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**Ube2i deletion in adipocytes causes lipoatrophy in mice**

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**ABSTRACT**

Objective: White adipose tissue (WAT) expansion regulates energy balance and overall metabolic homeostasis. The absence or loss of WAT occurring through lipodystrophy and lipoatrophy contributes to the development of hepatic steatosis and insulin resistance. We previously demonstrated that sole small ubiquitin-like modifier (SUMO) E2-conjugating enzyme Ube2i represses human adipocyte differentiation. The role of Ube2i during WAT development remains unknown.

Methods: To determine how Ube2i impacts body composition and energy balance, we generated adipocyte-specific Ube2i knockout mice (Ube2ia-KO). CRISPR/Cas9 gene editing inserted loxP sites flanking exons 3 and 4 at the Ube2i locus. Subsequent genetic crosses to Adipoq-Cre transgenic mice allowed deletion of Ube2i in white and brown adipocytes. We measured multiple metabolic endpoints that describe energy balance and carbohydrate metabolism in Ube2ia-KO and littermate controls during postnatal growth.

Results: Surprisingly, Ube2ia-KO mice developed hyperinsulinemia and hepatic steatosis. Global energy balance defects emerged from dysfunctional WAT marked by pronounced local inflammation, loss of serum adipokines, hepatomegaly, and near absence of major adipose tissue depots. We observed progressive lipoatrophy that commences in the early adolescent period.

Conclusions: Our results demonstrate that Ube2i expression in mature adipocytes allows WAT expansion during postnatal growth. Deletion of Ube2i in fat cells compromises and diminishes adipocyte function that induces WAT inflammation and ectopic lipid accumulation in the liver. Our findings reveal an indispensable role for Ube2i during white adipocyte expansion and endocrine control of energy balance.

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**Keywords** Ube2i; Lipodystrophy; Adipose tissue; Lipid metabolism

1. INTRODUCTION

White adipocytes sequester lipids and protect peripheral metabolic tissues from ectopic lipid accumulation. Consequently, the failure of integral lipid metabolism responses and reduced adipose tissue expandability during chronic states of positive energy balance contribute to the development of insulin resistance, obesity, and fatty liver disease [1]. Similar metabolic abnormalities develop in patients with lipodystrophy where a lack of adipose tissue does not allow storage of surfeit energy as lipids, leading to insulin resistance, hepatic steatosis, and dyslipidemia [2]. Therefore, healthy adipose tissue development mediates key aspects of metabolic homeostasis. Physiologic WAT expansion occurs through both increased adipocyte size (hypertrophy) and number (hyperplasia). White adipocyte differentiation requires a cascade of transcription factors that activate PPARγ, the master regulator of adipocyte differentiation [3]. Adipocyte differentiation requires PPARγ [4] to partner with distinct transcriptional co-regulators that coordinate brown and white adipocyte-specific gene expression [5,6]. Gene deletion or tissue-specific disruption of PPARγ impairs adipogenesis and results in severe lipodystrophy [4,7—9]. The mechanisms that enable adipose tissue development have broad basic implications for understanding energy balance disorders.

We previously showed Ube2i deletion in human subcutaneous pre-adipocytes accelerated fat cell differentiation [10]. In this study, we generated an adipocyte-specific Ube2i knockout (Ube2ia-KO) mouse to investigate how Ube2i regulates whole-body energy balance. Surprisingly, WAT mass failed to expand in male and female Ube2ia-KO mice. Progressive lipoatrophy and compromised adipocyte function likely provoked WAT inflammation and ectopic lipid accumulation leading to insulin resistance, impaired brown adipose tissue (BAT) function, and intolerance to cold temperatures. Taken together, our findings reveal critical roles for Ube2i in adipocyte function and expansion.

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Abbreviations

| Abbreviation | Description |
|--------------|-------------|
| a-KO         | adipocyte-specific knockout |
| BAT          | brown adipose tissue |
| CGL          | congenital generalized lipodystrophy |
| CLAMS        | Comprehensive Lab Animal Monitoring System |
| CRISPR       | Clustered Regularly Interspaced Short Palindromic Repeats |
| DAPI         | 4',6-diamidino-2-phenylindole |
| ELISA        | enzyme linked immunosorbent assay |
| FGF21        | fibroblast growth factor 21 |
| GFP          | green fluorescent protein |
| gWAT         | gonadal white adipose tissue |
| H/E          | Hematoxylin and Eosin |
| IP           | intraperitoneal |
| IxP          | locus of X-over P1 |
| PBS          | Phosphate Buffered Saline |
| PPARγ        | Peroxisome proliferator-activated receptor gamma |
| RER          | respiratory exchange ratio |
| sgRNA        | single guide RNA |
| IssDNA       | long single-stranded oligodeoxynucleotide donor |
| SUMO         | Small Ubiquitin-like Modifier |
| SVF          | stromal vascular fraction |
| TG           | triglyceride |
| Ube2i        | Ubiquitin conjugating enzyme E2 I |
| WAT          | White adipose tissue |

2. METHODS

2.1. Animals

All procedures with animals were approved by the Institutional Animal Care and Use Committee of Baylor College of Medicine (animal protocol AN-6411). Experimental animals received humane care according to criteria in the “Guides for the Care and Use of Laboratory Animals” (8th edition, revised 2011). Experimental animals were housed (no more than four per cage) in a barrier-specific pathogen-free animal facility with 12-h dark-light cycle and free access to water and normal chow (Harlan Laboratories 2920X). All experiments were conducted using littermate-controlled male and female mice maintained on a C57BL/6J (Harlan Laboratories 2920X). All experiments were conducted using a balance of male and female mice, with at least four per cage, in a barrier-specific pathogen-free animal facility with 12-h dark-light cycle and free access to water and normal chow (Harlan Laboratories 2920X). All experiments were conducted using littermate-controlled male and female mice maintained on a C57BL/6J background. At the end of experiments, mice were euthanized by cervical dislocation while under isoflurane anesthesia. After euthanasia, tissues were collected, flash-frozen in liquid N2, and stored at −80 °C until use. All experiments adhered to ARRIVE Guidelines.

2.2. Generation of a conditional Ube2i allele

Ube2irKO mice were generated by the Genetically Engineered Rodent Models Core at BCM using previously established methods [11]. We employed Cas9-initiated homology-driven repair (HDR) using a pair of single guide RNAs (sgRNAs) coupled with a long single-stranded oligodeoxynucleotide donor (lssDNA) to target events, only sgRNAs predicted to have off-target sites with at least 60% identity of the loxP sequences and 60% identity of the donor sequence were used to target Cas9 endonuclease activity to intronic sequences flanking exons 3 and 4. Two hundred C57BL/6J/C0 mice were crossed with CAG-CreER(T2) (Jackson Laboratory #028020) to generate adipocyte-specific Ube2iKO mice. iWAT and gWAT depots were digested in phosphate buffered saline (PBS) containing collagenase D (Roche, 1.5 U/mL) and dispase II (Sigma, 2.4 U/mL) supplemented with 10 mM CaCl2 at 37 °C for 45 min. The primary cells were filtered twice through 70 μm strainers and centrifuged to collect the SVF. For adenovirus transduction, SVF cells were incubated with adenoviral Cre recombinase or green fluorescent protein (GFP) in DMEM/F12 medium containing Glutamax (ThermoFisher) and 10% fetal bovine serum (FBS) for 24 h. After replacing the medium once, cells were cultured for 48 h before lystate preparation. Adenoviruses expressing Cre recombinase or GFP were provided by the BCM Gene Vector Core. Similarly, IxP SVF cells were isolated from CAG-CreEYFP(Ube2irKO) mice, treated with 1 μM 4-hydroxytamoxifen for 48 h followed by differentiation for eight days.

2.3. Genotyping

DNA extracted from mouse ear clips was used in PCR reactions with primers designed to detect the 5’ (P1 - AGGTAGGGGTGCTTAGAGG, P2 - GGTTTATTGTGCAATAGGG) and 3’ (P3 - CAAGTCCCAAGGATAGTCGTGC, P4 - CAGCTCAGACCTGGCCTTAC) IxP sequences and run on agarose gels. Cre transgenic mice were genotyped according to protocols provided by the Jackson Laboratory.

2.4. Antibodies and western blotting

Tissue and whole cell lysates were prepared in Protein Extraction Reagent (Thermo Fisher) supplemented with Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher). Immunoblotting was performed with lysates run on 4–12% Bis-Tris NuPage gels (Life Technologies) and transferred onto Immobilon-P Transfer Membranes (Millipore) followed by antibody incubation. Immunoreactive bands were visualized by chemiluminescence. The following antibodies were used for immunoblotting: α-HSP90 (Cell Signaling #4877), α-Ube2i (Cell Signaling #4786), α-ADPPOQ (Genetex #GTX112777), α-PPARγ (Cell Signaling #24433), α-Caspase-8 (Cell Signaling #4790), and α-Cleaved Caspase-8 (Cell Signaling #8592).

2.5. Cell culture

To validate Cre-inducible deletion of Ube2i, fibroblasts were isolated from the inguinal white adipose tissue (iWAT) stromal vascular fraction (SVF) of Ube2irKO mice. iWAT deposits were digested in phosphate buffered saline (PBS) containing collagenase D (Roche, 1.5 U/mL) and dispase II (Sigma, 2.4 U/mL) supplemented with 10 mM CaCl2 at 37 °C for 45 min. The primary cells were filtered twice through 70 μm strainers and centrifuged to collect the SVF. For adenovirus transduction, SVF cells were incubated with adenoviral Cre recombinase or green fluorescent protein (GFP) in DMEM/F12 medium containing Glutamax (ThermoFisher) and 10% fetal bovine serum (FBS) for 24 h. After replacing the medium once, cells were cultured for 48 h before lystate preparation. Adenoviruses expressing Cre recombinase or GFP were provided by the BCM Gene Vector Core. Similarly, IxP SVF cells were isolated from CAG-CreEYFP(Ube2irKO) mice, treated with 1 μM 4-hydroxytamoxifen for 48 h followed by differentiation for eight days.

2.6. Indirect calorimetry

Ube2irKO and littermate controls (Ube2ir+) were maintained on normal chow and housed at room temperature in Comprehensive Lab Animal Monitoring System (CLAMS) home cages (Columbus Instruments). Oxygen consumption, CO2 emission, energy expenditure, food and water intake, and activity were measured for six days (BCM Mouse Metabolic and Phenotyping Core). Mouse body weight was recorded,
and body composition examined by magnetic resonance imaging (Echo Medical Systems) prior to indirect calorimetry. Statistical analysis of energy balance was performed by ANCOVA with lean body mass as a co-variate using the CalR web-based tool [12].

2.7. Glucose and insulin tolerance tests
To determine glucose tolerance, mice were fasted for 16 h and glucose was administered (1.5 g/kg body weight) by intraperitoneal (IP) injection. To determine insulin tolerance, mice were fasted 4 h prior to insulin intraperitoneal injection (1.5 U/kg body weight). Blood glucose levels were measured by a handheld glucometer. Serum was collected after fasting and during glucose tolerance tests for insulin quantification.

2.8. ELISAs and lipid assays
Fed serum levels were used to measure insulin (#EZRMI-13K; Millipore), leptin (#90030; Crystal Chem), adiponectin (#KMP0041; Thermo Fisher), and free fatty acids (#SFA-1; ZenBio), and FGF21 (#MF2100; R&D Systems). Insulin levels during glucose tolerance tests were also measured by ELISA (Millipore). Hepatic triglyceride (TG) content was quantified by Thermo Fisher Scientific Triglycerides Reagent (#TR22421) and normalized per gram of liver tissue.

2.9. Histology
Formalin-fixed paraffin-embedded adipose and liver tissue sections were stained with hematoxylin and eosin (H/E) by the BCM Human Tissue Acquisition and Pathology Core. Images were captured using a Nikon Ci-L Brightfield microscope.

2.10. Fluorescence microscopy
Following differentiation, media was aspirated and 4% formaldehyde (Electron Microscopy Sciences) in PBS was immediately added for 30 min at room temperature. Excess paraformaldehyde was quenched with 100 mM ammonium chloride. Non-specific antibody blocking was bound by pre-incubating for 30 min in 2% bovine serum albumin in PBS/0.01% saponin (which was also used as an antibody diluent) at room temperature. Anti-perilipin antibody (GP-29, Progen) was diluted at a 1:1000 concentration in antibody diluent (Abcam) at room temperature. Anti-perilipin antibody binding was blocked by pre-incubating for 30 min in 2% bovine serum albumin in PBS/0.01% saponin (which was also used as an antibody diluent). AlexaFluor 647-conjugated anti-guinea pig secondary antibodies (Thermo Fisher) were used. Coverslips were then washed 3 times and incubated with secondary antibodies for 1 h at room temperature. AlexaFluor 647-conjugated anti-guinea pig secondary antibodies (Thermo Fisher) were used. Coverslips were then washed 3 times and incubated with lipid TOX green (1:1000, Thermo Fisher) and DAPI (10 μg/mL) in PBS for 45 min at room temperature. Slides were mounted with SlowFade Gold (ThermoFisher). Imaging was performed with the DeltaVision Core Image Restoration Microscope (GE Healthcare).

2.11. qPCR
Total RNA was extracted using the Direct-zol RNA MiniPrep kit (Zymo Research). cDNA was synthesized using iScript (Bio-Rad). Relative mRNA expression was measured with SsoAdvanced Universal Probes Supermix reactions read out with a QuantStudio 3 real-time PCR system (Applied Biosystems). TATA-box binding protein (Tbp) was the invariant control. Roche Universal Probe Gene Expression Assays were used as previously described [13].

2.12. Cold tolerance test
Six-month old male Ube2i<sup>+/−</sup> and Ube2i<sup>−/−</sup> mice were individually housed with water and exposed to cold temperature (4 °C) for 2.5 h. A temperature probe was placed subcutaneously on top of the intra-scapular BAT two days prior to the cold tolerance test. Temperature recordings were measured in duplicate at room temperature (time = 0) and during cold exposure every 30 min.

2.13. Statistical analyses
Statistical significance was assessed by unpaired Student’s t-test with a primary threshold for statistical significance set at p < 0.05. For gene expression data, statistical significance was assessed by multiple unpaired t-tests with a q-value < 0.05. Statistical analysis of energy balance was performed by ANCOVA with lean body mass as a co-variate and cumulative food intake by standard ANOVA using the CalR web-based tool [12]. All data are presented as mean ± standard error of the mean (SEM), unless otherwise stated.

3. RESULTS

3.1. Generation of conditional Ube2i knockout mice
Ube2i was expressed ubiquitously across all mouse tissues [14] and knockout strategies cause lethality and sterility [14–17]. To this end, we used CRISPR/Cas9 gene editing to generate a conditional Ube2i allele (Ube2i<sup>fl/fl</sup>) to explore tissue-specific roles for Ube2i. Loxp® sites flanking exons 3 and 4 of the Ube2i locus were introduced by homology-directed repair (Figure 1A) and the in vivo presence of loxP sites in the targeted regions was confirmed by genotyping of potential founder mice (Figure 1B). We verified that the loxP® sites targeted the Ube2i locus by transfecting Ube2i<sup>fl/fl</sup> iWAT-derived fibroblasts with adenovirus expressing Cre recombinase. Immunoblot analysis of whole cell lysates demonstrated near total deletion of UBE2I protein levels following Cre recombination compared to adenovirus GFP transductions (Figure 1C). To determine the cell autonomous effects of Ube2i deletion in adipocytes, CAG-Cre<sup>ER</sup> mice were crossed with Ube2i<sup>fl/fl</sup> mice and we isolated iWAT SVF cells for in vitro differentiation. Tamoxifen treatment knocked out UBE2I only in cells expressing CAG-Cre-Ube2i<sup>fl/fl</sup>®, regardless of differentiation status (Figure 1D). Although we observed efficient UBE2I protein depletion, inducible knockout of Ube2i left representative adipocyte differentiation genes unaffected (Figure 1E). However, we observed a dramatic increase in several brown/beige adipocyte marker genes, including Ucp1, Prdm16, Cidea, and Citld1, consistent with observations from human subcutaneous adipocytes [10]. Although Western blot and qPCR showed the expression of mature adipocyte markers, we observed fewer lipid droplets in Ube2i knockout adipocytes (Figure 1F). We also observed higher cleaved Caspase-8 levels indicative of dying adipocytes (Figure 1G), which suggests an important role for UBE2I in adipocyte survival.

To specifically study the in vivo effects of Ube2i deletion in mature fat cells, we generated adipose-specific Ube2i knockout mice (Ube2i<sup>−/−</sup>fat) by crossing Ube2i<sup>−/−</sup> animals with Adipoq-Cre transgenic mice (Figure 1H). Reproductive fitness and female nursing were unaffected by Ube2i<sup>−/−</sup>fat and all pups were viable and born at the expected Mendelian ratio. PCR analysis demonstrated Cre recombination of the Ube2i locus generated a deletion product (red arrow) in the gonadal WAT (gWAT) from Ube2i<sup>−/−</sup>fat mice that was absent in Ube2i<sup>−/−</sup> controls (Figure 1I). The full length, unrecombined product (green arrow) was also detected in Ube2i<sup>−/−</sup>fat, but at lower levels than control, suggesting contributions of Cre-negative cells in the SVF to the PCR product. Similarly, UBE2I protein was reduced in iWAT and brown adipose tissue (BAT) compared to Ube2i<sup>−/−</sup> controls (Figure 1J).

3.2. Adipocyte-specific Ube2i deletion impairs WAT expansion during postnatal growth
To determine the impact of adipocyte Ube2i loss in mice, we examined body weight (Figure 2A) and WAT mass (Figure 2B) at necropsy from
seven days to six months of age. As expected, Ube2i−/− control mice exhibited progressive expansion of WAT mass with increasing age (Figure 2B). Conversely, Ube2i+/− mice failed to expand WAT depots beginning at two months of age and by six months, WAT mass almost completely disappeared relative to Ube2i+/+ controls. Gene expression (Figure 2C) and histological analyses (Figure 2D) of WAT at one month of age revealed similar profiles between Ube2i+/− and Ube2i+/+ controls, suggesting adipocyte function and morphology are normal prior to impaired WAT expansion. Despite no effects on body weight gain (Figure 2E), magnetic resonance imaging showed reduced fat mass and increased lean mass in six-month-old Ube2i−/− male and female mice compared to controls (Figure 2F). Further examination of tissue weights at necropsy (Figure 2G) revealed grossly visible reductions (~80%) in iWAT and gWAT weights in male and female Ube2i−/− mice (Figure 2H). Reduced fat storage in primary WAT depots resulted in significantly higher liver weights associated with gross morphological changes indicative of ectopic lipid accumulation. Similarly, lipid accumulation in BAT was apparent at necropsy, with increased BAT weight detected in female Ube2i−/− mice. Collectively, these data demonstrate adipocyte-specific deletion of Ube2i impairs WAT expansion leading to redistribution of lipid storage.

3.3. Adipocyte-specific Ube2i deletion increases WAT inflammation and apoptosis

To assess the morphological changes associated with Ube2i knockout in adipose tissues, we performed H/E staining of gWAT and iWAT tissue sections in adult male and female mice. Pronounced immune cell infiltration was observed in gWAT of Ube2i−/− mice (Figure 3A), distinct from the commonly described crown-like structures typically associated with gWAT [18]. Similarly, we observed dispersed immune cell accumulation amongst stromal cells and large adipocytes in the iWAT of Ube2i−/− mice. Primary WAT depots from both sexes showed marked stroma invasion and few mature adipocytes. Consistent with reduced numbers of adipocytes, serum levels of adiponectin, leptin,
Figure 2: Adipocyte-specific Ube2i deletion impairs WAT expansion in male and female mice. (A) Body weight and (B) WAT weights (g) in combined male and female mice at 7 (n = 5–9), 30 (n = 4/group), and 180 (n = 23–24/group) days of age for Ube2i<sup>fl/fl</sup> (gray) and Ube2i<sup>a-KO</sup> (purple) mice. Data are presented as mean ± SEM; *p < 0.05. (C) Relative gene expression by qPCR in gWAT (left, yellow) and iWAT (right, cyan) from Ube2ia-KO (purple) and Ube2i<sup>fl/fl</sup> control (gray) mice for adipocyte maturation, lipid metabolism, inflammatory, and senescence genes represented as a heatmap of z-scores. Gray heatmap squares indicate outliers excluded based on Grubbs’ test. (D) Representative H/E stained gWAT and iWAT sections from one-month-old Ube2i<sup>a-KO</sup> and Ube2i<sup>fl/fl</sup> control mice. Scale bar 100 μm. (E) Ube2i<sup>a-KO</sup> and Ube2i<sup>fl/fl</sup> control mice were weighed for up to 23 weeks in males (n = 12–14/group, mean ± SD) and females (n = 6–8/group, mean ± SD). (F) Assessment of fat and lean mass (% body weight (left) and in grams (right); male n = 11–15/group, female n = 5–7/group). (G) Tissue weights (% body weight; male n = 15–16/group, female n = 8/group) at necropsy. (H) Corresponding necropsy images from six-month-old Ube2i<sup>fl/fl</sup> and Ube2ia-KO mice. Images of excised tissues demonstrate gross morphological increases in liver size, reductions in iWAT and gWAT, and lighter coloring of liver and BAT. (E–H) Gray = Ube2i<sup>fl/fl</sup> male and female controls, blue = male Ube2i<sup>a-KO</sup>, red = female Ube2i<sup>a-KO</sup>. Data are presented as mean ± SEM; *p < 0.05.
Figure 3: *Ube2ia* KO mice display WAT dysfunction with increased inflammation and apoptosis. (A) H/E-stained sections from gWAT and iWAT of male (top row) and female (bottom row) mice show substantial immune and stromal cell infiltration in *Ube2ia* KO mice. Scale bar 100 μm. (B) Fed serum adiponectin (ng/ml), leptin (ng/ml), and free fatty acids (mM) in male (blue/gray; n = 11–12/group) and female (red/gray; n = 7–9/group) *Ube2ia* KO mice compared to *Ube2i* fl/fl controls. Data are presented as mean ±/− SEM; *p < 0.05. (C) Relative gene expression by qPCR in gWAT (left) and iWAT (right) from male (blue) and female (red) *Ube2ia* KO (pink) and *Ube2i* fl/fl control (green) mice for adipocyte maturation, lipid metabolism, inflammatory, and senescence genes represented as a heatmap of z-scores (*p < 0.05, #p < 0.10). Gray heatmap squares indicate outliers excluded based on Grubbs’ test. (D) Tissue lysates from gWAT (left) and iWAT (right) of *Ube2i* fl/fl and *Ube2ia* KO mice were subjected to Western blot analysis of cleaved (p41/43, p18) and uncleaved Caspase-8. All analyses were performed in six-month-old adult mice.
and free fatty acids were significantly reduced in \( \text{Ube2f}^{-\text{Ko}} \) mice compared to controls (Figure 3B). Adipocyte-specific deletion of \( \text{Ube2f} \) reduced hallmark adipocyte differentiation (\( \text{Adipoq}, \text{Ppar}\gamma2, \text{Lep}, \text{Fabp4} \), lipogenesis (\( \text{Fasn} \), \( \text{Acaca} \), and lipolysis (\( \text{Atgl}, \text{Lipe} \)) genes in WAT. Immune cell markers (\( \text{F4/80}, \text{Tnf}, \text{Il1}\beta \)) and the collagen gene \( \text{Col1a1} \) were upregulated in both the gWAT and iWAT (Figure 3C), consistent with morphological changes. However, we noted divergent levels of inflammatory and lipogenesis genes in iWAT of males and females indicative of the known influence of sex on WAT [19]. Gene expression markers of senescence (\( \text{p16} \), \( \text{Nlrp3} \), \( \text{Il1}\beta \), \( \text{Ifng} \)) and the collagen gene \( \text{Col1a1} \) were upregulated in both the gWAT and iWAT (Figure 3C), indicating increased stress responses in the liver [21]. Accordingly, six-month-old \( \text{Ube2f}^{-\text{Ko}} \) mice displayed hepatic lipid droplet accumulation (Figure 4A) with significantly increased TG content (Figure 4B), indicating elevated stress responses in the liver [21]. Ad libitum fed glucose levels trended higher in \( \text{Ube2f}^{-\text{Ko}} \) adult mice while \( \text{Ube2f}^{-\text{Ko}} \) males exhibited only a 4-fold increase compared to controls. To test the consequences of adipose tissue loss on glucose homeostasis, we performed insulin (Figure 4F) and glucose tolerance tests (Figure 4G). \( \text{Ube2f}^{-\text{Ko}} \) male and female mice were insulin resistant relative to controls. However, with a prolonged fast, male \( \text{Ube2f}^{-\text{Ko}} \) showed improved glucose intolerance with a lower fasting glucose level.

3.4. Hepatosteatosis and insulin resistance in adult \( \text{Ube2f}^{-\text{Ko}} \) mice

The inability of WAT depots to sequester lipids causes ectopic accumulation of energy in peripheral organs and lipodystrophic phenotypes [20]. Accordingly, six-month-old \( \text{Ube2f}^{-\text{Ko}} \) mice developed fatty liver disease on a normal chow diet. Both male and female \( \text{Ube2f}^{-\text{Ko}} \) mice displayed hepatic lipid droplet accumulation (Figure 4A) with significantly increased TG content (Figure 4B). FGF21 levels were increased 4-fold in \( \text{Ube2f}^{-\text{Ko}} \) serum compared to controls (Figure 4C), indicating elevated stress responses in the liver [21]. Ad libitum fed glucose levels trended higher in \( \text{Ube2f}^{-\text{Ko}} \) male \( \text{Ube2f}^{-\text{Ko}} \) females and the consequences of adipose tissue loss on glucose homeostasis, we performed insulin (Figure 4F) and glucose tolerance tests (Figure 4G). \( \text{Ube2f}^{-\text{Ko}} \) male and female mice were insulin resistant relative to controls. However, with a prolonged fast, male \( \text{Ube2f}^{-\text{Ko}} \) showed improved glucose intolerance with a lower fasting glucose level. Glucose stimulated insulin secretion during glucose tolerance tests in

![Figure 4: Adipocyte-specific Ube2f knockout mice develop insulin resistance and hepatic steatosis. (A) Representative H/E stained liver sections from male (top row) and female (bottom row) \( \text{Ube2f}^{-\text{Ko}} \) and \( \text{Ube2f}^{+/+} \) control mice show lipid droplet accumulation in \( \text{Ube2f}^{-\text{Ko}} \) mice. Scale bar 100 \( \mu\text{m} \). (B) Hepatic triglycerides (TGs) per gram liver tissue (n = 7/group). (C) Fed serum FGF21 (pg/ml) (male n = 11–12/group; female n = 7–9/group). (D) Fed serum glucose (mg/dl) and (E) insulin (ng/ml) (male n = 11–12/group; female n = 7–9/group). (F) Insulin and (G) glucose tolerances tests were performed on \( \text{Ube2f}^{-\text{Ko}} \) and \( \text{Ube2f}^{+/+} \) male (n = 11–15/group) and female (n = 5–7/group) mice. Area under the curve is also shown. Values labeled on panels in (F) indicate the percentage decrease from initial fasting blood glucose. (H) Serum insulin during glucose tolerance test from \( \text{Ube2f}^{-\text{Ko}} \) and \( \text{Ube2f}^{+/+} \) male (n = 5/group) and female (n = 4–6/group) mice. Gray = \( \text{Ube2f}^{+/+} \) male and female controls, blue = male \( \text{Ube2f}^{-\text{Ko}} \), red = female \( \text{Ube2f}^{-\text{Ko}} \). Data are presented as mean \( \pm \) SEM. *p < 0.05 between groups, #p < 0.05 versus time zero. All analyses were performed in six-month-old adult mice.](image-url)
male Ube2i-KO mice trended higher than that in controls (Figure 4H), but importantly, demonstrated that the beta cell adapts properly during fasting compared to the fed state, as observed previously for other lipodystrophic mice [22]. Conversely, fasting glucose was elevated in female Ube2i-KO compared to controls, with nominal effects on overall glucose tolerance due to enhanced glucose stimulated insulin secretion (Figure 4H). In summary, female Ube2i-KO mice demonstrate fasting hyperglycemia and substantially impaired insulin sensitivity, in contrast to male Ube2i-KO that demonstrate modest insulin resistance with improved glucose clearance during glucose tolerance tests. The sex discrepancy in glucose tolerance may be partly explained by greater skeletal muscle mass available for glucose disposal [23], as suggested by 35% more lean mass in male Ube2i-KO than in female Ube2i-KO mice (Figure 2F).

3.5. Adipocyte-specific Ube2i deletion increases energy expenditure

We subsequently investigated the effects of Ube2i-KO on energy expenditure and BAT thermogenic functions. Histological assessment of H/E stained BAT sections from male Ube2i-KO compared to controls, with nominal effects on overall glucose tolerance due to enhanced glucose stimulated insulin secretion (Figure 4H). In summary, female Ube2i-KO mice demonstrate fasting hyperglycemia and substantially impaired insulin sensitivity, in contrast to male Ube2i-KO that demonstrate modest insulin resistance with improved glucose clearance during glucose tolerance tests. The sex discrepancy in glucose tolerance may be partly explained by greater skeletal muscle mass available for glucose disposal [23], as suggested by 35% more lean mass in male Ube2i-KO than in female Ube2i-KO mice (Figure 2F).

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as a fuel source during the light period (Figure 5D) as indicated by an elevated respiratory exchange ratio (RER). Increased energy expenditure was not associated with higher activity levels (Figure 5E), but rather poor metabolic flexibility likely stimulated greater food intake (Figure 5F,G) and consequently elevated energy expenditure in Ube2i-KO mice. To test the functional output of BAT, we performed a cold tolerance test in the absence of food. Before cold exposure, BAT temperature was reduced in Ube2i-KO mice compared to controls and dramatically dropped after 2.5 h at 4 °C (Figure 5H), demonstrating an inability of Ube2i-KO mice to defend body temperature. These experiments demonstrate that Ube2i-KO causes a hypermetabolic phenotype coupled with metabolic inflexibility and BAT thermogenic defects.

Collectively, adipocyte-specific deletion of Ube2i impairs WAT expansion fostering ectopic lipid accumulation in peripheral organs, compromising insulin sensitivity and thermogenesis.

4. DISCUSSION

Our previous work identified the E2 SUMO conjugating enzyme, Ube2i, as a negative regulator of fat cell differentiation in human subcutaneous preadipocytes [10]. In the present study, we generated adipocyte-specific Ube2i knockout (Ube2i-KO) mice to define the in vivo functions of Ube2i in mature fat cells. To our surprise, male and female Ube2i-KO mice developed a hypermetabolic phenotype and progressive WAT lipatrophy associated with immune cell infiltration and decreased circulating adipokines. Ube2i-KO mice exhibited additional hallmark features of lipodystrophy, including ectopic lipid deposition in the liver, insulin resistance, and metabolic inflexibility. Ube2i-KO WAT and BAT show broad dysfunction reflected by the expression of genes involved in energy storage and mobilization. These studies demonstrate previously unrealized functions of Ube2i in mouse WAT that are essential for mature adipocyte function and survival.

Patients with congenital general (CGL) or familial partial (FPLD) lipodystrophy exhibit profound insulin resistance, hepatic steatosis, and progressive WAT lipoatrophy associated with immune cell infiltration fostering ectopic lipid accumulation in peripheral organs, compromising insulin sensitivity and thermogenesis. As a consequence, adipocyte function, maturation, and survival.

A few lipodystrophic mouse models develop progressive lipodystrophy and hepatic steatosis, such as adipocyte-specific knockout of Akt1/Akt2 [29], insulin receptor [30], Raptor [31], and PPARγ [4]. Our model has many features similar to those of progressive lipodystrophy observed in PPARγ knockout models [4,8]. Lipodystrophy in fat-poor specific PPARγ knockout mice occurs because adipocytes lost lipids and shrank, consistent with the pivotal roles of PPARγ as a master regulator of adipose tissue formation [4,32]. PPARγ ablation in Sox2-expressing cells (PPARγΔSox) skews embryonic lethality and mice survive without WAT and BAT [8]. PPARγΔΔ show a hypermetabolic phenotype accompanied by higher energy expenditure and hyperphagia. Similar to Ube2i-KO mice, PPARγΔΔ mice show higher RER and glucose oxidation that derives from more lean mass. Alternatively, depletion of circulating leptin in lipodystrophy would increase hyperphagia and could underlie hyperinsulinemia and increased energy expenditure. However, at least one model demonstrated that increased food intake was independent of leptin levels in lipodystrophic mice [33]. The liver dominates mass-specific metabolic rates [34] and, consequently, the hepatomegaly and hyperinsulinemia present in Ube2i-KO likely contribute to greater glucose oxidation and metabolic inflexibility. Elevated energy expenditure and metabolic defects are similarly observed in other lipodystrophic mouse models [8,35,36]. Our observations expand on these studies and document a putative role for Ube2i and PPARγ interactions [10] in maintaining adipose tissue homeostasis during postnatal growth.

For reasons that remain unclear, Ube2i expression peaks early in differentiation of 3T3-L1 adipocytes, when adiponectin remains low, to regulate expression of the key adipogenic transcription factors PPARγ, C/EBPζ, and C/EBPβ [37]. Adiponectin expression occurs late in adipogenesis [38] and Adipoq-Cre mediated ablation of Ube2i occurs in relatively mature adipocytes. While impaired adipogenesis underlies the development of lipodystrophy in some models [8,35,36,39], it is unlikely to account for lipoatrophy in Ube2i-KO mice. The more dramatic lipodystrophy in older Ube2i-KO mice likely results from apoptosis and inflammation, consistent with the pivotal roles of Ube2i in cell survival across many tissue types [14]. Cell survival also depends on preservation of the nuclear architecture, evident by the embryonic lethality of Ube2i deficiency [16]. Additionally, Ube2i occupies transcriptional start sites of functional genes integral for cell growth and proliferation [40]. However, loss of Ube2i induces cell growth arrest, due in part, to impaired chromosome segregation, which contributes to reduced cell viability [40]. Along these lines, the lipodystrophy caused by induction of caspase 8 in Fabp4-positive adipocytes [33] mirrors some features of Ube2i-KO mice, including WAT inflammation and depletion of adipokines. However, recombination of alleles in other Fabp4-positive adipocytes and other tissues [41] likely contributes to some of the metabolic phenotype upon caspase-8 induction. Of note, the expression of NLRP3 inflammasome genes (Nlrp3, Il-1β, Pycard, Casp1) correlate with elevated cleaved Caspase-8 in WAT of Ube2i-KO suggesting a potential mechanism for adipocyte cell death. New studies that use temporal deletion strategies [27] will provide insight into whether acute deletion of Ube2i in adult mice causes lipodystrophy from fat cell death or restricted adipocyte turnover.

Ube2i exerts a diverse array of cellular functions through SUMOylation and protein—protein interactions that influence gene transcription among many others roles [43]. During adipocyte differentiation, Ube2i knockout de-represses transcription of the brown fat gene program, enabling the master transcription factor PPARγ to bind to UCP1 enhancers promoting uncoupled respiration [10]. Moreover, Ube2i interacts with the master transcription factor PPARγ to suppress DNA occupancy and ligand dependent activity in vitro [10]. In vivo, mice resistant to PPARγ SUMOylation at K107 exhibit better insulin sensitivity [44]. In some settings, SUMOylation prevents ubiquitination and protein turnover. For example, Cbx4 serves as SUMO E3 ligase that stabilizes PRDM16 to facilitate thermogenesis and glucose homeostasis [45], which may explain, at least in part, the cold intolerance and metabolic defects in Ube2i-KO mice.

We and others demonstrated that SUMOylation of PPARγ broadly inhibits metabolic functions of white adipocytes [10,44,46]. Based on
these data, we predicted greater beige fat thermogenesis and improved energy balance in \textit{Ube2i\textsuperscript{fl/fl}} mice relative to littermate controls. The lack of exact agreement between phenotypes observed in tissue culture versus whole animals is not surprising, but perhaps expected due to the importance of \textit{Ube2i} in cell survival [14,16]. \textit{Ube2i} knockout in adipocytes may also cause depletion of adipocyte progenitor cells coupled with Ucp1 deregulation and induction of the fibroinflammatory program seen in settings of accelerating ageing [47]. Regardless, the extensive roles of \textit{Ube2i} suggest the mechanism underlying lipoatrophy in \textit{Ube2i\textsuperscript{fl/fl}} is unlikely to be the result of a single SUMOylation event. It will now be important to use mass spectrometry and lineage tracing methods to identify the most critical metabolic and transcription factors downstream of \textit{Ube2i} functions that explain why \textit{Ube2i} allows WAT expansion during ageing.

Conditions of lipodystrophy and pathologic obesity suffer from a restricted capacity to expand white adipose depots to meet the demand for nutrient storage, which drives adipocyte dysfunction and WAT inflammation, along with ectopic lipid accumulation and insulin resistance [48]. Ultimately, identifying factors that enable healthy WAT expansion carries significant implications for treating diseases associated with fat storage and metabolism, including obesity and lipodystrophy. The striking phenotype of \textit{Ube2ip\textsuperscript{KO}} mice reveals the cell-autonomous necessity of \textit{Ube2i} in healthy adipose tissue maintenance and whole-body energy balance. As a rate-limiting E2 SUMO conjugating enzyme necessary for SUMOylation, this mouse model provides a valuable tool for understanding how the low-abundance post-translational modification SUMOylation [49] broadly affects mature adipocyte function and, more broadly, tissue development. In addition, \textit{Ube2ip\textsuperscript{KO}} mice add to a small list of mouse models to study systemic effects of WAT loss during postnatal development and fundamental aspects of energy balance.

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**AUTHOR CONTRIBUTIONS**

A.R.C. and S.M.H. conceptualized the study. P.M., N.C., A.R.C., and S.M.H. designed the experiments. A.R.C. and S.M.H. wrote the manuscript with editorial input from all authors. S.M.H. and A.R.C performed all experiments with assistance as noted. P.K.S. assisted with mouse phenotyping; A.R.C. performed qPCR analysis with assistance from J.B.F., N.C. and R.S.; D.D.M., S.M.B., and S.A.P. performed genotyping and troubleshooting; K.H.K. performed analysis of liver lipids. X.L. and Z.S. provided resources and support for body temperature experiments. All work was performed under the supervision of S.M.H. The authors thank Robb Moses (BCM) for reading drafts of the manuscript.

**CONFLICT OF INTEREST**

None declared.

**APPENDIX A. SUPPLEMENTARY DATA**

Supplementary data to this article can be found online at https://doi.org/10.1016/j.molmet.2021.101221.

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