Altered Lipid Metabolism in Residual White Adipose Tissues of Bsc12 Deficient Mice

Weiqin Chen1*, Hongyi Zhou1, Siyang Liu1, Cassie J. Fhaner2, Bethany C. Gross2, Todd A. Lydic2, Gavin E. Reid2,3

1 Department of Physiology, Medical College of Georgia at Georgia Regents University, Augusta, Georgia, United States of America, 2 Department of Chemistry, Michigan State University, East Lansing, Michigan, United States of America, 3 Department of Biochemistry and Molecular Biology, Michigan State University, East Lansing, Michigan, United States of America

Abstract

Mutations in BSCL2 underlie human congenital generalized lipodystrophy type 2 disease. We previously reported that Bsc12−/− mice develop lipodystrophy of white adipose tissue (WAT) due to unbridled lipolysis. The residual epididymal WAT (EWAT) displays a browning phenotype with much smaller lipid droplets (LD) and higher expression of brown adipose tissue marker proteins. Here we used targeted lipidomics and gene expression profiling to analyze lipid profiles as well as genes involved in lipid metabolism in WAT of wild-type and Bsc12−/− mice. Analysis of total saponified fatty acids revealed that the residual EWAT of Bsc12−/− mice contained a much higher proportion of oleic18:1n9 acid concomitant with a lower proportion of palmitic16:0 acid, as well as increased n3-polyunsaturated fatty acids (PUFA) remodeling. The acyl chains in major species of triacylglyceride (TG) and diacylglyceride (DG) in the residual EWAT of Bsc12−/− mice were also enriched with dietary fatty acids. These changes could be reflected by upregulation of several fatty acid elongases and desaturases. Meanwhile, Bsc12−/− adipocytes from EWAT had increased gene expression in lipid uptake and TG synthesis but not de novo lipogenesis. Both mitochondria and peroxisomal β-oxidation genes were also markedly increased in Bsc12−/− adipocytes, highlighting that these machineries were accelerated to shunt the lipolysis liberated fatty acids through uncoupling to dissipate energy. The residual subcutaneous white adipose tissue (ScWAT) was not browning but displays similar changes in lipid metabolism. Overall, our data emphasize that, other than being essential for adipocyte differentiation, Bsc12 is also important in fatty acid remodeling and energy homeostasis.

Introduction

Adipose tissue plays a key role in whole body energy homeostasis. Both obesity (excess fat) and lipodystrophy (lack of fat) cause dysfunction of adipose tissues which leads to the development of similar metabolic complications including dyslipidemia, diabetes, hypertension and cardiovascular diseases. Congenital generalized lipodystrophy (CGL) is an autosomal recessive disease characterized by a near total absence of body fat from birth or infancy associated with earlier diabetes onset and debilitating metabolic complications [1–3]. Mutations in the BSCL2 gene (also called seipin) in humans cause type 2, the most severe form of CGL [4]. Several studies have demonstrated the possible involvement of Bsc12 in adipogenesis, lipid metabolism and lipid droplet biogenesis and maintenance [5–9]. However, the function of Bsc12 remains mysterious. Recently, we and two other groups have independently generated Bsc12−/− mice which display massive loss of white adipose tissues and recapitulate most cell autonomous regulator of cyclic AMP (cAMP)/protein kinase A (PKA) mediated lipolysis and essential for terminal fat cell differentiation [12].

Central fat is more associated with the development of metabolic disorders [13]. Different from Bsc12−/− mice generated by other groups, we consistently observed about 3% residual EWAT which contained 56% of the DNA in our Bsc12−/− mice as compared to their wild-type littermates [12]. The residual Bsc12−/− EWAT displays a browning phenotype with much smaller lipid droplets (LD) and higher expression of brown adipose tissue marker genes [12]. The presence of visceral WAT has also been detected by different techniques in CGL2 patients [14,15]. Notably, adipose tissue is the main storage compartment for fatty acids with relatively slow turnover time in healthy humans. However, adipose tissue turnover may be influenced by the size of the depot. It is not known whether differences in adipocyte size, or changes in adipose function as observed in lipodystrophy, would affect adipose tissue total or TG fatty acid composition. Therefore, it is critical to understand the molecular events of fatty acid metabolism in residual adipose tissues in order to better control the progression of lipodystrophy.

To understand the effect of lipodystrophy on adipose tissue fatty acid composition, there are two metabolic routes to be considered:
de novo lipogenesis and the polyunsaturated fatty acid (PUFA) remodeling pathways [16]. Saturated fatty acids (SFAs), monounsaturated fatty acids (MUFA), and PUFAs are synthesized from dietary precursors (glucose, palmitic16:0, oleic18:1n9, linoleic18:2n6, α-linolenic18:3n3, eicosapentaenoic [EPA20:5n3], and docosahexaenoic [DHA22:6n3]) acids through a series of desaturation (Δ5-desaturase [Δ5D], Δ6-desaturase [Δ6D], or Δ9-desaturase [Δ9D]) and elongation (Elov1–7) reactions (Fig. 1). Using targeted lipidomics and gene expression profiling, here we identified substantial modifications in total fatty acid compositions and glycerolipid species in residual Bscl2−/− EWAT. The residual Bscl2−/− adipocytes from both EWAT and ScWAT had marked mRNA upregulation of elongases, desaturases, and TG synthesis enzymes as well as mitochondria and peroxisomal β-oxidation genes. These data suggest that in the absence of Bscl2, the residual Bscl2−/− adipocytes are still actively mobilizing dietary fatty acids through constant elongation, desaturation, TG remodeling, fatty acid oxidation and energy dissipation to counter uncontrolled lipolysis.

Materials and Methods

Ethics Statement

All animal experiments were done using protocols approved by the IACUC at Medical College of Georgia at Georgia Regents University (protocol No. 2012-0462). Mice were maintained under standard 12 h light and 12 h dark cycle at 70°F room temperature and fed with a normal chow diet (Teklad Global 18% Protein Rodent Diet 2018) ad libitum. Efforts were taken to prevent and ameliorate any suffering during the experiments and animals were sacrificed using direct cervical dislocation to avoid effect of anesthesia on lipid metabolism.

Figure 1. De novo lipogenesis and metabolic pathways of MUFA and PUFAs. Fatty acids are synthesized through de novo lipogenesis (DNL) or converted from dietary palmitic16:0, oleic18:1n9, linoleic18:2n6, and α-linolenic18:3n3 acids to long chain unsaturated fatty acids in vivo by a series of elongation by elongases (Elov1–7) and desaturation (Δ5 desaturase [Δ5D/Fad11], Δ6 desaturase [Δ6D/Fad2], or Δ9-desaturase [Δ9D/Scd1]). Fatty acids that accumulate in animal and human tissues are in solid boxes. Fatty acids derived from normal rodent chow diet are shaded in gray.

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Animals

6–10 week old male Bscl2+/+ and Bscl2−/− animals were used if not specifically indicated. All animals were sacrificed under nonfasting states at 10:00 am.

Tissue lipid analyses and thin layer chromatography (TLC)

Tissues were homogenized in standard PBS buffer. Lipids were extracted according to Bligh and Dyer [17]. Total lipids were dissolved in chloroform after being normalized to tissue weights and 5 μl samples or TLC standards (Nuchek, 18-1A and 18-6A) were loaded on Silica Gel 60 to separate the phospholipid (PL), triacylglyceride (TG), diacylglyceride (DG), nonesterified free fatty acid (NEFA), cholesterol (CHO) and cholesteryl ester (CE) fractions by one-dimensional thin-layer chromatography, using petroleum ether-diethyl ether-glacial acetic acid (85:20:1) and developed with iodine. For enzymatic analysis of triacylglycerides, total lipids were dissolved in 5% triton X-100 in PBS. Triacylglyceride concentration was measured using a triacylglyceride assay kit (Thermo Scientific) and normalized to tissue weights. Total triacylglyceride amounts were calculated based on total EWAT weights.

Isolation of adipocytes

Epididymal or subcutaneous white adipose tissues pooled from 3 animals were taken from 6 week old wild-type and Bscl2−/− mice, minced and digested with 2 mg/mL collagenase type IV in PBS with 2% BSA at 37°C for about 40 min, and the digest was filtered through a 100-μm mesh. Filtrates were then spun at 800 xg. The floating adipocytes were then collected and washed with PBS for RNA extraction and gene expression analysis.

Isolation and differentiation of mouse embryonic fibroblasts (MEFs)

MEFs were isolated and differentiated exactly as previously described [12].

Reverse transcription and real time PCR

Total RNA was extracted from cells or tissues with TRIzol (Invitrogen) and reverse-transcribed using MLV-V reverse transcriptase using random primers (Invitrogen). Real-time quantitative RT-PCR was performed on the Strategene MX3005. For tissue gene analysis, data were normalized to 2 house keeping genes (cyclophilin A and β-actin) based on the Genorm algorithm (http://medgen.ugent.be/genorm/) and were expressed as fold changes relative to wild-type controls. For cell gene expression analysis, data were normalized to cyclophilin A and expressed as fold changes relative to wild-type.

Tissue fatty acids analysis by RP-HPLC

Total tissue lipids were extracted and fatty acids were analyzed as described [18]. Briefly, an aliquot of total lipids from the same amount of tissues was saponified (0.4 N KOH in 80% methanol, 50°C for 1 h). Saponified fatty acids were acidified and extracted with diethyl ether and dissolved in methanol. Saponified free fatty acids were then introduced to the HPLC and fractionated using a YMC J-Sphere (ODS-H80) column, detected using ultraviolet absorbance and evaporative light scatter and quantitated by RP-HPLC [10]. Authentic fatty acid standards (Nu-Chek Prep) were used to generate calibration curves for verification and quantification of fatty acids. We presented data as mole% by dividing the amount of each fatty acid (mole) to the total amounts of detected fatty acids (mole) as previously described [10]. Unsaturation
index = (mole% x number of unsaturation of each fatty acid)/100 (mole% of total identified fatty acids).

**Mass spectrometry analysis of triacylglyceride and diacylglyceride lipids**

Lipids were diluted into isopropanol/methanol/chloroform (4:2:1 v/v) containing 20 mM NH₄Ac and 0.5 µM TG d5-(14:0/16:1/14:0) (Avanti Polar Lipids, Alabaster, AL) as an internal standard. All solvents used were filtered HPLC grade (methanol, MeOH) [J.T. Baker, Phillipsburg, NJ], ammonium acetate and chloroform (EMD Chemical, Gibbstown, NJ). Samples were centrifuged and loaded into Whatman Multichem 96-well plates (Fisher Scientific, Pittsburgh, PA), and sealed with Teflon Ultra-Thin Sealing Tape (Analytical Sales and Services, Pompton Plains, NJ). Preliminary mass spectrometry analysis of each lipid sample was acquired at a range of different dilutions to determine the range at which linearity in response for specific lipids was maintained, thus, the abundance of DG and TG molecular species at each m/z are reported as the % total lipid area for each lipokine. The browning of adipose tissues in both male and female Bsc12−/− mice by determining peak areas from the high resolution MS spectra was significantly increased (Fig. 3B). No palmitoleic acid, a potential lipokine derived from adipose tissue that contributes to the browning of adipose tissue, was observed (Fig. 3B). If calculated based on the total EWAT weights (0.55 ± 0.05 vs. 35.7 ± 0.9 mg, respectively), the overall TG content in Bsc12−/− EWAT was only 15% of that in Bsc12+/+ EWAT (0.55 ± 0.05 vs. 33.7 ± 3.5 mg) (Fig. 2D). Likewise, the absolute amounts of lipids other than TG would still be lower in Bsc12−/− EWAT. Nevertheless, Bsc12−/− EWAT display a shifted lipid profile toward increased abundance of lipids other than TG.

**Altered elongation and desaturation in total fatty acid compositions of visceral white adipose tissues from Bsc12++/− and Bsc12−/− mice**

We next analyzed the total adipose tissue fatty acid compositions of Bsc12++/− and Bsc12−/− mice by RP-HPLC. When expressed as mole% of total fatty acids, we observed a decrease in palmitic acid (molar% in contrast to an increase in oleic acid (molar% in Bsc12−/− EWAT, while no changes in stearic, linoleic, arachidonic acids were found between the two genotypes (Fig. 3A). Accordingly, the ratio of oleic to palmitic acids was significantly increased (Fig. 3B). No palmitoleic acid, a potential lipokine derived from adipose tissue that contributes to the browning of adipose tissue, was observed (Fig. 3B). If calculated based on the total EWAT weights (0.55 ± 0.05 vs. 33.7 ± 3.5 mg) (Fig. 2D). Likewise, the absolute amounts of lipids other than TG would still be lower in Bsc12−/− EWAT. Nevertheless, Bsc12−/− EWAT display a shifted lipid profile toward increased abundance of lipids other than TG.

**Figure 2. Residual EWAT and Bsc12−/− EWAT exhibit differential lipid profiles.** A) Inactivation of Bsc12 in mice causes massive loss of epididymal white adipose tissue (EWAT). B) Thin layer chromatography (TLC) analysis of total lipids extracted from EWAT of male non-fasting Bsc12++/− and Bsc12−/− mice. M: TLC standards. CE: cholesterol ester; TG: triacylglyceride; FF: free fatty acid, CHO: cholesterol; DG: diacylglyceride; PL: phospholipid. Total lipids from equal amounts of tissue for each genotype were loaded. B) Enzymatic analysis of EWAT triacylglycerides (TG). Results were expressed as µg TG per mg tissue. C) Total EWAT TG contents based on total EWAT weights. n = 6–7 each. **p < 0.005 between two genotypes. doi:10.1371/journal.pone.0082526.g002
Figure 3. Altered fatty acid compositions suggest increased rate of fatty acid mobilization in residual Bscl2−/− EWAT. A) Identification and quantification of changes in total adipose tissue saponified fatty acids by RP-HPLC. B) Ratio of oleic18:1n9/palmitic16:0 acids. C) End product/precursor ratio of DHA22:6n3/α-linolenic18:3n3 acids. D) Uncsaturation index based on the number of double bonds per fatty acyl residue. n = 3 with each sample pooled from EWAT fat pads from 2 animals. *: p<0.05; **: p<0.005.
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Marked changes in molecular species of glycerolipids from Bscl2+/+ and Bscl2−/− epididymal white adipose tissues

Since approximately 99% of adipose tissue is comprised of glycerolipids including DG and TG, we analyzed glycerolipids by nanoESI-MS, -MS/MS and -MS3. 95 TG species were identified with unique masses. Among the 46 TGs present at >0.1% total TG ion abundance in Bscl2+/+ EWAT, most exhibited relative decreases in short or very long fatty acyl chains in Bscl2−/− EWAT (Table 1). Interestingly, four abundant TGs (TG52:3, TG52:2, TG54:4, TG54:3) were elevated by 14.2%, 41%, 57% and 177%, respectively, in Bscl2−/− mice vs. wildtype littermates (Table 1). Most of the 49 lower abundant TGs (0.01–0.1% total TG ion abundance) were decreased by more than 50% in the regulation of global lipid homeostasis [24], was detectable in Bscl2−/− EWAT. However, two essential fatty acids, α-linolenic18:3n3, γ-linolenic18:3n6 acids, were decreased (Fig. 3A) as opposed to increased DHA22:6n3 in Bscl2−/− EWAT. Therefore, the ratio between the major end product of the α3-PUFA synthesis pathway (DHA22:6n3 relative to its precursor, α-linolenic18:3n3 acid) but not the n6-PUFA synthesis pathway (arachidonic20:4n6 acid relative to its precursor linoleic18:2n6 acid) was increased, suggesting enhanced α3-PUFA remodeling through elongation and desaturation in Bscl2−/− EWAT (Fig. 3C). Despite these changes, the unsaturation index (the number of double bonds per fatty acyl residue) remained similar between two genotypes (Fig. 3D).

Table 1. Lipidomic analysis of TGs by shotgun mass spectrometry of EWAT from Bscl2+/+ and Bscl2−/− mice.

| TG      | Bscl2+/+ | Bscl2−/− | TG      | Bscl2+/+ | Bscl2−/− |
|---------|----------|----------|---------|----------|----------|
| TG38:2  | 0.28±0.095 | 0.02±0.004** | TG51:2  | 0.19±0.003 | 0.17±0.02* |
| TG39:1  | 0.11±0.04  | 0.009±0.004** | TG52:6  | 0.33±0.02  | 0.21±0.04** |
| TG40:3  | 0.16±0.04  | 0.025±0.006** | TG52:5  | 3.33±0.14  | 2.34±0.28** |
| TG40:2  | 0.15±0.04  | 0.017±0.007** | TG52:4  | 12.62±0.46 | 10.55±0.78** |
| TG44:2  | 0.22±0.05  | 0.004±0.000** | TG52:3  | 16.2±0.26  | 18.5±0.11** |
| TG44:1  | 0.18±0.05  | 0.002±0.001** | TG52:2  | 7.09±0.27  | 10.01±0.94** |
| TG46:3  | 0.18±0.03  | 0.012±0.001** | TG53:4  | 0.21±0.01  | 0.18±0.03 |
| TG46:2  | 0.6±0.13   | 0.03±0.002**  | TG53:3  | 0.21±0.01  | 0.21±0.02 |
| TG46:1  | 0.47±0.11  | 0.03±0.004**  | TG54:7  | 0.89±0.08  | 0.24±0.03** |
| TG46:0  | 0.19±0.04  | 0.01±0.002**  | TG54:6  | 4.59±0.33  | 3.0±0.4** |
| TG48:4  | 0.22±0.02  | 0.033±0.004** | TG54:5  | 10.0±0.57  | 10.6±0.79 |
| TG48:3  | 0.75±0.09  | 0.18±0.011**  | TG54:4  | 10.2±0.28  | 16.0±0.3** |
| TG48:2  | 1.68±0.2   | 0.56±0.03**  | TG54:3  | 3.17±0.04  | 8.8±1.08** |
| TG48:1  | 1.37±0.21  | 0.46±0.05**  | TG54:2  | 0.51±0.01  | 0.26±0.02** |
| TG48:0  | 0.7±0.09   | 0.15±0.022**  | TG56:8  | 0.1±0.02   | 0.04±0.01** |
| TG49:2  | 0.12±0.001 | 0.07±0.011**  | TG56:7  | 0.24±0.04  | 0.14±0.04** |
| TG50:5  | 0.15±0.003 | 0.1±0.02**    | TG56:6  | 0.34±0.05  | 0.25±0.06** |
| TG50:4  | 1.3±0.04   | 0.92±0.122**  | TG56:5  | 0.47±0.06  | 0.34±0.05** |
| TG50:3  | 4.54±0.17  | 3.6±0.15**    | TG56:4  | 0.5±0.07   | 0.39±0.05 |
| TG50:2  | 8.46±0.51  | 6.44±0.03**  | TG56:3  | 0.31±0.07  | 0.25±0.04 |
| TG50:1  | 3.12±0.45  | 2.81±0.24    | TG58:5  | 0.17±0.09  | 0.12±0.02 |
| TG51:4  | 0.11±0.01  | 0.07±0.011**  | TG58:4  | 0.22±0.11  | 0.22±0.03 |
| TG51:3  | 0.24±0.01  | 0.2±0.03*    | TG58:3  | 0.10±0.03  | 0.11±0.02 |

TG species were determined using high resolution ESI-MS and confirmed via product ion scan mode CID-MS/MS and -MS3 as described in Methods (n = 3 pooled from 6 animals). Data are expressed as % total TG ion abundance in each genotype. Only the 46 TG species observed at >0.1% total TG ion abundance in Bscl2+/+ EWAT are listed. Data are presented as means ± SD. *: p<0.05; **: p<0.005. Arrows indicate upregulation or downregulation vs. Bscl2+/+ EWAT.
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Bosl2−/− EWAT (data not shown). DGs were also present but in much lower amounts and with less diversity of DG molecular species. A total of 18 DGs were identified, with most containing between 32-38 total acyl carbon atoms esterified to the glycerol backbone. 4 DGs were substantially diminished in Bosl2−/− EWAT including two major species, DG32:4 and DG36:6, along with the minor DG32:4 and DG36:5 species (Table 2). However, the percentage of DG36:2 was 38% higher in Bosl2−/− EWAT (14.97±1.6) as compared to Bosl2+/− EWAT (9.46±0.74). Interestingly, two DGs with very long fatty acid chains (DG48:1 and DG48:0) were only detectable in Bosl2−/− EWAT (Table 2). The possible combinations for fatty acid chains of major TG and DG species were listed in lipid identity excel File S1. Our data suggest that the residual Bosl2−/− adipocytes were actively involved in remodeling both DGs and TGs.

Enhanced expression of genes involved in fatty acid modification in Bosl2−/− adipocytes

Fatty acid changes in tissues are frequently mirrored by enzyme activities or gene expression differences in fatty acid remodeling enzymes such as elongases and desaturases. We next isolated adipocytes from EWAT of Bosl2+/− and Bosl2−/− mice and measured gene expression of elongases and desaturases by qRT-PCR analysis. Elovl1 is commonly regarded as a housekeeping gene in many tissues and is often seen expressed at steady levels [25]. We observed a significant increase in the mRNA levels of Elovl1 (1.56, P<0.05) in adipocytes from Bosl2−/− mice (Fig. 4A). Confirming the previous results with upregulated Elovl3 in browning Bosl2−/− EWAT, Elovl3 was increased by 35-fold in Bosl2−/− adipocytes compared to wild-type adipocytes. No difference was observed in Elovl5 gene expression. Elovl6, an important elongase that drives synthesis of stearic18:0 and oleic18:1n9 acids from palmitic16:0 acid [26], had a tendency toward higher expression that did not reach significance in Bosl2−/− adipocytes (Fig. 4A). In line with the increased n3-PUFA synthesis in Bosl2−/− EWAT, the mRNA levels of Δ5D (Fad3l) and Δ6D (Fad3l) were upregulated by 4.2 and 12 fold, respectively, in Bosl2−/− adipocytes vs. wildtype adipocytes. Interestingly, despite a much higher ratio of oleic18:1n9/palmitic16:0 acids in Bosl2−/− EWAT, the major Δ9 desaturase (Sce-1), which catalyzes the synthesis of monounsaturated fats like palmitoleic16:1n7 and oleic18:1n9 acids, was not different between Bosl2+/− and Bosl2−/− white adipocytes (Fig. 4B). We failed to observe any changes in genes involved in de novo lipogenesis, including Srebp1c, Acc1 and Fasn (Fig. 4C). Genes involved in direct fatty acid uptake including ap2, Cd36, Fatp1 and Cav1 were not altered. However, the mRNA expression of very low density lipoprotein receptor (Vldlr) was increased by about 3 fold (Fig. 4D), suggesting enhanced uptake of lipoproteins from blood stream by residual Bosl2−/− adipocytes. Furthermore, the mRNA levels of glycerol phosphate acyltransferase (Gpat) and acylglycerol-3-phosphate acyltransferase 2 (Agpat 2), the critical enzymes responsible for the first two steps of TG synthesis, were increased by 2.5 and 2.9 fold, respectively, in Bosl2−/− adipocytes. In contrast, we consistently observed an approximately 50% down-regulation of Dgat2, the enzyme responsible for the last step of TG synthesis, albeit with no change of Dgat1 gene expression in Bosl2−/− adipocytes (Fig. 4E).

Differential upregulation of thermogenic and fatty acid oxidation genes in isolated Bosl2−/− adipocytes in vivo and differentiating Bosl2−/− MEFs in vitro

Accelerated PKA mediated lipolysis underlies the failure of terminal adipocyte differentiation which ultimately leads to massive loss of white adipose tissue in Bosl2−/− mice. The residual adipocytes in Bosl2−/− EWAT are still undergoing rampant lipolysis [12]. To accommodate the increased release of free fatty acids, Cpt1α (a critical gene regulating fatty acid β-oxidation), and Cytochrome C (an electron transfer chain protein (CytC)) were upregulated along with a drastic increase in BAT specific Ucp1 gene expression, suggesting increased mitochondrial mediated β-oxidation and energy dissipation through uncoupling in Bosl2−/− EWAT [12]. Here we confirmed that Ucp1 was specifically upregulated in isolated Bosl2−/− adipocytes as expected (Fig. 5A). The expression of peroxisome proliferator-activated receptor α (Pparα), the important transcription factor that is activated by lipolytic products and regulates β-oxidation [27], was markedly elevated along with its target genes such as Cpt1α, Acyl-CoA thioesterase 2 (Acox2) and Acyl-CoA oxidase 2 (Acox2) in Bosl2−/− adipocytes vs. Bosl2+/− adipocytes (Fig. 5B); all of which are involved in fatty-acyl CoA turnover, mitochondria and peroxisomal fatty acid oxidation.

In vitro Bosl2−/− MEFs ultimately dedifferentiate into non-adipocytes. These cells do not express adipocyte specific transcription factors and their regulated genes [12]. Ucp1 and other BAT specific genes were also predominantly downregulated (data not shown). However, the residual Bosl2−/− adipocytes in vitro are most likely differentiating cells that are undergoing dynamic turn-over. One interesting question remains whether differentiating Bosl2−/− MEFs in vitro also undertake browning with induction of thermogenic and fatty acid oxidation genes. We next focused on D2-D5 differentiating Bosl2−/− MEFs before most Bosl2−/− cells turn over to nonadipocytes at D6 and afterwards (based on days after hormone cocktail induction). As shown in Fig. 5C, the

| DG   | Bosl2+/−  | Bosl2−/−  |
|------|-----------|-----------|
| DG32:5 | 0.82±0.59  | 0.19±0.06  |
| DG32:4 | 0.32±0.03  | 0.09±0.02† |
| DG32:3 | 0.0±0.0   | 0.63±0.96  |
| DG32:2 | 0.32±0.06  | 0.27±0.02  |
| DG32:1 | 1.14±0.6   | 0.61±0.18  |
| DG32:0 | 2.08±1.13  | 0.95±0.15  |
| DG34:3 | 3.95±1.69  | 4.31±0.18  |
| DG34:2 | 32.66±8.82 | 25.96±0.97†|
| DG34:1 | 13.73±0.66 | 15.46±1.79 |
| DG36:5 | 0.28±0.13  | 0.07±0.03† |
| DG36:4 | 11.57±0.88 | 8.54±0.93† |
| DG36:3 | 23.2±1.61  | 24.99±0.59 |
| DG36:2 | 9.45±0.74  | 14.97±1.61† |
| DG36:1 | 0.12±0.11  | 0.36±0.14† |
| DG38:4 | 0.12±0.13  | 1.29±0.16† |
| DG40:8 | 0.25±0.23  | 1.02±0.88  |
| DG40:1 | 0.0±0.0    | 0.11±0.03† |
| DG40:8 | 0.0±0.0    | 0.12±0.05† |

Table 2. Lipidomic analysis of DGs by shotgun mass spectrometry of EWAT from Bosl2+/− and Bosl2−/− mice.

Data are presented as ± SD. *: p<0.05; **: p<0.005. Arrows indicate upregulation or downregulation vs. Bosl2+/− EWAT.

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expression of Ucp1 tended to be higher in Bscl2<sup>−/−</sup> MEFs at D2. At D3 and D4, enhanced expressions of Ucp1 were obvious in Bscl2<sup>−/−</sup> differentiating MEFs despite its gradual reduction as differentiation progresses. By D5 this difference disappeared largely due to the dynamic turnover of Bscl2<sup>−/−</sup> MEFs. We did not find consistent changes in Elovl3 (Fig. 5D) and fatty acid oxidation genes Ppara and Cpt1a (data not shown). These data agree with a cell autonomous role of PKA activation in stimulating Ucp1 expression. It also suggests other cues may be missing for full browning of differentiating Bscl2<sup>−/−</sup> MEFs in vitro.

The residual Bscl2<sup>−/−</sup> subcutaneous white adipose tissues were not browning but had similar altered lipid metabolism

Subcutaneous white adipose tissue has been shown to have a greater thermogenic capacity and to be more susceptible to cold induced browning than other white adipose tissue depots [28]. Young adult Bscl2<sup>−/−</sup> mice retain 30% ScWAT, whose morphology is very similar to Bscl2<sup>−/−</sup> EWAT [12]. We next asked whether Bscl2<sup>−/−</sup> ScWAT is also browning, and undergoes similar altered lipid metabolism. Surprisingly, in contrast to drastic upregulation of Ucp1 in Bscl2<sup>−/−</sup> adipocytes from EWAT (Fig. 5A), Ucp1 mRNA expression was not different between Bscl2<sup>−/+</sup> and Bscl2<sup>−/−</sup> adipocytes from ScWAT (Fig. 6A) and was even downregulated in Bscl2<sup>−/−</sup> animals more than 12 week old (data not shown). A trend toward increased Elovl3 mRNA was detected in Bscl2<sup>−/−</sup> ScWAT adipocytes, however, with substantial variability (Fig. 6A). Ppara was slightly upregulated in Bscl2<sup>−/−</sup> adipocytes from ScWAT relative to controls, which did not result in a significant increase of downstream Cpt1a and Acox2 genes (Fig. 6A). Analysis of other genes involved in fatty acid elongation and desaturation, as well as TG synthesis, exhibited similar upregulation of Fads1, Fads2 and Gpat genes in Bscl2<sup>−/−</sup> adipocytes from ScWAT as observed in EWAT (Fig. 6B). TLC analysis further confirmed that Bscl2<sup>−/−</sup> ScWAT had decreased levels of TG in contrast to increased levels of other lipid classes, including CE, NEFA, DG, CHO, and PL, when normalized per mg tissue weight, an essentially similar finding as in Bscl2<sup>−/−</sup> EWAT (Fig. 2B). These data highlight a fat depot specific
Lipid Metabolism in Bscl2−/− Adipose Tissue

Browning feature albeit with similar lipid modifications between the two types of the residual Bscl2−/− WAT.

Discussion

Adipose tissue dysfunction has a profound impact on whole body lipid metabolism. We for the first time performed lipidomic analysis of a special browning adipose tissue from Bscl2−/− mice, a mouse model that recapitulates human congenital generalized lipodystrophy type 2 disease. Our findings reveal a substantial modification of fatty acid compositions and glycerolipid species in residual browning Bscl2−/− EWAT. These changes are correlated with markedly increased expression of genes regulating PUFA synthesis, TG remodeling, mitochondrial and peroxisomal β-oxidation associated with uncoupling. Our data emphasize that the residual Bscl2−/− adipocytes are actively mobilizing dietary fatty acids through constant lipolysis despite the much reduced adipocyte size and depot, and suggest that the presence of these residual adipocytes may still be able to contribute to whole body energy balance in CGL2 patients.

It is certain that the total content of different lipid classes in Bscl2−/− EWAT and ScWAT was largely reduced due to the massive loss of adipose tissues. However, when comparing the lipid profiles, we observed a shift towards more abundant phospholipids, NEFA, DG and CE in Bscl2−/− EWAT and ScWAT as compared to their wild-type counterparts containing predominantly TG (Fig. 2B). This is not surprising considering the marked differences between wild type and Bscl2−/− WAT in both structure and cell components, with the latter being more compact with much smaller adipocytes infiltrated with immune cell types including macrophages, a phenomena which also happens in lipodystrophy [29].

The fatty acid composition of adipose tissue has been considered as a gold standard to represent dietary fatty acids [30]. Indeed, the total saponified fatty acid compositions of both wild-type and Bscl2−/− EWAT are enriched with major dietary fatty acids present in the rodent chow diet (Fig. 1 and Fig. 3A). Dietary fatty acids have much greater impact on the fatty acid compositions of Bscl2−/− EWAT. Oleic18:1n9 acid, the most abundant dietary MUFA, was enriched in Bscl2−/− EWAT (Fig. 3A). The higher proportion of oleic18:1n9 acid could also be a result of increased conversion of palmitate to oleate through elongation and desaturation as Bscl2−/− EWAT has a higher ratio of oleic18:1n9/palmitic16:0 acids (Fig. 3B). The enrichment of dietary fatty acids in Bscl2−/− EWAT was also mirrored in its glycerolipids, which contain elevated proportions of TGs [such as TG52:3, TG52:2, TG54:4; TG54:3] and DGs (such as DG36:2) mainly consisting of oleyl-acyl chains and other dietary fatty acyl chains (highlighted in lipid identity excel File S1). Such enrichment was most likely a result of increased Vldl-TG uptake by Bscl2−/− adipocytes, an interesting phenomenon which may also help explain lack of hypertriglyceridemia in Bscl2−/− mice [11,12]. Notably, we measured the adipose tissue fatty acid compositions in nonfasting animals. As Bscl2−/− mice are hyperphagic [12], higher dietary intake in Bscl2−/− mice may contribute to the enrichment of dietary fatty acids. Interestingly, an essential fatty acid, α-linolenic18:3n-3 acid was downregulated in Bscl2−/− EWAT despite overfeeding, largely due to pronounced β-oxidation or enhanced conversion through n3-PUFA pathway to DHA22:6n3. Despite a relatively low accumulation of n3-PUFA in adipose tissue lipids, n3-PUFAs especially DHA22:6n3 have been found to be less adipogenic and able to prevent excessive growth of adipose tissue and induce mitochondrial biogenesis in adipocytes [31,32]. Thus far, it is premature to speculate whether such a minimal increased percentage of DHA22:6n3 may affect adipocyte differentiation and induce mitochondrial function in the browning Bscl2−/− EWAT. Nevertheless, these changes implicate that the residual Bscl2−/− adipocytes are actively metabolizing dietary fatty acids and may still contribute to whole body lipid metabolism.

It is known that the activities of the enzymes involved in both the elongation and desaturation of fatty acids appears to be regulated primarily at the transcriptional level, rather than by posttranslational protein modifications [25]. The increased

Figure 6. The residual Bscl2−/− subcutaneous white adipose tissues were not browning but had similar altered lipid metabolism. qPCR analyses of BAT specific genes Ucp1 and Elovl3, lipolytic product activated transcription factor Pparα and its targeted genes Cpt1α and Acox2 (A); and genes involved in elongation, desaturation and TG synthesis (B) in isolated adipocytes from ScWAT of Bscl2+/+ and Bscl2−/− mice. Each sample was pooled from 3-4 6-week-old nonfasting male wild-type and wild-type and [30]. Indeed, the total saponified fatty acid compositions of both...
oelc<sub>18:1n9</sub>/palmitic<sub>16:0</sub> acids ratio could be explained by increased elongation but not Δ9 desaturation as Scd1 gene expression was not perturbed in <em>Bscl2</em>−/− animals (Fig. 4A & 4B), suggesting other factors may be involved. Notably, a reduced Δ9-desaturase activity was observed in cultured human <em>Bscl2</em>−/− fibroblasts which leads to decreased unsaturation ratio [33]. This discrepancy with our result may arise from different cells or tissues measured in each study. In both EWAT and ScWAT of <em>Bscl2</em>−/− mice, elevated mRNA expression of <em>Δ5D</em> (Fads1) and <em>Δ6D</em> (Fads2) desaturases underlies the increased PUFA remodeling, as both are critical genes for PUFA synthesis pathways (Fig. 1). Elevation of <em>Δ5D</em> and <em>Δ6D</em> genes could be either due to overfeeding in <em>Bscl2</em>−/− mice as they are known to be nutritionally regulated [25] or through enhanced transcriptional regulation by lipid oxidation associated Ppar activation (Fig. 5B and [34]). Increased <em>Elov1</em> was also seen in <em>Hsd<sub>17β</sub></em>/−/− EWAT which was shown to be responsible for increased elongation of palmitic<sub>16:0</sub> to stearic<sub>18:0</sub> acids [35]. <em>Elov3</em> expression is not only important for TG formation in brown adipose tissue but also highly correlated with increased fatty acid oxidation as well as activation and recruitment of BAT [36,37]. However, in terms of lipid profile changes in the browning <em>Bscl2</em>−/− EWAT, our data did not detect an increase in C20:2/C24:2 very long chain saturated or monounsaturated fatty acids, the specific products of Elov3 [25]. These fatty acids could be present at low levels that are below the detection limit by RP-HPLC. Alternatively, the enhanced peroxisomal oxidation in <em>Bscl2</em>−/− EWAT, as indicated by induction of acyl-Coa oxidase 2 (Fig. 5B), the rate limiting enzyme of peroxisomal very long chain fatty acid (VLCFA) β-oxidation [38], may lead to higher turnover of these fatty acids in the residual <em>Bscl2</em>−/− EWAT. Nevertheless, DGs with very long fatty acyl chains (DG48:1 and DG48:0) were indeed found in <em>Bscl2</em>−/− EWAT, underpinning the presence of enhanced fatty acid elongation. Meanwhile, enhanced peroxisomal lipid oxidation also supports the increased conversion of DHA<sub>22:6n3</sub> which requires β-oxidation of tetracosahexaenoic<sub>24:6n3</sub> acid in peroxisomes.

Adipocytes with a single deletion of either Dgat1 or Dgat2 were capable of TG synthesis and LD formation [39]. Hence, in spite of a significant downregulation of Dgat2, <em>Bscl2</em>−/− adipocytes may still be able to synthesize TG normally in the presence of normal Dgat1 expression as highlighted by increased incorporation of major dietary fatty acids into TG species (Table 1). A prominent decrease in proportions of most TGs with either short or very long chain fatty acids can largely be attributed to rampant lipolysis that constantly hydrolyzes TGs in <em>Bscl2</em>−/− adipocytes. The increased expression of Gpat and Agpat2 suggests the intermediate steps of TG synthesis were probably heightened, which may generate higher proportions of lysophosphatidic acids (LPA) and phosphatidic acids (PA) (Fig. 2A). These data are in line with the previous findings showing increased PA levels in both yeast lacking BscI2 orthologue Fld1 and drosophila with dSeipin disrupted [40,41]. Recently, Sim et al demonstrated a critical role of BscI2 in targeting lipin 1 (PA phosphatase) to the ER, which is required for full Lipin1 function. They showed that differentiating 3T3-L1 cells with simultaneous Agpat2 overexpression and BscI2 knockdown contained more PA, which led to defective adipocyte differentiation in vitro [42]. Given the dynamic turnover of <em>Bscl2</em>−/− MEF cells undergoing differentiation in vitro, our lipidomic analyses of D4 differentiating <em>Bscl2</em>−/− MEF cells did not yield consistent changes in any lipid species (data not shown). Nevertheless, our findings with consistent mRNA upregulation of Gpat and/or Agpat2 in <em>Bscl2</em>−/− EWAT and ScWAT suggest BscI2 could act as an important regulator of lipid synthesis. However, further detailed studies are warranted to elucidate whether such changes in mRNA expression of TG synthesis genes and lipid species are secondary to lipolysis or in fact primary factors that mediate higher lipolysis and/or aborted differentiation in the absence of BscI2.

In conclusion, our study consists of the first lipidomic analysis of lipolytic adipose tissue and reveals dynamic lipid turnover and modification in adipocytes lacking BscI2. This data further highlights that, in addition to its known essential role in adipocyte differentiation, BscI2 is also important in fatty acid remodeling and energy homeostasis. Thus, dietary restriction or manipulation in BSC1L2 patients may have beneficial effects in alleviating metabolic disorders.

**Supporting Information**

Communication

**Conceived and designed the experiments: WC GER.** Performed the experiments: WC HZ SL CJF BCG GER. Analyzed the data: HZ TAL. Contributed reagents/materials/analysis tools: TAL. Wrote the paper: WG GER.

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Author/s:
Chen, W; Zhou, H; Liu, S; Fhaner, CJ; Gross, BC; Lydic, TA; Reid, GE

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