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The splicing factor SF3B1 is involved in brown adipocyte thermogenic activation

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ABSTRACT

The ability of alternative splicing mechanisms to control gene expression is increasingly being recognized as relevant for adipose tissue function. The expression of SF3B1, a key component of the SF3B complex directly involved in spliceosome formation, was previously reported to be significantly induced in brown adipose tissue under cold-induced thermogenic activation. Here, we identify that noradrenergic cAMP-mediated thermogenic stimulation increases SF3B1 expression in brown and beige adipocytes. We further show that pladienolide-B, a drug that binds SF3B1 to inhibit pre-mRNA splicing by targeting the SF3B complex, down-regulates key components of the thermogenic machinery (e.g., UCP1 gene expression), differentially alters the expression of alternative splicing-regulated transcripts encoding molecular actors involved in the oxidative metabolism of brown adipocytes (e.g., peroxisome proliferator-activated receptor-gamma co-activator-alpha [PGC-1α] and cytochrome oxidase subunit 7a genes), and impairs the respiratory activity of brown adipocytes. Similar alterations were found in brown adipocytes with siRNA-mediated knockdown of SF3B1 protein levels. Our findings collectively indicate that SF3B1 is a key factor in the appropriate thermogenic activation of differentiated brown adipocytes. This work exemplifies the importance of splicing processes in adaptive thermogenesis and suggests that pharmacological tools, such as pladienolide-B, may be used to modulate brown adipocyte thermogenic activity.

Abbreviations: AmA/AA, Antimycin A; ATP, Adenosine triphosphate; BAT, Brown adipose tissue; BSA, Bovine serum albumin; cAMP, Cyclic adenosine monophosphate; cDNA, Complementary deoxyribonucleic acid; Cox7b, Cytochrome *c* oxidase subunit 7B; CTRL, Control; DMEM, Dulbecco's modified Eagle's medium; ECAR, Extracellular acidification rate; EDTA, Ethylenediaminetetraacetic acid; eWAT, Epididymal white adipose tissue; FBS, Fetal bovine serum; FCCP, Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone; FGF21, Fibroblast growth factor-21; Gk, Glycerol kinase; GLUT1/4, Glucose transporter 1/4; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; iWAT, Inguinal white adipose tissue; Kng2-hmw, High molecular weight kininogen-2; Kng2-hmw, Low molecular weight kininogen-2; Ksrp, KH-type splicing regulatory protein; Lep, Leptin; mRNA, Messenger ribonucleic acid; NE, Norepinephrine; OCR, Oxygen consumption rate; Pgc1a/PGC-1α, Peroxisome proliferator-activated receptor-γ coactivator-1α; PLAD, Pladienolide-B; Plin5, Perilipin-5; PPAR, Peroxisome proliferator-activated receptor; PRDM16, PR domain containing protein-16; qRT-PCR, Quantitative reverse transcription polymerase chain reaction; Rot, Rotenone; RPLP0, Ribosomal protein lateral stalk subunit P0; SDS, Sodium dodecyl sulfate; SF3B1, Splicing factor 3B subunit 1; SEM, Standard error of the mean; SGBS, Simpson-Golabi-Behmel syndrome preadipocyte cell line; siRNA, Small interfering ribonucleic acid; Slc, Solute carrier; T3, Triiodothyronine; UCP1, Uncoupling protein-1; WAT, White adipose tissue.

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1. Introduction

Brown adipose tissue (BAT) is a main site of non-shivering thermogenesis in mammals. Brown adipocytes contain numerous mitochondria that are specialized for heat production due to the presence of uncoupling protein-1 (UCP1), a physiological proton conductance permeabilizer of the inner mitochondrial membrane that uncouples the mitochondrial respiratory chain from oxidative phosphorylation [1]. BAT activity is mainly regulated by sympathetic nervous system activation that occurs when norepinephrine interacts with beta-adrenergic receptors at the brown adipocyte surface to activate intracellular kinase pathways. Transcriptional regulation is a key mechanism by which the noradrenergic activation of brown adipocytes elicits a concerted induction of intracellular processes to favor thermogenic activity. UCP1 levels, which are key for non-shivering thermogenesis, are strongly regulated by control of *UCP1* gene transcription; UCP1 is up-regulated not only in response to the differentiation of brown adipocytes, enabling them to acquire their phenotypic identity, but also (and especially) in response to norepinephrine and other agents that acutely induce the thermogenic activation of differentiated brown adipocytes [2]. The mitochondrial respiratory equipment and lipid catabolism-related enzymatic activity are also governed by powerful transcriptional regulation in the context of brown adipocyte thermogenic activity [3].

In recent years, splicing has been increasingly recognized as being important for the control of metabolic adaptations. Alternative splicing enables the differential expression of enzymes and other proteins that allow an organism to adapt to metabolic needs by regulating the synthesis of specific transcripts encoding distinct protein products from a single gene. Alternative splicing is recognized to play a role in general adipogenesis, although the adipocyte differentiation-related splicing factors and events have not yet been thoroughly described [4]. Disturbed alternative splicing has been recognized in certain metabolic alterations, including obesity [5]. A few recent studies recognized the importance of alternative splicing specifically in beige and brown adipocyte differentiation. For example, some key enzymes and other factors related to the thermogenic machinery in brown adipocytes were shown to possess functionally distinct isoforms arising from alternative splicing of the encoding transcripts [6]. The *UCP1* gene has also been reported to possess alternative splicing-mediated transcript isoforms [7]. Moreover, the splicing factors and regulators Ksrp [8], Nova 1/2 [9], Srpk1 [10] and Acin1 [11], were reported to be involved in brown and beige adipogenesis. Most of the existing studies have tended to report a reciprocal relationship between the expression rate of splicing factors/regulators and brown/beige adipogenesis [12,13]. However, the extent to which splicing-driven processes are particularly involved in the thermogenic activation of BAT in response to acute environmental stimuli remains unknown. While analyzing changes of the BAT transcriptome in mice under cold-induced activation [14], we found a significant induction of *SF3B1* mRNA levels. This was concordantly found in an independent microarray-based transcriptome profiling analysis of BAT from mice acutely exposed to cold [15]. SF3B1 is a key spliceosome component; together with SF3a and the 12S RNA, it composes the U2 small nuclear ribonucleoprotein complex, which binds pre-mRNAs upstream of the intron branch site [16]. Mutations in SF3B1 have been related to several cancer pathologies [17] and researchers are actively seeking to identify synthetic and natural agents that interact with the SF3B complex to control carcinogenesis [18,19]. However, little information is available on the roles of SF3B1 in general adipocyte function or brown/beige thermogenic activity, or on potential pharmacological strategies that may be used to modulate these actions. Here, we report that SF3B1 is concertedly regulated with adrenergic thermogenic signaling in brown adipocytes and that this splicing factor is essential for the thermogenic responsiveness of brown adipocytes.

2. Materials and methods

2.1. Animal studies

Eight-week-old (26.5 ± 3.6 g) C57BL/6J male mice (Envigo, Huntington, United Kingdom) were maintained at thermoneutrality (29 °C) or exposed to cold (4 °C) for 24 h. All animals were maintained under a 12-h dark/light cycle with ad libitum access to food and water. After sacrifice, interscapular BAT, inguinal white adipose tissue (WAT), and epididymal WAT were frozen in liquid nitrogen for mRNA and protein analyses. All experiments were performed in accordance with European Community Council Directive 86/609/EEC, and the experiments and numbers of animals to be used were approved by the Institutional Animal Care and Use Committee of the University of Barcelona.

2.2. Adipocyte culture

Immortalized brown adipocytes from C57BL/6 J mice [20] were collected at passage 12, seeded in 6-well plates in Dulbecco's modified Eagle's medium (DMEM) containing 10 % fetal bovine serum (FBS), 20 mM HEPES, 1 nM triiodothyronine (T3), and 20 nM insulin, and grown at 37 °C in a humidified 95 % air/5% CO2 incubator until the cultures reached 70–80 % confluence. Then, 500 nM dexamethasone, 1 μM rosiglitazone, 125 μM indomethacin, and 500 μM 3-isobutyl-1-methylxanthine were added and cells were incubated for 2 days. Thereafter, the cells were cultured in medium containing only T3 and insulin until they were differentiated (day 7). Human SGBS pre-adipocytes at passage 10 (ATCC, PCS-210–010) were differentiated into mature beige adipocytes using established procedures [21]. Briefly, SGBS preadipocytes were maintained in DMEM:F12 plus 10 % FBS. When the cells reached confluence, differentiation was induced by incubation for 7 days in FBS-free medium containing 20 nM insulin, 0.2 nM T3, 100 nM cortisol, 25 nM dexamethasone, 500 μM 3-isobutyl-1-methyl-xanthine, and 2 μM rosiglitazone. The cells were then switched to DMEM:F12 plus 20 nM insulin, 0.2 nM T3, and 100 nM cortisol, and maintained for up to 14 days, during which time more than 90 % of the cells acquired a fully-differentiated multilocular adipocyte morphology. Where indicated, differentiated brown adipocytes were treated with 0.5 μM norepinephrine, 1 μM CL316,243, 1 mM dibutyryl-cAMP, or 10 nM pladienolide-B (PLAD). The utilized concentrations of these compounds, including PLAD, were chosen based on previous reports and were optimized to elicit proper biological responses without compromising cultured cell viability [13,20,22]. DMEM and FBS were obtained from ThermoFisher Scientific (Waltham, MA, USA). The other reagents and drugs used for adipocyte differentiation and treatments were obtained from Sigma-Aldrich (Darmstadt, Germany).

2.3. SF3B1 knockdown

For SF3B1 knockdown in differentiated brown adipocytes, Lipofectamine RNAiMAX (Invitrogen, Waltham, MA, USA) and Optimem (Life Technologies, Carlsbad, CA, USA) were used as directed to perform reverse transfection of random duplexes or an independent siRNA duplex designed to silence SF3B1 (duplex 1, final concentration 10 nM) (both obtained from Integrated DNA Technologies-TriFECTA). Forty-eight hours after transfection, the cells were treated as described above, collected for analysis of extracted RNA/protein, or seeded for Seahorse-based analysis of cell respiration.

2.4. RNA extraction and quantitative analysis of transcripts

RNA was extracted from tissues and cells using a NucleoSpin RNA Kit (Macherey-Nagel, Dueren, Germany), and mRNA levels were determined by quantitative reverse transcription–polymerase chain reaction (qRT–PCR). RNA (0.5 μg) was retrotranscribed using random hexamer primers (ThermoFisher Scientific) and, for qRT-PCR, reaction mixtures

containing 1 μL cDNA, and 10 μL TaqMan Universal PCR Master Mix (ThermoFisher Scientific) were used. To assess gene transcripts not associated with splicing isoforms, we used the following TaqMan probes targeting common transcript regions (250 nM probes and 900 nM of primers, ThermoFisher Scientific): *Sf3b1* (mouse, Mm01255457_m1)/*SF3B1* (human, Hs00961640_g1), *Ucp1* (Mm00494069_m1), *Fgf21* (Mm00840165_g1), *Prdm16* (Mm00712556_m1), leptin (Mm00434759_m1), *Slc2a1*/GLUT1 (Mm00441480_m1), *Slc2a4*/GLUT4 (Mm00436615_m1), and *Rplp0* (mouse, Mm00725448_s1)/*RPLP0* (human, Hs99999902_m1). For assessment of alternative splicing-driven isoforms, SYBR Green (ThermoFisher Scientific) was employed in conjunction with a StepOne PCR system (Life Technologies) using the following specific probes (5′-3′; forward (F) and reverse (R)): *Cox7b-001* (F: CGTCTCCAAGTTCGAAGCA; R: GTCATGGAAACTAGGTGCCC), *Cox 7b-002* (F: GTGAATTTGCACCAAGGCAG; R: CATAGGTCTCTTGGAGACGG), *Cox7b-003* (F: GTAGTCTGTGGAGTTGCTTTGC; R: GCCACCACTTGCTGAATGC), *Gk-004* (F: GTGTCAGCAACCAGAGGGAA; R: GAAGAGCCAGAGGGGACAAC), *Gk-006* (F: ACACTTGCTTTGTCTTGTCTGC; R: CAAGGCCCCAGCTTTCTGATA), *Kng2-hmw* (F: GACTGCCCAGAGAACAGAACC; R: CCTCTCCTGTATCTGTGTAGA), *Kng2-lmw* (F: GCAGGAACAACTAGGCTCCTA; R: CATCTCAGGATTCTTCTGCTCC), *PGC1a-1* (F: GGACATGTGCAGCCAAGACTCT; R: CACTTCAATCCACCCAGAAAGCT), *PGC1a-2* (F: CCACCAGAATGAGTGACATGGA; R: GTTCAGCAAGATCTGGGCAAA), *Plin5-001* (F: CCAGACCTGCTATAAGGACG; R: GAGAATCAGATCCCTGGGTG), *Plin5-002* (F: CTCAACTTTCCTGCCCGT; R: ACCGGACATTCTGCTGTG), and *Rplp0* (F: ACTGGTCTAGGACCCGAGAAG; R: TCAATGGTGCCTCTGGAGATT). Transcript levels of each gene of interest were normalized to that of the housekeeping reference gene, *RPLP0* (TaqMan Mm00725448_s1 and Hs00420895_gH), using the comparative $2^{-\Delta Ct}$ method.

2.5. Western blot analysis

Frozen cells were homogenized in lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 % v/v Triton X-100, 0.1 % SDS) containing a protease inhibitor cocktail (Roche, Sant Cugat del Vallès, Spain) and phosphatase inhibitors (2 mM sodium orthovanadate, 1 mM sodium pyrophosphate, 10 mM sodium fluoride). Lysates were centrifuged at 16,000 x g at 4 °C for 10 min. The utilized primary antibodies were rabbit anti-SF3B1 (ab39578, Abcam, Cambridge, United Kingdom) and rabbit anti-UCP1 (ab10983, Abcam) and horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (ab672, Abcam). Signals were detected using a chemiluminescence horseradish peroxidase substrate (Millipore, Darmstadt, Germany) on a LAS3000 system (Fujifilm, Tokyo, Japan). Images were digitalized and the data were quantified using Multi Gauge V3.0 (Fujifilm). Ponceau staining was performed as a protein loading control.

2.6. Cell respiration and glycolytic activity assessments

Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR, a surrogate measure of glycolytic activity toward lactate) were determined using the Agilent Seahorse XFe24 system (Agilent, Santa Clara, CA, USA) and procedures adapted for the specific assessment of mitochondrial respiration in brown adipocytes, as previously described [23]. Briefly, ~20,000 brown adipocytes/well were plated in a gelatin precoated Seahorse XFe24 Cell Culture Microplate. After 24 h, the medium was switched to Agilent Seahorse XF Base Medium supplemented with 2 % BSA fatty acid-free (A8806, Sigma-Aldrich), 5 mM glucose, 0.5 mM pyruvate, and 2 mM glutamine. For OCR determination, the following were sequentially added to cells for mitochondrial bioenergetic analysis: 5 μM oligomycin (O4876), as an ATP-synthase inhibitor; 2 μM carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone (FCCP; C2920) as a mitochondrial uncoupler; 5 μM rotenone (R8875), as a respiratory complex I inhibitor; and 15 μM antimycin A (A8674) (all from Sigma-Aldrich), as a respiratory complex III inhibitor. Where indicated, differentiated brown adipocytes were treated with 1 mM dibutyryl-cAMP or 10 nM PLAD for 24 h before the oxygen consumption assay.

2.7. Lipolytic activity quantification

Free glycerol levels in culture media, used to monitor intracellular lipolysis in adipocytes, were measured using the Free Glycerol reagent (F6428, Sigma-Aldrich) following the manufacturer's instructions.

2.8. Statistical analyses

Comparisons between two experimental conditions were made using two-tailed unpaired Student's *t*-tests, as applied with GraphPad Prism 8.0.1 (GraphPad, Boston, MA, USA). The threshold of statistical significance for all analyses was set to $p \leq 0.05$. Unless otherwise specified, all data are presented as means ± standard error of the mean (SEM) from $n = 3$ independent experiments in all cases.

3. Results

3.1. Expression of SF3B1 is induced by thermogenic activation in BAT, brown adipocytes, and beige adipocytes

Exposure of mice to a cold environment for 24 h and subsequent thermogenic activation of BAT led to significant induction of SF3B1 mRNA expression in BAT (Fig. 1A). Conversely, this cold-triggered induction did not occur in subcutaneous or visceral white adipose tissues (WAT; inguinal or epididymal WAT, respectively) (Fig. 1A). To determine whether SF3B1 induction under thermogenic stimulus occurred in a cell-autonomous manner, primary-culture mouse brown adipocytes were analyzed. Treatment of differentiated brown adipocytes with norepinephrine, as a standard mediator of thermogenic activation and UCP1 gene induction, significantly induced *Sf3b1* gene expression (Fig. 1B). cAMP, the main intracellular mediator of the noradrenergic stimulus of brown adipocytes, also significantly induced *SF3B1* expression (Fig. 1B). Immunoblot-based analysis confirmed that SF3B1 protein levels tended to be up-regulated under cAMP (Fig. 1C). However, other inducers of thermogenic activation and *Ucp1* gene expression in brown adipocytes, such as retinoic acid, the GPR120 receptor activator GW9508, and the PPARalpha activator GW7647 failed to induce *Sf3b1* gene expression despite their ability to significantly induce *Ucp1* levels (Fig. 1D). This suggests that SF3B1 gene expression is largely regulated by the noradrenergic cAMP-mediated pathway of thermogenic regulation. We also determined whether SF3B1 expression responded to thermogenic stimulation in a human model of beige adipocytes using SGBS adipocytes. Consistent with our findings in rodent brown adipocytes, norepinephrine or cAMP significantly induced SF3B1 mRNA expression in SGBS adipocytes (Fig. 1E). These results confirmed that brown and beige adipocytes exhibit enhanced expression of SF3B1 as a regulated event concordant with noradrenergic, cAMP-mediated, thermogenic induction, but not in relation to other inducers of thermogenic activation.

3.2. Experimental knockdown of SF3B1 represses thermogenic activation of brown adipocytes

To determine the role of SF3B1 in brown adipocyte function, we first used small interference RNA (siRNA) technology to knock down SF3B1 gene expression. Treatment of brown adipocytes with a SF3B1-targeting siRNA construct reduced the SF3B1 transcript and protein levels (Fig. 2A,B) to ~50 % or less than control levels. SF3B1-knockdown brown adipocytes exhibited a significant reduction of thermogenic activation, as marked by significant decreases of basal and cAMP-induced UCP1 mRNA expression levels (Fig. 2C) and a downward trend for cAMP-induced FGF21 mRNA expression. In addition to the

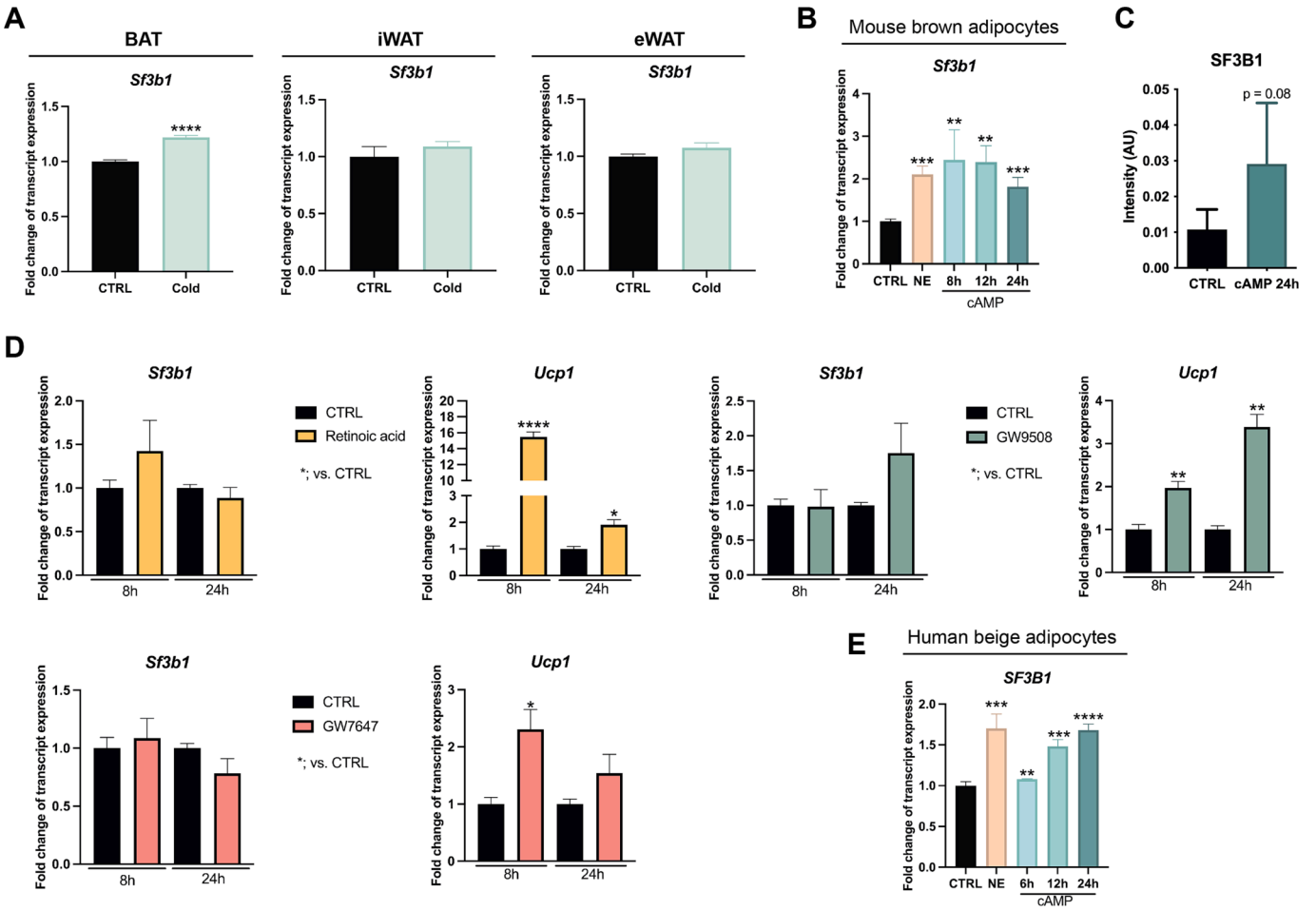

Fig. 1. SF3B1 expression in BAT, brown adipocytes, and beige adipocytes under thermogenic stimulation. (A) SF3B1 mRNA levels in interscapular BAT, inguinal WAT (iWAT), and epididymal WAT (eWAT) of mice maintained in an thermoneutral temperature of 29 °C (controls, CTRL) and after a 24-h exposure to 4 °C. (B) SF3B1 mRNA levels in differentiated murine brown adipocytes treated with 0.5 μM norepinephrine for 8 h (NE) or 1 mM dibutyryl-cAMP for 8, 12, or 24 h. (C) SF3B1 protein levels in differentiated murine brown adipocytes treated with dibutyryl-cAMP for 24 h. (D) Effects of 24-h treatment with retinoic acid (1 μM), GW9508 (100 μM), or GW7647 (1 μM) on SF3B1 and UCP1 mRNA expression levels in brown adipocytes. (E) SF3B1 mRNA levels in differentiated human SGBS beige adipocytes treated with 0.5 μM NE for 6 h or 1 mM dibutyryl-cAMP for 6, 12, or 24 h. Results are presented as means ± SEM of three replicate experiments. *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$ and ****: $p \leq 0.0001$ vs. CTRL. Unpaired *t-test.*

main known transcript encoding UCP1, we assessed recently reported alternative-spliced transcripts [7,24]; however, the non-canonical isoform levels were practically undetectable (data not shown). The transcript levels of PRDM16, which is a key factor for early stages of brown thermogenic differentiation unrelated to acute thermogenic activation [25], and leptin, a marker gene of white-versus-brown phenotype [26], were also reduced by SF3B1 knockdown under both basal and cAMP-induced conditions (Fig. 2C). Considering that SF3B1 is a key component of the splicing machinery, we next aimed to determine the effects of SF3B1 knockdown on the expression levels of transcript isoforms of brown adipocyte function-related genes known to be modulated by alternative slicing regulation. We analyzed alternative splicing-driven transcripts for the following: PGC-1α, a key transcriptional co-regulator involved in brown/beige thermogenic activation and mitochondrial biogenesis [27]; perilipin-5 (*Plin5*) [28], a key protein involved in coating lipid droplets in brown adipocytes; glycerol kinase (*Gk*), an enzyme that supports the increased uptake of glycerol in brown versus white adipocytes [29]; cytochrome oxidase subunit 7b (*Cox7b*), a component of mitochondrial respiratory chain [1]; and kininogen-2 (*Kng2*), a gene encoding high-molecular-weight (*hmw*) and low-molecular-weight (*lmw*) secreted kininogen through alternatively spliced transcripts [30]. We found that SF3B1 knockdown increased the cAMP-mediated induction of *PGC-1α-1* isoform expression and upregulated the levels of *PGC-1α-2* under basal and cAMP-stimulated conditions. It also tended to reduce the transcript isoform levels of *Plin5-001* and *Plin5-002* under cAMP stimulation; significantly reduced the *Gk-004* isoform but not the *Gk-006* levels; mildly induced *Cox7b-001*, did not change *Cox7b-003*, and dramatically induced *Cox7b-002*, irrespective of cAMP treatment; and tended to reduce the transcript level of *Kng2-lmw* but did not alter that of *Kng2-hmw* (Fig. 2D).

To assess the functional consequences of SF3B1 knockdown, we determined the mitochondrial respiration profiles of cultured brown adipocytes under basal and cAMP-treated conditions. The pattern of oxidative activity in SF3B1-knockdown brown adipocytes indicated that there were reductions of basal respiration, proton leakage, and ATP production (Fig. 3A,B). To assess how SF3B1 knockdown affected the glycolytic activity of brown adipocytes, we examined the extracellular acidification rate (ECAR) of the medium, which uses lactate release as a surrogate biomarker of glycolysis, and monitored the expression levels of glucose transporters GLUT1 and GLUT4. SF3B1 knockdown cells exhibited decreases in ECAR and the expression levels of both GLUT isoforms under basal and cAMP-stimulated conditions (Fig. 3C,D), suggesting that glycolytic activity was reduced. Finally, to evaluate effects on lipolysis, we examined glycerol release from brown adipocytes to culture media. We did not observe any overt alteration of this lipolysis marker in SF3B1-knockdown brown adipocytes (Fig. 3E).

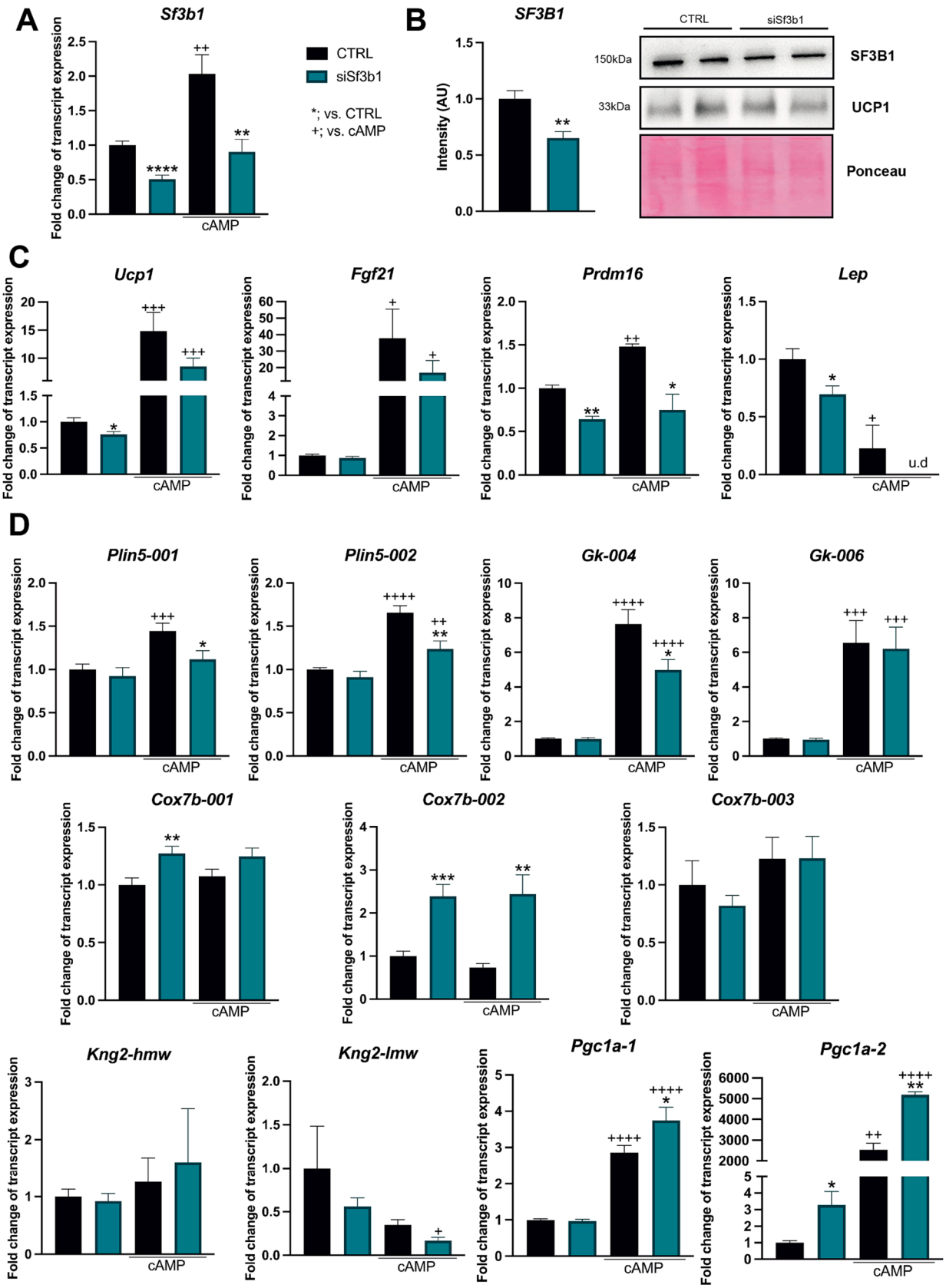

Fig. 2. Effects of SF3B1 knockdown on expression levels of thermogenesis-related genes and alternative splicing isoforms. SF3B1 mRNA (A) and protein (B) levels in brown adipocytes treated with scrambled siRNA (control) or SF3B1-targeting siRNA (10 nM). mRNA levels of canonical transcripts (C) and alternative splicing-driven transcript isoforms (D) in controls and SF3B1 knockdown brown adipocytes under basal and cAMP-stimulated (1 mM dibutyryl-cAMP, 12 h) conditions. Results are presented as means ± SEM of three replicate experiments. One symbol: $p \leq 0.05$, two symbols: $p \leq 0.01$, three symbols: $p \leq 0.001$, and four symbols: $p \leq 0.0001$. *: vs. CTRL and +: vs. non-cAMP treated cells. Unpaired *t-test.*

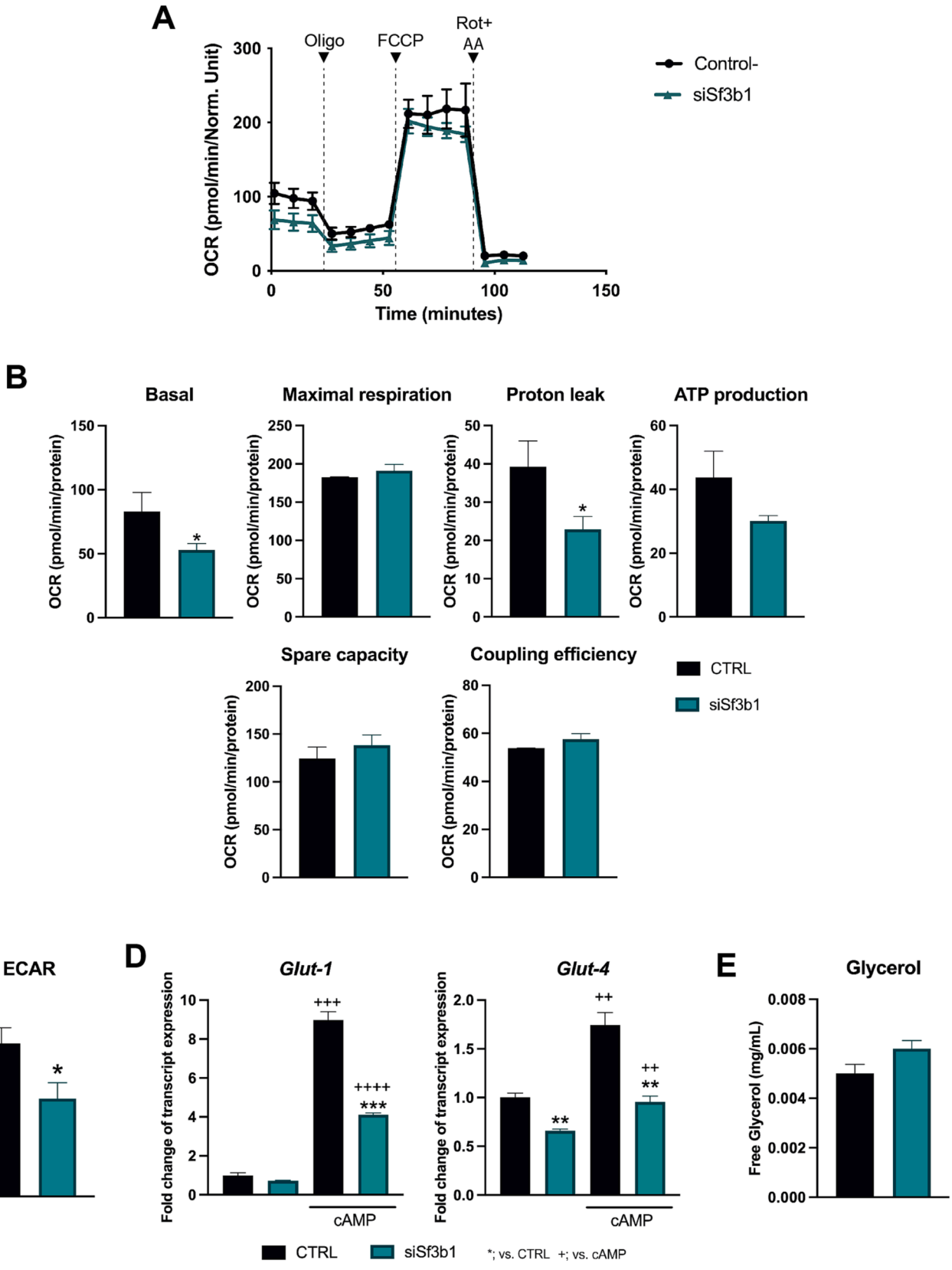

Fig. 3. Effects of SF3B1 knockdown on the respiratory activity of brown adipocytes. (A,B) Oxygen consumption rates (OCR) of differentiated brown adipocytes with or without siRNA-mediated SF3B1 knockdown and treated with oligo (5 μM oligomycin), FCCP (2 μM carbonyl cyanide-p-trifluoromethoxyphenylhydrazone), or Rot + AmA (5 μM rotenone and 15 μM antimycin A). (C) ECAR-assessed medium acidification in cultures of control and SF3B1-knockdown brown adipocytes. (D) mRNA levels of glucose transporters *Glut1* and *Glut4* in control and SF3B-knockdown brown adipocytes under basal and cAMP-stimulated (1 mM dibutyryl-cAMP, 12 h) conditions. (E) Glycerol medium content in control and SF3B1-knockdown brown adipocytes. Results are presented as means ± SEM of three replicate experiments. *: $p < 0.05$ vs. CTRL. Unpaired *t-test.*

3.3. Pharmacological inhibition of SF3B1 with pladienolide B represses the cAMP-induced thermogenic activation of brown adipocytes

To complement our SF3B1 transcript knockdown-based experiments, we next explored how pharmacological inhibition of SF3B1 activity affected the thermogenic activation of brown adipocytes. For this, we used pladienolide B (PLAD), which is a drug that targets SF3B1 and disrupts the conformation of the SF3B1 complex by interfering with its canonical splicing [31]. We treated differentiated brown adipocytes with 10 nM PLAD and determined gene expression under basal and cAMP-treated conditions. Our results revealed that PLAD reduced the basal transcript levels of the thermogenic markers UCP1 and FGF21, and dramatically repressed the capacity of cAMP to up-regulate these transcripts (Fig. 4A). Conversely, PLAD did not alter the expression level of PRDM16, which is a transcriptional regulator unrelated to acute thermogenic activation [25]. PLAD also reduced the mRNA levels of leptin, and mildly moderated the cAMP-mediated repression of its gene expression (Fig. 4A). Overall, our data indicated that the PLAD-mediated inhibition of SF3B1 trended to repress the transcript levels of key thermogenic components. For the brown adipocyte function-related alternative splicing-regulated transcripts (Fig. 4B), the basal and cAMP-stimulated expression levels of the main canonical transcript isoform, *PGC-1α-1*, were unaffected by PLAD, whereas the *PGC-1α-2* transcript isoform, the expression of which was hugely induced by cAMP, was down-regulated by PLAD in the cAMP-stimulated condition. *Plin5-001* and *Plin-5-002* were down-regulated by PLAD under the basal condition but not the cAMP-treated condition. The GK isoforms were not significantly altered by PLAD under the basal or cAMP-stimulated conditions. PLAD did not alter the level of *Cox7b-001* under the basal or cAMP-treated conditions, while dramatically increased that of *Cox7b-002* under both conditions, and increased that of *Cox7b-003* under the basal condition but not in the presence of cAMP. PLAD treatment increased the level of *Kng2-hmw* (encoding high molecular weight kininogen) and tended to increase that of *Kng2-lmw* (encoding low molecular weight kininogen) under the basal condition, but this effect was not seen under cAMP stimulation, especially for *Kng2-hmw*. Together, these data indicate that PLAD-mediated SF3B1 inhibition differentially alters the alternative splicing-mediated patterns of gene expression, especially for the *Cox7b* gene, in brown adipocytes under cAMP-mediated sympathetic stimulation.

We next decided to further quantify functional parameters in cAMP-stimulated brown adipocytes pharmacologically-challenged with PLAD. We found that PLAD treatment suppressed the ability of cAMP to increase the oxygen consumption, proton leakage, ATP production (Fig. 5A, B), and glycolytic activity (Fig. 5C) of brown adipocytes. PLAD treatment did not majorly alter glucose transporter expression levels except for its ability to downregulate GLUT4 under the basal condition (Fig. 5D), and had no discernable effect on the cAMP-induced release of glycerol to the cell medium (Fig. 5E).

4. Discussion

Here, we report that thermogenic activation of BAT involves the induction of SF3B1 expression via the intracellular cAMP-mediated noradrenergic activation of brown and beige adipocytes. Previous work showed that SF3B1 levels are up-regulated during the acute thermogenic activation of differentiated brown adipocytes, but does not occur in the long-term processes of brown or beige adipocyte differentiation *in vitro* or in white adipocyte browning [13]. This is similar to findings with other molecular actors, such as FGF21, which is up-regulated under acute thermogenic noradrenergic-mediated activation of brown/beige adipocytes but is unaltered (or even down-regulated) during the terminal differentiation of brown adipocytes [32].

Based on the results we obtained using two independent "loss-of-function" experimental approaches, namely siRNA-mediated knockdown of SF3B1 levels and pharmacological inhibition of SF3B1 by PLAD, we conclude that SF3B1 is essential for the appropriate transcriptional pattern and mitochondrial oxidative activity of thermogenically active brown adipocytes. SF3B1 contributes to controlling the transcript isoform levels of relevant thermogenesis-related genes that are regulated by alternative splicing. Moreover, although the key genes, UCP1 and FGF21, do not appear to undergo regulation via alternative splicing, they are strongly affected by SF3B1 levels, likely through indirect mechanisms.

The strategies of inhibiting SF3B1 through siRNA-mediated knockdown or PLAD treatment were concordant in reducing the transcript levels of key actors in thermogenesis (e.g., UCP1), differentially affecting the expression of alternative splicing-regulated transcript isoforms for genes involved in BAT thermogenic function (e.g., Cox7b, a component of the mitochondrial respiratory chain), and reducing oxidative activity. The two experimental approaches yielded some discrepancies in the intensity and type of parameters affected. For example, PGC1α transcripts were up-regulated by siRNA-mediated SF3B1 knockdown but not PLAD treatment. The reasons for this is unclear, but may reflect differences in the extent to which the biological activity of SF3B1 was impaired: PLAD is considered to be a powerful inhibitory drug [22], whereas our siRNA-mediated knockdown decreased the SF3B1 protein level to approximately half its control level. Alternatively (or in addition to) this, differences due to distinct molecular interventions at a prominent core site of splicing control may have led to an expansive set of consequential ramifications on the whole pattern of gene expression in brown adipocytes.

Overall, our findings suggest that SF3B1 critically mediates a pattern of alternative splicing-mediated gene expression that enables optimal glycolytic and mitochondrial oxidative function and adaptation to enhance thermogenic activity. This may involve several layers of direct and indirect effects to ultimately impact thermogenesis-related gene products that are even not regulated by alternative splicing, such as UCP1. However, the lack of existing knowledge on the differential functions of alternative splicing-determined isoforms makes difficult achieving a precise picture on how the functional consequences of changes in SF3B1 levels and/or activity occur. For example, the role of different isoforms of *Cox7b* and *Gk* genes on determining cytochrome oxidase and glycerol kinase activities, respectively, is not fully known [29], as it is yet unknown how transcript isoforms of the *Plin5* gene may affect differentially perilipin-mediated lipid droplet biology. Thus, a limitation of our current study is the limited provision of a comprehensive picture of the molecular mechanisms by which SF3B1 controls the thermogenic functional processes in brown adipocytes. Further research will be needed to establish the precise mechanisms by which SF3B1-mediated splicing regulation targets specifically the pattern of thermogenic gene expression and function in brown adipocytes.

Notably, our observation that PLAD treatment of brown adipocytes represses most basal and cAMP-induced indicators of thermogenic activation may suggest future applications. Traditionally, research on the mechanisms responsible for regulating BAT activity have focused on identifying tools to activate BAT as an anti-obesity treatment, since the low BAT activity associated with obesity leads to an impaired energy expenditure that favors an obesogenic positive energy balance [33]. However, over-activation of BAT is also a pathological event in some situations, such as certain cancers [34] and in burn patients [35]; in these cases, BAT over-activation leads to cachectic and hypermetabolic conditions. Our findings may shed light on the potential use of PLAD to reduce brown fat activity through its ability to repress SF3B1. PLAD has been developed as an efficient drug to treat certain oncologic pathologies, such as hepatogastric cancer, subcutaneous squamous cell

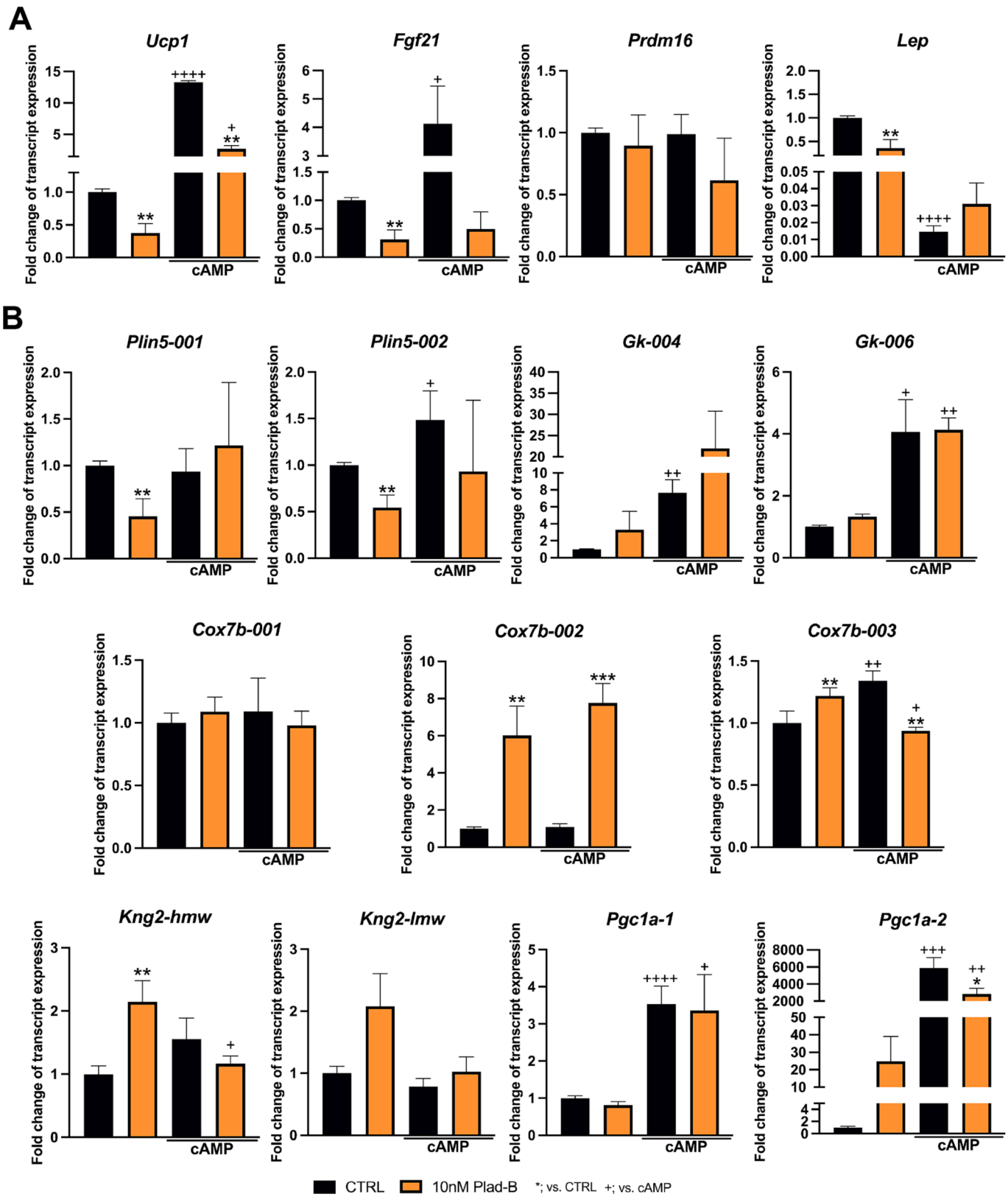

Fig. 4. Effects of PLAD on expression levels of thermogenesis-related genes and alternative splicing isoforms. Differentiated brown adipocytes were treated with 10 nM PLAD and treated with or without 1 mM dibutyryl-cAMP. Bars show levels of (A) canonical transcripts and (B) alternative splicing-driven transcript isoforms of the indicated genes. Results are presented as means ± SEM of three replicate experiments. One symbol: $p \leq 0.05$, two symbols: $p \leq 0.01$, three symbols: $p \leq 0.001$, and four symbols: $p \leq 0.0001$. *: vs. CTRL and +: vs. non-cAMP treated cells. Unpaired *t-test.*

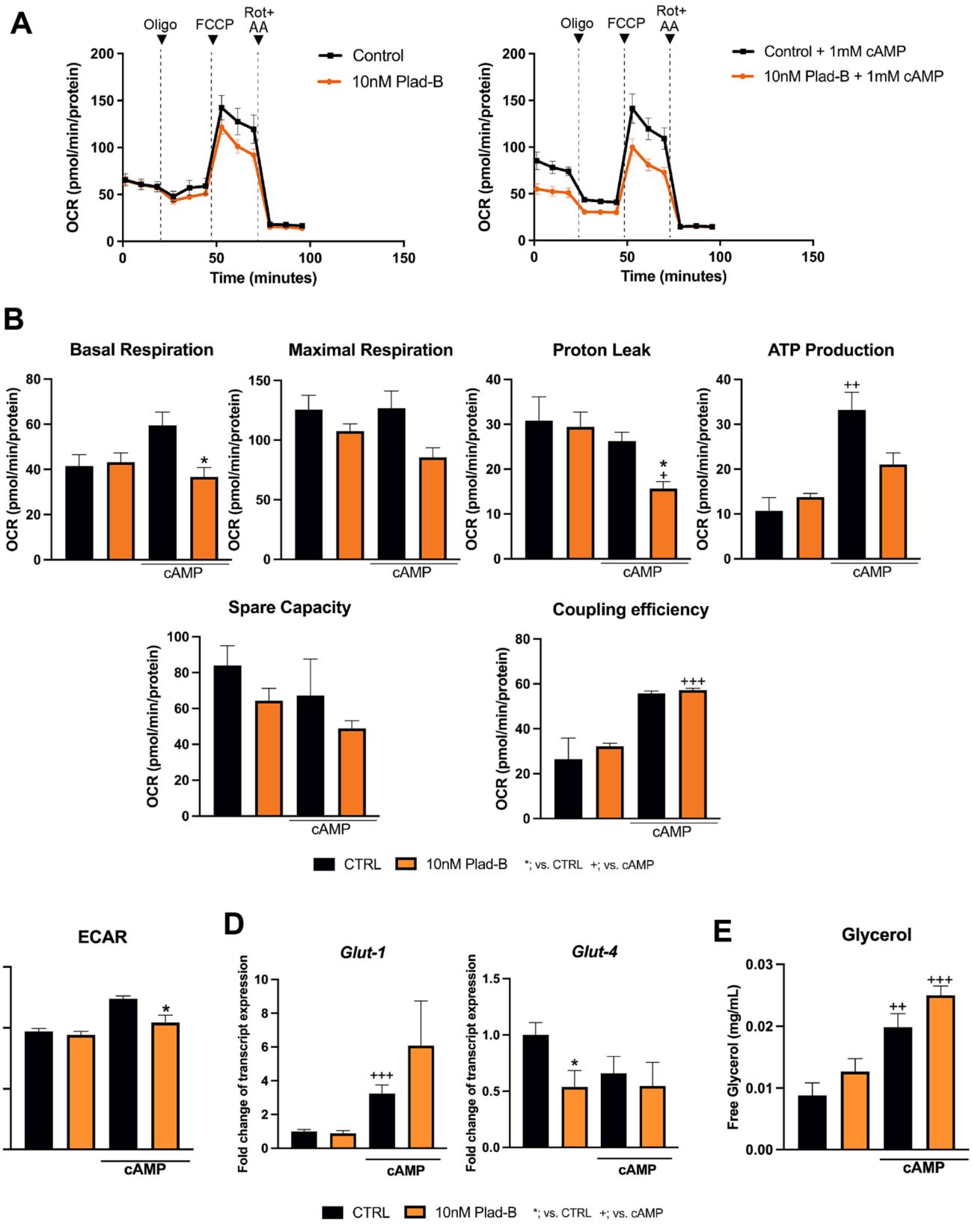

Fig. 5. Effects of PLAD on the respiratory activity of brown adipocytes. Differentiated brown adipocytes were treated with 10 nM PLAD and treated with or without 1 mM dibutyryl-cAMP. (A,B) Oxygen consumption rates (OCR) of control or PLAD-treated differentiated brown adipocytes treated with oligo (5 μM oligomycin), FCCP (2 μM carbonyl cyanide-p-trifluoromethoxyphenylhydrazone), or Rot + AmA (5 μM rotenone and 15 μM antimycin A). (C) ECAR-assessed medium acidification in cultured control and SF3B1 knockdown brown adipocytes. (D) mRNA levels of glucose transporters Glut1 and Glut4 in control and SF3B1-knockdown brown adipocytes under basal and cAMP-stimulated (1 mM dibutyryl-cAMP, 12 h) conditions. (E) Glycerol medium content in control and SF3B1-knockdown brown adipocytes. Results are presented as means ± SEM of three replicate experiments. *: $p < 0.05$ vs. CTRL. Unpaired *t*-test.

carcinoma, and leukemia [22,36]. Our present findings that SF3B1 contributes to brown adipocyte thermogenic activation open the possibility that PLAD could potentially be used in patients to ameliorate uncontrolled BAT activation or modulate it as a pharmacological target.

CRediT authorship contribution statement

Moisés Castellá: Conceptualization, Data curation, Formal analysis, Methodology, Visualization, Writing – original draft, Writing – review & editing. **Alberto Mestres-Arenas:** Investigation, Methodology, Writing – review & editing. **Aleix Gavaldà-Navarro:** Formal analysis, Investigation, Methodology, Supervision, Writing – review & editing. **Albert Blasco-Roset:** Investigation, Methodology, Writing – review & editing. **Tania Quesada-López:** Data curation, Investigation, Methodology, Software, Visualization, Writing – review & editing. **Inés Romero-Carramiñana:** Formal analysis, Investigation, Methodology, Writing – review & editing. **Marta Giralt:** Conceptualization, Funding acquisition, Supervision, Writing – original draft, Writing – review & editing. **Francesc Villarroya:** Conceptualization, Funding acquisition, Supervision, Writing – original draft, Writing – review & editing. **Rubén Cereijo:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Supervision, Visualization, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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