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Inactivation of glycogen synthase kinase-3β (GSK-3β) enhances skeletal muscle oxidative metabolism

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ABSTRACT

Background: Aberrant skeletal muscle mitochondrial oxidative metabolism is a debilitating feature of chronic diseases such as chronic obstructive pulmonary disease, type 2 diabetes and chronic heart failure. Evidence in non-muscle cells suggests that glycogen synthase kinase-3β (GSK-3β) represses mitochondrial biogenesis and inhibits PPAR-γ co-activator 1 (PGC-1), a master regulator of cellular oxidative metabolism. The role of GSK-3β in the regulation of skeletal muscle oxidative metabolism is unknown.

Aims: We hypothesized that inactivation of GSK-3β stimulates muscle oxidative metabolism by activating PGC-1 signaling and explored if GSK-3β inactivation could protect against physical inactivity-induced alterations in skeletal muscle oxidative metabolism.

Methods: GSK-3β was modulated genetically and pharmacologically in C2C12 myotubes *in vitro* and in skeletal muscle *in vivo*. Wild-type and muscle-specific GSK-3β knock-out (KO) mice were subjected to hind limb suspension for 14 days. Key constituents of oxidative metabolism and PGC-1 signaling were investigated.

Results: In vitro, knock-down of GSK-3β increased mitochondrial DNA copy number, protein and mRNA abundance of oxidative phosphorylation (OXPHOS) complexes and activity of oxidative metabolic enzymes but also enhanced protein and mRNA abundance of key PGC-1 signaling constituents. Similarly, pharmacological inhibition of GSK-3β increased transcript and protein abundance of key constituents and regulators of mitochondrial energy metabolism. Furthermore, GSK-3β KO animals were protected against unloading-induced decrements in expression levels of these constituents.

Conclusion: Inactivation of GSK-3β up-regulates skeletal muscle mitochondrial metabolism and increases expression levels of PGC-1 signaling constituents. *In vivo*, GSK-3β KO protects against inactivity-induced reductions in muscle metabolic gene expression.

1. Introduction

Impairments in skeletal muscle oxidative phenotype (OXPHEN), defined as the collective of cellular features determining fatigue resistance and capacity for mitochondrial substrate oxidation, are frequently observed in chronic diseases such as chronic obstructive pulmonary disease (COPD) [1,2], type 2 diabetes mellitus [3] and chronic heart failure [4], and are also observed during aging [5]. Interestingly,

Abbreviations: Acadvl, very long-chain specific acyl-CoA dehydrogenase; AKT, protein kinase B; AMPK, AMP-activated protein kinase; BL, base-line; CHIR, CHIR99021; COPD, chronic obstructive pulmonary disease; *COX*, Cytochrome c oxidase; CS, citrate synthase; *Cyc1*, cytochrome C1; *CycloA*, cyclophilin A; DM, differentiation media; DMEM, Dulbecco's modified Eagle's medium; *Errα*, estrogen-related receptor α; FAO, fatty acid β-oxidation; FBS, fetal bovine serum; GFP, green fluorescent protein; *Glut*, glucose transporter; GM, growth media; GS, glycogen synthase; GSK-3α/β, glycogen synthase kinase-3α/β; HAD, β-hydroxyacyl-CoA dehydrogenase; HBSS, Hank's balanced salt solution; *HkII*, hexokinase II; HLS, hind limb suspension; *Hprt*, hypoxanthine-guanine phosphoribosyltransferase; IGF-I, insulin-like growth factor-I; KO, knock-out; LiCl, lithium chloride; mtDNA, mitochondrial DNA; *Ndufb3*, NADH:Ubiquinone Oxidoreductase Subunit B3; *Nrf-1/2α*, nuclear respiratory factor-1/2α; OXPHEN, oxidative phenotype; OXPHOS, oxidative phosphorylation; *Perm1*, PGC-1 and ERR-induced regulator in muscle 1; *Pfkfb3*, 6-phosphofructo-2-kinase/fructose-2;6-biphosphatase 3; *Pgc-1α/β*, PPAR gamma coactivator-1α/β; *PI3K*, phosphoinositide-3-kinase; *Pparα/δ*, peroxisome proliferator-activated receptor α/δ; *Rpl13a*, ribosomal protein L13a; RT, room temperature; *Sdhb*, Succinate Dehydrogenase Complex Iron Sulfur Subunit B; siRNA, small interfering RNA; *Sirt1*, sirtuin 1; *Tfam*, mitochondrial transcription factor A; WT, wild-type; *β-2M*, β-2 microglobulin

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these abnormalities, commonly associated with progressive disability and reduced quality of life in these conditions, are also often associated with conditions of muscle mass loss such as cachexia and sarcopenia [6–8]. Although the exact molecular mechanisms and triggers underlying muscle abnormalities in these conditions remain incompletely understood, physical inactivity and disuse of skeletal musculature likely constitute an important contributing factor [9,10].

Besides the well-known exercise-induced potentiation of mitochondrial metabolism and muscle contractile function [11,12], a wealth of evidence demonstrates that anabolic stimuli like insulin or insulin-like growth factor-I (IGF-I) can induce muscle hypertrophy [13]. It is clear that the IGF/phosphoinositide-3-kinase (PI3K)/protein kinase B (Akt) pathway regulates muscle mass by controlling protein turnover by modulation of protein synthesis and protein breakdown [13–15]. However, whether or not Akt signaling is involved in the control of muscle mitochondrial metabolism is largely unexplored. Glycogen synthase kinase-3β (GSK-3β) is a direct target of Akt which phosphorylates GSK-3β on Ser^9 reducing its kinase activity. Previous, our group reported that GSK-3β inactivation is essential for muscle mass regulation as GSK-3β has a pivotal role in the determination of muscle mass by controlling muscle protein- and myonuclear turnover [15–17].

Intriguingly, in recent years, evidence emerged indicating a role for GSK-3β in the regulation of mitochondrial metabolism in non-muscle cells. More specifically, GSK-3β inhibition using lithium chloride (LiCl) potently induced mitochondrial biogenesis in endothelial and neuronal cells [18,19], which was accompanied by an increase in activity of the peroxisome proliferator-activated receptor (PPAR)-γ co-activator 1 (PGC-1) signaling network, a key signaling pathway controlling cellular oxidative metabolism. In line with these results, PGC-1α protein levels increased in vascular smooth muscle after treatment with LiCl [20]. Whether or not GSK-3β plays a role in the regulation of mitochondrial metabolism in muscle thus far is unexplored.

PGC-1α, and to a lesser extent its family member PGC-1β, is a key player in the regulation of fatty acid (FA) β-oxidation (FAO), oxidative phosphorylation (OXPHOS), mitochondrial biogenesis as well as mitochondrial DNA (mtDNA) replication [21]. PGC-1 acts on these processes by co-activating several nuclear proteins with transcriptional activity such as estrogen-related receptors (ERRs), PGC-1 and ERR-induced regulator in muscle 1 (PERM1) [22], PPARs [23] and nuclear respiratory factor 1/2α (NRF-1/2α) [24]. Also, PGC-1 co-activates transcription of the promoter of the gene encoding mitochondrial transcription factor A (TFAM) [24], a key protein involved in transcription of the mitochondrial genome and mitochondrial biogenesis. Impairments in this regulatory molecular network have been shown in muscle biopsies of diabetic [25] and COPD [26] patients. In addition, studies using different experimental modalities of inactivity or limb immobilization have demonstrated that physical inactivity-induced loss of muscle mitochondrial capacity is associated with decreased abundance/activity of key constituents of the PGC-1 signaling pathway in both mouse and human [27,28].

In the present study, we genetically and pharmacologically modulated GSK-3β in C2C12 myotubes and investigated the impact on mitochondrial metabolism and its regulation by the PGC-1 signaling network. Furthermore, as a proof of concept, we subsequently explored the *in vivo* physiological relevance of a potential role of GSK-3β in the regulation of muscle mitochondrial metabolism by subjecting wild-type (WT) and skeletal muscle-specific GSK-3β knock-out (KO) mice to a 14-day hind limb suspension (HLS) protocol known to detrimentally impact muscle mitochondrial capacity and its regulation by PGC-1 [28,29].

2. Materials and methods

2.1. Cell culture

C2C12 murine myoblasts were cultured as previously described [15]. Briefly, myoblasts were cultured in low-glucose (1 g/l) Dulbecco's modified Eagle's medium (DMEM) (GIBCO, Carlsbad, CA, USA) growth medium (GM) enriched with 9% (vol/vol) fetal bovine serum (FBS) (PAA Laboratories, Pasching, Austria) and antibiotics (50 U/ml penicillin and 50 μg/ml streptomycin; both from GIBCO). The cells were plated in BD Matrigel-coated 6-well plates at a density of $1 * 10^4$ cells/cm^2. Cells were cultured for 24 h in GM before initiation of differentiation, by growth factor withdrawal [30] using differentiation medium (DM), which consisted of low-glucose DMEM + GlutaMAX™ (GIBCO) with pyruvate, supplemented with 0.5% heat-inactivated FBS (30 min at 56 °C) and antibiotics (50 U/ml penicillin and 50 μg/ml streptomycin). The differentiating cells were provided with fresh DM on day 1, 3 and 5 of differentiation.

2.2. Treatment

GSK-3β (MSS226316, Thermo Scientific, Boston, MA, USA) or GSK-3α (MSS286308, Invitrogen, Carlsbad, CA, USA) mRNA expression was silenced in C2C12 myotubes that had undergone differentiation for 5 days and were grown in 6-well plates. The myotubes were transiently transfected using stealth RNAi small interfering (si)RNAs (Invitrogen) according to the manufacturer's guidelines. The transfection mix was prepared in Opti-MEM reduced serum medium (Invitrogen), and Lipofectamine RNAiMAX (Invitrogen) was used as transfection agent. The transfection mixture, for both universal control siRNA (low GC scrambled control; Invitrogen) and target siRNA (final concentration both at 10 nM), was incubated at room temperature (RT) for 20 min, and added to the culture plates containing fresh DM. CHIR99021 (CHIR) (#361559, Calbiochem, San Diego, CA, USA), a specific GSK-3α/β inhibitor, was used to pharmacologically inhibit GSK-3α/β with a final concentration of 7 μM. Adenoviral over-expression was used to over-express GSK-3β or green fluorescent protein (GFP) as a control in C2C12 myotubes that had undergone differentiation for 5 days and were grown in 6-well plates after new DM was supplied.

2.3. Cell processing

C2C12 myotubes were washed twice with pre-cooled 1X Hank's balanced salt solution (HBSS) and harvested in RLT buffer (Qiagen, Hilden, Germany) containing β-mercaptoethanol (1:100) for RNA isolation, whole cell lysates buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1% Nonidet P40) with PhosSTOP and complete protease inhibitors (both Sigma-Aldrich, St. Louis, MO, USA) for Western Blotting, DNA lysis buffer (0.1 M Tris/HCl pH 8.5, 5 mM EDTA pH 8.0, 0.2% SDS and 0.2 M NaCl) for DNA isolation or 0.5% Triton X in HBSS for enzyme activity assays using cell scrapers (rubber policemen). For enzymatic activity assays, samples were spun down for 2 min at maximal speed at 4 °C and dissolved in 1% BSA in lysis buffer before storing at − 80 °C.

2.4. Animals

The animal study described here was approved by the Institutional Animal Care Committee of Maastricht University (DEC-2009-074). Male skeletal muscle-specific GSK-3β KO animals on a C57/Bl6 background were generated by breeding GSK-3βfl/fl MLC1f-Cre +/− (muscle-specific GSK-3β KO) with GSK-3βfl/fl MLC1f-Cre −/− (Wild-type Ctrl/WT) mice [31]. Double Cre-negative litter mates served as 'WT' controls. At the start of the experiment mice were 13 weeks old (young adults). Animals were housed in a temperature-controlled room (21–22 °C) with 12:12 h light–dark cycle, and had *ad libitum* access to standard chow pellets and water. Animals were randomly divided into 2 groups for each genotype, and were subsequently subjected to no experimental procedures (baseline (BL)) or 14 days of HLS. The group size for the BL-groups was n = 9 and for the HLS-groups n = 8 [32]. In brief, a tail harness was placed while mice were lightly anesthetized using isoflurane inhalation. HLS was accomplished using a tail suspension device consisting of a plastic-coated iron wire taped around the mouse's tail and connected to a swivel hook to allow circular motility.

Table 1
qPCR primer sequences.

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)
Gsk-3β	CCACATGCTCGGATTCAGGC	TGTCCACGGTCTCCAGCATTAGTAT
Gsk-3α	CCCTCACCACTTCCTACAACCCA	TTGTGGCATCAGATGGCTGC
Ndufb3	ACAGACAGTGGAAAATTGAAGGG	GCCCATGTATCTCCAAGCCT
Sdhb	AATTTGCCATTTACCGATGGGA	AGCATCCAACACCATAGGTCC
Cyc1	GCATTCGGAGGGGTTTCCAG	CCGCATGAACATCTCCCCA
HkII	AACCTCAAAGTGACGGTGGGC	AAGGACACGTCACATTTCGGAGC
Pfkfb3	AGAACTTCCACTCTCCCACCCAAA	AGGGTAGTGCCCATTGTTGAAGGA
Glut1	TGACCATCGCCCTGGCCT	GGACCAGGGCCTACTTCAAAGAAG
Glut4	GATGCCGTCGGGTTTCCAGCA	TGAGGGTGCCTTGTGGGATGG
Cox II	CCATCCCAGGCCGACTAA	ATTTCAGAGCATTGGCCATAGAA
Pdk-4	AGGTTATGGGACAGACGCTATCATCTACT	AGACTGGGAGCTTTTCTACAGACTCAGA
Acadvl	AGACGGAGGACAGGAATCGG	ACCACGGTGGCAAATTGATC
Pgc-1α	CAACAATGAGCCTGCGAACA	CTTCATCCACGGGGAGACTG
Pgc-1β	ACCCTGAGAAAGCGCAATGA	CCCAGATGAGGGAAGGGACT
Errα	GGCGGACGGCAGAAGTACAAA	GCGACACCAGAGCGTTCAC
Perm1	GCTTAACAGTCAGGAAGGCAGGG	ACAGCCCAAGTGGCAAAGGCAAC
Pparα	ACTACGGAGTTCACGCATGTG	TTGTCGTACACCAGCTTCAGC
Pparδ	AGGCCCGGAGCATCCTCA	TGGATGACAAAGGGTGCGTTG
Nrf-1	AGCCACATTGGCTGATGCTT	GGTCATTTCACCGCCCTGTA
Nrf-2α	TGCTGCACTGGAAGGCTACA	TTACCCAAACCACCCAATGC
Tfam	CCGGCAGAGACGGTTAAAAA	TCATCCTTTGCCTCCTGGAA
Sirt1	ACAGTGAGAAAATGCTGGCCTAA	CCTCAGCACCGTGGAATATGT
Reference genes		
β2M	CTTTCTGGTGCTTGTCTCACTGA	GTATGTTCGGCTTCCCATTCTC
Rpl13a	CACTCTGGAGGAGAAACGGAAGG	GCAGGCATGAGGCAAACAGTC
CycloA	TTCCTCCTTTCACAGAATTATTCCA	CCGCCAGTGCCATTATGG
Hprt	TGGATATGCCCTTGACTATAATGAGTAC	AGGACTCCTCGTATTTGCAGATTC

Gsk-3α/β: glycogen synthase kinase-3α/β, *Ndufb3*: NADH:Ubiquinone Oxidoreductase Subunit B3, *Sdhb*: Succinate Dehydrogenase Complex Iron Sulfur Subunit B, *Cyc1*: cytochrome C1, *COX*: Cytochrome c oxidase, *Acadvl*: very long-chain specific acyl-CoA dehydrogenase, *HkII*: hexokinase II, *Pfkfb3*: 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3, *Glut*: glucose transporter, *Ppara/δ*: peroxisome proliferator-activated receptor α/δ, *Pgc-1α/β*: PPAR gamma coactivator-1α/β, *Errα*: estrogen-related receptor α, *Nrf-1/2α*: nuclear respiratory factor-1/2α, *Tfam*: mitochondrial transcription factor A, *Sirt1*: sirtuin 1, *Perm1*: PGC-1 and ERR-induced regulator in muscle 1, *β-2M*: β-2 microglobulin, *Rpl13a*: ribosomal protein L13a, *CycloA*: cyclophilin A and *Hprt*: hypoxanthine-guanine phosphoribosyltransferase.

The latter was attached to a Teflon-coated PVC ring, which slid over an iron rod spanning the length of the cage to allow longitudinal motility. The mice were raised so as to prevent the hind limbs from touching the cage floor or sides. In this way, four HLS mice could be housed in one standard cage. During the whole experiment body weight and water consumption were monitored. After euthanasia with sodium pentobarbital at BL or after HLS, *M. gastrocnemius* muscle was dissected using standardized dissection methods, cleaned of excess fat and tendon/connective tissue, pair weighed on an analytical balance, snap frozen in liquid nitrogen, and stored at − 80 °C for further processing.

2.5. Quantitative real-time PCR

cDNA was synthesized from 400 ng mRNA using the tetro cDNA synthesis kit (Bioline, Singapore, Singapore) according to manufacturer's guidelines. cDNA was diluted (1:50) in nuclease-free H_2O and stored at 4 °C, cDNA stocks were stored at − 20 °C. Each qRT-PCR reaction contained 4.4 μl diluted cDNA, 5 μl SyBr-green qPCR mastermix (Bioline) and 0.6 μl primers containing 5 μM of both the forward and the reverse primer per sample. The samples were run in a white 384-well plate (Roche, Basel, Switzerland), which was sealed and spun down. qRT-PCR was performed on a Roche LightCycler480 system for 10 min at 95 °C followed by 45 cycles of 15 s at 95 °C and 30 s at 60 °C. Melt curves were made using a gradual increase in temperature of 0.11 °C/s with 5 acquisitions/s and a temperature range of 60 °C to 90 °C. The melt curves were checked for double peaks and the negative controls were checked using the LightCycler480 software (Roche). Gene expression of genes of interest was analyzed using LinRegPCR software. The expression of these genes of interest was normalized with a correction factor calculated by GeNorm [33] based on the expression levels of *Rpl13a*, *Hprt*, *CycloA* and *β2M* as reference genes. The sequences of the used primers are listed in Table 1.

2.6. DNA isolation

Proteinase K was added (final concentration is 0.2 mg/ml) to the lysates and incubated overnight at 55 °C. Lysates were spun down for 5 min at full speed and DNA from the supernatants was precipitated with isopropanol. DNA pellets were washed twice with 70% ethanol. When all ethanol was evaporated DNA pellets were dissolved in 250 μl 10 mM Tris/HCl pH 8.0 and heated to 55 °C for 2 h followed by overnight incubation at 4 °C. When dissolved, the DNA was diluted 100 × in TE buffer and *CoxII* and *CycloA* levels were determined as described in Section 2.5.

2.7. Western blot analysis

Samples were thawed, spun down for 2 min at maximal speed at 4 °C and total protein content was assessed in the supernatant with the Thermo Scientific Pierce BCA protein assay kit (Pierce) according to manufacturer's guidelines. The supernatants were diluted in Laemmli sample buffer to 1 × as final concentration (0.25 M Tris·HCl pH 6.8; 8% (wt/vol) SDS; 40% (vol/vol) glycerol; 0.4 M DTT and 0.04% (wt/vol) bromophenol blue), adjusted to 0.5–1 μg/μl and boiled for 5 min at 95 °C. The samples of both the *in vitro* as the *in vivo* work were loaded, between 5 and 15 μg protein per lane (depending on the antibody), on a Criterion XT Precast 4–12% Bis-Tris gel (BioRad, Hercules, CA, USA). Proteins were separated by electrophoresis with 100-140 V. The proteins on the gel were transferred to a 0.45-μm Whatman Protran Nitrocellulose Transfer membrane (Whatman) by electroblotting (BioRad Criterion Blotter) with 100 V for 1 h. Ponceau S staining (0.2% Ponceau S in 1% acetic acid; Sigma-Aldrich) was performed and captured using the Amersham 600 imager (GE Healthcare, Chalfont St. Giles, UK), to quantify total protein loading. Blots were blocked for 1 h using 3% non-fat dry milk (FrieslandCampina, Amersfoort, the

Netherlands) and incubated overnight at 4 °C with primary antibodies directed to: GSK-3β (610201, BD Biosciences, San Jose, CA, USA), GSK-3α and PGC-1α (24259 and 516557, Merck Millipore, Billerica, MA, USA), total OXPHOS (Mitosciences, Eugene, OR, USA), phosphorylated eukaryotic translation initiation factor 2β (p-eIF2Bε), phosphorylated glycogen synthase (p-GS) and hexokinase II (HKII) (all from Cell Signaling Technology, Beverly, MA, USA) diluted in Tris-buffered saline with 0.1% Tween 20. The blots were probed with a peroxidase-conjugated secondary antibody (no. PI-1000) (Vector Laboratories, Burlingame, CA, USA) and visualized using SuperSignal West Pico or SuperSignal West Femto Chemiluminescent Substrate (both Pierce Biotechnology) according to the manufacturer's instructions. The Western blots were digitalized using the Amersham 600 imager, and subsequent quantification was done using ImageQuant TL software (GE Healthcare).

2.8. Enzyme activity assays

Total protein content was assessed by Thermo Scientific Pierce BCA protein assay kit (Pierce Biotechnology) and used as a correction factor. Enzymatic activities of citrate synthase (CS; EC 2.3.3.1) [34] and β-hydroxyacyl-CoA dehydrogenase (HAD; EC 1.1.1.35) [35] were analyzed spectrophotometrically at 412 nm and 340 nm, respectively as previously described using a Multiskan Spectrum plate reader (Thermo Labsystems, Breda, The Netherlands) at 37 °C with a 30 s interval for a total duration of 30 min.

2.9. Statistical analysis

Data were analyzed using an unpaired student's *t*-test or one-way ANOVA with a Bonferroni as post-hoc test with GraphPad Prism 5.0 software (GraphPad, Prism, La Jolla, CA, USA). Significant differences are indicated as $p < 0.05$ in each figure. Data are expressed as mean ± SEM. Graphs were made using GraphPad Prism 5.0 software and representative blots were added using Adobe illustrator CS6 software (Adobe Systems Incorporated, San Jose, CA, USA).

3. Results

3.1. GSK-3β knock-down increases mitochondrial content

Knock-down of GSK-3β, verified by a 80–90% decrease in GSK-3β mRNA and protein abundance as well as reduced phosphorylation status of a direct downstream target of GSK-3β (eIF2Bε) (Fig. S1), significantly enhanced myotube mitochondrial oxidative metabolism. This was evidenced by an increased mitochondrial DNA copy number (Fig. 1A) and elevated CS enzyme activity (Fig. 1B). Moreover, enzymatic activity and protein- and mRNA transcript abundance of key constituents of the electron transport chain (nuclear- and mitochondrial-encoded OXPHOS sub-units) and mitochondrial fatty acid β-oxidation pathway (HAD and *Acadvl*: very long-chain specific acyl-CoA dehydrogenase) were significantly up-regulated after knock-down of GSK-3β (Fig. 1C, D). This apparent enhancement of cellular oxidative metabolism after knock-down of GSK-3β protein was also associated with increased elevated HKII protein levels and increased mRNA transcript abundance of the glycolytic genes *HkII*, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (*Pfkfb3*), glucose transporter-1 (*Glut-1*) and *Glut-4*. Pyruvate dehydrogenase kinase-4 (*Pdk-4*) mRNA expression also increased after GSK-3β knock-down (Fig. S2).

3.2. GSK-3β knock-down increases expression levels of key components of the PGC-1 signaling network

Enhancement of mitochondrial content after knock-down of GSK-3β was associated with increased PGC-1α protein abundance and a potent 8–10-fold increase in *Pgc-1α* mRNA transcript levels (Fig. 2A, B). Additionally, knock-down of GSK-3β also resulted in a marked increase in transcript levels of several PGC-1-co-activated transcription factors heavily involved in fatty acid oxidation, fatty acid transport and mitochondrial substrate metabolism including *Pparα*, *Pgc-1β*, *Errα* and the recently identified PGC-1-ERRα-co-activated transcription factor *Perm1* (Fig. 2D).

3.3. Pharmacological GSK-3 inhibition enhances myotube oxidative gene expression

Interestingly, we observed that knock-down of the GSK-3β protein resulted in increased mRNA- and protein abundance of the GSK-3α isoform, which coincided with an increased phosphorylation status of the well-known GSK-3 phosphorylation target glycogen synthase (GS) (Fig. S3). To explore a potential role for GSK-3α in the regulation of muscle mitochondrial metabolism, but also to investigate whether or not pharmacological inhibition of GSK-3 activity exerts similar effects on myotube oxidative gene expression compared to specific physical absence of the GSK-3β protein (as shown in Figs. 1 and 2), we designed experiments in which the presence and/or activity of the 2 GSK-3 isoforms was modulated.

Firstly, pharmacological inhibition of both GSK-3 isoforms by CHIR, as shown by a potent decrease in phosphorylated eIF2Bε (Fig. S4), significantly increased transcript levels of genes involved in OXPHOS (CII and III sub-units) and fatty acid oxidation (*Acadvl*) (Fig. 3A, B). In addition, CHIR also up-regulated expression levels of key constituents of the PGC-1 signaling network (*Pgc-1α*, *Errα* and *Perm1*) (Fig. 3C). Similar to the findings obtained following knock-down of GSK-3β, pharmacological inhibition of GSK-3 with CHIR also resulted in increased mRNA expression of several genes involved in glycolysis (*HkII, Pfkfb3* and *Glut-1*) (Fig. S5). Pharmacological inactivation of GSK-3 with CHIR, however, did not further enhance siGSK-3β-induced increases in mRNA levels of constituents and regulators of myotube oxidative metabolism (data not shown). As depicted in Fig. S6, specific knock-down of the GSK-3α protein resulted in increased transcript levels of *Pgc-1α* although this was less pronounced compared to GSK-3β knock-down. Furthermore, knock-down of GSK-3α did not affect transcript levels of genes involved in OXPHOS (CI, CII and CIII sub-units), glycolysis (*HkII* and *Glut-1*), PGC-1 or PGC-1 co-activated transcription factors (*Pgc-1β*, *Pparα*, *Errα* and *Perm1*) showing that although pharmacological inhibition of GSK-3α/β recapitulates the effects of knock-down of GSK-3β on metabolic gene expression, GSK-3β appears to be the predominant GSK-3 isoform involved in suppression of mitochondrial energy metabolism.

3.4. PGC-1α is required for increased OXPHOS gene expression induced by GSK-3 inhibition

In order to explore whether increases in metabolic gene expression, induced by GSK-3 inhibition, are PGC-1α-dependent, we knocked-down *Pgc-1α* in C2C12 myotubes and subsequently inhibited GSK-3 using CHIR. Knock-down of *Pgc-1α* (Fig. 4A) prevented CHIR-induced increases in *Pgc-1α* mRNA as well as inductions of known PGC-1α-dependent target genes including genes encoding for OXPHOS sub-units (CII and CIII) [36] (Fig. 4B) as well as *Errα* [37,38] (Fig. 4C).

3.5. Over-expression of GSK-3β in myotubes does not affect oxidative gene expression

Next, in order to explore whether over-expression of GSK-3β has opposite effects on myotube metabolic gene expression compared to knock-down of the protein, we used an adenoviral system to over-express GSK-3β. Over-expression of GSK-3β resulted in an approximate 3.5 fold increase in *Gsk-3β* mRNA and protein abundance as well as increased phosphorylation levels of its down-stream target eIF2Bε (Fig.

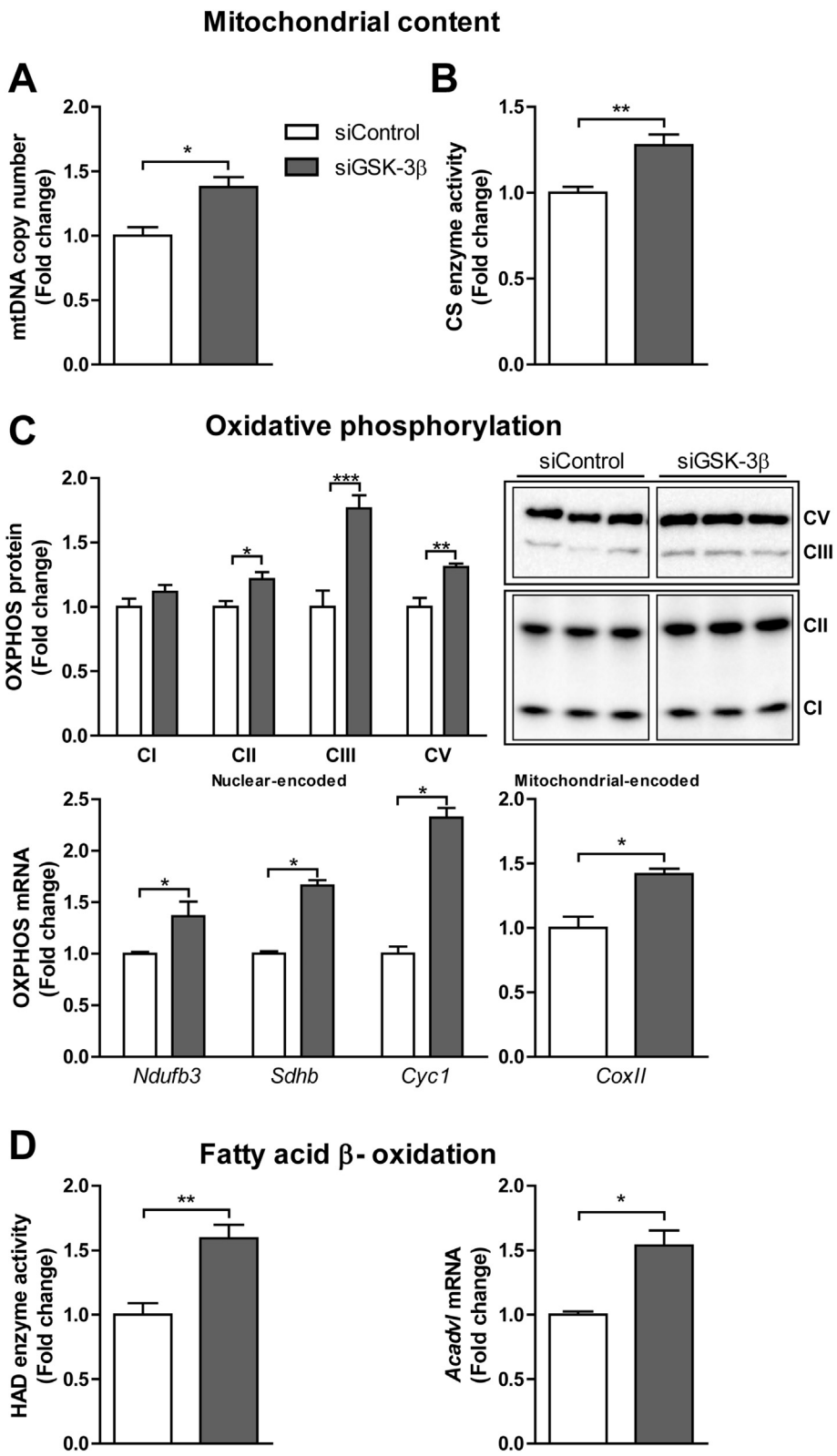

Fig. 1. GSK-3β knock-down increases mitochondrial content. C2C12 myotubes were differentiated for 5 days and transfected with siRNA constructs targeting GSK-3β (grey) or a low GC scrambled control (white). Myotubes were harvested at different time-points post-transfection (48 h for mitochondrial DNA copy number, transcript- and protein levels or 72 h for enzymatic activities). (A) Mitochondrial DNA copy number, (B) CS activity, (C) protein levels of different complexes of the respiratory chain and gene expression of nuclear-encoded sub-units (*Ndufb3*, *Sdhb* and *Cyc1* respectively Complex I, II and III) and the mitochondrial-encoded sub-unit (*CoxII*, respectively Complex IV) and (D) HAD enzyme activity and mRNA levels of *Acadvl* all are given in fold changes compared to control. Representative pictures of the blots are shown and Western blot data is corrected for total protein loading assessed by Ponceau S staining. Black boxes around the representative pictures indicate that they are cut from the same Western blot. All data are expressed as means ± SEM from n = 4/experimental condition (mRNA) or n = 6/experimental condition (mitochondrial DNA copy number, protein and enzyme) (n = 3 experiments), *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 *vs.* siControl. GSK-3β: glycogen synthase kinase-3β, mtDNA: mitochondrial DNA, CS: citrate synthase OXPHOS: oxidative phosphorylation, *Ndufb3*: NADH:Ubiquinone Oxidoreductase Subunit B3, *Sdhb*: succinate dehydrogenase complex iron sulfur subunit B, *Cyc1*: cytochrome *c* 1, *CoxII*: Cytochrome c oxidase II, HAD: β-hydroxyacyl-CoA dehydrogenase and *Acadvl*: very long-chain specific acyl-CoA dehydrogenase.

S7). However, GSK-3β over-expression did not alter the expression of any of the metabolic genes or genes involved in the PGC-1α signaling pathway (Fig. S8A-D) that were investigated. Moreover, over-expression of GSK-3β did not change protein content of OXPHOS subunits (Complex, I, II, III and V) compared to myotubes over-expressing GFP as control (Fig. S8A).

3.6. GSK-3β knock-out protects against disuse-induced loss of oxidative gene expression in skeletal muscle

Having established that the physical absence or pharmacological inhibition of the GSK-3β protein in cultured myotubes enhanced cellular mitochondrial metabolism and up-regulated PGC-1 signaling, we

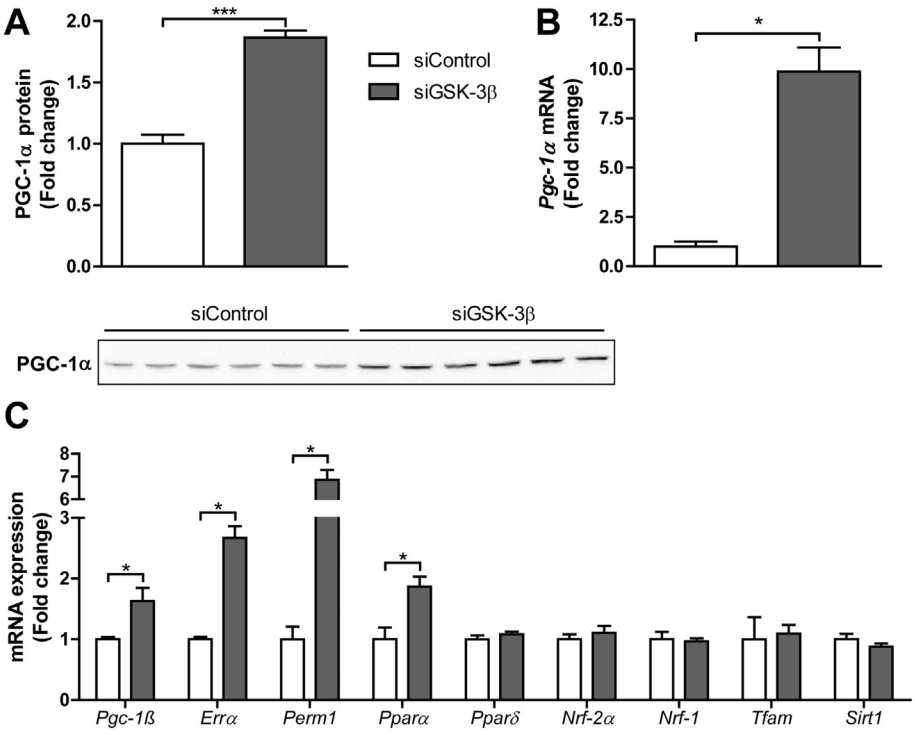

Fig. 2. GSK-3β knock-down increases transcript levels of key components of the PGC-1 signaling network. C2C12 myotubes were differentiated for 5 days and transfected with siRNA constructs targeting GSK-3β (grey) or a low GC scrambled control (white). Myotubes were harvested 48 h post-transfection. (A) Protein content of PGC-1α, (B) mRNA expression of *Pgc-1α* and (C) mRNA expression of *Pgc-1β* and transcription factors involved in PGC-1α signaling. A representative picture of a blot is shown and Western blot data is corrected for total protein loading assessed by Ponceau S staining. All data are expressed as means ± SEM from n = 4/experimental condition (mRNA) or n = 6/experimental condition (protein) (n = 3 experiments), *p ≤ 0.05, ***p ≤ 0.001 *vs.* siControl. GSK-3β: glycogen synthase kinase-3β, *Ppara/δ*: peroxisome proliferator-activated receptor α/δ, *Pgc-1α/β*: PPAR gamma coactivator-1α/β, *Errα*: estrogen-related receptor α, *Nrf-1/2α*: nuclear respiratory factor-1/2α, *Tfam*: mitochondrial transcription factor A, *Sirt1*: sirtuin 1 and *Perm1*: PGC-1 and ERR-induced regulator in muscle 1.

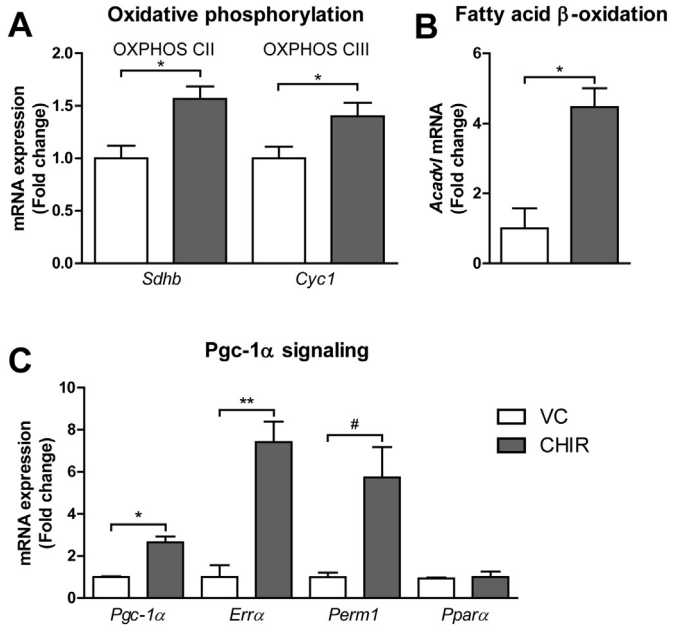

Fig. 3. Pharmacological inactivation of GSK-3 increases oxidative metabolic gene expression in myotubes. C2C12 myotubes were differentiated for 5 days and treated with CHIR99021 (grey) (final concentration: 7 μM) or DMSO as vehicle (white) and transfected with a low GC scrambled control siRNA. Myotubes were harvested 48 h post-treatment. Gene expression of (A) OXPHOS sub-units CII (*Sdhb*) and CIII (*Cyc1*), (B) *Acadvl* and (C) *Pgc-1α* and transcription factors involved in PGC-1α signaling. All data are expressed as means ± SEM from n = 3/experimental condition (n = 3 experiments), *p ≤ 0.05, **p ≤ 0.01, #p ≤ 0.1 *vs.* VC. OXPHOS: oxidative phosphorylation, *Sdhb*: succinate dehydrogenase complex iron sulfur subunit B, *Cyc1*: cytochrome *c* 1, *Acadvl*: very long-chain specific acyl-CoA dehydrogenase, *Ppara*: peroxisome proliferator-activated receptor α, *Pgc-1α*: PPAR gamma coactivator-1α, *Errα*: estrogen-related receptor α and *Perm1*: PGC-1 and ERR-induced regulator in muscle 1.

subsequently explored the physiological relevance of muscle-specific ablation of GSK-3β *in vivo* with regard to mitochondrial metabolism and its regulation of the PGC-1 signaling network. To address this, we submitted WT and skeletal muscle-specific GSK-3β KO animals to a 14-day HLS protocol.

Previously, for both genotypes 10% body weight loss was reported after the 14-day HLS protocol, although body weight loss was significantly less in the GSK-3β KO animals on day 2 of HLS. Furthermore, BL *M. gastrocnemius* muscle mass was similar between WT and GSK-3β KO animals and HLS comparably decreased muscle mass in both WT (16.9% ± 2.7%) and GSK-3β KO (16.3% ± 2.2%) animals [39]. Fourteen days of HLS reduced protein and mRNA levels of OXPHOS complex sub-units in WT animals (Fig. 5A, B), while this was not observed in GSK-3β KO animals. Glycolytic protein and gene expression were largely unaltered by the unloading protocol in both genotypes (Fig. S9A, B). In addition, *Pdk-4* mRNA expression was potently reduced after 14 days of HLS in GSK-3β KO animals (Fig. S9B) while gene expression of *Acadvl* was found to decrease in *M. gastrocnemius* after HLS in both genotypes (Fig. 5C).

3.7. GSK-3β ablation protects against unloading-induced changes in the PGC-1 signaling network

Besides the protective effect of GSK-3β KO on unloading-induced decrements in protein and mRNA levels of OXPHOS subunits, muscle-specific GSK-3β KO animals were also protected against unloading-induced decrements in expression levels of key regulators of mitochondrial biogenesis. This was evidenced by a significant reduction of NRF-1 protein in WT animals after unloading, which was not detected in GSK-3β KO animals, PGC-1α and TFAM protein levels did not change significantly in both genotypes after 14 days of HLS (Fig. 6A). However, transcript levels of *Pgc-1β*, *Tfam*, *Pparα* and *Errα* were significantly reduced in WT animals after 14 days of HLS, whereas these unloading-induced decreases were not present in muscle-specific GSK-3β KO animals (Fig. 6B). In addition, other constituents involved in the PGC-1 signaling network, namely *Nrf-1*, *Nrf-2α*, *sirtuin 1* (*Sirt1*) and *Pparδ* were unaltered after HLS in WT animals, while expression levels of these genes were significantly induced in muscle-specific GSK-3β KO animals. Furthermore, *Perm1* gene expression tended to decrease in GSK-3β KO animals after HLS (Fig. 6B).

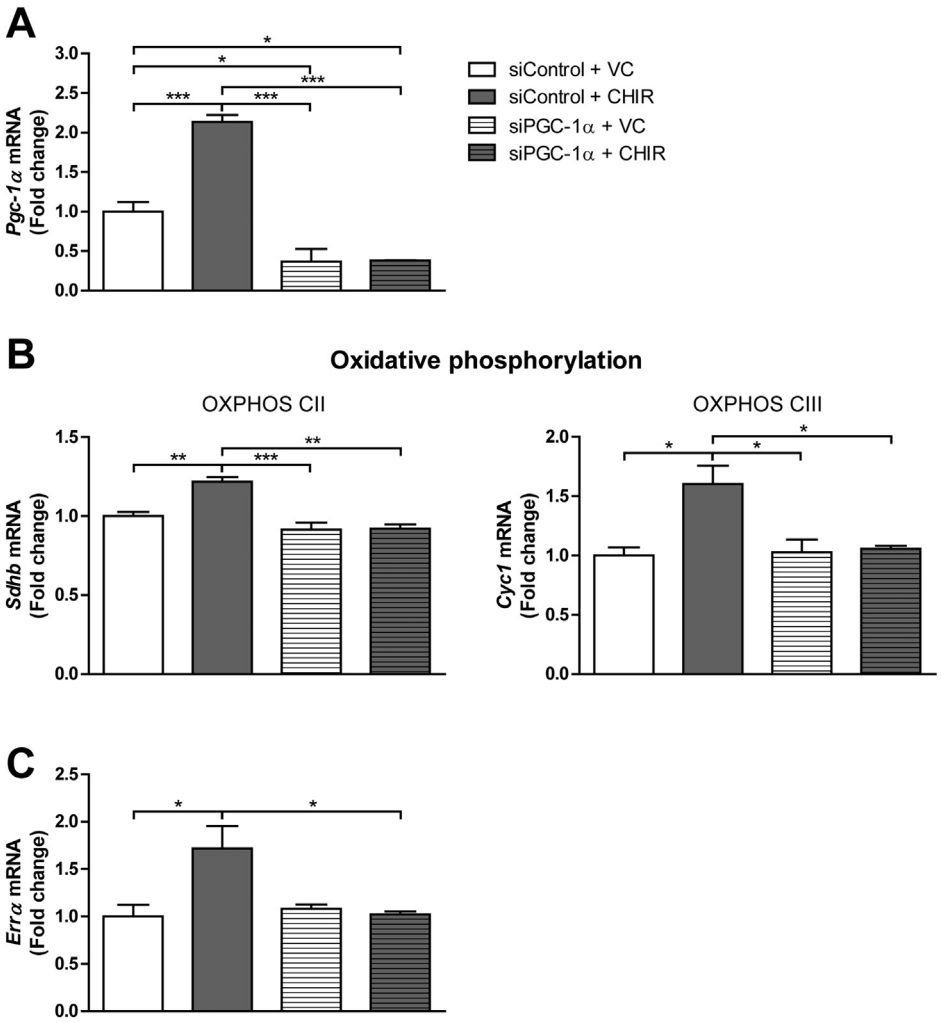

Fig. 4. Pharmacological inactivation of GSK-3 increases oxidative gene expression in a *Pgc-1α*-dependent manner. C2C12 myotubes were differentiated for 5 days and treated with siRNA constructs targeting PGC-1α or a low GC scrambled control and treated with CHIR99021 (final concentration: 7 μM) or DMSO as vehicle on day 6 of differentiation. Myotubes were harvested 48 h post CHIR treatment. Gene expression of (A) *Pgc-1α*, (B) OXPHOS sub-units CII (*Sdhb*) and CIII (*Cyc1*) and (C) *Errα* is expressed as means ± SEM from n = 3/experimental condition (n = 3 experiments), *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001. OXPHOS C: oxidative phosphorylation complex, *Pgc-1α*: PPAR gamma coactivator-1α, *Sdhb*: succinate dehydrogenase complex iron sulfur subunit B, *Cyc1*: cytochrome *c* 1 and *Errα*: estrogen-related receptor α.

4. Discussion

In skeletal muscle, GSK-3β has been appreciated for its regulatory role in glycogen metabolism and protein turnover (for review see [40]). However, the role of GSK-3β in muscle mitochondrial oxidative metabolism has not been studied thus far. In this study, we show for the first time that knock-down as well as pharmacological inhibition of the GSK-3β protein kinase stimulates oxidative phenotype and its regulation by the PGC-1 signaling network in cultured C2C12 myotubes which was evidenced by increases in abundance and activity levels of key constituents of mitochondrial substrate metabolism and increased expression levels of PGC-1 signaling constituents. Furthermore, we showed that knock-down of *Pgc-1α* prevented increases in mRNA expression of OXPHOS sub-units induced by pharmacological GSK-3 inhibition. In contrast, over-expression of GSK-3β failed to result in any alterations in metabolic gene expression and PGC-1-co-activated events, suggesting that in homeostatic conditions endogenous GSK-3β levels are sufficient to exert maximal control over the regulation of oxidative energy metabolism. In addition to our *in vitro* data showing that knock-down of GSK-3β enhances mitochondrial metabolism and the PGC-1 signaling network in cultured C2C12 myotubes, muscle-specific GSK-3β KO *in vivo* protected against unloading-induced decrements in expression levels of key constituents of muscle mitochondrial metabolism and the PGC-1 signaling network.

In our study we show that genetic or pharmacological inactivation of GSK-3β, a direct downstream phosphorylation target of Akt, stimulates muscle cell oxidative phenotype. The PI3K/Akt/GSK-3 signaling axis is highly conserved amongst species and well-known for its regulatory role in muscle protein synthesis, growth and survival [41]. Interestingly, although the exact molecular mechanisms remain elusive to date, recent reports suggest involvement of the PI3K/Akt/GSK-3 signaling axis in the regulation of cellular energy metabolism. For example, in skeletal muscle, Akt-dependent secretion of the myokine Musclin increases mitochondrial biogenesis in response to physical activity [42]. In addition, recently, one study in skeletal muscle showed that IGF-I/Akt-mediated phosphorylation and subsequent activation of ATP citrate lyase improved mitochondrial function in skeletal muscle [43]. These studies suggest that activation of Akt, potentially partly *via* inactivation of GSK-3β, can improve oxidative metabolism in skeletal muscle. This notion is in line with our data as we show for the first time potent inductions in OXPHOS protein and mRNA abundance upon inactivation GSK-3β in myotubes. Interestingly, evidence in non-muscle cells support our findings as these studies indicate that inhibition of GSK-3β similarly resulted in enhanced mitochondrial biogenesis. For example, in adipocytes OXPHOS protein abundance was shown to increase upon inhibition of GSK-3β with LiCl [44]. Furthermore, LiCl treatment increased expression levels of mitochondrial- and nuclear-encoded subunits of OXPHOS complexes in cortical neuronal [19] and aortic endothelial cells [18].

In addition to increases in transcript and protein abundance and activity of key proteins involved mitochondrial oxidative substrate metabolism, we also observed increased protein levels of HKII and increased transcript abundance of genes heavily involved in glycolysis following either knock-down or pharmacological inhibition of

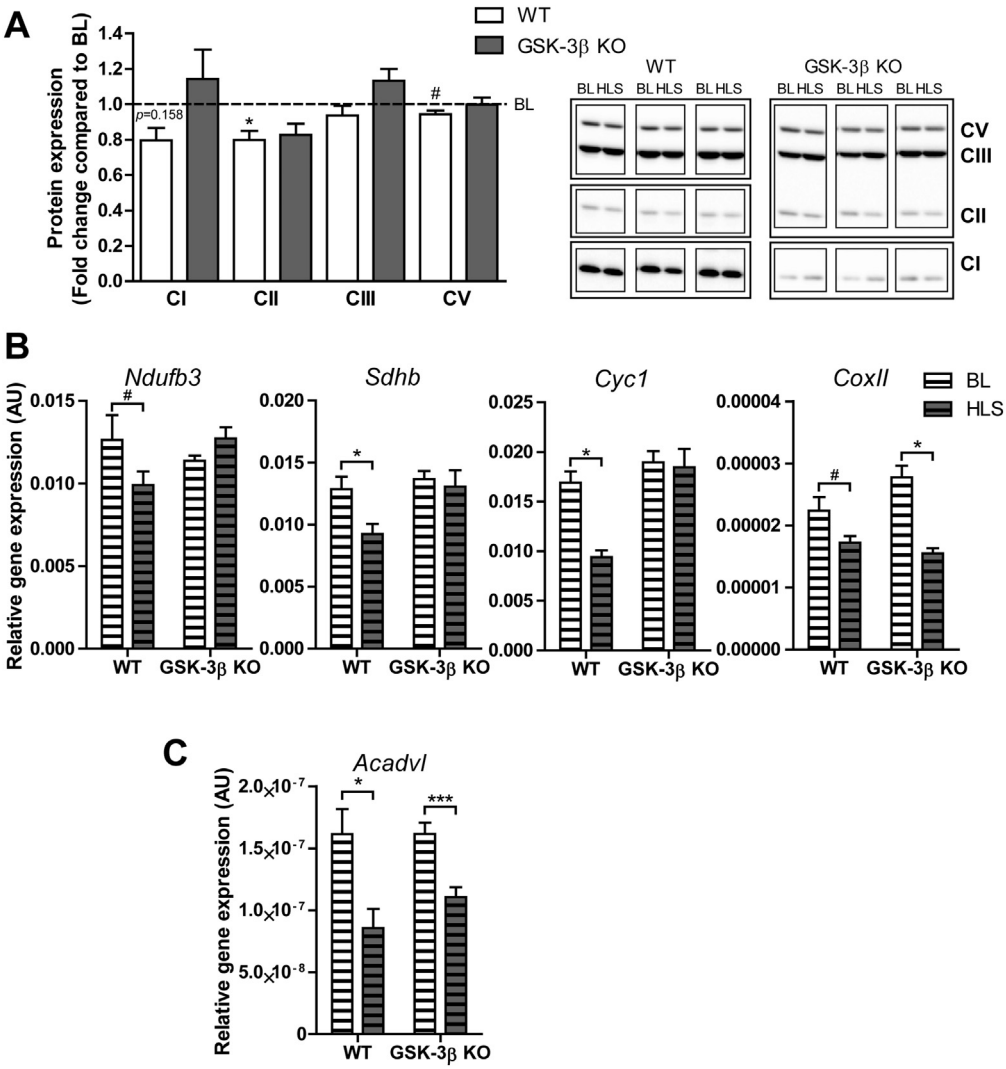

Fig. 5. GSK-3β knock-out partially protects against unloading-induced decline in oxidative gene expression. Muscle-specific GSK-3β KO mice and WT littermates were subjected to HLS for 14 days. *M. gastrocnemius* was prepared for western blotting and qPCR analysis. (A) Protein abundance of OXPHOS complexes of muscle-specific GSK-3β KO (grey bars) and WT (white bars) mice subjected to HLS are compared to BL (—). (B) Gene expression of nuclear-encoded sub-units (*Ndufb3*, *Sdhb* and *Cyc1* respectively Complex I, II and III) and the mitochondrial-encoded sub-unit (*CoxII*, respectively Complex IV) and (C) *Acadvl* under BL (striated white bars) and HLS state (striated grey bars) in muscle-specific GSK-3β KO and WT mice are expressed as arbitrary units (AU) in *M. gastrocnemius* muscle. Representative pictures of the blots are shown and Western blot data is corrected for total protein loading assessed by Ponceau S staining. Black boxes around the representative pictures indicate that they are cut from the same Western blot. Significant differences are tested with an unpaired Student's *t*-test within genotype comparing BL (n = 9/group) and HLS (n = 8/group), #p ≤ 0.1, *p ≤ 0.05, ***p ≤ 0.001. GSK-3β: glycogen synthase kinase-3β, OXPHOS: oxidative phosphorylation, *Ndufb3*: NADH:Ubiquinone Oxidoreductase Subunit B3, *Sdhb*: succinate dehydrogenase complex iron sulfur subunit B, *Cyc1*: cytochrome *c* 1, *CoxII*: Cytochrome c oxidase II, *Acadvl*: very long-chain specific acyl-CoA dehydrogenase, BL: baseline, HLS: hind limb suspension, WT: wild-type and KO: knock-out.

GSK-3. This is in line with available literature that showed that Akt activation increased glycolysis in cancer cells and immortalized mouse kidney epithelial cells [45,46]. Moreover, increased Akt activity resulted in higher mitochondrial-bound HKII by direct phosphorylation and inhibition of GSK-3β. The mitochondrial-bound HKII forms a link between glycolysis and oxidative phosphorylation through better accessibility to generated ATP [47]. However, we also report a higher *Pdk-4* gene expression upon both knock-down or pharmacological inhibition of GSK-3, suggesting an increased block on pyruvate input in the TCA cycle originating from glycolysis. PGC-1α is known to regulate *Pdk-4* gene expression *via* co-activation of ERRα [48], which could explain the increase in *Pdk-4* mRNA observed in our study. Increased *Pdk-4* is indicative of inhibition of oxidative processing of glucose and a switch to FA use as a substrate for mitochondrial ATP production [49]. We did not measure FAO following GSK-3β knock-down in the current study. However, we did show increased activity of HAD, an important enzyme involved in FAO, after knock-down of GSK-3β in myotubes. Measuring HAD activity has been reported to be reflective of FAO activity [50]. As such, these data highlight the potential role of GSK-3β inactivation in active fuel selection in skeletal muscle. However, why inactivation of GSK-3β was not only associated with increased *Pdk-4* mRNA levels and increased expression or activity of key constituents of fatty acid β-oxidation but also with increased expression of glycolytic genes remains unclear. Although PDK-4 is mainly controlled at the transcriptional level, we did not directly measure PDK-4 protein abundance or activity or glycolytic input in the TCA cycle precluding conclusions regarding this.

Mitochondrial biogenesis is heavily controlled by PGC-1 and the downstream co-activation of several nuclear receptors [21,51]. In our study we showed a 10-fold increase in *Pgc-1α* gene expression and a significant increase in PGC-1α protein levels after knock-down of GSK-3β. Additionally, we report increased expression levels of several other key components of the PGC-1 signaling network, like ERRα and PERM1, in C2C12 myotubes after either knock-down of the GSK-3β protein or pharmacological inhibition of GSK-3 with CHIR. This is in line with the effect of LiCl on *Pgc-1α* mRNA expression in various other cell types. For example, LiCl treatment in aortic endothelial cells resulted in increased *Pgc-1α* transcript levels [18]. Furthermore, in vascular smooth muscle, GSK-3β inhibition resulted in increased PGC-1 signaling [20]. In addition, during megakaryocyte development, GSK-3β inhibition using LiCl not only increases mitochondrial density and mtDNA copy number, but also increases important constituents of the PGC-1 signaling cascade [52]. In line with these findings, other studies reported that activation of Akt (thereby inhibiting GSK-3) resulted in similar findings [53]. For example, Ling et al. showed increased *Pgc-1α* transcription following insulin treatment, known to activate Akt [54]. Moreover, the ginsenoside Rg3 significantly increased insulin-stimulated Akt phosphorylation and *Pgc-1α* gene expression in C2C12 myotubes [55]. However, Kim et al. [55] did not report the phosphorylation status of GSK-3. In contrast, one study showed a 3-fold decrease in *Pgc-1α* mRNA expression upon expression of constitutively active Akt in

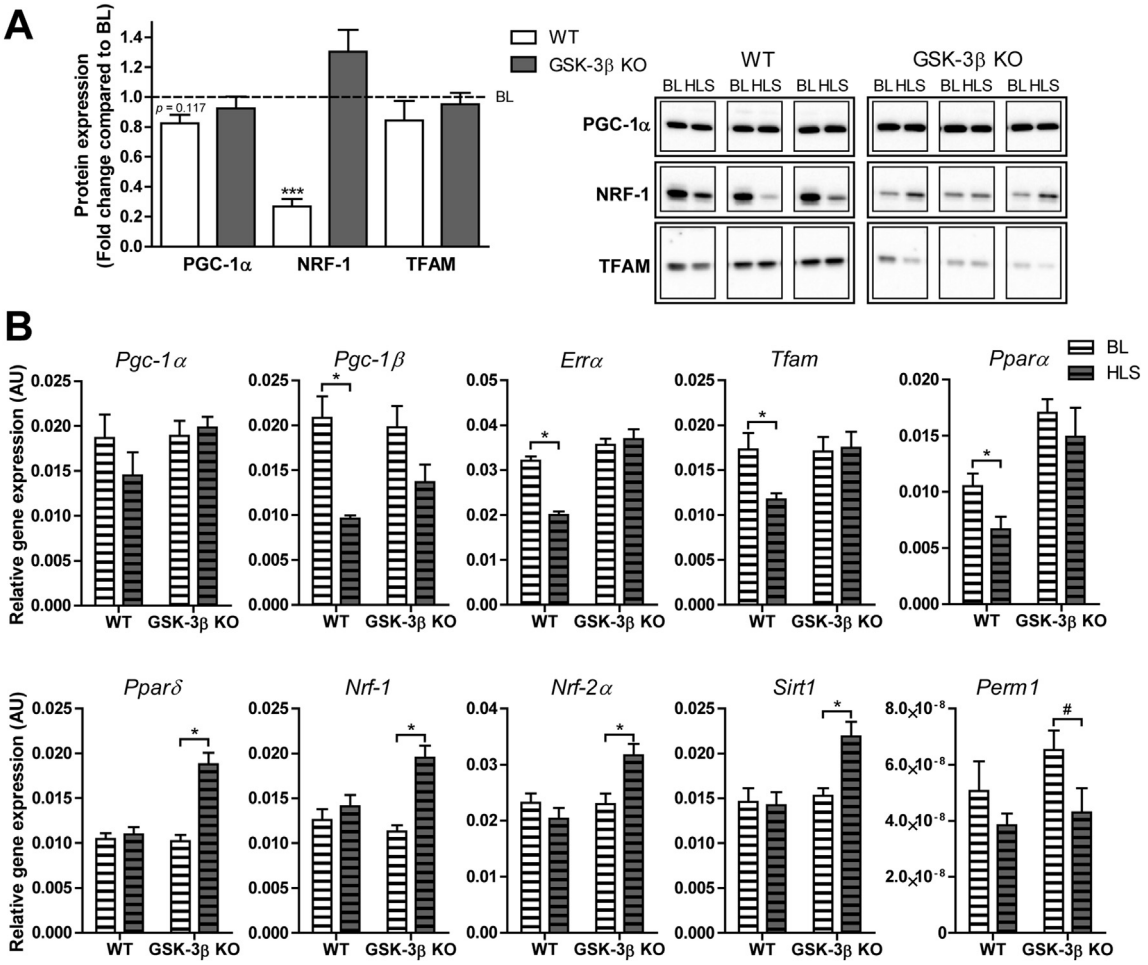

Fig. 6. GSK-3β knock-out protects against unloading-induced decline in PGC-1α signaling constituents. Muscle-specific GSK-3β KO mice and WT littermates were subjected to HLS for 14 days. *M. gastrocnemius* was prepared for western blotting and qPCR analysis. (A) Protein abundance of PGC-1α, NRF-1 and TFAM of GSK-3β knock-out (grey bars) and WT (white bars) mice subjected to HLS are compared to BL (—). (B) Gene expression of *Pgc-1α* and gene involved in the PGC-1 signaling network under BL (striated white bars) and HLS state (striated grey bars) in GSK-3β KO and WT mice are expressed as arbitrary units (AU) in *M. gastrocnemius* muscle. Representative pictures of the blots are shown and Western blot data is corrected for total protein loading assessed by Ponceau S staining. Black boxes around the representative pictures indicate that they are cut from the same Western blot. Significant differences are tested with an unpaired Student's *t*-test within genotype comparing BL (n = 9/group) and HLS (n = 8/group), #p ≤ 0.1,*p ≤ 0.05, ***p ≤ 0.001. GSK-3β: glycogen synthase kinase-3β, *Pparα/δ*: peroxisome proliferator-activated receptor α/δ, *Pgc-1α/β*: PPAR gamma coactivator-1α/β, *Errα*: estrogen-related receptor α, *Nrf-1/2α*: nuclear respiratory factor-1/2α, *Tfam*: mitochondrial transcription factor A, *Sirt1*: sirtuin 1 and *Perm1*: PGC-1 and ERR-induced regulator in muscle 1, BL: baseline, HLS: hind limb suspension, WT: wild-type and KO: knock-out.

cardiac muscle [56]. We used knock-down of *Pgc-1α* to investigate if the effects of pharmacological inhibition of GSK-3 on cellular energy metabolism are mediated *via* PGC-1α. As expected, we show initial evidence that increased expression of genes involved in OXPHOS (CII and CIII sub-units) and the PGC-1 signaling network (*Errα*) are dependent on GSK-3 inactivation-mediated increase in *Pgc-1α*. Previously, it was reported that the gene expression of *Errα* [37], *Sdhb* and *Cycs* [36] are under control of PGC-1α.

Although we did not include this in the present study, the activity of the PGC-1α protein can be altered by post-translational modifications. An array of kinases can modulate the activity of PGC-1, amongst others Akt which can directly inhibit PGC-1α activity by phosphorylating Ser^{570} [57]. In addition, in skeletal muscle AMP-activated protein kinase (AMPK) is a well-known positive regulator of post-transcriptional regulation of PGC-1α activity [58]. Interestingly, one study showed that GSK-3β can phosphorylate PGC-1α thereby labeling it for intra-nuclear proteasomal degradation, suggesting stabilization of PGC-1α resulting in increased protein abundance upon GSK-3β inactivation [59]. This suggests that the kinase activity of GSK-3β is an important aspect of the molecular mechanism by which GSK-3β regulates oxidative gene expression and expression of constituents of the PGC-1 signaling network. Another example of post-transcriptional regulation of the PGC-1α protein is activation *via* SIRT-dependent deacetylation in response to high NAD + levels [60]. However, in our study we did not investigate phosphorylation or acetylation status or intra-nuclear levels of PGC-1α. In addition, future research targeting transcription factors known to increase *Pgc-1α* gene expression could reveal the potential mechanism of how GSK-3β knock-down increases transcription of *Pgc-1α* and its downstream targets. In this regard, as AMPK activation is known to increase transcription of the PGC-1α gene [61] and interplay between AMPK and GSK-3β has been described [62,63], AMPK activation may also play a role. Interestingly, GSK-3 can inhibit AMPK by the inhibitory phosphorylation of Thr^{479} of the α-subunit of AMPK [64]. Therefore it is not unlikely that GSK-3β inactivation-mediated activation of AMPK plays a role in the regulation of the transcription of the PGC-1α gene as well as regulation of the function of the PGC-1α protein in skeletal muscle.

Our data reveals a predominant role for the GSK-3β isoform in the regulation of skeletal muscle oxidative phenotype compared to GSK-3α. Specific knock-down of the GSK-3α isoform resulted in less pronounced effects on *Pgc-1α* gene expression while changes in *e.g.* mRNA expression of OXPHOS sub-units were absent. This suggest that inactivation of GSK-3β, and not GSK-3α, is sufficient to stimulate an oxidative phenotype in skeletal muscle cells and shows that besides PGC-1α, ERRα

and PERM1 are most likely important to induce increases in cellular oxidative energy metabolism. ERRs have an important function in the regulation of skeletal muscle oxidative metabolism. For example, ERRα is able to induce expression of genes involved in mitochondrial biogenesis and OXPHOS [37]. ERRγ regulates metabolic and mitochondrial gene expression and is necessary to increase exercise capacity in skeletal muscle [65]. *Perm1* has recently been discovered to be a potent muscle-specific regulator downstream of PGC-1/ERR mediated signaling, as it was shown that PERM1 levels are related to physical activity levels and that knock-down of the PERM1 protein in C2C12 myotubes decreased respiratory capacity, OXPHOS, mitochondrial biogenesis and FAO [22]. Recently, over-expression of PERM1 in murine skeletal muscle revealed increases in mitochondrial content and oxidative capacity *in vivo* [66]. These data are in line with our observations.

In vivo, we observed that GSK-3β KO animals were protected against unloading-induced decreases in nuclear-encoded gene expression of OXPHOS sub-units, while the mitochondrial-encoded sub-unit of Complex IV (*CoxII*) was decreased after HLS in both genotypes. It is not unlikely that protective effect of GSK-3β knock-out upon HLS is limited to gene expression in the nucleus, as known regulators of OXPHOS gene expression are nuclear proteins, such as PGC-1α, NRF-1/2α and ERRs [21].

The PI3K/Akt/GSK-3β signaling axis historically has been appreciated for its role in the regulation of muscle mass. For example, GSK-3β is required for the induction of muscle atrophy *in vitro* [15]. Also, it was reported that pharmacological inactivation of GSK-3β with SB216763 protected against LPS-induced loss of muscle mass [67]. However, muscle-specific GSK-3β KO animals were previously shown not to be protected against inactivity-induced loss of *M. gastrocnemius* mass [39]. GSK-3β has been shown to modulate both protein synthesis [68] and protein degradation pathways [15]. Besides these known effects of GSK-3β on muscle protein turnover, in the current study, we now show for the first time, not only that inactivation of GSK-3β enhances myotube mitochondrial metabolism but also that muscle-specific KO of GSK-3β protects against decrement in metabolic gene expression and expression of the PGC-1 signaling network. Taken together, this suggests that GSK-3β represents a nodal point interconnecting regulatory pathways involved in the regulation of mitochondrial energy metabolism on the one hand and pathways controlling muscle mass on the other hand. Recent reports in literature support the notion that these pathways are intertwined [69]. For example, PGC-1α controls mitochondrial biogenesis and oxidative metabolism, but also protects against atrophy when over-expressed [70,71]. Furthermore, besides its regulatory role in mitochondrial oxidative metabolism, PGC-1 has also been implicated in the regulation of muscle mass. Not only has it been demonstrated that PGC-1α transcript and protein levels sharply decrease after atrophy induced by well-established muscle atrophy models by denervation, fasting and inactivity but it has also been shown that over-expression of the PGC-1α protein protects against muscle atrophy after denervation, fasting or inactivity [70,71]. Next to PGC-1α, Akt-dependent activation of ATP citrate lyase boosts skeletal muscle oxidative metabolism, besides the well-known effects of Akt on the regulation of muscle hypertrophy and atrophy [43]. Previously our group reported that muscle fibre type distribution was largely preserved in GSK-3β KO muscle compared to WT animals, [39]. Investigations into *in vivo* functional changes such as skeletal muscle oxygen consumption or the contractile properties of the musculature were not the primary goal of this study, therefore we did not include these measurements. This, however, is an interesting aspect for future research.

In conclusion, we report a novel interaction between inactivation or loss of the GSK-3β protein and cellular energy metabolism which, in concert with available literature, highlights an intricate link between pathways involved in the regulation of skeletal muscle energy production and those controlling muscle mass. However, if the effects of inactivation of GSK-3β on mitochondrial metabolism and biogenesis are mediated solely *via* PGC-1 remains to be further investigated. In addition, it remains unclear if inactivation of GSK-3β can enhance OXPHEN in skeletal muscle of patients with altered muscle mitochondrial metabolism such as seen in COPD, type 2 diabetes or chronic heart failure. Gaining more insight in the regulation of muscle oxidative metabolism is vital to improve understanding of the molecular mechanisms governing oxidative energy metabolism during muscle maintenance and is required for optimizing available exercise and nutritional interventions and to develop proficient new strategies improving skeletal muscle oxidative metabolism and related physical functioning in chronic disease management and prevention.

Transparency document

The http://dx.doi.org/10.1016/j.bbadis.2017.09.018 associated with this article can be found, in the online version.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbadis.2017.09.018.

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