) μCT analyses of mid-tibial cortical bone area (Ct. BA/TA) and cortical bone mineral density (Ct. BMD). Histological images shown at 100x magnification; scale bar, 50 μm. Data presented as mean ± S.D. * indicates significance at $p < 0.05$.

# Figure 4.6

**Figure 4.6.** *β-cat^{-/-}* **mice are protected from diet-induced obesity and metabolic dysfunction.** (**A**) Growth curve over time of 32-week-old *β-cat^{fl/fl}* and *β-cat^{-/-}* mice fed 60% HFD for 24 weeks. (**B**) Body composition analysis of 28-week-old mice. (**C-D**) Glucose tolerance test and area under the curve analysis in 28-week-old mice. (**E**) Blood glucose concentrations in random-fed and 16 h fasted mice. (**F**) Insulin tolerance test in 30-week-old mice. (**G**) Serum insulin concentrations in random-fed mice or in 16 h fasted mice at indicated times after intraperitoneal glucose injection (1 mg/kg body weight). Serum (**H**) TAG, (**I**) total cholesterol, and (**J**) adiponectin in 32-week-old mice. (**K**) Tissue weights at time of sacrifice. (**L**) Representative histological images of H&E-stained tissues from *β-cat^{fl/fl}* and *β-cat^{-/-}* mice fed HFD for 24 weeks; 200x magnification; scale bar, 100 μm. (**M**) Quantification of liver TAG in 32-week-old mice. Data shown from male mice; *β-cat^{fl/fl}*: n = 5, *β-cat^{-/-}*: n = 11. Data presented as mean ± S.D. * indicates significance at $p < 0.05$.

**Supplemental Figure 4.6**

**Supplemental Figure 4.6.** (**A-D**) Lipogenic gene expression in whole eWAT, iWAT, liver, or isolated eWAT adipocytes of $\beta$-cat$^{fl/fl}$ and $\beta$-cat$^{-/-}$ mice (n = 8). (**E**) Expression of immune, endothelial, and stromal cell markers in SVF isolated from eWAT of $\beta$-cat$^{fl/fl}$ and $\beta$-cat$^{-/-}$ mice (n = 6). (**F**) Expression of inflammatory markers in whole eWAT of $\beta$-cat$^{fl/fl}$ and $\beta$-cat$^{-/-}$ mice (n = 6; n = 10). (**G-H**) Expression of cell markers and Wnt target genes in SVF sub-populations isolated by FACS analysis (3 mice per sample; n = 3 samples). RNA expression normalized to PPIA. Data presented as mean $\pm$ S.D. * indicates significance at p < 0.05.

**Figure 4.7**
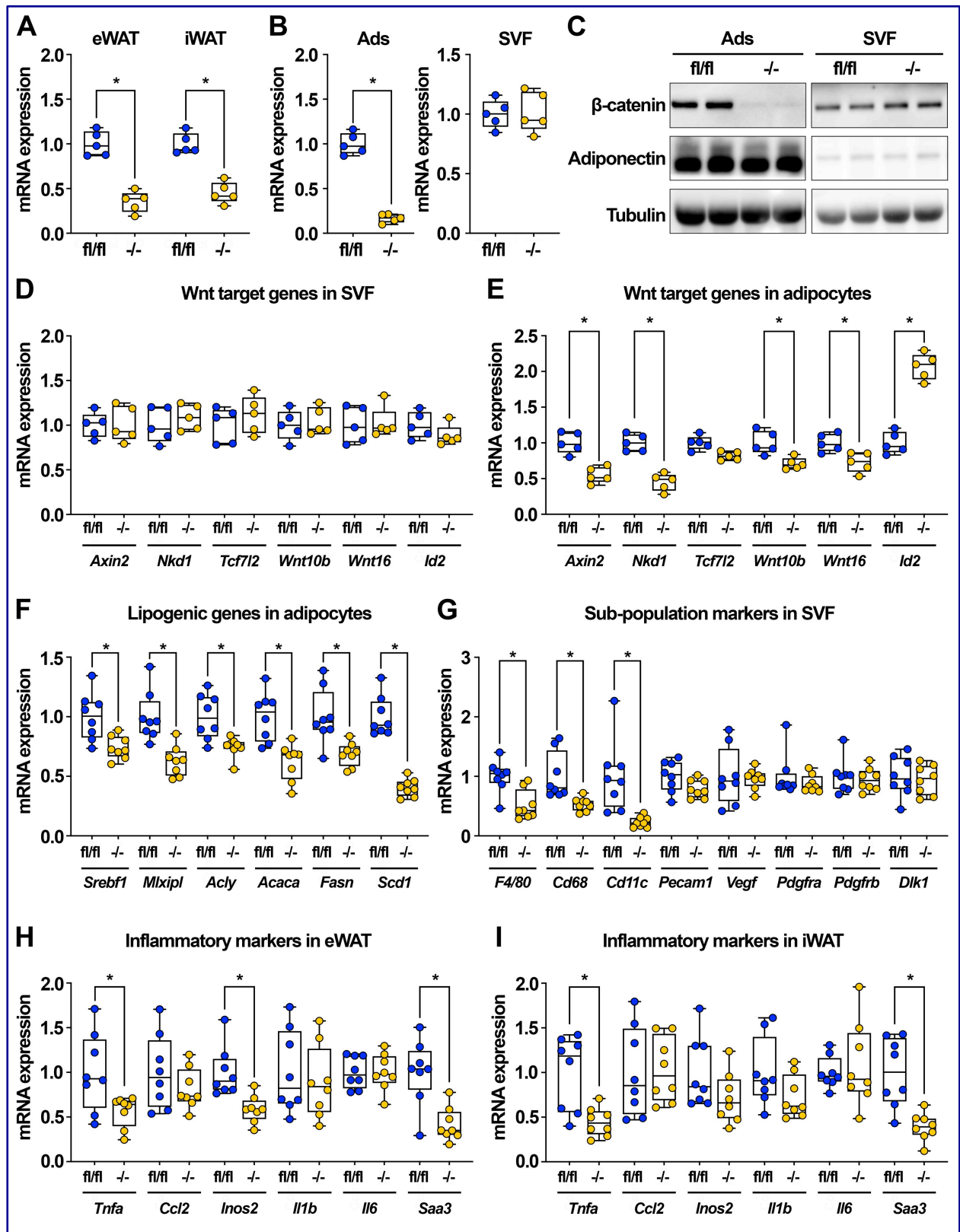
**Figure 4.7. Diet-induced obesity overcomes compensatory up-regulation of Wnt/β-catenin signaling in SVF of knockout mice.** (**A**) *Ctnnb1* mRNA expression in eWAT and iWAT of *β-cat*$^{fl/fl}$ and *β-cat*$^{-/-}$ mice fed HFD for 28 weeks (n = 5). (**B**) *Ctnnb1* mRNA expression in isolated eWAT adipocytes and SVF of HFD-fed *β-cat*$^{fl/fl}$ and *β-cat*$^{-/-}$ mice (n = 5). (**C**) β-catenin protein expression in isolated eWAT adipocytes and SVF of *β-cat*$^{fl/fl}$ and *β-cat*$^{-/-}$ mice fed HFD; adiponectin and laminin shown as controls. (**D-E**) Wnt target gene expression in SVF and adipocytes isolated from eWAT of obese *β-cat*$^{fl/fl}$ and *β-cat*$^{-/-}$ mice (n = 5). (**F**) Lipogenic gene expression in eWAT adipocytes isolated from HFD-fed *β-cat*$^{fl/fl}$ and *β-cat*$^{-/-}$ mice (n = 8). (**G**) Expression of immune, endothelial, and stromal cell markers in SVF isolated from eWAT of obese *β-cat*$^{fl/fl}$ and *β-cat*$^{-/-}$ mice (n = 8). (**H-I**) Expression of inflammatory markers in whole eWAT and iWAT of *β-cat*$^{fl/fl}$ and *β-cat*$^{-/-}$ mice fed HFD (n = 8). RNA expression normalized to PPIA. Data presented as mean ± S.D. * indicates significance at $p < 0.05$.

**Supplemental Figure 4.7**



**Supplemental Figure 4.7.** (**A**) Daily food intake of male $\beta$-cat$^{fl/fl}$ and $\beta$-cat$^{-/-}$ mice fed HFD for 24 weeks. (**B**) ITT area under the curve analysis of male $\beta$-cat$^{fl/fl}$ and $\beta$-cat$^{-/-}$ HFD-fed mice. (**C**) Pancreas weights at time of sacrifice of HFD-fed mice (n = 5, n = 11). (**D-E**) mRNA expression of adipokines in eWAT and iWAT of HFD-fed mice (n = 8). (**F**) Immunoblot and densitometry quantification of UCP1 and $\beta$-catenin protein expression in BAT isolated from HFD-fed $\beta$-cat$^{fl/fl}$ and $\beta$-cat$^{-/-}$ mice (n = 4). Data presented as mean $\pm$ S.D. * indicates significance at $p < 0.05$.

**Figure 4.8.**
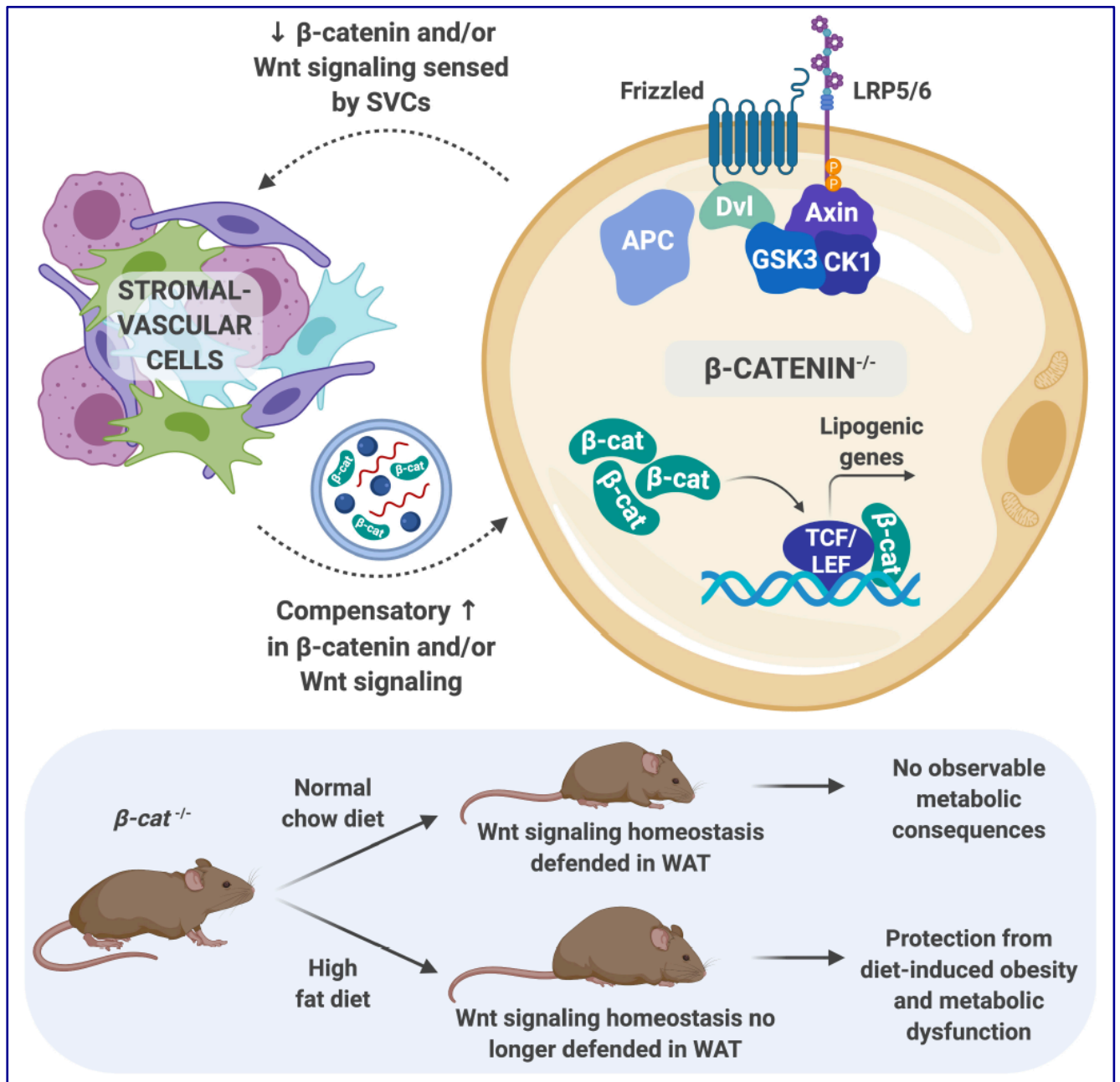


**Figure 4.8. Schematic depicting the consequences of adipocyte-specific deletion of β-catenin.** Canonical Wnt/β-catenin signaling is required for coordinate regulation of adipocyte lipogenesis and fatty acid desaturation. In chow-fed mice, loss of adipocyte β-catenin is sensed and compensated for by neighboring stromal cells to defend tissue-wide canonical Wnt signaling homeostasis. Chronic overnutrition overrides this compensatory mechanism, revealing that adipocyte-specific β-catenin knockout mice are protected from diet-induced obesity and metabolic dysfunction.

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# CHAPTER V
## Discussion and Future Perspectives

The role of Wnt signaling in mesenchymal stem cell fate determination and differentiation is well-established[1-4]. Indeed, the preponderance of data within the field of adipose biology to date has characterized Wnt inhibition of adipogenesis and promotion of other cell fates, including osteoblastogenesis, chondrogenesis, and myogenesis[5-10]. However, increasing genetic evidence in humans has linked various Wnt pathway members to body fat distribution, obesity, and metabolic dysfunction[11-23]. In recent years, a flurry of studies in mice has uncovered compelling evidence suggesting that Wnt signaling plays important roles in adipocyte metabolism, particularly under obesogenic conditions[24-27]. Perhaps unsurprisingly, given the breadth and complexity of this pathway and differences in experimental models, approaches and results, the exact functional roles of the Wnt pathway and its underlying molecular mechanisms in this context remain unclear.

Thus, against this backdrop, my doctoral work presented herein set out to unravel the unique contributions of Wnt pathway members to adipocyte metabolism. To this end, I generated novel cultured cell and mouse models to functionally characterize the differential roles of two key pathway members, Wntless (*Wls*) and β-catenin (*Ctnnb1*), in terminally-differentiated adipocytes. Deletion of Wntless, a dedicated intracellular chaperone for Wnts, allowed me to investigate the functional roles of both canonical and non-canonical Wnts secreted from adipocytes, whereas loss of β-catenin allowed me to specifically interrogate the contribution of canonical Wnt signaling to adipocyte function.

These studies revealed for the first time that loss of adipocyte-derived Wnts or canonical Wnt/β-catenin signaling in adipocytes coordinately down-regulates lipogenic gene expression, resulting in impaired *de novo* lipogenesis (DNL) and fatty acid

monounsaturation. Further, effects on lipid metabolism are mediated by repression of *Srebf1* and *Mlxipl*, known transcriptional regulators of DNL enzymes. *In vivo*, Wntless and β-catenin do not appear to influence global metabolism in mice maintained on normal chow diet (NCD). However, deeper investigation reveals that adipose tissues are able to defend adipocyte-specific loss of Wntless or β-catenin by compensatory up-regulation of Wnt signaling in surrounding stromal-vascular cells (SVC). Finally, long-term overnutrition overrides this compensatory mechanism, such that both *Wls*^{-/-} and *β-cat*^{-/-} mice are resistant to diet-induced obesity and protected from metabolic dysfunction. These novel findings underscore the critical importance of Wnt signaling in adipocyte metabolism, and also raise some fascinating questions and lines of inquiry worthy of future investigation.

**Does Wnt signaling directly regulate lipogenic gene transcription or are the effects mediated indirectly by *Srebf1* and *Mlxipl* activity?**

Our studies reveal for the first time that both Wntless and β-catenin are required for coordinate expression of a network of lipogenic genes, including *Acly*, *Acaca*, *Fasn*, *Scd1*, *Elovl6*, and *Elovl7*. Extensive studies in the liver have identified SREBP1c and ChREBP as key upstream transcriptional regulators of many DNL enzymes[28-30], and genome-wide ChIP-seq analysis in white adipose tissues (WAT) recently identified ChREBP binding sites on many genes related to metabolism, including *Acaca*, *Fasn*, and *Scd1*[31]. Thus, we hypothesized that effects of Wnt signaling on lipogenesis are also mediated by these transcription factors. We found that mRNA and protein levels of both *Srebf1* and *Mlxipl* are suppressed in *Wls*^{-/-} and *β-cat*^{-/-} adipocytes, whereas other transcription factors involved in adipogenesis and adipocyte metabolism, including *Pparg* and *Cebpa*, are unaffected. It is important to note that the consistent results observed in both Wntless and β-catenin knockout models suggest that adipocyte-derived Wnts largely signal through the canonical pathway to mediate effects on lipid metabolism. Further, overexpression of ChREBP or SREBP1c is sufficient to induce SCD1 in *β-cat*^{fl/fl} adipocytes, and to partially rescue SCD1 mRNA and protein levels in *β-cat*^{-/-} cells. Thus, loss of Wnt signaling results in impaired DNL at least in part due to suppression of *Mxlipl* and *Srebf1*.

A compelling question raised by these results is whether Wnt signaling directly regulates lipogenic gene transcription, or whether the effects are mediated indirectly by *Srebf1* and *Mlxipl* activity. Since β-catenin activates TCF/LEF proteins to mediate target gene transcription, a few possibilities exist. Perhaps β-catenin/TCF/LEF complexes bind directly to lipogenic genes to promote transcription; indeed, Geoghegan *et al.* reported Tcf7l2 binding sites within 1 kilobase of the transcriptional start site of many lipogenic genes, including *Acly*, *Fasn*, and *Scd1*[24]. It is also possible that β-catenin/TCF/LEF complexes bind to and activate *Srebf1* and *Mlxipl*, which in turn regulate transcription of lipogenic genes. Analyses of the promoter regions of *Srebf1* and *Mlxipl*, as well as DNL enzymes like *Fasn* and *Scd1*, for potential TCF binding motifs may provide important clues regarding Wnt signaling regulation of these genes. ChIP-seq analyses of β-catenin binding sites in adipocytes will be required to identify the genes that are directly transcriptionally regulated by Wnt signaling. Decreased promoter occupancy of these genes in *β-cat*^{-/-} adipocytes and impaired expression of *Srebf1*, *Mlxipl*, or lipogenic genes following mutation of β-catenin binding sites will definitively prove the mechanisms underlying Wnt-mediated transcriptional regulation of lipid metabolism in adipocytes.

Pharmacological activation of Wnt signaling or enforced expression of β-catenin in *Srebf1*^{-/-} or *Mlxipl*^{-/-} adipocytes will also provide valuable insights into the requirement of these transcription factors for Wnt regulation of DNL enzymes. Experiments to determine whether there is increased expression of DNL enzymes above baseline with Wnt pathway activation in SREBP1c- or ChREBP-deficient cells would test if these transcription factors are required for basal or Wnt regulation of lipogenic genes in adipocytes. However, if Wnt activation does not up-regulate lipogenic gene expression in the absence of SREBP1c- or ChREBP, it is more likely that these transcription factors exclusively mediate the effects of Wnt signaling on DNL enzymes. These experiments and others will be necessary to more clearly define the mechanisms underlying Wnt regulation of lipogenesis in adipocytes.

**Are other adipocyte functions regulated by Wnt signaling?**

To obtain a global view of the transcriptional pathways regulated by canonical Wnt signaling in terminally differentiated cells, we performed RNA-seq analyses of $\beta$-cat$^{fl/fl}$ and $\beta$-cat$^{-/-}$ adipocytes. Gene set enrichment analyses (GSEA) of differentially expressed genes identified several metabolic pathways as being down-regulated with β-catenin deletion, including those involved in glycolysis, oxidative phosphorylation, and fatty acid, cholesterol, and bile acid metabolism. Pathways up-regulated by loss of β-catenin are largely related to inflammatory response, including TNFα, interferon-α, interferon-γ, and IL6/JAK/STAT3 signaling. Of note, Geogheghan *et al.* recently reported that Tcf7l2 also regulates many metabolic pathways in WAT, including cholesterol, fatty acyl-CoA and TAG biosynthesis and amino acid, carbohydrate, and peroxisomal lipid metabolism[24].

Since lipogenesis and lipid accumulation are two specialized functions of adipocytes, we focused our investigations on down-regulation of the DNL pathway in $\beta$-cat$^{-/-}$ and *Wls*$^{-/-}$ adipocytes. Although we did not observe effects on lipolysis, β-oxidation, or glucose uptake in $\beta$-cat$^{-/-}$ or *Wls*$^{-/-}$ cells, it is likely that Wnt signaling influences other metabolic pathways in adipocytes. Indeed, interactions between effects on distinct pathways may, in part, explain differences observed between reported phenotypes of impaired Wnt signaling in adipocytes[24-27]. Thorough characterization of the effects of impaired Wnt signaling on other cellular functions, including inflammation, glycolysis, mitochondrial activity, and cholesterol synthesis, may provide particularly important insights into the protective phenotypes observed in knockout mice with long-term high fat diet (HFD) feeding.

One pathway found to be down-regulated in both $\beta$-cat$^{-/-}$ adipocytes and preadipocytes that may be of particular interest is mTORC1 signaling. mTORC1, which is a central integrator of several distinct signaling molecules and pathways, activates SREBP1c to promote lipogenesis in adipocytes[32,33]. Thus, it is possible that Wnt signaling regulation of *Srebf1* and downstream lipogenic genes is mediated in part by mTORC1 activity. Evaluation of basal mTORC1 signaling in $\beta$-cat$^{-/-}$ and *Wls*$^{-/-}$ adipocytes compared to control cells will reveal whether this pathway is indeed

suppressed by impaired Wnt signaling. If enforced expression of mTORC1 signaling is sufficient to restore *Srebf1* levels to that of control cells, this pathway may be an important mediator of Wnt pathway effects on adipocyte lipid metabolism.

**What is the compensatory mechanism by which stromal-vascular cells sense and respond to loss of adipocyte-specific Wnt signaling?**

To date, virtually all models of impaired Wnt signaling in adipose tissues have reported a lack of phenotype in mice maintained on chow diet[24-26]. Consistent with these prior studies, adipocyte-specific Wntless or β-catenin deletion does not overtly influence body composition or whole-body metabolism. Surprisingly, given the effects of impaired Wnt signaling on lipogenesis in cultured adipocytes, DNL gene expression is not influenced in whole WAT or isolated adipocytes from *β-cat*^{-/-} and *Wls*^{-/-} mice.

Although previous studies did not probe further into a lack of detectable phenotype in chow-fed mice, our experiments have uncovered a striking compensatory mechanism by which adipose tissues defend adipocyte-specific loss of Wnt signaling. In *Wls*^{-/-} adipocytes, despite efficient ablation of Wntless at the DNA, RNA and protein levels, downstream Wnt signaling and lipogenic gene expression is maintained. Unexpectedly, surrounding SVF cells broadly up-regulate mRNAs of Wnts and their transcriptional targets, including *Cmyc*, *Tcf7l2*, and *Ppard*. Our observations in the β-catenin model are even more interesting: although β-catenin is completely recombined at the genomic level in *β-cat*^{-/-} adipocytes, *Ctnnb1* mRNA is only reduced by ~50% and over 80% of β-catenin protein remains. Paradoxically, we found dramatic induction of β-catenin mRNA and protein within the SVF of *β-cat*^{-/-} mice. Consistent with β-catenin expression patterns, many Wnt targets, including *Axin2*, *Nkd1*, *Tcf7l2*, *Wnt10b*, and *Wnt16*, were not altered in *β-cat*^{-/-} adipocytes; in contrast, these genes were significantly up-regulated in the SVF with β-catenin deficiency. Data from these two independent models support the compelling conclusion that impaired Wnt signaling in adipocytes is sensed by and compensated for by surrounding cells. Indeed, we contend that this compensatory mechanism may explain the lack of observable phenotypes under standard conditions in other mouse models that have been developed thus far to study Wnt signaling in adipocytes.

A fascinating unanswered question raised by our observations is undoubtedly the exact underlying mechanism by which Wnt signaling is sensed and up-regulated in the SVF of Wntless or β-catenin knockout animals. A number of possibilities emerge to explain this phenomenon. For example, impaired Wnt signaling may alter SVF cellular composition, enriching for subpopulations that have highly active Wnt signaling. Indeed, we observe elevated expression of macrophage markers, including *F4/80*, *Cd68*, and *Cd11c*, in SVF of *β-cat^{-/-}* mice, suggesting increased macrophage numbers. Flow cytometry analyses using CD45 and CD31 antibodies did not yield observable differences in proportions of immune or endothelial cells, respectively, between control and knockout SVF in either model; however, these cell surface markers may not be specific enough to highlight the regulated cell population. Thus, future experiments using more specific markers, including *F4/80* or *Cd11c*, will be required to more accurately quantify macrophage numbers in SVF of *β-cat^{-/-}* and *Wls^{-/-}* mice. Immunofluorescence staining using antibodies for F4/80 or CD68 to visualize macrophages may also provide suitable quantification of cell numbers in WAT tissue sections from control and knockout mice.

Alternatively, a particular cell type within the SVF may detect loss of Wnt signaling from adipocytes and respond by increasing its own Wnt or β-catenin production to maintain signaling homeostasis within the tissue. Evaluation of gene expression within CD45^{+}, CD31^{+}, and CD45^{-}/CD31^{-} populations isolated by fluorescence-activated cell sorting reveals that CD45^{-}/CD31^{-} stromal cells, which include adipose stem cells, committed preadipocytes and pericytes, are responsible at least in part for the observed compensation. Since β-catenin and Wnt proteins are broadly expressed, further studies using unbiased single-cell RNA-seq analysis of SVF sub-populations from control and knockout mice may prove to be more successful in specifically identify the cell populations that are up-regulating their Wnt signaling.

Of course, it is also possible and perhaps likely that the compensatory mechanism is more complex, involving cross-talk between multiple cell populations. For example, Chen *et al.* reported that in HFD-fed mice, β-catenin deletion suppresses WAT expression of Saa3, an adipokine known to activate macrophages[26]. Further, they contend that down-regulation of Saa3 results in impaired macrophage recruitment and

decreased preadipocyte proliferation[26]. Although we found that macrophage markers and Saa3 are down-regulated in WAT of HFD-fed *β-cat*^{-/-} mice, we observed only mild up-regulation in eWAT and no change in iWAT of chow-fed mice. Thus, Saa3 probably does not mediate the compensatory effects observed in SVF of *β-cat*^{-/-} and *Wls*^{-/-} mice. However, unbiased transcriptome analyses of WAT and isolated SVF from chow-fed control and knockout animals may provide insights into secreted factors that are involved in cross-talk between adipocytes and surrounding cell populations. These experiments will shed much-needed light on the mechanism by which adipocyte-specific loss of Wnt signaling is sensed and defended.

Interestingly, we also found that diet-induced obesity overrides this compensatory mechanism in both models: SVF of HFD-fed *Wls*^{-/-} and *β-cat*^{-/-} mice no longer exhibit elevated Wnt signaling, and consequently, isolated adipocytes from these mice have decreased Wnt target and lipogenic gene expression. Additionally, β-catenin mRNA and protein expression is negligible in knockout adipocytes from HFD-fed mice. Long-term HFD feeding has well-established effects on adipose tissue inflammation, remodeling and SVF composition [34-36]. These changes likely contribute to the loss of compensation in HFD-fed knockout mice, but further experiments are required to fully understand why this occurs, revealing a phenotype in obese mice.


## Is β-catenin delivered back to deficient adipocytes from stromal-vascular cells by extracellular vesicles?

Although secretion of Wnts is blocked in *Wls*^{-/-} adipocytes, the remaining signaling machinery, including β-catenin, is intact. Thus, a plausible mechanism for tissue-wide compensation in this model emerges readily. One might imagine that loss of adipocyte-derived Wnts is sensed by neighboring cells, which in turn respond by increasing their own Wnt production and secretion. Wnts from SVF cells are then able to act in a paracrine fashion to maintain downstream signaling in deficient adipocytes.

A model of compensation in β-catenin-deficient adipocytes is more complicated. The relatively high levels of β-catenin mRNA and protein observed in knockout adipocytes suggests the intriguing possibility that β-catenin is being delivered back to deficient cells, perhaps by SVF-derived small extracellular vesicles (sEV). Many

different cell types are known to secrete sEVs containing proteins, lipids, and genetic material; these sEVs mediate a unique mechanism for intercellular communication and have diverse and intricate effects on recipient cells[37,38]. This is of particular interest in the context of adipose biology, since WAT-derived sEVs have widespread effects[39-42]. sEVs are also secreted by various SVCs, including macrophages, endothelial cells, and stromal cells, and can have profound effects on metabolism[43-45]. Thus, sustained expression of β-catenin in knockout adipocytes may be the result of mRNA transfer and subsequent translation, or more direct trafficking of protein.

The possibility of direct β-catenin protein transfer is supported by recent findings by Crewe *et al.*, who reported that sEVs derived from endothelial cells mediate cross-talk between adipocytes and SVF cells populations[45]. Indeed, they described a phenomenon strikingly similar to the one we observe in chow-fed *β-cat*$^{-/-}$ mice. Despite efficient genetic ablation of *Cav1* (caveolin-1), cav1 protein is readily detectable in deficient adipocytes; this was found to be due to sEV-mediated trafficking of cav1 protein from surrounding endothelial cells back to *Cav1*$^{-/-}$ adipocytes. In addition to cav1, proteomic analyses of isolated endothelial sEVs identified members of the Wnt signaling pathway, including β-catenin.

Thus, it is possible that a similar mechanism explains the sustained expression of β-catenin protein in knockout adipocytes. Evaluation of β-catenin expression in sEVs secreted from cultured SVF of control and knockout mice will provide insight into the potential trafficking of β-catenin mRNA or protein. If sEVs from SVF of *β-cat*$^{-/-}$ mice contain higher amounts of β-catenin, this will provide compelling evidence for the delivery of β-catenin protein back to deficient adipocytes. Alternatively, SVF of knockout mice may secrete larger quantities of sEVs in order to maintain β-catenin expression in *β-cat*$^{-/-}$ adipocytes.

Once a potential SVC subpopulation responsible for compensation is identified, studies ablating β-catenin from that particular cell type will be required to definitively establish a causal relationship. For example, if future studies provide compelling evidence for macrophages being the source of β-catenin up-regulation, experiments using different Cre drivers to simultaneously delete β-catenin from both adipocytes and

macrophages will be required; loss of compensation in these studies will conclusively define an adipocyte-SVF axis of Wnt regulation.

The animal models presented in this dissertation both used constitutively expressed adiponectin-Cre. We had originally bred *β-cat*$^{fl/fl}$ mice to a tamoxifen-inducible adiponectin-Cre$^{ER}$ model, but sustained β-catenin expression in knockout mice led us to believe that a lack of phenotype in chow-fed mice was due to inefficient tamoxifen-induced gene recombination. Thus, all future studies were conducted using the constitutively active adiponectin-Cre. In hindsight, it is likely that we were observing the same compensatory mechanism later found with constitutive loss of β-catenin or Wntless.

Indeed, inducible deletion of adipocyte-specific Insig-1, a negative regulator of SREBP1c transcriptional activity, blocks DNL gene expression acutely[46]. However, subsequent diminished efflux of citrate from mitochondria triggers production of reactive oxygen species, which activate mTORC1. Thus, an mTORC1-mediated compensatory mechanism rapidly restores SREBP1c and downstream lipogenic enzyme expression less than 72 hours after gene deletion. This study highlights the importance of maintaining lipogenic gene expression in adipocytes and may shed light on possible reasons that adipose tissues rigorously defend Wnt signaling. Thus, future studies using mice with inducible loss of Wntless or β-catenin may provide critical insights into the timing and mechanism of compensation.


**How does adipocyte-specific loss of Wnt signaling provide functional protection from diet-induced obesity?**

To date, studies of Wnt signaling in mature adipocytes have consistently revealed metabolic phenotypes with long-term HFD feeding[24-26]. Indeed, we found that both *Wls*$^{-/-}$ and *β-cat*$^{-/-}$ mice are resistant to diet-induced obesity and are protected from subsequent metabolic dysfunction. Specifically, HFD-fed Wntless knockout mice are characterized by decreased fat and increased lean mass, improved glucose homeostasis, and increased circulating insulin levels. *β-cat*$^{-/-}$ mice fed HFD demonstrate decreased weight gain and fat mass, significantly improved glucose homeostasis, and decreased circulating TAG and glucose-stimulated insulin release. Both knockout

models have decreased lipogenic gene expression within WAT depots and are protected from hepatosteatosis.

A point of interest is the mechanism underlying protection from diet-induced obesity and metabolic dysfunction that is afforded to mice lacking adipocyte-specific Wnt signaling. Although both *Wls*$^{-/-}$ and *β-cat*$^{-/-}$ mice exhibit decreased fat mass and liver TAG accumulation compared to their control counterparts, food intake is unaffected. If energy intake is the same but it is not being stored in WAT or liver, logic dictates that *Wls*$^{-/-}$ and *β-cat*$^{-/-}$ mice utilize more energy compared to controls. Thus, energy expenditure, oxygen consumption, and physical activity should be quantitatively measured in future cohorts of HFD-fed mice using the Comprehensive Lab Animal Monitoring System (CLAMS).

Another possible explanation for decreased energy storage in HFD-fed mice may be increased heat loss via the skin; global *Scd1*$^{-/-}$ and keratinocyte-specific *Scd*$^{-/-}$ mice are resistant to diet-induced obesity in part due to decreased insulation and thus increased heat loss through the skin[47-49]. Since obese *Wls*$^{-/-}$ and *β-cat*$^{-/-}$ mice exhibit decreased SCD1 expression in WAT and isolated adipocytes, it is possible that SCD1 expression is also suppressed in dermal WAT, leading to up-regulation of thermogenic pathways at the expense of nutrient utilization. Quantification of dermal adipocyte lipids and measurement of thermogenic genes will provide insights into the contribution of dermal WAT lipogenesis to the observed phenotype. Although unlikely since a difference in fat mass only emerges after at least 20 weeks of HFD, it is also possible that *Wls*$^{-/-}$ and *β-cat*$^{-/-}$ mice absorb less lipids from the intestine. The presence of a malabsorptive phenotype can be tested by measuring fecal lipids in knockout mice either under basal conditions or after intragastric fat challenge.

In addition to improved glucose tolerance, *β-cat*$^{-/-}$ mice also demonstrate lower glucose-stimulated insulin concentrations and a trend toward improved systemic insulin sensitivity. Thus, protection from metabolic dysfunction in β-catenin knockout mice may be due to greater peripheral insulin sensitivity and glucose uptake. Measurement of phosphorylated AKT in WAT and muscle after insulin injection in HFD-mice may answer this question: increased pAKT levels in peripheral tissues of *β-cat*$^{-/-}$ mice will suggest

improved peripheral insulin sensitivity. Hyperinsulinemic-euglycemic clamps can also be used to quantitatively assess insulin sensitivity in tissues of control and knockout mice.

In contrast to *β-cat*^{-/-} mice, Wntless knockout mice exhibit increased circulating random-fed and glucose-stimulated insulin concentrations; pancreas weights are not altered. Interestingly, *Wls*^{-/-} mice also demonstrate increased liver weight, despite less hepatosteatosis observed by histological analysis. One possible explanation for this constellation of findings is the existence of a Wnt-regulated adipokine that signals to the pancreas to suppress insulin secretion under normal conditions. When this signal is removed by loss of adipocyte-derived Wnt secretion, elevated circulating insulin may act upon the liver to suppress gluconeogenesis, stimulating glycogen synthesis and storage, and increase liver weight. Thus, quantitative measurements of pancreatic beta cell mass and number, liver TAG and glycogen levels, and hepatic cell size and number in future HFD cohorts may uncover mechanisms by which Wntless deficiency improves glucose homeostasis in obese mice.

**Why does ablation of Wnt signaling confer resistance to diet-induced obesity and metabolic dysfunction?**

Characterization of two independent animal models has revealed that Wnt signaling homeostasis is rigorously defended by adipose tissues under standard nutritional conditions. This finding suggests that Wnt signaling is critically important for the proper function of mature adipocytes. Indeed, RNA-seq and GSEA analyses revealed that loss of β-catenin up-regulates inflammatory pathways and down-regulates many metabolic pathways, including those related to mitochondrial function and lipid and cholesterol metabolism.

Our molecular studies in cultured adipocytes also revealed that Wnt signaling regulates lipogenesis and fatty acid desaturation, two essential adipocyte functions. Indeed, Wnt signaling is required for expression of a network of lipogenic enzymes, including FASN and SCD1. FASN mediates synthesis of palmitic acid (C16:0) and SCD1 catalyzes its desaturation to palmitoleic acid (C16:1). Adipocytes are largely responsible for *in vivo* production of palmitoleic acid (C16:1), a fatty acid with insulin-sensitizing and anti-inflammatory effects[50,51]. In contrast, cytokine signaling and

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inflammation, predicted to be increased with β-catenin deletion, are associated with adipose tissue dysfunction, dyslipidemia, and insulin resistance[34-36,52]. Thus, one might reasonably expect that functional loss of Wnt signaling with HFD would have catastrophic effects on vital adipocyte functions, including lipid synthesis and storage, and promote WAT inflammation and whole-body metabolic dysfunction.

However, we and others have found that adipocyte-specific deletion of β-catenin or Wntless in mice provides protection from diet-induced obesity and subsequent metabolic dysfunction[26]. Perhaps the most compelling question that has emerged from my doctoral work arises from these counter-intuitive findings and is partly philosophical in nature: why does adipocyte-specific loss of Wnt signaling confer resistance to diet-induced obesity and metabolic function?

Canonical Wnt signaling has been identified as a significant regulator of adipocyte lipogenesis and fatty acid desaturation. Thus, the answer to this question may lie in clearer understanding of the relative physiological importance of adipocyte DNL in global metabolism, particularly under obesogenic conditions. Unfortunately, genetic manipulation of DNL enzymes in adipocytes has thus far produced conflicting effects on systemic metabolism. Global *Scd1*^{-/-} mice fed HFD are characterized by reduced weight gain and fat accumulation and increased oxygen consumption and insulin sensitivity compared to controls; these effects persist despite significantly increased food intake by knockout mice[47]. Surprisingly, adipocyte-specific SCD1 deletion does not confer resistance to diet-induced obesity or otherwise appear to influence systemic metabolism[53]. Although mouse models of *Srebf1* deletion in adipose tissues have not been reported to date, adipocyte-specific loss of ChREBP results in altered WAT fatty acid composition, impaired glucose transport and profound insulin resistance[54]. Inactivation of *Acaca* using the aP2-Cre causes reduced WAT accumulation in a fasting/refeeding paradigm[55]; however, this Cre is known to leak outside of adipose tissues and consistent with this, *Acaca*^{-/-} mice also exhibit growth retardation, confounding these results.

Mice with adipocyte-specific deletion of FASN are characterized by increased energy expenditure, browning of WAT, and resistance to diet-induced obesity[56]. Indeed, loss of FASN in WAT of HFD-fed mice using an inducible adiponectin-Cre promotes

browning and UCP1 expression in iWAT and is accompanied by improved glucose homeostasis. Effects on browning are mediated by increased sympathetic outflow, as evidenced by elevated tyrosine hydroxylase (NH) and neuropeptide Y (NPY) protein expression in iWAT. Interestingly, both *Th* (tyrosine hydroxylase) and *Npy* (neuropeptide Y) were identified by our RNA-seq dataset as up-regulated in $\beta$-cat^{−/−} adipocytes. Although we do not observe obvious signs of browning in histological sections of iWAT from HFD-fed $\beta$-cat^{−/−} mice, staining for UCP1, TH, and NPY and measurement of protein levels by immunoblotting will provide more quantitative assessments of browning in WAT of these mice.

The discordant results observed in these different mouse models of impaired adipose tissue DNL highlight the complexities of animal physiology. Nevertheless, detailed analyses of cytokines, circulating fatty acid species like palmitoleic acid, and measurement of oxygen consumption and energy expenditure in HFD-fed mice may yield clues as to the mechanisms by which loss of Wnt signaling confers protection from metabolic dysfunction. Further, an unbiased view of gene expression changes in control and knockout *Wls* and $\beta$-cat mice may shed light on novel metabolic pathways that are influenced *in vivo*.

Thus, although the roles of adipocyte Wnt signaling and lipogenesis in systemic metabolism remain unclear, the findings presented in this dissertation contribute novel knowledge to our burgeoning understanding of Wnt signaling in mature adipocyte function. Further, given the evolutionary conservation and ubiquitous nature of this pathway, it is highly likely that our findings will be widely applicable in the biology of diverse cell types.

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