ORIGINAL ARTICLE

Kruppel-like factor 15 regulates fuel switching between glucose and fatty acids in brown adipocytes

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ABSTRACT

Aims/Introduction: Brown adipose tissue (BAT) utilizes large amounts of fuel for thermogenesis, but the mechanism by which fuel substrates are switched in response to changes in energy status is poorly understood. We have now investigated the role of Kruppel-like factor 15 (KLF15), a transcription factor expressed at a high level in adipose tissue, in the regulation of fuel utilization in BAT.

Materials and Methods: Depletion or overexpression of KLF15 in HB2 differentiated brown adipocytes was achieved by adenoviral infection. Glucose and fatty acid oxidation were measured with radioactive substrates, pyruvate dehydrogenase complex activity was determined with a colorimetric assay, and gene expression was examined by reverse transcription and real-time polymerase chain reaction analysis.

Results: Knockdown of KLF15 in HB2 cells attenuated fatty acid oxidation in association with downregulation of the expression of genes related to this process including *Acox1* and *Fatp1*, whereas it increased glucose oxidation. Expression of the gene for pyruvate dehydrogenase kinase 4 (PDK4), a negative regulator of pyruvate dehydrogenase complex, was increased or decreased by KLF15 overexpression or knockdown, respectively, in HB2 cells, with these changes being accompanied by a respective decrease or increase in pyruvate dehydrogenase complex activity. Chromatin immunoprecipitation showed that *Pdk4* is a direct target of KLF15 in HB2 cells. Finally, fasting increased expression of *KLf15*, *Pdk4* and genes involved in fatty acid utilization in BAT of mice, whereas refeeding suppressed *Klf15* and *Pdk4* expression.

Conclusions: Our results implicate KLF15 in the regulation of fuel switching between glucose and fatty acids in response to changes in energy status in BAT.

INTRODUCTION

Brown adipose tissue (BAT) functions as a thermogenic organ, and is important for basal and inducible energy expenditure. Given that thermogenesis is a highly energetic process, BAT requires a readily available energy substrate[1–3]. The energy-expending properties of BAT and its discovery in human adults have made it an attractive target for the treatment of obesity and metabolic disorders, including type 2 diabetes.

Brown adipose tissue has a high capacity for uptake of fuels, including fatty acids and glucose, from the circulation[4,5]. Fatty acids are a major fuel substrate for thermogenesis in BAT. Fatty acids taken up by brown adipocytes are converted to acyl-carnitine for transport into mitochondria through the carnitine shuttle and then become substrates for β-oxidation. Glucose is

also an important fuel substrate for BAT thermogenesis[6,7]. After its uptake by brown adipocytes, glucose is metabolized to pyruvate by glycolysis. Pyruvate is converted to acetyl coenzyme A by the pyruvate dehydrogenase complex (PDC), and acetyl coenzyme A then enters the citric acid cycle for mitochondrial oxidation. Despite the importance of fatty acids and glucose as fuels for BAT thermogenesis, however, the transcriptional regulation of fuel utilization is poorly understood. Furthermore, the regulation of fuel switching between fatty acids and glucose in response to changes in energy status in BAT remains uncharacterized.

Kruppel-like factor 15 (KLF15), a member of the zinc-finger family of transcription factors, is highly expressed in metabolically active organs including skeletal and cardiac muscle, liver and adipose tissue[8,9]. It plays key roles in the regulation of energy metabolism – including the metabolism of glucose, lipids and amino acids – in response to changes in energy demand[10–14]. KLF15 is abundant in both white adipose tissue (WAT) and BAT[15]. Although KLF15 has been found to be an important regulator of lipid metabolism in WAT as well as of adipocyte differentiation in this tissue[16,17], the physiological role of KLF15 in BAT has remained unknown.

To study the role of KLF15 in brown adipocytes, we carried out adenovirus-mediated gain- and loss-of-function experiments for KLF15 in differentiated brown adipocytes of the mouse cell line HB2. Here, we show that KLF15 promotes the oxidation of fatty acids and suppresses that of glucose in these cells through transcriptional control of genes related to fatty acid utilization, as well as the gene for pyruvate dehydrogenase kinase 4 (PDK4), a negative regulator of glucose flux. We also provide evidence implicating KLF15 as a regulator of fuel switching between fatty acids and glucose in response to changes in energy status in BAT.

MATERIALS AND METHODS
Mice
C57BL/6 mice were obtained from CLEA Japan, and were housed at 21–25°C and maintained on a 12-h light–dark cycle in the animal facility at Kobe University Graduate School of Medicine. All animal experiments were carried out following the national guidelines and the relevant national laws on the protection of animals, and they were approved by and carried out in accordance with the guidelines of the animal ethics committees of Kobe University Graduate School of Medicine.

Cell culture
HB2 cells were cultured and induced to differentiate as described previously[18]. In brief, the cells were seeded on collagen-coated plates and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin until confluence. For the induction of differentiation, they were cultured for 2 days in DMEM supplemented with 0.5 mmol/L 3-isobutyl-1-methylxanthine, 1 µmol/L dexamethasone, 10% FBS and 1% penicillin-

streptomycin and then for 3–5 days in DMEM supplemented with insulin (10 µg/mL), 50 nmol/L triiodothyronine, 10% FBS and 1% penicillin–streptomycin. The cells were used for experiments at 5–7 days after the induction of differentiation.

Adenoviral infection
Adenovirus infection was carried out as described previously[11]. In brief, for KLF15 knockdown, differentiated HB2 cells were infected with an adenovirus encoding a short hairpin ribonucleic acid (RNA) for KLF15 (shKLF15) or with a control virus containing the U6 gene promoter alone (shCont). For KLF15 overexpression, the cells were infected with an adenovirus encoding KLF15 or β-galactosidase (LacZ) as a control. Cells were used for experiments at 48–72 h after adenovirus infection.

Assay of PDC activity
PDC activity in lysates prepared from cells in phosphate-buffered saline containing detergent and protease and phosphatase inhibitor cocktails was measured with the use of a kit (Abcam, Cambridge, UK).

RNA isolation, reverse transcription and real-time polymerase chain reaction analysis
Total RNA was extracted from cells with the use of an RNeasy Minikit (Qiagen, Hilden, Germany) and was subjected to reverse transcription with a high-capacity reverse transcription kit (Applied Biosystems, Foster City, CA, USA). The resulting complementary deoxyribonucleic acid was subjected to real-time polymerase chain reaction (PCR) analysis with SYBR Green PCR Master Mix in an ABI StepOne Plus Real-Time PCR system (Applied Biosystems). Relative expression levels were determined with the standard curve method and with the use of 36B4 messenger RNA (mRNA) for normalization of target mRNA abundance. The PCR primer sequences are listed in Table 1.

Immunoblot analysis
Cells were homogenized on ice in lysis buffer (20 mmol/L Tris-HCl [pH 7.5], 150 mmol/L NaCl, 2 mmol/L ethylenediaminetetraacetic acid, 1% Nonidet P-40, 10% glycerol) supplemented with protease and phosphatase inhibitors. Lysates were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the separated proteins were transferred to a nitrocellulose membrane for immunoblot analysis with antibodies to KLF15 as described previously[9] or with those to tubulin (Abcam).

Chromatin immunoprecipitation analysis
Chromatin immunoprecipitation (ChIP) analysis was carried out with the use of a ChIP assay kit (Merck Millipore, Burlington, MA, USA) as described previously[19]. In brief, after cross-linking with 1% formaldehyde, the nuclear fraction was isolated from differentiated HB2 cell extracts, lysed and then subjected to ultrasonic treatment to shear chromatin followed by immunoprecipitation with antibodies to KLF15 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or with control

Table 1 | Sequences of polymerase chain reaction primers

Gene	Forward (5′→3′)	Reverse (5′→3′)
Klf15	ACCGAAATGCTCAGTGGGTTACCTA	GGAACAGAAGGCTTGCGAGTCA
Acox1	TGACCTGCCGAGCCAGCGTAT	GACAGAAGTCAAGTTCCACGCCACT
Fatp1	TCTGTTCTGATTCGTGTTCGG	CAGCATATACCACTACTGGCG
Cox8b	GCTGGCTGGACTCTGTCATT	GTACCAGGGCCTGCATAGTG
Cpt1a	CCTGCATTCCTTCCCATTTG	TGCCCATGTCCTTGTAATGTG
Cpt1b	GCTGCCGTGGGACATTC	CTTGGCTACTTGGTACGAGTTCTC
Pdk4	AGGGAGGTCGAGCTGTTCTC	GGAGTGTTCACTAAGCGGTCA
Pdk2	AGGGGCACCCAAGTACATC	TGCCGGAGGAAAGTGAATGAC
Pdh	TGGTGCTGCTAATCAGGGTC	CCATAGCGGTTGTTCTCACAGA
Hk1	AACGGCCTCCGTCAAGATG	GCCGAGATCCAGTGCAATG
Hk2	TGATCGCCTGCTTATTCACGG	AGACCAATCTCGCAGTTCTGA
Pfk1	TGCAGCCTACAATCTGCTCC	GTCAAGTGTGCGTAGTTCTGA
Pkm2	GCCGCCTGGACATTGACTC	CCATGAGAGAAATTCAGCCGAG

immunoglobulin G (Santa Cruz Biotechnology). Immunoprecipitated deoxyribonucleic acid and input deoxyribonucleic acid were purified, and the promoter region and 3′ untranslated region of *Pdk4* were amplified by real-time PCR analysis. The amount of the target regions in immunoprecipitates was normalized by that in the input samples and is presented as fold enrichment relative to the corresponding immunoprecipitate prepared with control immunoglobulin G. ChIP primer sequences (forward and reverse, respectively) for *Pdk4* are as follows: promoter, 5′-AGTCCTAGCGACCTGGGATC-3′ and 5′-TCACTAGAAAGGCCTGGCA-3′; 3′-untranslated region, 5′-TCTGTCAAGACAGCTCCAGT-3′ and 5′-GAACTC-GACTTTCTGAGGTC-3′.

Assays of glucose and fatty acid oxidation

Assays of glucose and fatty acid oxidation were carried out as described previously[3]. In brief, differentiated HB2 cells in six-well plates were incubated for 2 or 4 h in DMEM supplemented with 2% FBS for measurement of glucose and fatty acid oxidation, respectively. The cells were then incubated at 37°C for 1 h with KRB-HEPES buffer containing 2% bovine serum albumin, 5mmol/L glucose, 200 nmol/L adenosine and [1-^{14}C] glucose (0.5 μCi/mL) for assay of glucose oxidation or with KRB-HEPES buffer containing 2% bovine serum albumin, 5mmol/L glucose, 0.1 mM oleic acid, 100 μmol/L carnitine and [1-^{14}C]oleic acid (0.5 μCi/ml) for assay of fatty acid oxidation. Hydrogen peroxide was then added to each well, and the plates were sealed with wipe smears supplemented with 1 mol/L benzethonium hydroxide solution. Radioactivity trapped in the wipe smears was determined with a liquid scintillation counter (PerkinElmer, Waltham, MA, USA).

Statistical analysis

Quantitative data are presented as the mean ± standard error of the mean, and were compared between groups with the two-tailed Student's *t*-test. A *P*-value of <0.05 was considered statistically significant.

RESULTS

KLF15 regulates fatty acid and glucose oxidation in HB2 cells

Consistent with previous results[8,9], we found that KLF15 mRNA was abundant in metabolically active organs of C57BL/6 mice, especially in the liver, BAT and WAT (Figure 1). To study the role of KLF15 in the function of brown adipocytes, we investigated the effect of KLF15 knockdown or overexpression on fuel utilization, including glucose and fatty acid oxidation, in HB2 differentiated brown adipocytes. Infection of the cells with an adenovirus encoding shKLF15 resulted in depletion of KLF15 mRNA by >80%, whereas that with an adenovirus encoding KLF15 increased the amount of KLF15 mRNA by a factor of ~10 (data
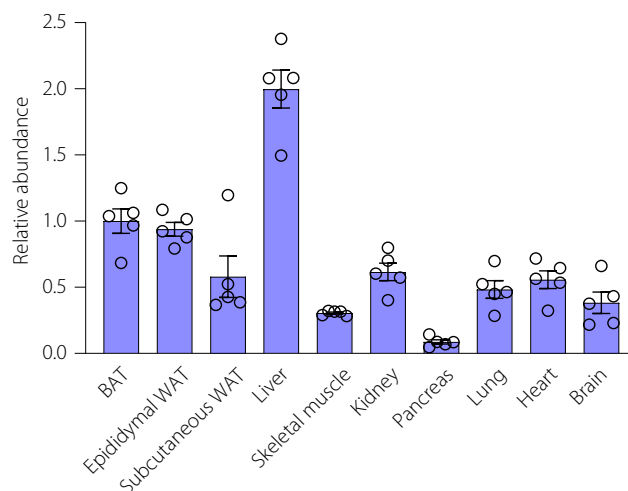
Figure 1 | Tissue distribution of Kruppel-like factor 15 messenger ribonucleic acid in mice. The abundance of Kruppel-like factor 15 messenger ribonucleic acid in the indicated organs or tissues of C57BL/6 mice (male, 8 weeks-of-age) was determined by reverse transcription and real-time polymerase chain reaction analysis. The expression level in each tissue relative to that in brown adipose tissue (BAT) is shown. Data are the mean ± standard error of the mean (*n* = 5). WAT, white adipose tissue.
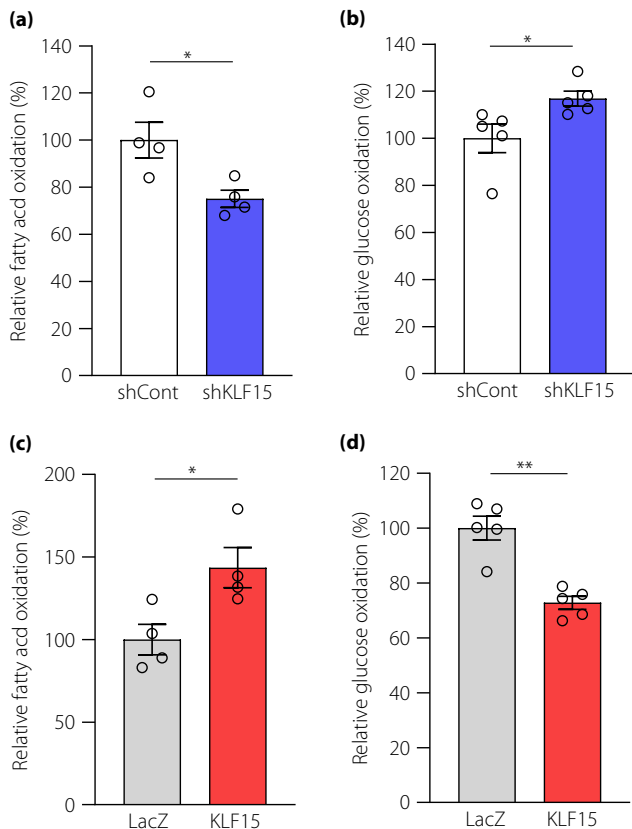
Figure 2 | Effects of Kruppel-like factor 15 (KLF15) knockdown or overexpression on glucose and fatty acid oxidation in HB2 differentiated brown adipocytes. (a,c) Fatty acid oxidation and (b,d) glucose oxidation were measured in HB2 cells infected with an adenovirus encoding short hairpin ribonucleic acid for KLF15 (shKLF15) or (a,b) with a control virus containing the U6 gene promoter alone (shCont) as well as (c,d) with adenoviruses encoding KLF15 or LacZ. The amounts of fatty acid and glucose oxidation were calculated as radioactivity (cpm) per microgram of cellular protein, and were presented as relative fatty acid oxidation or relative glucose oxidation to each control. Data are the mean ± standard error of the mean (n = 4–5 biologically independent samples). **P < 0.01 (Student's t-test).

not shown). Such knockdown of KLF15 significantly attenuated fatty acid oxidation (Figure 2a) and significantly increased glucose oxidation (Figure 2b), whereas overexpression of KLF15 significantly increased fatty acid oxidation (Figure 2c) and attenuated glucose oxidation (Figure 2d), implicating KLF15 in the positive and negative regulation of fatty acid and glucose oxidation in differentiated HB2 cells, respectively.

KLF15 regulates the expression of genes related to fuel utilization in HB2 cells

To elucidate the molecular mechanism by which KLF15 regulates fuel utilization, we examined the effects of gain- or loss-of-function of KLF15 on the expression of genes related to fatty acid or glucose oxidation in differentiated HB2 cells. Infection of the cells with an adenovirus encoding shKLF15 again suppressed KLF15 mRNA abundance by >80% and KLF15 protein amount by >50%, whereas that with an adenovirus encoding KLF15 increased the amount of KLF15 mRNA by a factor of ~10 and of KLF15 protein by 30–40-fold (Figure 3a). Among genes related to fatty acid oxidation, KLF15 knockdown attenuated the expression of *Acox1 and Fatp1*, whereas KLF15 overexpression increased the expression of these genes and *Cox8b* (Figure 3b). The expression of *Cpt1a* and *Cpt1b* was inhibited by KLF15 knockdown, but unaffected by KLF15 overexpression (Figure 3b). These results suggested that KLF15 upregulates the expression of various genes related to fatty acid utilization and thereby increases fatty acid oxidation in HB2 cells. Among genes related to glucose oxidation, the expression of *Pdk4* was significantly attenuated in KLF15-depleted cells compared with control cells, whereas that of *Hk1*, *Hk2*, *Pfk1*, *Pkm2*, *Pdk2* and *Pdh* was unaffected by KLF15 knockdown (Figure 3c). Overexpression of KLF15 increased the expression of *Pdk4*, as well as that of *Hk1* and *Hk2*, whereas it inhibited that of *Pfk1* (Figure 3c). Thus, among genes related to glucose utilization, only *Pdk4* expression was reciprocally regulated by gain or loss of function of KLF15 in HB2 cells.
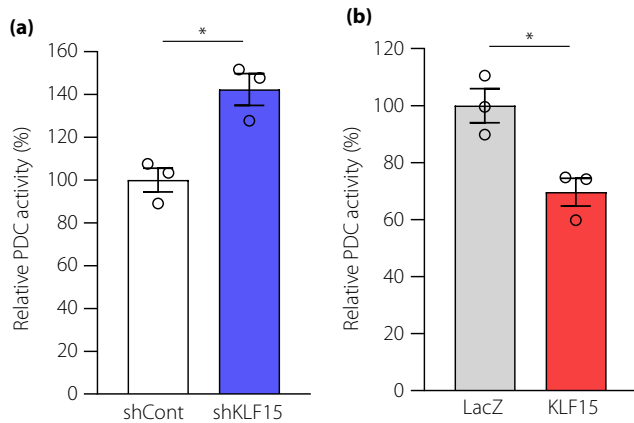
KLF15 regulates PDC activity in HB2 cells

PDK4 phosphorylates and thereby inhibits the activity of PDC, which catalyzes the oxidative decarboxylation of pyruvate to acetyl coenzyme A to provide fuel for Krebs cycle[20]. To investigate whether control of *Pdk4* expression by KLF15 might contribute to the regulation of PDC activity, we examined the effects of KLF15 depletion or overexpression on PDC activity in differentiated HB2 cells. Consistent with its stimulation of glucose oxidation, knockdown of KLF15 significantly increased PDC activity in these cells (Figure 4a), whereas overexpression of KLF15 attenuated PDC activity (Figure 4b). These results suggested that KLF15 suppresses glucose oxidation by increasing *Pdk4* expression and thereby inhibiting PDC activity.

KLF15 binds to the *Pdk4* promoter region in HB2 cells

To investigate whether *Pdk4* is a direct target for transcriptional regulation by KLF15, we searched the promoter region of the gene for consensus binding sites for KLF family members (5′-CACCC-3′). Several such sites were identified in the mouse *Pdk4* promoter region, with one of these sites located at position −238–234 bp relative to the transcription start site also being fully conserved in the human *PDK4* promoter (Figure 5a). We then carried out a ChIP assay with antibodies to KLF15 to examine whether KLF15 directly binds to this putative binding site in differentiated HB2 cells. We detected significant enrichment of KLF15 binding in this *Pdk4* promoter region (Figure 5b), indicating that *Pdk4* is
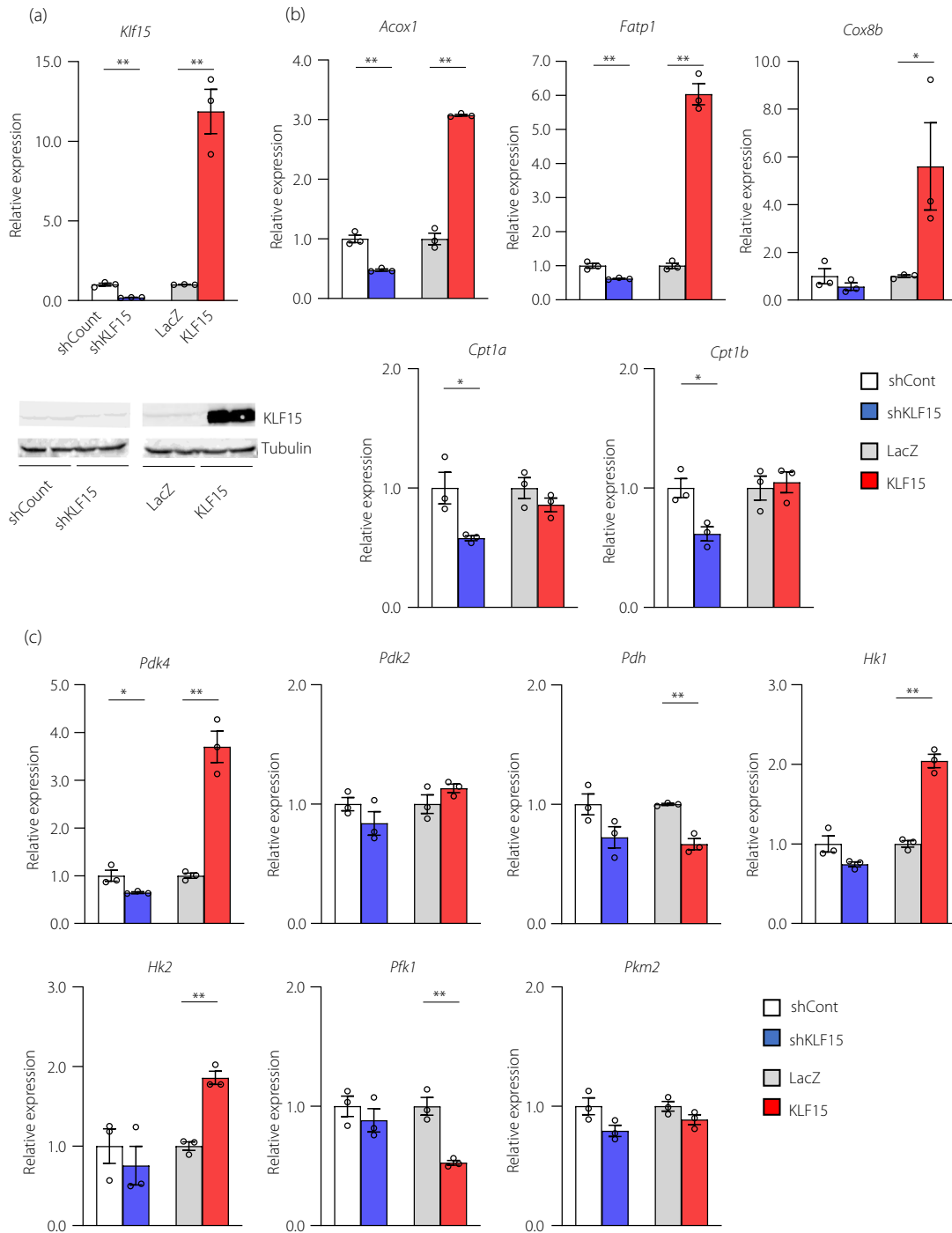
Figure 3 | Effects of Kruppel-like factor 15 (KLF15) knockdown or overexpression on the expression of various genes related to energy utilization in HB2 differentiated brown adipocytes. (a) The amount of KLF15 messenger ribonucleic acid as well as the protein amount of KLF15 and tubulin were determined by reverse transcription and real-time polymerase chain reaction analysis and by immunoblot analysis, respectively, in HB2 cells infected with an adenovirus encoding short hairpin ribonucleic acid for KLF15 (shKLF15), a control virus containing the U6 gene promoter alone (shCont) or adenoviruses encoding KLF15 or LacZ. The amount of messenger ribonucleic acids derived from genes related to (b) fatty acid or (c) glucose utilization in HB2 cells infected with adenoviruses described in (a) were determined by reverse transcription and real-time polymerase chain reaction analysis. Data are the mean ± standard error of the mean (*n* = 3), except for the panel of immunoblot analysis in (a) (*n* = 2). *$P < 0.05$, **$P < 0.01$ (Student's *t*-test).
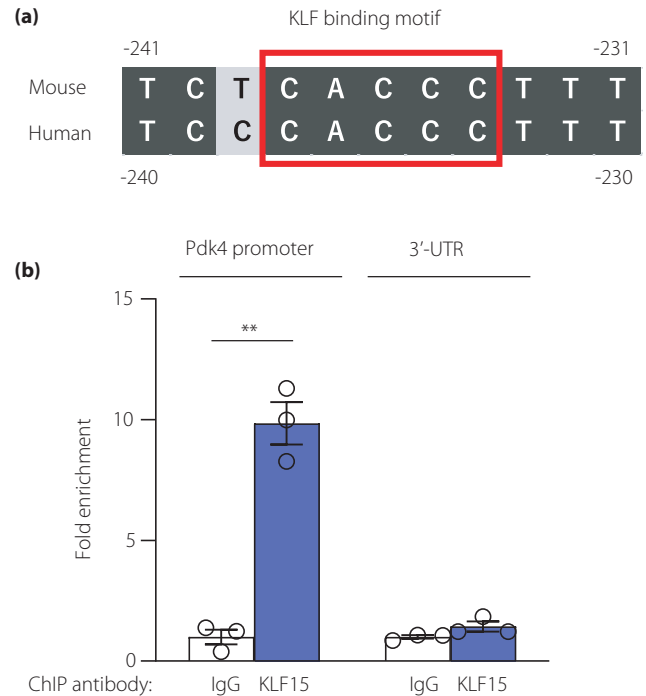
Figure 4 | Effects of Kruppel-like factor 15 (KLF15) knockdown or overexpression on pyruvate dehydrogenase complex (PDC) activity in HB2 differentiated brown adipocytes. PDC activity was measured in HB2 cells (a) infected with an adenovirus encoding short hairpin ribonucleic acid for KLF15 (shKLF15) or with a control virus containing the U6 gene promoter alone (shCont) or (b) in those infected with adenoviruses encoding KLF15 or LacZ. PDC activity was evaluated as the change in absorbance units (ΔA) per milligram of cellular protein per minute, and was presented as relative PDC activity to control. Data are the mean ± standard error of the mean ($n = 3$ biologically independent samples). $*P < 0.05$ (Student's t-test).

indeed a direct target for transcriptional regulation by KLF15.

Role of KLF15 as a metabolic switch for glucose and fatty acid utilization in BAT

To study the physiological relevance of KLF15 in the regulation of fuel utilization, we examined the possible effects of food deprivation and refeeding on *Klf15* expression in BAT of mice. The abundance of KLF15 mRNA in BAT was upregulated after fasting (Figure 6a), and downregulated after refeeding (Figure 6c). These changes in *Klf15* expression were accompanied by corresponding changes in *Pdk4* expression (Figure 6b,d), suggesting that KLF15 is an important regulator of glucose oxidation in BAT in response to changes in energy status. The expression of genes related to fatty acid utilization – including *Acox1*, *Fatp1*, *Cpt1a* and *Cpt1b* – was also increased after fasting in association with the upregulation of KLF15 mRNA (Figure 6b), suggesting that KLF15 might increase the expression of these genes to promote fatty acid utilization in BAT under the fasting condition. In contrast, the expression of these genes related to fatty acid utilization was unaltered or increased after refeeding (Figure 6d), suggesting that transcription factors other than KLF15 might contribute to the regulation of fatty acid utilization in response to refeeding. The expression of genes related to thermogenesis and BAT function including *Ucp1*, *Cidea*, *Ppargc1a* and *Prdm16* in BAT was significantly increased after refeeding (Figure 6d), whereas only *Prdm16* expression was increased after fasting (Figure 6b).

Figure 5 | Chromatin immunoprecipitation (ChIP) analysis of Kruppel-like factor 15 (KLF15) binding to the *Pdk4* promoter region in HB2 differentiated brown adipocytes. (a) Alignment of the promoter regions of the mouse and human PDK4 genes. The red box contains a consensus binding motif for KLF family members. (b). ChIP analysis of KLF15 binding to the promoter region containing the putative KLF binding site shown in (a) or to the 3′-UTR (negative control) of pyruvate dehydrogenase kinase 4 (*Pdk4*) in HB2 cells. Immunoprecipitation was carried out with antibodies to KLF15 or with control immunoglobulin G (IgG; negative control). Data are the mean ± standard error of the mean for triplicates of an experiment representative of a total of three independent determinations. $**P < 0.01$ (Student's t-test).

DISCUSSION

BAT requires fuel substrates for thermogenesis, with fatty acids and glucose being the major substrates for this process. We have here studied the physiological role of KLF15 in the regulation of fuel utilization in brown adipocytes. We found that KLF15 increases fatty acid oxidation and the expression of genes related to fatty acid utilization, whereas it inhibits glucose oxidation in association with direct upregulation of *Pdk4* expression and attenuation of PDC activity, in HB2 differentiated brown adipocytes. Given that *Klf15* expression in BAT was upregulated in response to fasting and downregulated after subsequent refeeding in mice, and that these changes were accompanied by alterations in the expression of genes related to glucose and lipid utilization, KLF15 might play an important role in the regulation of fuel switching between glucose and fatty acids in response to changes in energy status in BAT.
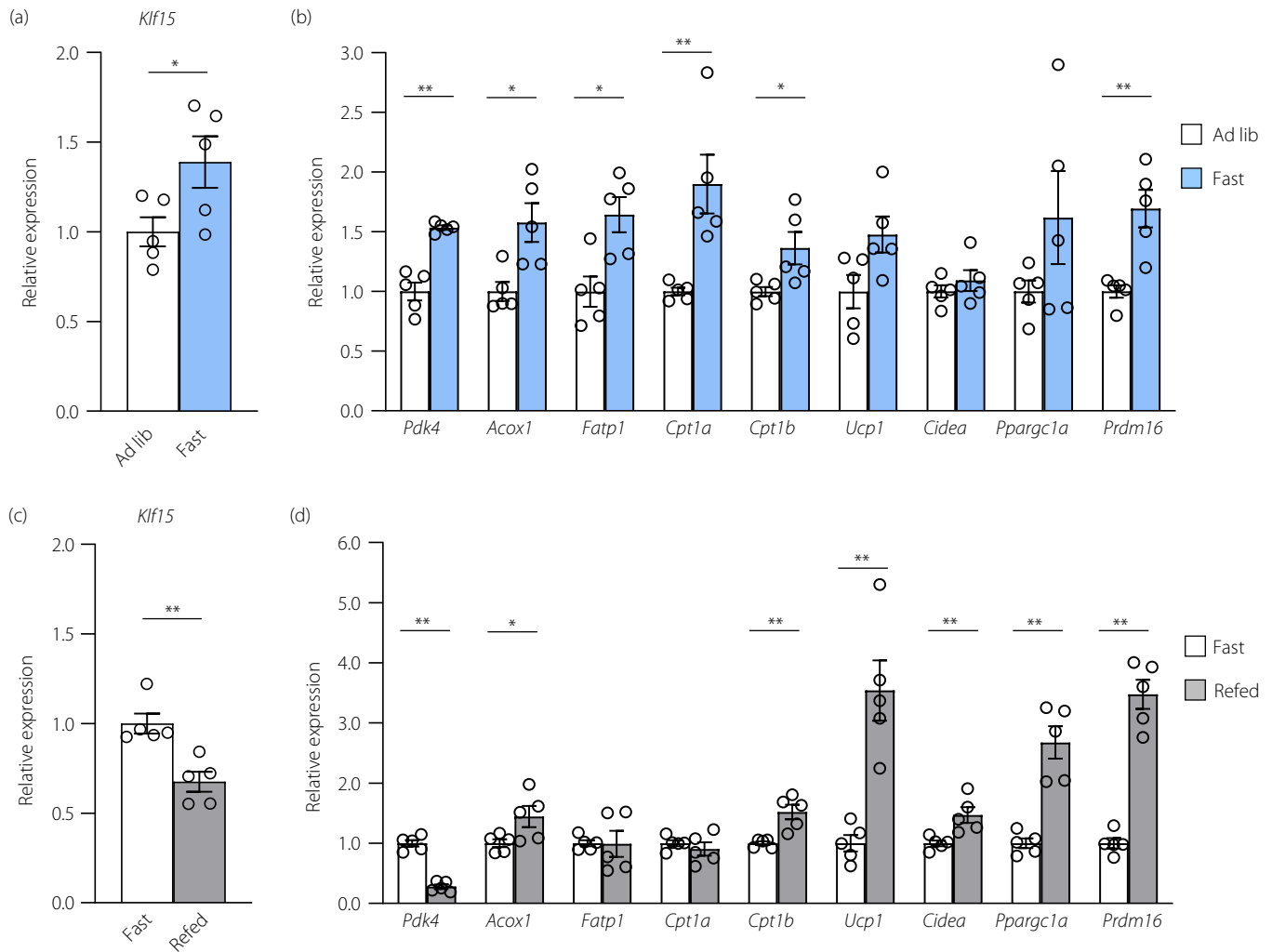
Figure 6 | Role of Kruppel-like factor 15 (KLF15) in regulation of gene expression related to energy utilization in brown adipose tissue in response to fasting and refeeding in mice. The expression of *Klf15* and the indicated genes in brown adipose tissue of C57BL/6 mice in the randomly fed state (Ad lib) or after food deprivation (Fast) for (a) 3 h or for (b) 6 h, as well as (c,d) after an overnight fast without (Fast) or with subsequent refeeding for 3 h (Refed) was determined by reverse transcription and real-time polymerase chain reaction analysis. Data are the mean ± standard error of the mean ($n = 5$). *$P < 0.05$, **$P < 0.01$ (Student's *t*-test).

In the present study, we found that *KLF15* expression in BAT was regulated by fasting and refeeding. Our previous study showed that KLF15 expression is upregulated by hyperglycemia in skeletal muscle of diabetic animals in a manner of post-transcriptional regulation[21]. Although such post-transcriptional regulation of KLF15 protein is observed in the hyperglycemic pathophysiological condition, the alteration of *KLF15* mRNA expression in BAT is observed in the normal glycemic physiological condition. Thus, the regulation of *KLF15* mRNA expression after fasting and refeeding is unlikely to be mediated through glucose concentration. Given that KLF15 expression is upregulated by the activation of cAMP signaling and is downregulated by insulin in mouse liver or hepatocytes[9], it is possible that insulin and glucagon is involved in the regulation of KLF15 expression during fasting and refeeding in other tissues including BAT.

KLF15 has been implicated in the regulation of energy metabolism in metabolically active organs including muscle, liver and WAT, but the physiological role of this transcription factor in BAT has remained unknown. Our present results suggest that KLF15 plays an important role in the regulation of fuel utilization in brown adipocytes. Consistent with the present findings, fatty acid utilization was previously found to be suppressed, whereas glucose utilization was enhanced in cardiac muscle from KLF15 knockout mice[13]. Similarly, endurance exercise capacity was found to be reduced as a result of impaired utilization of fatty acids in skeletal muscle of KLF15 knockout mice[12]. These observations suggest that KLF15 similarly regulates the utilization of fatty acids and glucose in both muscle and BAT. The establishment of BAT-specific KLF15 knockout mice should provide a useful tool for further

characterization of the role of KLF15 in the regulation of fuel utilization for BAT thermogenesis, as well as in the regulation of nutrient flux between BAT and other tissues.

The present findings suggest that KLF15 might regulate fuel switching between glucose and fatty acids in response to changes in energy status in BAT. Under the physiological condition, humans switch from the predominant use of carbohydrate during the postprandial state to that of lipids during the fasted state. A reduction in such metabolic flexibility has been suggested to be a primary defect leading to insulin resistance[22–24]. Our data implicating KLF15 in the mechanism underlying fuel switching between fatty acids and glucose in BAT might therefore provide a basis for the development of a new strategy for the treatment of insulin resistance and related disorders.

In summary, the present results implicate KLF15 as an important regulator of fuel switching between glucose and fatty acids in response to changes in energy status in BAT. This role of KLF15 appears to be mediated both by the control of genes related to fatty acid utilization, as well as by that of PDC activity in a manner dependent on direct regulation of *Pdk4* expression in HB2 differentiated brown adipocytes. Our findings suggest that modulation of KLF15 function in BAT warrants further investigation as a potential strategy to promote metabolic flexibility.

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DISCLOSURE

The authors declare no conflict of interest.

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