The transcriptional co-regulator LDB1 is required for brown adipocyte function

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ABSTRACT

Objective: Brown adipose tissue (BAT) is critical for thermogenesis and glucose/lipid homeostasis. Exploiting the energy uncoupling capacity of BAT may reveal targets for obesity therapies. This exploitation requires a greater understanding of the transcriptional mechanisms underlying BAT function. One potential regulator of BAT is the transcriptional co-regulator LIM domain-binding protein 1 (LDB1), which acts as a dimerized scaffold, allowing for the assembly of transcriptional complexes. Utilizing a global LDB1 heterozygous mouse model, we recently reported that LDB1 might have novel roles in regulating BAT function. However, direct evidence for the LDB1 regulation of BAT thermogenesis and substrate utilization has not been elucidated. We hypothesize that brown adipocyte-expressed LDB1 is required for BAT function.

Methods: LDB1-deficient primary cells and brown adipocyte cell lines were assessed via qRT-PCR and western blotting for altered mRNA and protein levels to define the brown adipose-specific roles. We conducted chromatin immunoprecipitation with primary BAT tissue and immortalized cell lines. Potential transcriptional partners of LDB1 were revealed by conducting LIM factor surveys via qRT-PCR in mouse and human brown adipocytes. We developed a Ucp1-Cre-driven LDB1-deficiency mouse model, termed Ldb1\(^{-/}\)BAT, to test LDB1 function in vivo. Glucose tolerance and uptake were assessed at thermoneutrality via intraperitoneal glucose challenge and glucose tracer studies. Insulin tolerance was measured at thermoneutrality and after stimulation with cold or the administration of the \(\beta\)-adrenergic receptor (\(\beta\)-AR) agonist CL316,243. Additionally, we analyzed plasma insulin via ELISA and insulin signaling via western blotting. Lipid metabolism was evaluated via BAT weight, histology, lipid droplet morphometry, and the examination of lipid-associated mRNA. Finally, energy expenditure and cold tolerance were evaluated via indirect calorimetry and cold challenges.

Results: Reducing Ldb1 in vitro and in vivo resulted in altered BAT-selective mRNA, including Ucp1, Elov13, and Dio2. In addition, there was reduced Ucp1 induction in vitro. Impacts on gene expression may be due, in part, to LDB1 occupying Ucp1 upstream regulatory domains. We also identified BAT-expressed LIM-domain factors Lmo2, Lmo4, and Lhx8, which may partner with LDB1 to mediate activity in brown adipocytes. Additionally, we observed LDB1 enrichment in human brown adipose. In vivo analysis revealed LDB1 is required for whole-body glucose and insulin tolerance, in part through reduced glucose uptake into BAT. In Ldb1\(^{-/}\)BAT tissue, we found significant alterations in insulin-signaling effectors. An assessment of brown adipocyte morphology and lipid droplet size revealed larger and more unilocular brown adipocytes in Ldb1\(^{-/}\)BAT mice, particularly after a cold challenge. Alterations in lipid handling were further supported by reductions in mRNA associated with fatty acid oxidation and mitochondrial respiration. Finally, LDB1 is required for energy expenditure and cold tolerance in both male and female mice.

Conclusions: Our findings support LDB1 as a regulator of BAT function. Furthermore, given LDB1 enrichment in human brown adipose, this co-regulator may have conserved roles in human BAT.

Keywords LDB1; Transcriptional co-regulator; Brown adipocyte; Thermogenesis; Glucose; Lipid; Ucp1

1. INTRODUCTION

Obesity contributes to metabolic dysregulation, including glucose intolerance and ectopic lipid deposition, ultimately affecting multiple organ systems. Brown adipose tissue (BAT) produces heat from stored energy and, thus, has potential as a novel therapeutic target for obesity-related diseases. Moreover, BAT expansion via transplantation or drug-induced stimulation improves glucose tolerance and insulin sensitivity in rodent models [1–3]. BAT is present in adult humans and is activated by cold exposure, with BAT activity dampened in obese individuals [4,5]. These observations suggest that modulating BAT function is a potential therapeutic target against metabolic dysregulation.

Brown adipocytes are densely packed with mitochondria containing uncoupling protein-1 (UCP1), an inner membrane anion carrier that uncouples mitochondrial respiration, to dissipate energy as heat [6].

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UCP1 is almost exclusively restricted to thermogenic adipose and is robustly increased by stimuli including cold exposure and a high-fat diet [6]. Global Ucp1 deficiency results in cold intolerance and increased obesity susceptibility [7,8]. Ucp1 expression and BAT function are regulated by transcriptional complexes comprised of transcription factors (TFs) and interacting transcriptional co-regulators [9]. However, many BAT TFs are also expressed in white adipocytes, placing greater importance on identifying BAT-enriched transcriptional co-regulators required for tissue-specific gene expression. Co-regulators, including PGC1α and PRDM16, promote thermogenic gene expression by stabilizing complexes in the regulatory regions of brown adipocyte targets, including Ucp1. For example, PRDM16 is required for brown adipocyte development and function, and a loss of which results in cold intolerance and altered brown adipocyte identity, in part, through the reduced expression of genes that include Ucp1, Cicda, Ppca1, and Dio2 [10]. Though many TFs and co-regulators have been identified in brown adipocyte differentiation, the transcriptional complexes maintaining mature brown adipocyte function are not fully elucidated. A greater understanding of gene regulatory mechanisms governing normal brown adipose function will enhance the development of targeted therapies against metabolic dysregulation. We previously identified LIM domain-binding protein 1 (LDB1) as a potential regulator of BAT function [11]. This co-regulator acts as a dimerized scaffold and directly binds LIM-domain factors, including LIM-domain TF and LIM-only (LMO) adaptor proteins [12]. Ldb1 is widely expressed, including in BAT, and regulates metabolic function [11-13]. We assessed whole-body metabolism in global Ldb1 heterozygous (i.e., $Ldb^{1+/−}$) lean and obese mice to determine how a mild genetic insult affects energy expenditure and glucose metabolism [11]. $Ldb^{1+/−}$ mice displayed reduced energy expenditure at both ambient and reduced temperatures, suggesting thermogenic impairments. Thermogenic mRNAs, including Elovl3, involved in cellular lipid homeostasis, were reduced in $Ldb^{1+/−}$ BAT. Chromatin immunoprecipitation (ChIP) analysis revealed LDB1 occupancy at an upstream Elovl3 control domain, indicating LDB1 may directly regulate this BAT gene [11]. Due to the global LDB1 heterozygosity employed in our prior study, we could not assess the direct roles of BAT-expressed LDB1. Here, we examined the specific role of LDB1 as a novel effector of BAT function. In vitro experiments revealed Ldb1-deficient adipocytes had reduced BAT-selective mRNAs as well as adipogenic- and lipid-associated markers. Additionally, Ldb1-deficient adipocytes were unable to induce Ucp1 expression upon [3-AR agonism. Effects on Ucp1 may be due, in part, to the LDB1 occupancy of Ucp1 regulatory domains. Additionally, we found that BAT expresses several LIM-domain factors, which could serve as potential transcriptional partners mediating LDB1 action. LDB1 expression was also enriched in human adipose biopsies, suggesting LDB1 may have analogous roles in humans. In vivo studies using a BAT-specific LDB1 knockout mouse model, termed $Ldb^{ΔBAT}$, revealed significant impacts on glucose and insulin tolerance. $Ldb^{ΔBAT}$ mice had reduced BAT glucose uptake and altered lipid utilization, suggesting LDB1 regulates substrate metabolism in brown adipocytes. Finally, reductions in LDB1 resulted in significant impairments in thermogenesis and energy expenditure. Overall, our data support the regulatory importance of LDB1 in BAT function.

2. MATERIALS AND METHODS

2.1. Mice

All mice were fed a standard chow (4.7 kcal%, #7917, Envigo, Indianapolis, IN). The conditional BAT $Ldb^{1}$ knockout ($Ldb^{ΔBAT}$) model was generated by crossing floxed $Ldb^{1}$ (Ldb$^{flx}$ defined as CTL) [14,15] and transgenic Ucp1-Cre mice (B6.FVB-Tg(Ucp1-cre)1Evdri/J, Jax stock #024670) [16]. All mouse lines were maintained on a mixed (C57BL/6J) background. Mice were single- or group-housed on a 12:12 h light/dark cycle (lights on from 0600 to 1800 h) near thermoneutrality at 28 °C ± 1 °C [17] and constant humidity with ad libitum access to food and water, except when noted. All studies were approved and performed according to the University of Alabama at Birmingham (UAB) Institutional Animal Care and Use Committee guidelines.

2.2. Stromal Vascular Fraction (SVF) induction and adenovirus transduction

The stromal vascular fraction containing preadipocytes was isolated from interscapular mouse BAT depots after dissection, mincing, collagenase digestion, and centrifugation [18]. The SVF pellet was resuspended in a maintenance media containing DMEM, fetal bovine serum, penicillin, and streptomycin (1% solution, Gibco by Life Technologies, # 15140-122) and seeded onto a 12-well dish. Adherent cells were switched to differentiation media (maintenance media supplemented with 20 nM insulin, 1 nM T3, 125 uM indomethacin, 0.5 μM dexamethasone, 0.5 μM IIBX, 5 μM rosiglitazone) and cultured for up to 6 d, with feeding every 2 d. For the transduction of progenitors or mature brown adipocytes, adenovirus expressing LacZ or Cre was added to cultured $Ldb^{ΔBAT}$ cells before induction at MOI = 10–100 (for 50,000 cells) for 48 h. A subset of cells was treated with the [3-AR agonist [19] (CL316,243, 10 μM, #50-476-10001 EMD Millipore/Calbiochem) or DMSO vehicle for 24 h.

2.3. Tissue isolation

Ad libitum-fed $Ldb^{1BAT}$ and CTL $Ldb^{ΔBAT}$ mice were anesthetized with isoflurane then euthanized via cervical dislocation. Tissues were harvested from brown, epididymal, and inguinal adipose deposits, flash-frozen, and stored at −80 °C until analysis. For the isolation and assessment of mature adipocytes, tissue was extracted, followed by collagenase digestion (Collagenase P, Roche, # 11249002001, 1.5 mg/ml) and centrifugation at 10,000 × g at 4 °C for 15 min. The top layer containing mature adipocytes was collected for analysis.

2.4. Cell lines

siRNA transfections [20] were conducted with the Ucp1-expressing X9 adipocyte cell line as previously described [21]. These cells were induced similarly to the SVF experiment detailed above. ChIP was performed using chromatin extracted from an immortalized brown preadipocyte cell line generously provided by Dr. Kai Ge (Adipocyte Biology and Gene Regulation Section, NIDDK) [22]. This cell line was also induced similarly to the SVF experiment detailed above.

2.5. Body composition and indirect calorimetry

Weekly body weights were collected on male and female CTL and $Ldb^{ΔBAT}$ mice. Body composition was measured immediately before indirect calorimetry using noninvasive nuclear magnetic resonance spectroscopy (EchoMRI; Echo Medical Systems) at the UAB Nutrition Obesity Research Center Small Animal Phenotyping Core. The energy expenditure (EE), food intake, and respiratory quotient were simultaneously measured using a combined indirect calorimetry system (Comprehensive Laboratory Animal Monitoring System; Columbus Instruments) as previously described [11,23]. O₂ consumption and CO₂ production were measured every 15 min to determine the respiratory quotient and energy expenditure. Home-cage locomotor activity was determined using a multidimensional infrared light-beam system.
2.6. Western blotting

Western blotting was performed as described previously [20,24]. Polyvinylidene difluoride (PVDF) membranes were probed using the following primary antibodies: rabbit α-LDB1 (1:1000, generously provided by Dr. Paul Love, NIH), rabbit α-UCP1 (1:1000, Cell Signaling #14670S (D9D6X)), mouse α-Actin (1:1000, Abcam ab32820), rabbit α-pAKT (1:1000, Cell Signaling #9271S), rabbit Pan AKT (1:3000, Cell Signaling #46915S), rabbit α-phospho-p44/42 MAPK (1:2000, Cell Signaling #4226) (1:1000, Cell Signaling #4370S), and rabbit α-p44/42 MAPK (1:2000, Cell Signaling #4224) (1:1000, Cell Signaling #4695S).

2.7. In vivo metabolic analysis

Mice underwent a 6-hour fast prior to intraperitoneal glucose tolerance tests (PGITT) and intraperitoneal insulin tolerance tests (iPTTT) as previously described [11,20]. Mice were then injected intraperitoneally with 2.0 g/kg body weight of glucose or 0.5 U/kg body weight of insulin. Core body temperature was assessed hourly during a 6-hour fast before an intraperitoneal glucose injection (2.5 g/kg). Thirty minutes after the injection, mice were sacrificed, and tissues were snap-frozen in liquid N2. Sample tissues were lysed in 1 N NaOH at 80 °C, followed by neutralization (~pH 7.5) with 12 N HCl. Supernatants were extracted with Ba(OH)2·ZnSO4 (i.e., Somogyi method [26]). Reported 2-deoxyglucose phosphate was defined as the difference between the radioactivity of the neutralized and extracted supernatant. Core BAT tissue was harvested for insulin signaling 10 min after an intraperitoneal injection of 1 U/kg body weight of insulin. Core body temperature was assessed hourly during a 4 h cold challenge using a rodent rectal probe (Microtherm, Thermoworks). After collecting basal core body temperature at 28 °C, mice were individually separated into empty boxes without food, water, or bedding, then placed at 4 °C.

2.8. Histology

The gross morphology of BAT tissue was imaged with a 16 MP camera. BAT was fixed for 6 h in 4% paraformaldehyde diluted in PBS at 4 °C, then embedded in paraffin for the histological analysis. Sections were cut at 6 μm for hematoxylin and eosin (H&E) staining. Slides were imaged using an Olympus IX81 fluorescence microscope and processed using the count and measure feature on CellSens Dimension software version 1.12 (Olympus). Lipid droplet size was quantified from male mice (n = 3–4) using CellSens Dimension version 1.12 software from duplicate or triplicate slides.

2.9. Quantitative real-time PCR

RNA was isolated from cultured cells or the whole tissue using the RNeasy Lipid Mini Kit (Qiagen; #74136) according to the manufacturer’s instructions. Complementary DNA was synthesized by RT-PCR using a Bio-Rad SuperScript III. Single-gene quantitative PCR was performed with iTaq SYBR Green (Bio-Rad; #172-5124) using a LightCycler 480 II (Roche Applied Science) or a CFX96 Real-Time System (Bio-Rad). Data were analyzed using the 2^ -ΔΔCT method and normalized to the housekeeping gene TATA-Box Binding Protein (TBP, for mice), ACTIN (human), or gapdh (cell line). See Supplemental Tables 1 and 2 for primer sequences.

2.10. Chromatin Immunoprecipitation (ChIP)

BAT from C57Bl/6 mice was dissected, minced, fixed with 1% formaldehyde diluted in PBS for 20 min at room temperature, quenched with 125 mM glycine for 5 min at room temperature, then homogenized using a tissue homogenizer (PowerGen 125; Fisher Scientific). Samples were then centrifuged at 4000 × g for 5 min at 4 °C, the middle formaldehyde/PBS layer was removed and washed three times with PBS. Lysis buffer containing 1% SDS, 10 mM EDTA, and 50 mM Tris pH 8 was added, and protein–DNA fragments were sonicated to a mean size of ~500 bp four times using 5 min intervals with 30 s on/off (Misonix Sonicator 3000). Sonicated samples were centrifuged at 10,000 × g for 5 min at 4 °C, and the supernatant was collected. Chromatin was then incubated overnight at 4 °C with α-LDB1 (Santa Cruz; sc-11198x) or control immunoglobulin G (Bethyl; Cat. F50-100). Chromatin complexes were precipitated with Protein G Dynabeads (invitrogen by Thermo Fisher, #10003D) at 4 °C for 4 h, washed with a series of high salt, low salt, LiCl and TE buffer solutions, and eluted with an elution buffer consisting of 0.1 M NaHCO3, 0.2% SDS, 5 M NaCl, and Proteinase K, and then crosslinks were reversed. Quantitative PCR was performed on the ChiP DNA using an SYBR Green PCR master mix (Bio-Rad). Enriched ChiP DNA was normalized to Albumin promoter DNA enrichment and calculated relative to IgG, set as one-fold (ΔΔCT) [11,20,24]. ChiP experiments were performed with independent chromatin preparations. Primers were designed to flank putative A/T-rich homeodomain binding elements (i.e., core TAAT/ATTAC in the Ucp1 proximal promoter and distal enhancer region. See Supplemental Table 2 for primer sequences.

2.11. Statistical analysis

All data are represented as mean ± standard error of the mean (SEM). Statistical significance was calculated using either unpaired Student’s t-tests or one- and two-way analysis of variance (ANOVA), where appropriate. This analysis was followed by multiple-comparison Tukey and Sidak post-tests, respectively. All statistical measures were completed using GraphPad Prism version 8.0 (GraphPad Software). Statistical significance was assigned at P < 0.05.

3. RESULTS

3.1. LD1B is required for the expression of thermogenic BAT genes in vitro

We assessed two in vitro models of Ldb1 deficiency to examine the LDB1 effects on brown adipocyte gene expression. We first conducted an siLdb1 knockdown in the UCP1 + X9 adipocyte cell line (20). After differentiation, an Ldb1-specific siRNA transfection resulted in significant Ldb1 mRNA reduction, compared to scramble (SiScr) controls (Figure 1A). We employed a β3-AR agonist CL-316,243 (CL) to induce thermogenic gene expression and Ucp1 transcription compared to the DMSO vehicle (V). There was no difference in Ucp1 mRNA in siLdb1 cells treated with CL or V (Figure 1B), suggesting LDB1 is required for Ucp1 induction upon agonist treatment. We next transduced cultured primary brown adipocytes isolated from Ldb1 ± Flox mice with either an adenovirus expressing Cre recombinase to elicit Ldb1 recombination or a LacZ control. Transduced cells were cultured in differentiation media then CL-treated before harvesting (Figure 1C). Cre-transduced adipocytes had reduced Ldb1 and Ucp1 protein and mRNA compared to LacZ controls (Figure 1D–F). LacZ and Cre-transduced adipocytes were treated with a CL agonist to evaluate impacts on Ucp1 induction. CL-treatment in control cells (Lacz + CL) significantly induced Ucp1 mRNA (Figure 1F). However, Ldb1-deficient adipocytes treated with the agonist (Cre + CL) had impaired Ucp1 induction, both relative to LacZ control and the fold induction to Cre + Vehicle control (Figure 1F). Next, we assessed whether LDB1 effects are specific to brown adipocytes or general adipocyte differentiation in vitro independent of CL
Figure 1: LDB1 is required for the expression of thermogenic BAT genes in vitro. (A–B) Ldb1 and Ucp1 mRNA quantification from siLdb1 or control siScramble (siScr) transfected and vehicle (V) or agonist (CL) treated X9 cells reveals that knockdown of Ldb1 results in significantly reduced Ucp1 mRNA in agonist treated cells (n = 7–9). Right, inset of Ucp1 fold change in X9 cells comparing siLdb1 knockdown with vehicle (V) and CL agonist treatment. (C) Schematic showing experimental design of Ldb1 recombination in primary cell culture. (D) Western blot assessing LDB1 and UCP1 protein levels as compared to Actin loading control upon LacZ- or Cre-expressing adenoviral transduction and agonist (CL) or DMSO vehicle treatment of primary Ldb1Flox cells (n = 4). Right panel is the quantification of western blots via densitometry of LDB1 and UCP1 protein band intensity normalized to Actin. (E–F) Ldb1 and Ucp1 mRNA quantification upon LacZ- or Cre-expressing adenoviral transduction and agonist (CL) or vehicle treatment of primary Ldb1Flox cells (n = 4). Right, inset of Ucp1 fold change of Cre-expressing adenoviral transduced primary brown adipocytes treated with vehicle (V) or CL agonist. ns, not significant via Student’s t-tests. (G) qRT-PCR analyses of adipocyte and differentiation markers in Adeno-LacZ or Cre-treated cells (n = 3–4); *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 via Student’s t-tests or one- and two-way ANOVA, where appropriate.
Ldb1-deficient cells had significant reductions in brown adipocyte markers Elovl3, Cidea, Pgc1a, and Dio2 (Figure 1G). We also observed reductions of pan-adipocyte markers Pparg and Ppara, WAT-selective markers Lep and Hoxc9 and the Myf5 progenitor differentiation marker (Figure 1G). Additionally, we found mRNA involved in lipid uptake (e.g., Lpl and Cd36) and fatty acid oxidation, including Acadl, Acadm, and Cpt1b, reduced upon Ldb1 loss (Sup Fig. 1). This finding suggests that without CL induction, LDB1 may be required for the expression of brown selective markers related to adipocyte differentiation and basal function in vitro. However, not all markers related to adipocyte maturation and differentiation were reduced (Figure 1G, Sup Fig. 1), highlighting the specificity of LDB1 effects on brown adipocyte genes, rather than a general loss of transcription.

3.2. Expression of thermogenic related mRNA requires LDB1 in vivo

We employed a conditional knockout strategy via the recombination of an Ldb1Flox allele driven by Ucp1-Cre, termed Ldb1D BAT (Figure 2A), to directly test the role of LDB1 in regulating BAT function in vivo. An assessment of body weight over time showed no difference between

![Figure 2](image-url)
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$Ldb^{ΔBAT}$ and CTL mice for both sexes (Figure 2B). For the characterization of this model, all mice were housed long-term at 28 °C, near thermoneutrality [17]. As BAT is the primary adipose depot expressing $Ucp1$ at this temperature, a cold-induced Cre expression in other adipose depots should be limited [16]. Mature adipocytes were isolated from CTL and $Ldb^{ΔBAT}$ mice to assess the efficiency of $Ldb1$ loss. Compared to CTL, $Ldb1$ mRNA and protein were significantly reduced by >60% in $Ldb^{ΔBAT}$ mice (Figure 2C–D, Sup Fig. 2). This moderate but not complete loss could be due to the relatively large distance between LoxP sites (approximately 3 kb) within the $Ldb1$ locus or incomplete penetrance of the Cre transgene. In addition, without sorting $Ldb1$-deficient mature brown adipocytes, we could not reveal potentially greater recombination efficiency in purified $Cre^+$ cells. $Ldb1$ mRNA levels were compared between BAT, epididymal, and inguinal white adipocyte depots from CTL and $Ldb^{ΔBAT}$ male mice to determine the tissue specificity of the recombination. $Ldb1$ reductions were restricted to brown adipocytes (Figure 2D). Even with a 60% loss of $Ldb1$ in BAT (Figure 2C–D), mRNA-encoding BAT-selective thermogenic regulators, including $Ucp1$, $Elovl3$, $Dio2$, and $Fgca1$, were reduced, whereas the brown adipocyte marker $Zic1$ was unchanged (Figure 2E). This finding corroborates the in vitro results above (Figure 1) and our previous $Ldb1^{−/−}$ study. Markers for adipogenesis, myogenesis, and differentiation were evaluated to determine if the reductions in specific thermogenic mRNA were due to altered adipocyte differentiation. Adipogenic markers $Pparg$ and $Ppasa$ (both of which are involved in lipid metabolism in mature adipocytes) were unchanged in $Ldb^{ΔBAT}$ mice (Figure 2E). White adipocyte selective markers $Lep$ and $Hoxc9$ were also unchanged, whereas the muscle-associated structural gene $Myh3$ was reduced (Figure 2F). Transcriptional regulators $Myf5$ and $Prdm16$, both associated with progenitor differentiation, were unchanged, whereas $Egr2$, a late marker of brown adipocyte maturation, was slightly reduced (Figure 2F). Overall, these data suggest that $Ldb1$ is necessary for the maintenance of the thermogenic identity of mature brown adipocytes.

3.3. $Ldb1$ occupies upstream $Ucp1$ regulatory domains, has several potential BAT-expressed partners, and is enriched in human supraclavicular adipose

Having observed the impacts of $Ldb1$ on $Ucp1$ expression, we hypothesized that $Ldb1$ might occupy $Ucp1$ regulatory regions to control thermogenic gene expression. We examined whether $Ldb1$ occupies the previously described $Ucp1$ promoter and/or enhancer regions [27]. As $Ldb1$ lacks DNA-binding capacity, we designed ChIP primers flanking one A/T-rich putative LIM-homeodomain (HD) binding element within the $Ucp1$ proximal promoter and two within the enhancer (Figure 3A). With chromatin from either induced immortalized mature adipocytes [22] or primary BAT, we observed significant $Ldb1$ enrichment in the $Ucp1$ enhancer, spanning HD elements 2 or 3, and within the proximal promoter spanning HD element 1, compared to IgG control (Figure 3B–C). Given that $Ldb1$ forms large transcriptional complexes, we next wanted to assess potential LIM-factor interacting partners required to elicit $Ldb1$ function. A LIM-factor survey in BAT revealed an enrichment of LIM-only factors (LMO), Lmo2 and Lmo4, in addition to $Lhx8$ (Figure 3D). An assessment of BAT extracted at 28 °C, and 4 °C revealed $Ldb1$, $Lhx8$, and $Lmo2$ mRNA levels were reduced in response to cold, whereas $Lmo4$ remained unchanged (Figure 3E). Compared to preadipocytes in the SVF fraction, $Ucp1$, $Ldb1$, and $Lmo4$ were enriched, $Lhx8$ was decreased, and $Lmo2$ remained unchanged in mature brown adipocytes (Figure 3F). Elevations of $Ldb1$ and $Ucp1$ protein upon differentiation were also observed in immortalized brown preadipocytes (Figure 3G).

3.4. Glucose tolerance and uptake are reduced upon loss of $Ldb1$, in part via altered insulin signaling

Given the known importance of glucose to brown adipocyte function, we hypothesized that $Ldb1$ might regulate glucose utilization, altering whole-body glucose homeostasis. Though the fasting blood glucose was unchanged between genotypes (Figure 4A), an intraperitoneal glucose tolerance test (IPGTT) revealed impaired glucose tolerance in male $Ldb1^{ΔBAT}$ mice (Figure 4B). Given that glucose intolerance was only observed in males, we further conducted an intraperitoneal insulin tolerance test (IPITT) in male $Ldb1^{ΔBAT}$ mice. Under thermoneutral conditions, insulin tolerance was similar between CTL and $Ldb1^{ΔBAT}$ mice (Figure 4C). However, when the IPITT was performed after a cold challenge or CL injection, $Ldb1^{ΔBAT}$ mice exhibited reduced glucose clearance, suggesting that $Ldb1$ affects insulin action in active BAT (Figure 4D–E). An assessment of 2DG uptake uncovered impaired BAT glucose uptake in $Ldb1^{ΔBAT}$ male mice (Figure 4F). This effect was specific to BAT, as uptake in other metabolic tissues (including the gastroc and heart) were similar regardless of genotype. Additionally, there was no difference in fasting plasma insulin at thermoneutrality between $Ldb1^{ΔBAT}$ and CTL mice (Figure 4G), which led us to assess changes in insulin signaling. The quantification of insulin-stimulated AKT phosphorylation revealed decreased pAKT$\text{Ser}^{473}$, with increased pERK1/2 in BAT of $Ldb1^{ΔBAT}$ mice (Figure 4H–G), supporting that mildly impaired glucose tolerance in $Ldb1^{ΔBAT}$ mice may be due, in part, to altered insulin signaling.

3.5. $Ldb1$ impacts brown adipocyte morphology and lipid metabolism

Brown adipocytes utilize intracellular and circulating lipids to fuel thermogenesis and maintain tissue function. Based on our observations, we predicted that $Ldb1$ might regulate brown adipocyte lipid utilization and storage. Despite no overt differences in gross BAT morphology between CTL and $Ldb1^{ΔBAT}$ mice, BAT weight was significantly increased in female $Ldb1^{ΔBAT}$ mice, with a trending increase in males (Figure 5A–B). An assessment of adipocyte morphology and lipid droplet size at thermoneutrality revealed a trend toward slightly larger adipocytes and lipid droplets between groups (Figure 5C–D). After an acute 3 h cold challenge, lipid droplets of $Ldb1^{ΔBAT}$ brown adipocytes were significantly larger than CTL (Figure 5E–F). Except for the increased plasma TG levels in $Ldb1^{ΔBAT}$ mice at thermoneutrality, an assessment of plasma TG, free fatty acids (FFA), and cholesterol levels during a cold challenge revealed no differences between groups (Sup Fig. 3). This finding suggests that impairments in lipid handling may be specific to BAT. We assessed changes in BAT mRNA involved in fatty acid uptake, synthesis, and
Figure 3: LDB1, which occupies the Ucp1 regulatory domains and is enriched in human supraclavicular adipose, may partner with several BAT expressed LIM factors to regulate gene expression. (A) Schematic of putative homeodomain (HD) binding sites in the Ucp1 promoter and enhancer region with primer locations and amplicon size denoted. (B–C) LDB1 ChIP of enhancer and promoter regions of Ucp1 using chromatin isolated from a brown adipocyte cell line (n = 3) or whole BAT (n = 4). (D) LIM factor survey assessing mRNA levels of LIM-HD and LMO transcriptional regulators relative to TBP in BAT (n = 4). Ct values for all mRNA tested are shown. (E) Comparison of mRNA markers using BAT from mice housed at 28°C or after a 3-hour 4°C cold challenge (n = 4). (F) Assessment of mRNA levels from SVF or mature adipocytes isolated from BAT of mice housed at 28°C (n = 4–7). (G) Western blot showing protein expression of LDB1 and UCP1 before and after preadipocyte differentiation (n = 3). (H) Gene expression data from human adipose biopsies taken from subcutaneous WAT (black bar n = 2) and supraclavicular fat (i.e., thermogenic adipose, white bars n = 3). *p < 0.05; **p < 0.01; ***p < 0.001 via Student’s t-tests.
oxidation to explore this effect further. Except for Cd36, which was significantly reduced in Ldb1ΔBAT mice, most markers of fatty acid uptake, including Lpl and Fatp1, were unchanged. An assessment of mRNA associated with fatty acid synthesis (including Fasn) and oxidation (including Acadl, Acadm, Cpt1a, and Cpt1b) revealed reductions in the Ldb1ΔBAT mice compared to CTL. Additionally, several mRNA associated with mitochondrial respiration, including Cox, Nduf, Cytc, Coxdb, and Cox7a, were also reduced in the Ldb1ΔBAT mice. Together these data suggest that LDB1 regulates pathways involved in fatty acid oxidation in brown adipocytes.

Figure 4: Glucose tolerance and uptake are reduced upon loss of LDB1, in part via altered insulin signaling. (A) Plasma blood glucose from male (n = 5) and female (n = 11) CTL and Ldb1ΔBAT mice after a 6-h fast. (B) Intraperitoneal glucose tolerance tests (IPGTT) after injection of 2.0 g/kg body weight of glucose for CTL and Ldb1ΔBAT mice raised at 28 °C for female mice (CTL n = 11 and Ldb1ΔBAT n = 12) and male mice (CTL n = 5 and Ldb1ΔBAT n = 4) aged 2–4 months. Inset shows Area Under the Curve (AUC). (C) Intraperitoneal insulin tolerance tests (IPITT) to assess changes in blood glucose upon injection of 0.5U/kg body weight of insulin in CTL and Ldb1ΔBAT male mice at 28 °C (n = 5–6). (D–E) IPITT conducted in male mice 15 min after an acute 4 °C cold challenge or CL316,243 injection, respectively (n = 5–6; aged 3-month-old). Genotype effect found on lower right corner calculated via 2-way ANOVA. (F) Glucose uptake via 2DG glucose tracer in male mice (n = 5–7; 8–9-month-old). (G) Plasma insulin assessed via ELISA from CTL and Ldb1ΔBAT male mice at 28 °C (n = 6). (H) Assessment via western for pan or pAKT (Ser473) protein (n = 3); Bottom panel is densitometry of pAKT (Ser473) protein band intensity normalized to total AKT. (I) Assessment via western of total and p-Erk1/2 protein (n = 3); Bottom panel is densitometry of p-Erk1/2 protein band intensity normalized to total Erk1/2 protein. * p < 0.05; **p < 0.01; ***p < 0.001; ns, not significant via Student’s t-tests or two-way ANOVA, where appropriate.
3.6. LDB1 is required for energy expenditure and thermogenic function

Having observed that LDB1 is required to maintain BAT gene expression and glucose/lipid homeostasis, we next examined whether LDB1 impacts BAT-driven EE and thermogenesis. EE was measured in CTL and \textit{Ldb1}^{\Delta BAT} mice via indirect calorimetry at 26 °C, 24 °C, and 22 °C to assess if EE changes with temperature (Figure 6A–B). Both female and male cohorts followed a typical circadian EE pattern at all temperatures. For female mice, there was no EE difference observed between genotypes at 26 °C or 24 °C (Figure 6A). However, at 22 °C,
when the thermogenic activity of BAT is higher, there was a significant reduction in EE in Ldb1ΔBAT female mice compared to CTL (Figure 6A). Additionally, there was no temperature-dependent difference in EE in Ldb1ΔBAT females, suggesting LDB1 is required for brown adipocyte activation (Figure 6A). Compared to CTL mice, EE was reduced at all temperatures in Ldb1ΔBAT male mice (Figure 6B). These changes were independent of body composition, respiratory quotient, food intake, and locomotor activity, which were similar between genotypes (Sup Fig. 4). This finding suggests that BAT-expressed LDB1 is required for maintaining whole-body EE. Due to the EE impairments and observed reductions of BAT-selective mRNA, we hypothesized that BAT-mediated thermogenesis would be impaired upon LDB1 loss. We tested this hypothesis by conducting a 4°C cold challenge with core body temperature measured at baseline (time = 0) and every hour thereafter, for up to 4 h. Strikingly, both female and male Ldb1ΔBAT mice exhibited profound reductions in core body temperature over time, compared to CTL mice (Figure 6C–D). These data strongly support that LDB1 regulates brown adipocyte-driven EE and thermogenesis.

4. DISCUSSION

Through complementary in vitro and in vivo deficiency models, we uncovered novel regulatory roles for LDB1 in brown adipocyte function. BAT-specific reductions of LDB1 resulted in impaired whole-body EE, cold intolerance, and altered lipid metabolism in male and female mice, as well as glucose intolerance in male mice. Cold intolerance in Ldb1ΔBAT mice is consistent with other models of UCP1 deficiency, either through Ucp1 knockout [7,8] or loss of Ucp1 transcriptional regulators, including PRDM16 [10]. Though LDB1 may regulate Ucp1 to affect cold tolerance, reductions in additional gene targets involved in lipid metabolism and thyroid signaling (e.g., Elovl3, Dio2) likely

Figure 6: LDB1 is required for energy expenditure and thermogenic function. (A–B) Indirect calorimetry on female and male Ldb1ΔBAT and CTL littermates. Energy expenditure (EE) was measured at three temperatures, 26 °C, 24 °C, and 22 °C, in chow-fed mice (n = 4–6), aged 5–6 months. Right panel is total EE quantified at 26 °C, 24 °C, and 22 °C. Genotype effect found on lower right corner calculated via 2-way ANOVA. (C–D) Four-hour cold challenge was conducted at 4 °C on (C) female mice (CTL n = 9 and Ldb1ΔBAT n = 14) and (D) male mice (CTL n = 8 and Ldb1ΔBAT n = 11) aged 2–4 months. *, p < 0.05; **, p < 0.01; ***, p < 0.001; ****, p < 0.0001; via Student’s t-tests or two-way ANOVA, where appropriate.

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contribute to the severe cold intolerance phenotype. Substrates like glucose and fatty acids are either oxidized as fuel for thermogenesis or used to replenish intercellular lipid pools [34,35]. Given the importance of metabolites for adipocyte thermogenesis, models affecting substrate uptake and utilization, including an adipose triglyceride lipase (ATGL) knockout model, also result in cold intolerance phenotypes [36]. Ldb1ΔBAT male mice have impaired glucose uptake, whereas both male and female Ldb1ΔBAT mice have apparent changes in fatty acid oxidation. Therefore, we hypothesized that the severe cold intolerance phenotype observed is due, in part, to changes in glucose and/or lipid metabolism. In support of this hypothesis, we found altered insulin signaling in male Ldb1ΔBAT mice. Reductions in p- Akt[Ser473], a substrate of the IRS-1/PI3K/AKT pathway, may suggest Ldb1 regulates pathways involved in insulin-induced lipid synthesis and Ucp1 induction [37]. However, upon reductions in Ldb1, we also found significant increases in p-Erk1/2, a substrate of the Grb-2/Ras/MAPK pathway, suggesting Ldb1 may negatively regulate pathways involved in adipocyte proliferation [37]. This finding suggests that Ldb1 can be both a positive and negative regulator of brown adipocyte function. Future studies will further elucidate brown adipocyte gene expression and cellular pathways, including those regulated by Ldb1.

This study also revealed sex-dependent effects of BAT-expressed Ldb1, consistent with prior studies characterizing BAT physiology. Previously, differences in BAT thermogenesis between sexes have been reported in rodents, with females showing a greater uncoupling capacity, higher mitochondrial content, and greater intracellular lipid content [38–41]. In humans, sex differences in energy metabolism and BAT activity have also been described, with females exhibiting lower EE and higher BAT activity than males [4,42,43]. In the Ldb1ΔBAT model, we observed sex differences in both BAT EE and thermogenesis upon Ldb1 loss. Compared to Ldb1ΔBAT male mice, which had significant reductions in EE at all temperatures, female Ldb1ΔBAT mice only displayed reduced EE at 22 °C. Additionally, a cold challenge revealed that male Ldb1ΔBAT mice displayed the greatest reduction in core body temperature at 4 h. Sex differences in thermogenesis may be attributed to variations in basal uncoupling capacity, sympathetic activity, fat mass composition, and hormone signaling [41,44]. This difference also extends to metabolic parameters, like glucose and lipid homeostasis [45]. Previous studies highlighted differences in insulin sensitivity, insulin-stimulated glucose uptake, and lipogenesis in rodent adipocytes, with females being more resistant to metabolic dysregulation [46–48]. Additionally, genetic models utilizing HFD highlight the differential outcomes of sex on disease development [49,50]. For example, a model of Id1 overexpression, a negative regulator of Pgc1α and brown adipose thermogenesis, showed that males but not females were susceptible to HFD-induced obesity. These sex differences are consistent with our model in which, despite reductions in Ldb1 in both sexes, glucose homeostasis was maintained in females. Future studies will investigate the genes and signaling pathways differentially regulated in male and female Ldb1-deficient brown adipocytes in more depth.

As a transcriptional co-regulator, Ldb1 directly interacts with LIM-homeodomain and LIM-only TF sub-classes to regulate gene targets [12,51]. The BAT LIM-factor survey revealed an enrichment of several LIM-factor mRNA, including Lmo2, Lmo4, and Lhx8. Though Lhx8 is a known BAT marker (27–29), its role in brown adipose is largely unknown. Previously, Lhx8 mRNA levels were shown to decrease in brown adipocytes in response to cold [52], as our findings confirm. In addition to Lhx8, Lmo proteins, including Lmo2 and Lmo4, are transcriptional adapters that affect gene expression through interactions within larger transcriptional complexes. In human visceral adipocytes, Lmo3 promotes adipocyte differentiation via the regulation of PPARγ activity [53]. In mice, Lmo4, the highest expressed factor in our LIM survey, was implicated in regulating preadipocyte proliferation and adipogenesis [54]. Though these data support a potential role for Ldb1-Lmo factor complexes in regulating BAT function, LIM-factor antibody limitations precluded us from assessing which LIM factor(s) mediate Ldb1 action in BAT.

The range of interacting partners affords Ldb1-containing complexes a broader potential to regulate brown adipocyte transcription. Through Lmo adapters, Ldb1 can also indirectly interact with basic helix-loop-helix (bHLH) and GATA TF classes to affect gene expression [12]. For example, GATA2, a potential Ldb1 interacting partner, has been linked to brown adipocyte differentiation [55]. Additionally, we found Ldb1 occupying Ucp1 regulatory regions enriched by sequences of other critical BAT TFs and co-regulators, including PPARγ, Pgc1α, and C/EBP [9,27]. Many of these transcriptional regulators participate in large multi-protein complexes, which regulate Ucp1 expression. Examples include PRDM16 and Pgc1α co-regulators, which stabilize transcriptional complexes involving PPAR TFs [56]. The occupancy of Ldb1 in the Ucp1 enhancer and promoter regions may allow for potential interaction with these regulators. Future studies utilizing mass spectrometry may help to define BAT-specific Ldb1-interacting partners. Throughout this study, various models of Ldb1 loss highlighted the requirement of Ldb1 for brown adipocyte function and gene expression. Reductions in thermogenic mRNA levels could be due to the loss of stability of Ldb1-mediated multi-protein complexes. Given the detrimental effects observed upon reductions in Ldb1, it is tempting to propose the over-expression of this co-regulator as a potential mechanism to improve BAT function. However, genetic models employing the over-expression of Ldb1 or interacting LIM-HD TF interactors alone have resulted in dominant negative effects on cellular processes [57–59]. The misexpression of Ldb1 or Lmo2 resulted in the inhibition of erythroid differentiation [57,60]. Additionally, the over-expression of the Drosophila ortholog of Ldb1 (known as Chip) resulted in similar wing formation defects in knockout mutants, likely due to disruptions in protein complex stoichiometry [61]. These single-gene over-expression defects were rescued upon the co-expression of Ldb1 and the Drosophila ortholog of Lhx2 (known as apterous), thus restoring the stoichiometry of the complex. This LIM stoichiometry consists of a tetramer of two dimerized Ldb1 proteins bound directly by two LIM-factors to achieve proper function [12]. Negative impacts conferred by both gain- and loss-of-function models highlight the role of Ldb1 stoichiometry in maintaining the integrity of multi-protein complexes and, therefore, normal cellular processes [57,62]. Given that Ldb1 is required to maintain brown adipocyte function, identifying factors that trans-activate Ldb1 expression would allow for more specific therapeutic targeting of Ldb1-containing complexes. However, there are no known regulators of the Ldb1 gene described in brown adipocytes.

Establishing Ldb1 in brown adipose thermogenesis and substrate utilization serves as an entry point for the future assessment of this co-regulator in other cellular processes. Given reductions in several mRNAs involved in adipocyte differentiation in primary brown adipocytes (Figure 1), utilizing an Myf5-Cre mouse model may further clarify the role of Ldb1 in preadipocyte differentiation. Additionally, models utilizing a high-fat diet may provide a greater understanding of Ldb1 regulation under conditions of metabolic dysregulation. The apparent enrichment of Ldb1 and the putative partner Lhx8 in human BAT supports the supposition that Ldb1 may have conserved roles across species. The identification of LIM-factor regulators acting in BAT or the development of small molecules that can stabilize Ldb1-containing
complexes may enhance the therapeutic potential of LDB1 for the treatment of metabolic diseases.

**AUTHOR CONTRIBUTIONS**

JDK and CSH directed the study. JDK and CSH wrote and edited the manuscript. *In vitro* studies were completed by JDK, TK, and YL. GR provided reagents and guidance on experimental design for adenoviral transfection. *In vivo* experiments were conducted by JDK. Indirect calorimetry was conducted by MY, with analysis performed by JDK and KMH. Analysis of human adipose tissue was conducted by CC, JWJ, and AMC.

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**CONFLICT OF INTEREST**

The authors have declared that no conflict of interest exists.

**APPENDIX A. SUPPLEMENTARY DATA**

Supplementary data to this article can be found online at https://doi.org/10.1016/j.molmet.2021.101284.

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