Regulation of skeletal muscle mitochondrial biogenesis by GSK-3β



Wessel Theeuwes

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Cover design: Wessel Theeuwes (*Design and Painter*)
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Layout: Wessel Theeuwes
Printed by: ProefschriftMaken || www.proefschriftmaken.nl
ISBN: 978-94-6380-738-8

The research described in this thesis was performed at the Department of Respiratory Medicine, NUTRIM School of Nutrition and Translational Research in Metabolism at Maastricht University Medical Center+, Maastricht, The Netherlands.

The research was financially supported the Lung Foundation Netherlands (5.2.13.067).

Regulation of skeletal muscle mitochondrial biogenesis by GSK-3β

Proefschrift

Ter verkrijging van de graad van doctor aan de Universiteit Maastricht,
op het gezag van de Rector Magnificus, Prof. dr. Rianne M. Letschert,
volgens het besluit van het College van Decanen,
in het openbaar te verdedigen op vrijdag
3 april 2020 om 16:00 uur.

door

Wessel Frank Theeuwes
Geboren te Breda op 6 januari 1992, Nederland

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Chapter 1

General Introduction

Introduction

Skeletal muscle tissue of healthy humans comprises approximately 40% of the total body weight [1], is the major site for glucose disposal [2] and vital for physical movement. Large mitochondrial networks with a high capacity for mitochondrial biogenesis characterize tissues with high rates of aerobic metabolism such as skeletal muscle [3]. Skeletal muscle is composed of several fiber types of which slow-twitch oxidative fibers (type I and IIA) are denser in mitochondrial content compared to fast-twitch glycolytic fibers [4]. Skeletal muscle mitochondrial density is largely regulated by a process called mitochondrial biogenesis. Mitochondrial biogenesis is activated by endurance exercise and increases during muscle differentiation [5, 6] and *vice versa* is decreased in the musculature of patients in several chronic disorders and during ageing [7-10].

Regulation of skeletal muscle oxidative phenotype and mitochondrial biogenesis

Oxidative phenotype

Oxidative phenotype (OXPHEN) is determined by the proportion of oxidative fibers (type I, IIA), activity and abundance of enzymes involved in the tricarboxylic acid (TCA) cycle and fatty acid β-oxidation, mitochondrial quantity and abundance of oxidative phosphorylation (OXPHOS) complexes involved in the electron transport chain (ETC). Skeletal muscle OXPHEN plays a crucial role in adequate muscle function [11]. For example, loss of muscle OXPHEN is often associated with reduced muscle endurance [12], while enhanced muscle OXPHEN is associated with increased resistance to muscle fatigue [13].

Mitochondria

Mitochondria are double-membrane organelles that are most familiar as the 'powerhouses' of eukaryotic cells. Mitochondria are thought to have originated from endocytosis of aerobic prokaryotic cells by anaerobic cells, resulting in endosymbiosis [14]. Indicative of this endocytosis event, mitochondria preserved specific bioenergetic pathways and possess their own genome, namely mitochondrial DNA (mtDNA). The inner mitochondrial membrane is highly folded into several cristae resulting in its well-known and distinct morphology. Engulfed by the inner mitochondrial membrane the mitochondrial matrix contains the mtDNA and is the main site for mitochondrial metabolic pathways such as fatty acid β-oxidation and the TCA cycle. The mtDNA encodes 37 genes of which 13 are polypeptides involved in the OXPHOS system

which is located on the inner mitochondrial membrane [15]. OXPHOS is defined as the oxygen consuming process in which energy obtained by electron transport is used to synthesize adenosine triphosphate (ATP) by phosphorylation of adenosine diphosphate (ADP) or adenosine monophosphate (AMP). Both nuclear-encoded as well as mitochondrial-encoded sub-units of OXPHOS proteins contribute to OXPHOS protein complex assembly (essential for oxidative energy production), underscoring the complexity of the regulation of mitochondrial bioenergetic pathways.

Mitochondrial oxidative phosphorylation

Fat, carbohydrates (*i.e.* glucose) and protein are the three main fuels of our cells of which fat and carbohydrates are predominantly used to produce energy. Glucose can be converted into pyruvate via a multi-step process called glycolysis, yielding two ATP and two nicotinamide adenine dinucleotide (NADH). In the absence of oxygen, pyruvate can subsequently be reduced to lactate (using NADH) in the cytosol. In the presence of oxygen on the other hand, upon transport to the mitochondrial matrix via mitochondrial pyruvate carriers [16], pyruvate can be converted to acetyl-coenzyme A (CoA) yielding one additional NADH molecule. Next to its formation from the catabolism of glucose, acetyl-CoA can also be formed from fatty acid breakdown. Upon fatty acid activation, a process that converts fatty acids to fatty acetyl-CoA, carnitine binds to fatty acyl-CoA allowing transport to the mitochondrial matrix. This is catalyzed by carnitine palmitoyltransferase I and trans-membrane transport is facilitated by acylcarnitine transferase. Within the mitochondrial matrix, fatty acyl-CoA, released from carnitine by carnitine palmitoyltransferase II, serves as input for β-oxidation [17]. Fatty acid β-oxidation is a multistep mitochondrial process where fatty acyl-CoA is oxidized, yielding several acetyl-CoA, NADH and flavin adenine dinucleotide ($FADH_2$) molecules [18]. Once inside the mitochondrial matrix, acetyl-CoA derived from the catabolism of either glucose or fatty acids is converted into citrate which serves as input for the TCA cycle. The TCA cycle comprises a multi-step process of enzymatic reactions yielding one $FADH_2$, one guanosine triphosphate and three NADH molecules and in the process produces carbon dioxide.

NADH and $FADH_2$ are electron carriers that donate electrons to the mitochondrial ETC. NADH and $FADH_2$ deliver electrons to OXPHOS Complex I and II, respectively. In this process, Complex I transfers protons to the mitochondrial intermembrane space. Electrons from Complex I and II are transferred to ubiquinone, which transports the electrons to Complex III. More hydrogen ions are pumped across the membrane as electrons pass through Complex III. The electrons are then passed on to cytochrome C, shuttling the electrons to Complex IV. In turn, Complex IV produces water by donating the electrons to oxygen and hydrogen, and thus is the main site in the

mitochondria where oxygen is used. Summarized, OXPHOS complexes I, III, and IV use the energy released from electrons moving from higher to lower energy levels to move protons from the matrix into the mitochondrial intermembrane space, generating an energy-rich proton gradient [19]. Complex V, or ATP synthase, then uses the energy from this proton gradient to produce energy in the form of ATP by chemiosmosis. ATP can subsequently be used to fuel a multitude of energy consuming cellular processes. Together, the ETC and chemiosmosis form the OXPHOS system. Next to the abundance and activity of components of mitochondrial bioenergetic pathways, mitochondrial biogenesis plays a crucial role in maintaining normal mitochondrial function. Mitochondrial biogenesis controls the skeletal muscle mitochondrial pool and hence contributes to alterations in skeletal muscle OXPHEN.

Mitochondrial dynamics

Mitochondrial turnover is the balance between mitochondrial biogenesis and mitophagy and suggested to be essential for skeletal muscle health [20]. Mitochondrial biogenesis controls the growth of pre-existing mitochondria and the formation of new mitochondria. Thereby it increases the mitochondrial mass and hence the oxidative capacity of the musculature. The transcriptional co-activator molecule peroxisome proliferator-activated receptor (PPAR)γ co-activator-1α (PGC-1α) is at the heart of a molecular signaling network that controls mitochondrial biogenesis [21] (see next section for more details). In order to selectively degrade parts of the mitochondrial network dysfunctional parts of the organelle are budded-off by a process called mitochondrial fission, mainly regulated by dynamin-related protein 1 (DRP1) and fission protein 1 (FIS1) [22, 23]. Mitochondrial autophagy, often referred to as mitophagy, then selectively clears these defective parts of the mitochondrial network. The ubiquitin 26S-proteasome system (UPS) selectively clears damaged mitochondrial proteins [24], while the autophagy-lysosomal pathway (ALP) degrades small parts of the mitochondrial membrane that bud-off from the mitochondrial surface [24]. A process called mitochondrial fusion accommodates merging of mitochondria. Mitofusins1 (MFN1) and MFN2 regulate fusion of the inner mitochondrial membrane while optic atrophy 1 (OPA1) is essential for mitochondrial fusion of the outer mitochondrial membrane [24]. Together mitochondrial fusion and fission accommodate a constantly changing mitochondrial network. Although all processes involved in mitochondrial dynamics serve muscle function, this thesis focusses on mitochondrial biogenesis.

Regulation of mitochondrial biogenesis by PGC-1α

PGC-1α signaling network

PGC-1α is a key regulator controlling many facets of skeletal muscle OXPHEN. These include fatty acid β-oxidation, OXPHOS, mitochondrial biogenesis and gene expression of structural proteins comprising slow-twitch oxidative fibers [21, 25]. To date, several PGC-1α isoforms have been identified [26]. PGC-1β and PGC-1-related co-activator (PRC) are family members of PGC-1α and, although studied to a lesser extent, also regulate multiple features of muscle OXPHEN [27]. Acute exercise induces skeletal muscle PGC-1α levels in humans [28] and rodents [29]. Illustrative of the importance of PGC-1α in the regulation of skeletal muscle mitochondrial biogenesis, muscle-specific overexpression of PGC-1α increased the proportion of oxidative type I myofibers [30, 31] and increased mitochondrial biogenesis in mice resulting in an enhanced aerobic capacity [31]. Likewise, transgenic overexpression of PGC-1α-b, a PGC-1α isoform, increased mitochondrial biogenesis, exercise capacity and oxygen uptake [32]. On the other hand, skeletal muscle-specific PGC-1α knock-out (KO) mice exhibited decreased expression of OXPHOS genes [33]. In addition, skeletal muscle PGC-1α/β-deficiency results in reduced muscle oxidative capacity, defects in mitochondrial biogenesis and diminished exercise performance [34, 35]. In addition, exercise was unable to increase OXPHOS complex activity in these PGC-1α/β-deficient mice [35] indicating that PGC-1 is important in fully exploiting beneficial effects of exercise on muscle OXPHEN.

It has been convincingly shown that PGC-1α exerts control over skeletal muscle OXPHEN and mitochondrial biogenesis via co-activation of a wide variety of transcription factors and nuclear receptors, such as estrogen-related receptors (ERRs) [36], PPARs [37] and nuclear respiratory factors (NRFs) [38]. To facilitate activation of specific gene programs, PGC-1α binds to histone acetyltransferase-containing proteins [39] and docks to several mediator complexes [40] which are able to acetylate histones. This results in remodeling of the chromatin structure allowing gene activation. PGC-1α can physically interact with amongst others the above mentioned transcription factors initiating specific gene programs regulating several aspects of OXPHEN and mitochondrial biogenesis in the skeletal muscle [37]. In general, PGC-1α regulates fatty acid β-oxidation, a glycolytic to oxidative fiber type shift and gene programs involved in the regulation of OXPHOS via PPARs, ERRs and NRFs [41, 42]. Moreover, NRFs regulate the expression of mitochondrial transcription factor A (TFAM) via co-activation by PGC-1α [38]. Furthermore, PGC-1 and ERR-induced regulator in muscle 1 (*Perm1*) expression increases via co-activation of ERRα by PGC-1α in skeletal muscle [43]. Several studies exploring the effect

of KO or overexpression of the above mentioned transcription factors on muscle OXPHEN revealed their importance in the molecular regulation of oxidative substrate metabolism and mitochondrial biogenesis, which is described below.

Estrogen-related receptors

The role of ERRα and ERRγ in skeletal muscle has been extensively studied. For example, it has been shown that, coinciding with the induction of mitochondrial biogenesis, their expression increases in response to physical activity in muscle [44]. Moreover, muscle-specific overexpression of ERRα increased exercise capacity, oxygen consumption, mitochondrial content and glycogen content in mice [45]. Likewise, muscle-specific overexpression of ERRγ induced a glycolytic to oxidative fiber type shift and increased skeletal muscle exercise capacity, mitochondrial biogenesis and angiogenesis [44, 46]. *Vice versa*, ERRα KO animals display reduced exercise tolerance and impaired expression of genes involved in the TCA cycle and OXPHOS [47]. In addition, ERRα is required for adequate myocyte differentiation [48] and muscle regeneration [49]. Co-activation of ERRα by PGC-1α increases pyruvate dehydrogenase kinase 4 gene expression [50] which is associated with metabolic shifting from carbohydrate use towards the use of fatty acids in the production of ATP. Transgenic overexpression of the ERRα target PERM1 in skeletal muscle is associated with increased maximal respiration, activity and abundance of OXPHOS proteins, citrate synthase activity, mtDNA content and delayed time to muscle fatigue [51]. Taken together, this underscores a prime role for ERR transcription factors in the regulation of fatty acid β-oxidation and OXPHOS in skeletal muscle.

Peroxisome proliferator-activated receptors

PGC-1α was initially identified as co-activator of PPARγ in brown adipose tissue [52]. Interestingly, exercise increases the expression of PPARs in skeletal muscle [53]. Although PPARγ is expressed in low quantity in skeletal muscle [54], specific KO revealed that PPARγ is important in the maintenance of skeletal muscle insulin sensitivity [55]. However, PPARγ agonist treatment did not enhance endurance capacity [56]. On the other hand, overexpression of PPARδ increased running time and distance, while PPARδ null mice displayed impaired exercise capacity [57]. Moreover, muscle-specific PPARδ overexpression resulted in a fiber type shift toward more oxidative fibers [57, 58] and increased gene expression and protein activity of several proteins involved in oxidative substrate metabolism [58]. In addition, PPARδ agonist treatment initiated gene programs involved in fatty acid transport, fatty acid β-oxidation and mitochondrial respiration [59], resulting in an increased β-oxidation rate [59, 60] and enhanced palmitate-fueled respiration and running endurance [61]. In addition, muscle-specific overexpression of PPARα increases fatty acid β-oxidation

rates [62]. Collectively, these studies demonstrate that besides ERRs, PPARs are also key regulators of skeletal muscle OXPHEN and exercise capacity [36].

Nuclear-respiratory factors and mitochondrial transcription factor A

It is well-known that physical activity increases the expression of NRFs [29, 56]. In addition, co-activation of NRF-2α by PGC-1α is associated with increased expression of nuclear-encoded OXPHOS sub-units [63]. In skeletal muscle, overexpression of NRF-1 increased protein levels of several OXPHOS proteins [64]. In addition via co-activation of NRFs, PGC-1α initiates gene expression of TFAM [38, 65], a key protein involved in transcription of the mitochondrial-encoded genes, mtDNA duplication and mitochondrial biogenesis. Following migration into mitochondria, TFAM increases mtDNA duplication and expression of mitochondrial-encoded OXPHOS sub-units. Indeed, TFAM protein abundance is directly correlated with the amount of mtDNA, *i.e.* knock-down of TFAM results in mtDNA depletion [66] while overexpression of TFAM increases mtDNA copy number [67]. An overview of processes regulated by PGC-1α-mediated co-activation of several transcription factors is shown in Fig. 1.

Transcriptional control of Pgc-1α gene expression

As outlined above, PGC-1α sits at the center of the molecular signaling network that controls skeletal muscle OXPHEN. Transcription of the *Pgc-1α* gene is stimulated by external stimuli such as cold exposure and physical exercise [68] and is well-known to be controlled by several transcription factors that are able to bind the PGC-1α promoter. Cyclic AMP response element binding protein (CREB) [69], myocyte enhancer factor (MEF)2 [70], myoblast determination protein 1 (MyoD) [71], forkhead box class O (FoXO)1 [72], p53 [73], ERRα [74] and members of the microphthalmia/TFE (MiT) family of transcription factors [75, 76] are all able to bind and activate the PGC-1α promoter. MEF2 transcription factors can bind specific MEF2-binding sites located on the PGC-1α promoter resulting in PGC-1α promoter activation [70, 77]. Moreover, overexpression of MyoD or sirtuin 1 (SIRT1) has been associated with enhanced PGC-1α promoter activation [71]. In addition, a positive auto-feedback loop where PGC-1α activates its own promoter via MEF2 or MyoD was previously described [71, 77]. Furthermore, a CRE-binding site is located on the PGC-1α promoter [78]. CREB binding on this CRE-binding element activates *Pgc-1α* transcription, which is accompanied by increased respiration and mitochondrial biogenesis [69]. Additionally, transcription factor binding to IGHM enhancer 3 (TFE3) and transcription factor EB (TFEB) are both members of the MiT family that are able to bind coordinated lysosomal expression and regulation (CLEAR)-regions located on the PGC-1α promoter [75, 76]. Mutagenesis of these CLEAR-regions prevented PGC-1α promoter activation mediated by TFE3 overexpression [75]. Interestingly, some of these transcription factors are associated with physical activity or

exercise-mediated induction of *Pgc-1α* gene expression [79, 80]. A schematic overview of several transcription factors known to be able to activate PGC-1α transcription is provided in Fig. 2.

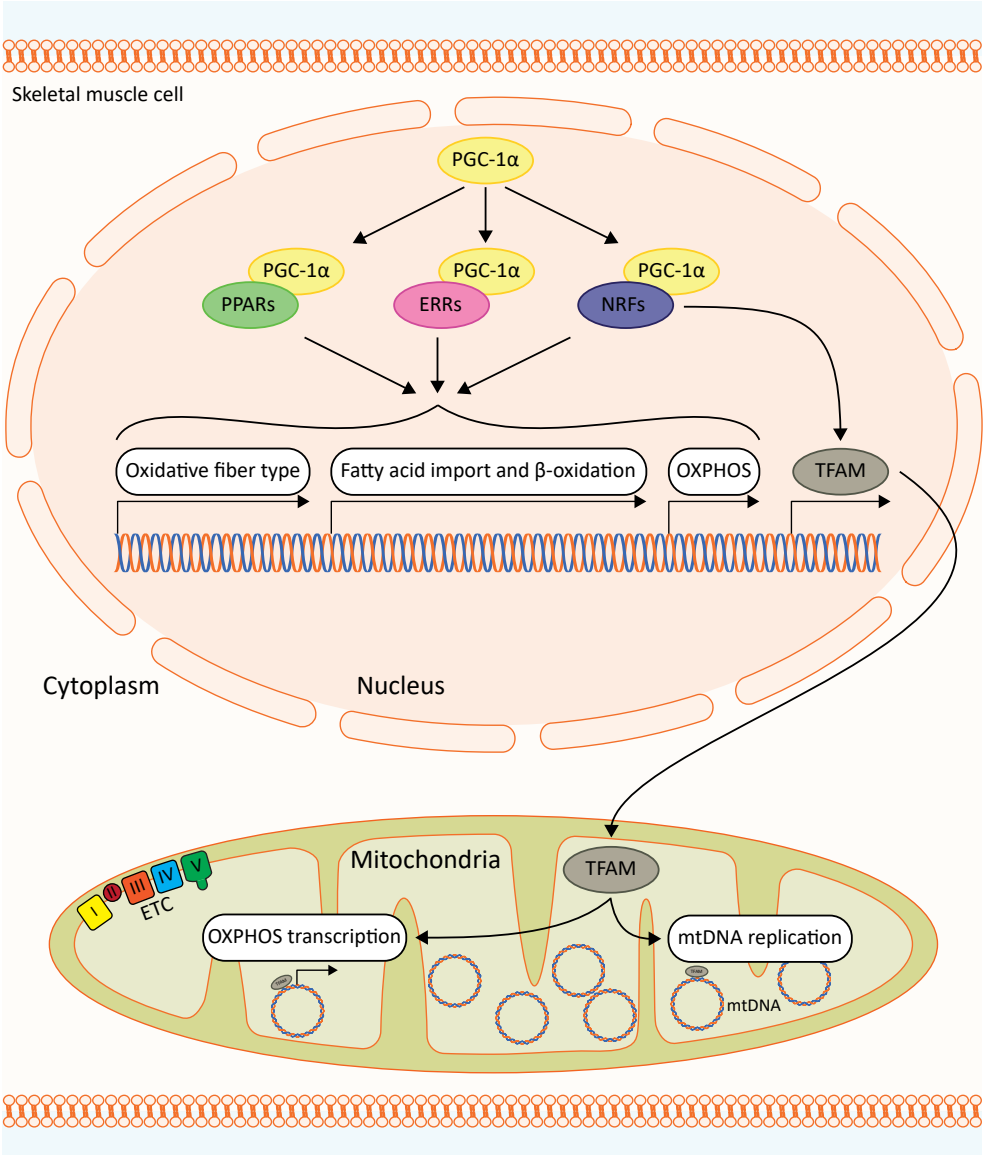


Figure 1. PGC-1α regulates skeletal muscle oxidative substrate metabolism and mitochondrial biogenesis. Peroxisome proliferator-activated receptor (PPAR)γ co-activator-1α (PGC-1α), estrogen-related receptors (ERRs), nuclear respiratory factors (NRFs), mitochondrial transcription factor A (TFAM), oxidative phosphorylation (OXPHOS), electron transport chain (ETC) and mitochondrial DNA (mtDNA).

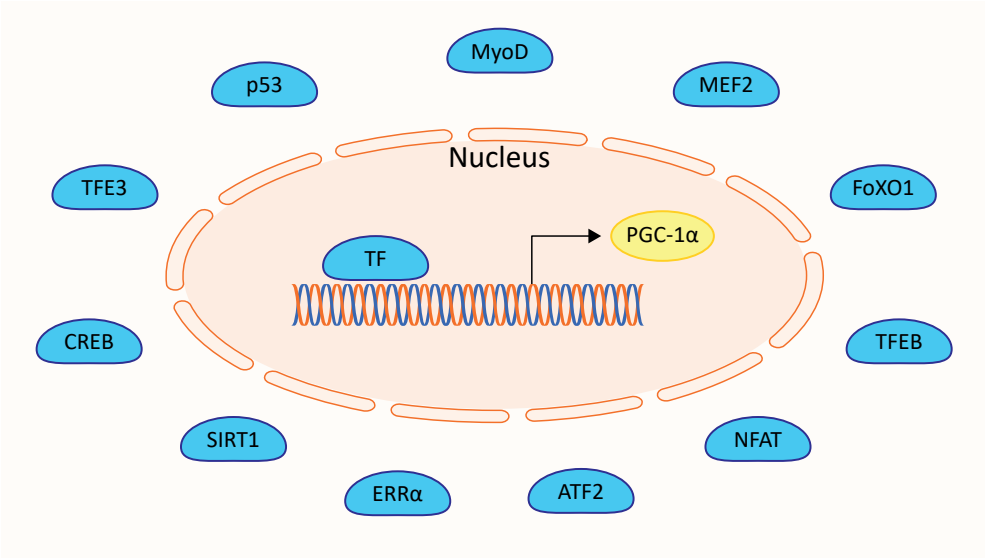


Figure 2. Transcription factors involved in the transcriptional regulation of the *Pgc-1α* gene. Peroxisome proliferator-activated receptor-γ co-activator-1α (*Pgc-1α*) gene expression is controlled by several transcription factors (TFs). Amongst others, cyclic AMP response element binding protein (CREB), transcription factor binding to IGHM enhancer 3 (TFE3), p53, myoblast determination protein 1 (MyoD), myocyte enhancer factor 2 (MEF2), forkhead box class O1 (FoXO1), transcription factor EF (TFEB), nuclear-factor of activated T-cells (NFAT), activating transcription factor 2 (ATF2), sirtuin 1 (SIRT1) and estrogen-related receptorsα/γ (ERRα/γ).

PGC-1α isoforms

Recent advances in the field revealed that transcription of the *Pgc-1α* gene is controlled by two distinct promoter regions, namely the proximal PGC-1α promoter and the alternative PGC-1α promoter (for review see [26]). Several different splice variants of PGC-1α are known to originate from activation of these two promotor regions. More specifically, *Pgc-1α-a* is transcribed from the proximal PGC-1α promoter, while the alternative PGC-1α promoter controls transcription of *Pgc-1α-b* and *Pgc-1α-c*. Additionally, truncated variants of these three isoforms are known [26]. It has been proposed that *Pgc-1α* isoforms transcribed from the proximal promoter coordinate basal PGC-1α levels [26] while the alternative promoter is predominantly activated by external stimuli such as exercise [81, 82]. Furthermore, PGC-1α4 a truncated isoform transcribed from the alternative promoter increases in response to exercise and regulates skeletal muscle hypertrophy [83].

Post-transcriptional modifications of PGC-1α

Next to regulation at the transcriptional level, the activity of the PGC-1α protein is regulated by post-transcriptional modifications. For example, the signaling molecule AMP-activated protein kinase (AMPK) is activated by an increased AMP/ATP ratio and is a major factor involved in post-transcriptional modifications of the PGC-1α protein. AMPK can directly phosphorylate PGC-1α thereby increasing its activity [84]. In addition, AMPK activates PGC-1α indirectly via SIRT1-mediated de-acetylation [85]. Furthermore, p38MAPK and protein kinase A can also increase PGC-1α activity through phosphorylation [86, 87]. In addition, PGC-1α can be tagged for intranuclear proteasomal degradation by glycogen synthase kinase (GSK)-3β-mediated phosphorylation [88]. Interestingly in this regard is that inactivation of GSK-3β resulted in activation of signaling pathways involved in the regulation of OXPHEN and increased levels of PGC-1α in several non-muscle cells [89, 90]. However, the role of GSK-3β in the regulation of OXPHEN in skeletal muscle is thus far unexplored.

Glycogen synthase kinase-3

GSK-3 is an ubiquitously-expressed serine/threonine (Ser/Thr) kinase originally identified as a regulator of glycogen metabolism [91]. In muscle, GSK-3β inhibits glycogen deposition by phosphorylation and subsequent inhibition of glycogen synthase. In mammals, two homologue GSK-3 isoforms have been identified namely GSK-3α and GSK-3β. Of all kinases, GSK-3 has the most predicted targets [92] and functions in several cellular signaling pathways including canonical Wnt-signaling [93] and the insulin-like growth factor-I (IGF-I)/protein kinase B (AKT) pathway. AKT is a nodal point in the regulation of muscle protein turnover that negatively regulates GSK-3 activity through phosphorylation of Ser^{9} or Ser^{21}, of GSK-3β and GSK-3α respectively [94]. Unlike the majority of kinases, GSK-3 is constitutively active and exerts its function in several cellular processes via regulatory inactivation of its substrates. GSK-3β recognizes a specific Ser/Thr motif (Ser/Thr-X-X-X-Ser~P/Thr~P; where X can be any amino acid), often pre-phosphorylated at the C-terminal site by other kinases [95]. GSK-3β then phosphorylates the Ser/Thr four residues downstream of the pre-phosphorylated Ser/Thr. Many GSK-3β substrates contain adjacent copies of this motif, resulting in multiple phosphorylation sites for GSK-3β [96]. For example, the GSK-3β consensus motif is present on glycogen synthase [96], transcription factors of the MiT family [97] and eukaryotic translation initiation factor 2B (eIF2B) [98]. Because of its constitutively active nature and involvement in several cellular signaling cascades that have been shown to be dysregulated

in several chronic diseases, GSK-3β is an interesting target for pharmacological inhibition. Lithium was the first GSK-3 inhibitor discovered [99]. The mode of action of lithium is competition for magnesium ions which are required for GSK-3 activity [100]. Hence, lithium inhibits not only GSK-3 but also other magnesium-dependent kinases [101]. Therefore, several small synthetic peptides specifically inhibiting GSK-3 have been developed, amongst others CHIR99021 [102]. Interestingly, in skeletal muscle GSK-3β has been shown to modulate both protein synthesis [98] and protein degradation pathways [103] as well as myogenic differentiation [104, 105], all of which are involved in the regulation of skeletal muscle mass.

Regulation of skeletal muscle mass

Skeletal muscle tissue has a remarkably high plasticity in response to changes in physical activity or muscle loading [106]. Next to the capacity to regulate OXPHEN, muscle plasticity also involves the coordinated regulation of skeletal muscle mass. The regulation of skeletal muscle mass involves post-natal myogenesis and protein turnover.

Post-natal myogenesis

Myonuclear turnover involves the loss and accretion of myofiber nuclei and relies on post-natal myogenesis as an important regulatory mechanism in the control of muscle regeneration and mass. Furthermore, satellite cells are thought to be essential in muscle hypertrophy which is defined as post-natal muscle growth [107]. In adult muscle, satellite cells are in a quiescent state but upon external stimuli, they become activated and proliferate thereby expanding the precursor pool. This is part of the skeletal muscle regenerative response (*i.e.* post-natal myogenesis) [107]. Upon proliferation, a subset of satellite cells repopulate the quiescent satellite cell reservoir while others commit to post-natal myogenesis as myoblasts. Myoblasts differentiate and fuse with pre-existing myofibers donating amongst others their myonuclei (a process referred to as myonuclear accretion) (Fig. 3). Upon accretion of myonuclei, muscle-specific gene expression further drives myogenic differentiation aiding muscle fiber regeneration. Inhibition of GSK-3β has been shown to accelerate the induction of the myogenic differentiation program *in vitro* [104, 105]. Interestingly, recovery of disuse-induced atrophy is accompanied by a potent induction of post-natal myogenesis [108, 109] and inactivation of GSK-3β [109-112]. Collectively these observations suggest a role for inactivation of GSK-3β in the regulation of post-natal myogenesis.

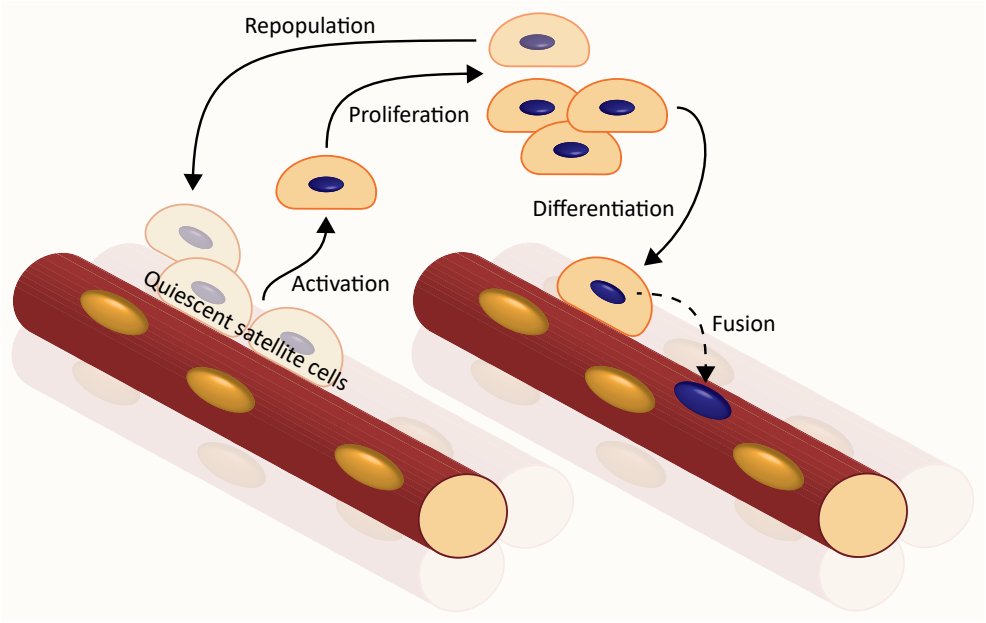


Figure 3. Satellite cell function in skeletal muscle regeneration, muscle mass maintenance and hypertrophy. In response to activation, satellite cells proliferate. Upon proliferation, a subset of satellite cells repopulate the quiescent satellite cell reservoir while others commit to post-natal myogenesis as myoblasts. Myoblasts differentiate and fuse with pre-existing myofibers donating their myonuclei (myonuclear accretion).

Protein turnover

Protein turnover is the balance between protein synthesis and protein breakdown. An anabolic state as required for hypertrophy is driven by relatively higher levels of protein synthesis over protein breakdown. *Vice versa*, the rate of protein breakdown exceeds protein synthesis in a catabolic state resulting in muscle atrophy. The IGF-I/AKT signaling network is one the most important signaling cascades that controls protein turnover (Fig. 4). In short, binding of IGF-I to the IGF receptor activates AKT. Through phosphorylation of its substrates, AKT activates mammalian target of rapamycin (mTOR) and inhibits GSK-3β and FoXO. Activation of mTOR signaling and the AKT-mediated inhibitory phosphorylation of GSK-3β initiate mRNA translation [98], stimulating skeletal muscle protein synthesis [113]. AKT also inhibits FoXO, repressing protein breakdown by reducing gene expression involved in both the UPS and ALP proteolytic pathways. In contrast, in catabolic conditions FoXO increases proteolysis (Fig. 4), due to the lack of AKT-mediated repression. Interestingly, GSK-3β has been shown to also modulate protein degradation pathways [103].

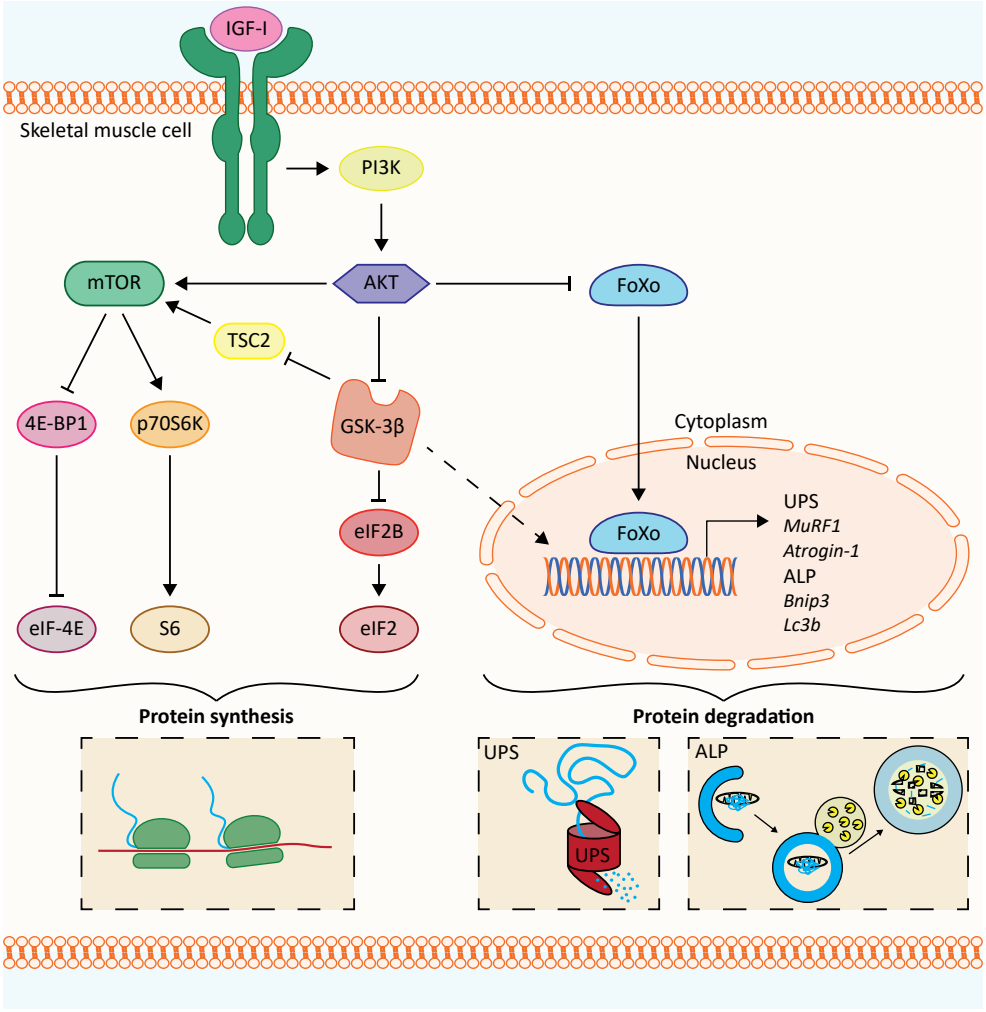


Figure 4. Simplified schematic representation of the molecular pathways involved in muscle protein turnover (protein synthesis/breakdown) controlling muscle mass. Anabolic factors such as insulin-like growth factor-I (IGF-I) activate phosphoinositide 3-kinase (PI3K). In turn, protein kinase B (AKT) is activated by PI3K. AKT stimulates protein synthesis via activation of mammalian target of rapamycin (mTOR) and inhibition of glycogen synthesis kinase-3β (GSK-3β). Upon activation, mTOR-mediated inhibition of eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) results in dissociation of eukaryotic translation initiation factor 4E (eIF-4E). In addition, mTOR activates S6 via activation of ribosomal protein S6 kinase beta-1 (p70S6K). In parallel, due to inhibition of GSK-3β, eukaryotic initiation factor 2B (eIF2B) is relieved from its inhibitory phosphorylation and activates eIF2. Furthermore, AKT inhibits forkhead box class O (FoXO)-mediated atrogene expression. In catabolic conditions AKT-mediated phosphorylation of FoxO is suppressed, resulting in nuclear translocation which controls the transcription of the E3-ligases like atrogin-1/muscle atrophy F-box (atrogin-1) and muscle-specific RING finger protein 1 (MuRF1) involved in the ubiquitin (Ub) 26S-proteasome system (UPS). Moreover, FoXO initiates gene expression of constituents of the autophagy-lysosomal pathway (ALP), including BCL2 Interacting Protein 3 (Bnip3) and microtubule-associated protein light chain 3B (Lc3b). The UPS and ALP are the major processes in control of protein breakdown. Note that, AKT stimulates protein synthesis and inhibits proteolysis indirectly via inactivation of GSK-3β. A dashed arrow indicates a not fully understood path.

A potential role for GSK-3β in mitochondrial biogenesis

Skeletal muscle homeostasis encompasses the regulation of molecular pathways controlling protein turnover, post-natal myogenesis, mitochondrial biogenesis and oxidative substrate metabolism. In skeletal muscle, it is currently unknown how the energy-demanding processes of hypertrophy or muscle recovery are fueled. Because skeletal muscle plasticity includes both the regulation of OXPHEN and muscle mass, it is not unlikely that the molecular pathways regulating muscle mass are also involved in the regulation of muscle OXPHEN. Illustrative of this, it was recently reported that downstream of AKT (the main hub in muscle mass regulation; Fig. 4), ATP citrate lyase is involved in the regulation of muscle mitochondrial metabolism in hypertrophic conditions [114]. As described above, GSK-3β (another direct downstream target of AKT) has been implicated in the regulation of myogenesis and protein turnover. More specifically, inactivation of GSK-3β increases expression of genes involved in myogenic differentiation [104, 105], indices of protein synthesis [115] and concomitantly inhibits protein degradation signaling [103] in skeletal muscle cells. Interestingly, in non-muscle cells pharmacological inactivation of GSK-3 resulted in increased levels of the master regulator of mitochondrial biogenesis, PGC-1α. In more detail, GSK-3β inhibition using lithium chloride (LiCl) potently induced mitochondrial biogenesis in endothelial and neuronal cells [89, 90], which was accompanied by increased levels of constituents of the PGC-1α signaling network. In line with these results, PGC-1α protein levels increased in vascular smooth muscle after treatment with LiCl [116]. Whether or not GSK-3β plays a role in the regulation of PGC-1α and hereby affects mitochondrial biogenesis and oxidative energy metabolism in skeletal muscle thus far is unexplored.

Aim and outline of this thesis

The overall aim presented in this thesis is to investigate the effect of GSK-3β on skeletal muscle oxidative substrate metabolism and mitochondrial biogenesis. This overall aim is divided into four specific aims, which will be investigated within this thesis.

1. To elucidate whether recovery of muscle OXPHEN and muscle mass following disuse-induced muscle atrophy requires inactivation of GSK-3.

2. To investigate whether GSK-3β inactivation regulates mitochondrial biogenesis and oxidative energy metabolism in adult skeletal muscle cells and protects against disuse-induced decrements in mitochondrial biogenesis and oxidative energy metabolism in muscle.

3. To elucidate whether inactivation of GSK-3β regulates mitochondrial biogenesis and oxidative energy metabolism in skeletal muscle cells during myogenic differentiation and regeneration after muscle unloading.

4. To reveal the molecular mechanism underlying the upregulation of PGC-1α levels mediated by inactivation of GSK-3β in skeletal muscle cells.

To investigate the above-mentioned aims several experimental models were used which are described in more detail in the next section. In the following paragraphs, the outline of this thesis is first described.

In **Chapter 2**, we hypothesize that GSK-3β inhibition is a prerequisite for activation of the signaling pathways controlling the recovery of muscle OXPHEN and muscle mass upon reloading of the skeletal musculature after a period of inactivity. We address this hypothesis using whole-body constitutively active (C.A.) GSK-3α/β knock-in (KI) mice and wild-type animals under baseline conditions, directly after 14 days of hind-limb suspension (HLS; as a disuse atrophy model) and during reloading of the hind-limbs. We measure *M. soleus* mass, fiber cross-sectional area, OXPHEN (abundance of sub-units of OXPHOS complexes and fiber type composition) as well as the expression level of their main regulators (protein synthesis/degradation, myogenesis and PGC-1α signaling).

In **Chapter 3**, we explore whether inactivation of GSK-3β increases PGC-1α levels and subsequently induces mitochondrial biogenesis and expression of sub-units

of OXPHOS complexes in skeletal muscle. To address this hypothesis, we use pharmacological and genetic approaches to inactivate GSK-3β in fully differentiated C2C12 murine myotubes (as an analogue of *in vivo* myofibers). We measure several parameters involved in mitochondrial energy metabolism (gene expression and protein abundance of sub-units of OXPHOS complexes and activity of enzymes involved in the TCA cycle and fatty acid β-oxidation), mtDNA copy number and the expression of key constituents of the PGC-1α signaling network in these C2C12 myotubes. In addition, to assess whether absence of GSK-3β protects against unloading-induced loss of muscle OXPHEN we measure key players involved in the PGC-1α signaling network and expression of OXPHOS sub-units in *M. gastrocnemius* of muscle-specific GSK-3β KO mice at baseline and directly after 14 days of HLS.

In **Chapter 4**, we investigate if inactivation of GSK-3β potentiates the PGC-1α signaling cascade and subsequent mitochondrial biogenesis and mitochondrial function during myogenic differentiation of C2C12 myoblasts into multi-nucleated myotubes and during recovery of inactivity-induced atrophied muscle. To address this, we use pharmacological and genetic approaches to inactivate GSK-3β in C2C12 cells at the beginning of the myogenic differentiation program and follow these cells during the process of myogenic differentiation. We measure several parameters involved in mitochondrial energy metabolism (gene expression and protein abundance of sub-units of OXPHOS complexes and activity of enzymes involved in the TCA cycle and fatty acid β-oxidation), mtDNA copy number, mitochondrial respiration and the expression of key constituents of the PGC-1α signaling network. Furthermore, we use muscle-specific GSK-3β KO mice and measure mRNA expression of important constituents of the PGC-1α signaling network and expression of OXPHOS sub-units in *M. gastrocnemius* in response to reloading (recovery) after 14 days of HLS.

To elucidate the effect of inactivation of GSK-3β on the molecular regulation of skeletal muscle mitochondrial biogenesis, we aim to address how GSK-3β regulates *Pgc-1α* gene expression in **Chapter 5**. In order to investigate this, we use fully differentiated C2C12 myotubes and measure activation of the proximal PGC-1α promoter following inhibition of GSK-3β. Furthermore, several transcription factors known to be involved in the transcriptional control of PGC-1α will be inactivated (genetic or pharmacological inhibition) in order to reveal possible involvement in the transcriptional regulation of PGC-1α mediated by inactivation of GSK-3β.

Finally, in **Chapter 6**, an outline of the outcomes of the study described in this thesis is given and discussed in a broader context and in light of recent literature.

Experimental models used in this thesis

Within this thesis, HLS is used as an acute skeletal muscle disuse model. Reloading of the hind-limbs after 14 days of HLS is used to study skeletal muscle recovery. As described in more detail below, HLS is a well-known trigger that negatively influences muscle OXPHEN and muscle mass while reloading reverses these unloading-induced decrements in muscle OXPHEN and muscle mass. To elucidate the role of GSK-3β in the regulation of muscle OXPHEN, we use several genetic models with altered GSK-3β content or activity, *i.e.* muscle-specific GSK-3β KO (**Chapter 3 and 4**) and whole-body C.A. GSK-3α/β KI mice (**Chapter 2**) and subject these mice to a HLS/ reloading protocol.

Hind-limb suspension in mice

Inactivity is a well-known trigger for skeletal muscle dysfunction through impaired muscle OXPHEN and loss of skeletal muscle mass. Skeletal muscle unloading by means of HLS is a well-established rodent model for *in vivo* physical inactivity, which is known to result in decreased muscle mass [109] and loss of muscle OXPHEN. The loss of muscle OXPHEN upon HLS is illustrated by decreased activity of OXPHOS sub-units [117] and reductions in *Pgc-1α* gene expression [118]. Likewise, reduced mitochondrial respiration and OXPHOS protein abundance have been reported in response to skeletal muscle unloading in mice [119]. Translated to humans, acute inactivity reduces skeletal muscle mitochondrial content [120] and decreases expression of several genes involved in OXPHOS, mitochondrial biogenesis, fatty acid β-oxidation and carbohydrate metabolism [121]. Thus, inactivity reduces skeletal muscle OXPHEN both in mice as well as in humans. During early stages of skeletal muscle unloading levels of phosphorylated GSK-3β decrease [122, 123]. This is suggestive of a potential role for GSK-3β during skeletal muscle unloading, as a reduced phosphorylation status of GSK-3β at Ser^{9} is indicative of less inactivated GSK-3β.

Skeletal muscle reloading in mice

Reloading of the hind-limbs after unloading reverses unloading-induced decrements in muscle mass and muscle OXPHEN. For example, previous reports showed that reloading restores unloading-induced loss of mitochondrial mass [124]. In addition, mitochondrial biogenesis occurs in parallel with muscle regeneration [125]. Next to OXPHEN regulation, reloading of unloaded muscle increases muscle protein synthesis, dampens proteolysis and potently induces a myogenic regenerative response [109]. Consistently, exercise has been shown to be associated with similar changes in protein turnover [126]. Muscle mass is therefore maintained by several

processes, including post-natal myogenesis and protein turnover [127]. Indicative of involvement of GSK-3β in molecular pathways controlling muscle recovery is the fact that GSK-3β is phosphorylated, and thus inactivated, during muscle reloading after a period of unloading [109-112].

In vitro myogenic differentiation

In **Chapter 4**, myogenic differentiation of skeletal muscle myoblasts into myotubes is used as an *in vitro* model of post-natal myogenesis. The effects of muscle recovery on several molecular pathways controlling energy metabolism largely overlap the effect of *in vitro* myogenic differentiation on these processes. For example, gene expression of PGC-1α and several constituents of the PGC-1α signaling network increases during *in vitro* myogenic differentiation [128] as well as during muscle regeneration [129]. Increased abundance of constituents of the PGC-1α signaling network is associated with increased protein abundance of OXPHOS proteins, mtDNA copy number and activity of enzymes involved in the TCA cycle and fatty acid β-oxidation [128]. Knock-down of PGC-1α blocked *in vitro* myotube formation, illustrating the importance for mitochondrial biogenesis in adequate myogenic differentiation [130].

Fully differentiated myotubes

Within this thesis, C2C12 myotubes (that are at the end of the myogenic differentiation program) are used as *in vitro* analogues of myofibers. Indeed, myotube cultures are a well-established *in vitro* model for muscle fibers. In this thesis, myotubes are used to study the effects of inactivation of GSK-3β on skeletal muscle mitochondrial biogenesis and oxidative substrate metabolism (**Chapter 3 and 5**).

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Chapter 2

Recovery of muscle mass and muscle oxidative phenotype following disuse does not require GSK-3 inactivation

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Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease. 2020 Feb 19; Epub ahead of print.

Abstract

Background

Physical inactivity contributes to muscle wasting and reductions in mitochondrial oxidative phenotype (OXPHEN), reducing physical performance and quality of life during aging and in chronic disease. Previously, it was shown that inactivation of glycogen synthase kinase (GSK)-3β stimulates muscle protein accretion, myogenesis, and mitochondrial biogenesis. Additionally, GSK-3β is inactivated during recovery of disuse-induced muscle atrophy.

Aim

Therefore, we hypothesize that GSK-3 inhibition is required for reloading-induced recovery of skeletal muscle mass and OXPHEN.

Methods

Wild-type (WT) and whole-body constitutively active (C.A.) $Ser^{21/9}$ GSK-3α/β knock-in mice were subjected to a 14-day hind-limb suspension/14-day reloading protocol. Soleus muscle mass, fiber cross-sectional area (CSA), OXPHEN (abundance of sub-units of oxidative phosphorylation (OXPHOS) complexes and fiber-type composition), as well as expression levels of their main regulators (respectively protein synthesis/degradation, myogenesis and peroxisome proliferator-activated receptor-γ co-activator-1α (PGC-1α) signaling) were monitored.

Results

Subtle but consistent differences suggesting suppression of protein turnover signaling and decreased expression of several OXPHOS sub-units and PGC-1α signaling constituents were observed at baseline in C.A. GSK-3 *versus* WT mice. Although soleus mass recovery during reloading occurred more rapidly in C.A. GSK-3 mice, this was not accompanied by a parallel increased CSA. The OXPHEN response to reloading was not distinct between C.A. GSK-3 and WT mice. No consistent or significant differences in reloading-induced changes in the regulatory steps of protein turnover, myogenesis or muscle OXPHEN were observed in C.A. GSK-3 compared to WT muscle.

Conclusion

This study indicates that GSK-3 inactivation is dispensable for reloading-induced recovery of muscle mass and OXPHEN.

Introduction

Skeletal muscle tissue has a remarkably high plasticity in response to changes in physical activity both in terms of the regulation of its mass as well as in its capacity for oxidative substrate metabolism. In chronic diseases [1-4] and during normal physiological aging [5, 6], physical inactivity or reduced loading of the musculature contributes significantly to muscle wasting and reductions in muscle oxidative phenotype (OXPHEN; most important determinants being the proportion of oxidative muscle fibers and the abundance of oxidative phosphorylation (OXPHOS) complexes) [7, 8]. Collectively, these changes culminate in a reduced physical performance, diminished quality of life [9] and even increased mortality [10]. Resuming physical activity or reloading the disused muscle by means of exercise convincingly showed to be a potent stimulus for improving functionality of the musculature after a period of inactivity, including muscle growth and an enhanced OXPHEN [11, 12].

Upon reloading of disuse-atrophied muscle, multiple intra-cellular signaling cascades are induced resulting in activation or suppression of regulatory molecules [11], including the protein glycogen synthase kinase-3β (GSK-3β). GSK-3β activity is regulated at multiple levels, including phosphorylation. The inhibitory Ser^{9} phosphorylation of GSK-3β can be mediated by an array of kinases including protein kinase B (Akt) [13], protein kinase A [14], protein kinase C [15], p70 S6 kinase [16], mitogen-activated protein kinase [17] and cGMP-dependent protein kinase II [18]. In addition, GSK-3β phosphorylation decreases during the early stages of skeletal muscle unloading resulting in an increased activity level of GSK-3β [19, 20]. GSK-3β is directly involved in the signaling pathways controlling myogenesis [11, 21] and protein turnover [21-23]. Interestingly, GSK-3β has recently also been implicated in the regulation of skeletal muscle mitochondrial energy metabolism by governing control over the peroxisome proliferator-activated receptor (PPAR)-γ co-activator-1α (PGC-1α) signaling network [24, 25].

Myonuclear turnover involves the loss and accretion of myofiber nuclei and relies on post-natal myogenesis as an important regulatory mechanism in the control of muscle mass and regeneration. Indeed, satellite cells are essential for muscle recovery in response to atrophy as they serve as donors of myonuclei [26, 27]. Accretion of new myonuclei into existing myofibers requires activation of quiescent satellite cells [28], characterized by increased PAX7 expression [29], and enhanced satellite cell proliferation, illustrated by increased Ki-67 protein content [30]. Upon accretion of myonuclei, muscle-specific gene expression, regulated by muscle regulatory factors (MRFs) including *MyoD* and *Myogenin* [31, 32], further drives myogenic

differentiation aiding muscle regeneration. Inactivation of GSK-3β has been shown to increase myogenesis-associated gene expression during reloading of atrophied muscle [11, 21].

Muscle protein synthesis is mainly regulated by insulin-like growth factor-I (IGF-I) which activates Akt [33-35]. Akt subsequently activates mammalian target of rapamycin (mTOR) [36, 37] and inhibits GSK-3β [13], resulting in de-repression eIF2Bε [38, 39] which facilitates initiation of mRNA translation [40, 41] and stimulates protein synthesis. mTOR-raptor complex 1 (mTORC1) hyper-phosphorylates eIF4E binding protein 1 (4E-BP1), effectively releasing the brake on translation initiation, and phosphorylates p70S6K1 to stimulate translation capacity [37, 42-44]. Conversely, inactivation of GSK-3β inhibits muscle protein degradation in septic rats and dexamethasone-treated myotubes [45]. Muscle protein breakdown involves various proteolytic systems, including the ubiquitin (Ub) 26S-proteasome system (UPS) and the autophagy-lysosomal pathway (ALP) [46-48]. The UPS selectively degrades proteins that are poly-ubiquitinated by *e.g.* muscle-specific E3 ligases atrogin-1/ muscle atrophy F-box (*Atrogin-1*) and muscle-specific RING finger protein 1 (*MuRF1*) [49]. Inhibition of GSK-3β reduces *Atrogin-1* and *MuRF1* expression in myotubes [23]. The ALP, initiated or aided by proteins such as LC3 and BNIP3 [50], clears long-lived proteins and dysfunctional organelles through autophagosome formation [51-53], but its regulation by GSK-3 in muscle has not been assessed.

The PGC-1 signaling network plays a central role in the molecular control of oxidative substrate metabolism, mitochondrial biogenesis and gene expression of structural protein of slow-twitch fibers [54, 55]. PGC-1 proteins are essential drivers of this network as they co-activate several nuclear proteins with transcriptional activity such as estrogen-related receptors (ERRs) [56], PPARs [57] and nuclear respiratory factor (NRFs) [58]. Collectively, these transcription factors drive the expression of essential components of mitochondrial metabolic pathways including, for example, sub-units of protein complexes involved in OXPHOS in the electron transport chain and exert a coordinated control over mitochondrial biogenesis via mitochondrial transcription factor A (TFAM) [58]. Different experimental models of inactivity or limb-immobilization 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 [59, 60]. *Vice versa*, reloading-induced recovery of muscle mitochondrial function is associated with activation of this pathway [61]. Interestingly, we recently showed not only that GSK-3β inactivation potently increased expression of PGC-1α in cultured myotubes [24], but also that inactivation of GSK-3β enhanced cellular oxidative capacity in muscle cells [24, 25].

Collectively, these findings implicate GSK-3 as a negative regulator of skeletal muscle mass [62] and mitochondrial function [25]. As GSK-3 activity decreases during muscle recovery following disuse-induced atrophy [11], we hypothesized that GSK-3 inhibition (through phosphorylation of Ser^{9} and Ser^{21} for GSK-3β and GSK-3α, respectively) is a prerequisite for the recovery of muscle mass and oxidative energy metabolism upon reloading of the musculature after a period of inactivity. We addressed this hypothesis using whole-body constitutively active (C.A.) GSK-3α/β knock-in mice and their wild-type (WT) counterparts under baseline (BL) conditions, directly after HLS (as a disuse atrophy model) and during reloading of the hind-limbs.

Material and Methods

Animals

The mouse study described here was approved by the Institutional Animal Care Committee of Maastricht University (DEC-2009-074). Homozygous whole-body constitutively active GSK-3α/$\beta^{21A/21A/9A/9A}$ knock-in mice (hereafter referred to as C.A. GSK-3) and wild-type (WT) male mice were used [63]. The age (average ± SD) of the mice at the start of the experiment was 15.6±0.7 weeks for WT and 15.4±0.8 weeks for C.A. GSK-3. Animals were housed in a temperature-controlled room (21-22 °C) with a 12:12h light:dark cycle and received *ad libitum* standard chow pellets and water. Animals were randomized into 7 different groups for each genotype, and were subsequently subjected to: no experimental procedures (BL), 14 days of hind limb suspension (HLS) or HLS followed by reloading (RL) for 1, 2, 3, 5, or 14 days (RL1, RL2, RL3, RL5 and RL14, respectively). Group size was n=8 except for WT baseline (n=9) unless indicated otherwise. Group size calculations were based on paired soleus muscle weight as the primary read-out parameter. The unloading/reloading model has previously been described [64]. HLS was accomplished using a tail harness which was applied under isoflurane anesthesia. The harness consisted of a plastic-coated iron wire taped around the mouse's tail connected to a swivel hook allowing circular motility. The latter was attached to a Teflon-coated PVC ring, able to slide over an iron rod spanning the length of the cage allowing longitudinal motility. Using this suspension device, the mice were raised to prevent the hind-limbs from touching the cage floor or sides, while front limb weight bearing was sustained. In this way, four HLS mice were socially housed in adapted cages. For reloading, the mice were anesthetized and released from the tail harness after 14 days of HLS, allowing to resume normal cage activity. During the experimental period, food intake, water consumption, and general appearance of the mice were

monitored daily. After euthanasia by intra-peritoneal administration of an overdose sodium pentobarbital at the indicated time-points, hind-limb lower leg muscles were dissected using standardized dissection methods, cleaned of excess fat and tendon/connective tissue, and pair-weighed on an analytical balance. Dissected muscles were snap-frozen in liquid nitrogen and stored at −80 °C for RNA and protein extraction or embedded in OCT (Optimal Cutting Temperature compound; Tissue-Tek®. Sakura, Finetek, Zoeterwoude, the Netherlands) for histological analyses.

Western Blot

M. soleus was grinded into powder and homogenized in 400 µl IP lysis buffer (50 mM Tris, 150 mM NaCl, 10% glycerol, 0.5% Nonidet P40, 1 mM EDTA, 1 mM Na_3VO_4, 5 mM NaF, 10 mM β-glycerophosphate, 1 mM $Na_4O_7P_2$, 1 mM dithiothreitol, 10 µg/ml leupeptin, 1% aprotenin, 1 mM PMSF, pH 7.4) with a Polytron PT 1600 E (Kinematica). After homogenization, samples were incubated for 15 min on a rotating wheel at 4 °C and spun for 30 min at maximum speed (20,817 × g) in a centrifuge cooled to 4 °C. Supernatant was aliquoted, snap-frozen, and stored at −80 °C until analysis. A portion of the supernatant was saved to determine total protein content with the Pierce BCA protein assay kit (Thermo Fisher Scientific). The other supernatants were diluted in Laemmli sample buffer to 1× as final concentration (0.25M Tris-HCl pH 6.8; 8% (w/v) SDS; 40% (v/v) glycerol; 0.4M DTT and 0.04% (w/v) Bromophenol Blue) and boiled for 5 min at 95 °C before storing at −80 °C. The samples were loaded, with 10 µg protein per lane, on a Criterion XT Precast 4–12% Bis-Tris gel (BioRad, Hercules, CA, USA). Proteins were separated by electrophoresis with 100-140 V until full separation as indicated by the protein ladder. The proteins on the gel were transferred to a 0.45 µm Whatman Protran Nitrocellulose Transfer membrane (Whatman, Maidstone, UK) by electroblotting (BioRad Criterion Blotter) at 100 V for 1 h. The blots were blocked for 1-2 h at room temperature (RT) in 5% (w/v) NFDM (non-fat dried milk) (ELK, Campina, the Netherlands) diluted in Tris-buffered saline with 0.05-0.1% Tween 20 (TBST). Nitrocellulose blots were washed in TBST, followed by overnight incubation at 4 °C with specific antibodies directed against: p-mTOR (Ser^{2448}) (#2971), mTOR (#2983), p-Akt (Ser^{473}) (#9271), Akt (#9272), p-GSK-3α (Ser^{21}) (#9316), GSK-3α (#4337), p-GSK-3β (Ser^{9}) (#9336), GSK-3β (#9332), p-p70S6K (Thr^{389}) (#9206), p70S6K (#2708), 4E-BP1 (#9452) and GAPDH (#2118), all from Cell Signaling Technology, Inc. (Danvers, MA), total OXPHOS (Mitosciences, Eugene, OR, USA), PGC-1α (516557, Calbiochem), NRF-1 (ab55744, Abcam, Cambridge, UK) and TFAM (DR1071, Millipore) diluted in TBST with 5% BSA/NFDM. The blots were washed with TBST and probed with a peroxidase-conjugated secondary antibody (Vector Laboratories, #PI-1000) diluted in TBST with 3-5% NFDM for 1 h at RT. According to the manufacturers' instructions,

the blots were visualized by chemiluminescence using Supersignal® WestPico Chemiluminescent Substrate (Pierce Biotechnology, Inc.). The blots were exposed to film (Biomax light film, KODAK) and subsequent quantification was done using the Quantity One analysis software (Bio-rad). For analyses, gels were loaded with both WT and C.A. GSK-3 samples, and samples of the same group were distributed over multiple gels to allow comparisons to HLS after factor correction [65].

RNA isolation, cDNA synthesis and RT-qPCR

M. soleus (approximately 5-10 mg) was ground and lysed in RLT buffer containing 1% (v/v) β-mercaptoethanol. According to manufacturer's guidelines, RNA was isolated with the RNeasy fibrous tissue Mini Kit (Qiagen, Venlo, the Netherlands) including DNase treatment. RNA was dissolved in 50 µl RNase-free water and stored at −80 °C. RNA concentrations were measured using a Nanodrop® ND-1000 UV-Vis spectrophotometer. RNA was diluted >5× in ddH_2O and 400 ng of RNA was reverse-transcribed to cDNA using the Transcriptor first strand cDNA synthesis kit (Roche Diagnostics GmbH, Mannheim, Germany) with anchored oligo-dT primers according to the manufacturers' conditions for generating cDNA fragments of 4 kb in a final reaction volume of 20 µl. cDNA was used for quantification of transcript levels of interest by real-time quantitative PCR (RT-qPCR) analysis. RT-qPCR reactions (16 µl final volume) contained SensiMix SYBR Hi-ROX Kit (Quantace-Bioline, London, UK) with 300 nM primers and were run in a 384-well MicroAmp Optical 384-Well Reaction Plate (Applied Biosystems, Nieuwerkerk a/d IJssel, the Netherlands) on a 7900HT Fast Real-Time PCR System (Applied Biosystems). Relative cDNA starting quantities for the samples were derived using standard curves generated from serial dilutions of pooled cDNA samples. Starting quantities were obtained by extrapolating Ct values on the standard curve. The expression of the genes of interest was normalized with a correction factor derived by GeNorm [66], based on the expression levels of GAPDH, RPL13A, ARBP, Calnexin, and β2M as reference genes.

Immunohistochemistry

Frozen OCT-embedded M. soleus sections were cut (5 µm), air-dried, treated with 0.5% Triton X-100 in phosphate-buffered saline (PBS), incubated with primary anti-myosin heavy chain (MyHC)-I (Developmental Studies Hybridoma Bank (DSHB), University of Iowa, Ames, IA), anti-MyHC-IIa (DSHB), and anti-laminin (Sigma, Zwijndrecht, the Netherlands) followed by secondary antibodies labeled with Alexa Fluor 555, Alexa Fluor 488, and Alexa Fluor 350 (Invitrogen, Breda, the Netherlands). Unstained fibers were considered type IIX/B fibers. After staining, all images were digitally captured using fluorescence microscopy (Nikon Instruments Europe). Image processing and

quantitative analyses were done using the Lucia 4.81 software package (Laboratory Imaging, Prague, Czech Republic). The mean muscle fiber cross-sectional area (CSA) of each muscle was calculated by analysis of on average >200 fibers per muscle. Additionally, frozen *M. soleus* sections were cut (5 µm), air-dried, treated with 0.5% Triton X-100 in PBS, incubated with primary anti-laminin and Ki-67 (Biocare medical, Duiven, the Netherlands) followed by secondary antibodies labeled with Alexa Fluor 488 and Alexa Fluor 350 and propidium iodide (PI) for nuclear staining. After staining, all images were digitally captured using fluorescence microscopy and quantified using the Lucia 4.81 software package. Double stained (PI and Ki-67) nuclei were counted as proliferating nuclei and divided by the number of fibers per muscle section analyzed to determine the proliferation level for that specific muscle.

Statistical analysis

Data is expressed as MEAN ± SEM and depicted as fold change compared to baseline of WT animals in bar charts and fold changes compared to unloading of both genotypes for line graphs. Statistical data analyses were performed to allow comparisons between C.A. GSK-3 and WT mice at specific time-points, and within genotypes between a specific time-point vs. HLS. Data was analyzed using IBM SPSS Statistics (Statistical Package for the Social Sciences, Version 25.0 for Windows; SPSS; Chicago, IL). The distribution was tested and in general, the data was not normally distributed. Therefore, data were analyzed with a non-parametric Kruskal-Wallis test and where necessary Mann-Whitney-U tests to compare groups (both over time between the genotypes and within the genotype over the time-points), $p<0.05$ or smaller as indicated in each figure. Graphs were made using GraphPad Prism 5.0 software (GraphPad, Prism, La Jolla, CA, USA) and representative pictures of the blots were added using the Adobe illustrator CS6 software (Adobe Systems Incorporated, San Jose, CA, USA).

Results

Recovery of muscle mass does not require GSK-3 inactivation

Baseline characterization of C.A. GSK-3 and WT mice showed similar *M. soleus* (Fig. 1A) weight and no difference in fiber cross-sectional area distribution (Fig. 1B, C), while bodyweight (Fig. S1A) was slightly higher in C.A. GSK-3 mice. Unloading decreased the bodyweight of animals from both genotypes, (Fig. S1A). During reloading, compared to the unloaded condition, bodyweight only increased significantly in C.A. GSK-3 animals resulting in a higher bodyweight of C.A. GSK-3 vs WT animals during the course of the reloading protocol (Fig. S1B). Food intake per gram of bodyweight was slightly higher in C.A. GSK-3 animals compared to WT during early reloading, but was similar during the last phase of the reloading protocol (Fig. S1C). The absence of the regulatory Ser21 and Ser9 residues of GSK-3α/β in the C.A. GSK-3 mice were confirmed in skeletal muscle (Fig. S2A-C). In WT and C.A. GSK-3 mice, soleus muscle mass decreased significantly in response to unloading (Fig. 1A), which was accompanied by a similar left-ward shift of the fiber size distribution although average fiber CSA was only significantly decreased in C.A. GSK-3 mice (Fig. 1D, E). At the end of the reloading protocol (RL14), significant increases in average CSA compared to HLS were absent in either genotype (Fig. 1F). However, reloading resulted in a rightward shift in myofiber CSA distribution, indicating recovery of muscle fiber size at the end of the reloading protocol (Fig. 1G). In addition, the inhibitory phosphorylation status of GSK-3β at Ser9 increased during early reloading (RL2) in WT animals (Fig. S2B, D). Remarkably, reloading-induced muscle mass recovery was potentiated in C.A. GSK-3 mice compared to WT animals for M. soleus (Fig. 1H), indicating that inactivation-resistant GSK-3 differently affects muscle mass and fiber size recovery.

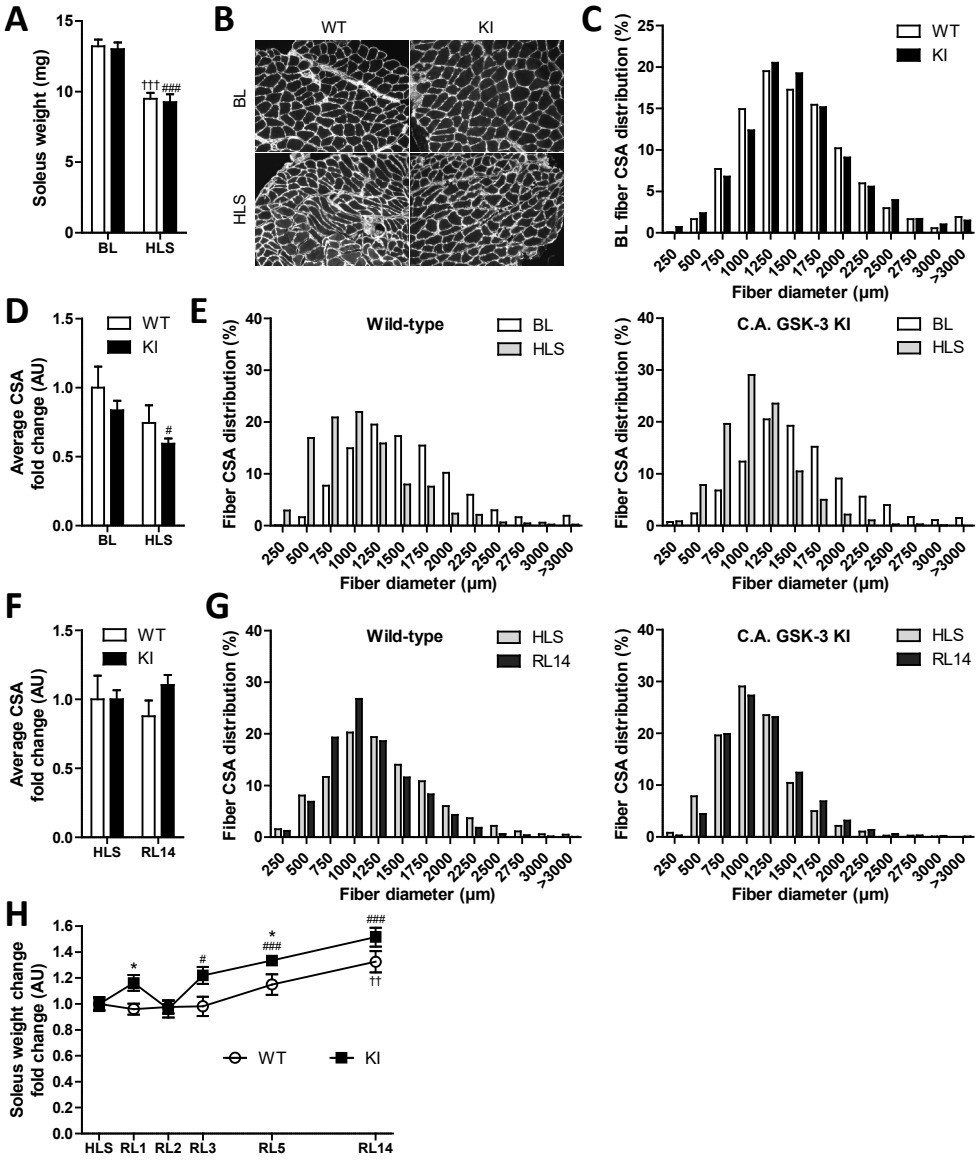


Figure 1. Recovery of muscle mass does not require GSK-3 inactivation. (A) Paired *M. soleus* weight of wild-type (WT) and C.A. GSK-3 (KI) mice at baseline (BL) and following hind-limb suspension (HLS). (B) Cross-sectional area (CSA) at BL and after HLS, and (C) CSA distribution at BL. (D) averaged fiber CSA of *M. soleus* at BL and after HLS. (E) *M. soleus* CSA distribution at BL and after HLS for both WT (left) and KI (right). (F) Averaged fiber CSA of *M. soleus* after HLS and 14 days of RL. (G) CSA distribution of *M. soleus* after HLS and 14 days of reloading for both WT (left) and KI (right). (H) *M. soleus* mass during reloading. MEAN ± SEM from n=8-9 (muscle weights) or n=3-4 (CSA) are presented. * indicates WT vs KI at a specific time-point, † indicates time effect compared to BL (bar charts) or HLS (line graphs) for WT and # indicates time effect compared to BL (bar charts) or HLS (line graphs) for KI; 1 symbol equals $p \leq 0.05$, 2 symbols equal $p \leq 0.01$, and 3 symbols equal $p \leq 0.001$.

Rapid induction of cell proliferation and myogenesis during muscle reloading does not require GSK-3 inactivation

Next, we investigated whether molecular processes controlling muscle mass were differentially regulated between WT and C.A. GSK-3 in *M. soleus* upon reloading. To characterize the reloading-response in terms of satellite cells activation, cell proliferation and myogenic differentiation, we assessed the expression of multiple genes known to be involved in these processes. Transcript levels of genes involved in satellite cell activation (*Pax7*), cell proliferation (*Cyclin D1*) and myogenic differentiation (*MyoD*, *Myogenin* and *Myf-5*) as well as Ki-67 positive nuclei were not significantly different at baseline between both genotypes (Fig. 2A, B, D, F, H and Fig. S3A, E). The only exception, *Pcna* gene expression was lower in C.A. GSK-3 animals compared to WT at baseline (Fig. S3C). Overall, markers of cell proliferation were largely comparable after unloading (Fig. 2B and Fig. S3C), whereas an induction of *Pax7* mRNA expression, as a marker for satellite cell activation, was apparent, although only significantly in C.A. GSK-3 animals (Fig. 2D). Additionally, *MyoD* and *Myf-5* (but not *Myogenin*) mRNA levels, as markers for myogenesis, were significantly induced by unloading in WT animals, a response that was absent in C.A. GSK-3 animals (Fig. 2F, H and Fig. S3E). Early reloading (RL1-3) was accompanied by an increase in the number of Ki-67 positive fibers (Fig. 2A, C), and induction of mRNA expression of genes involved in cell proliferation (*Cyclin D1* and *Pcna*) and myogenesis (*MyoD* and *Myogenin*, but not *Myf-5*). Reloading-induced increases in these markers were largely similar between genotypes with the exception of *Cyclin D1*, which was more potently induced during late reloading (RL5-RL14) in the WT animals (Fig. 2G, I and Fig. S3B, D, F). Collectively, these data indicate that inactivation of GSK-3 is dispensable for the rapid induction of cell proliferation and myogenesis during muscle reloading.

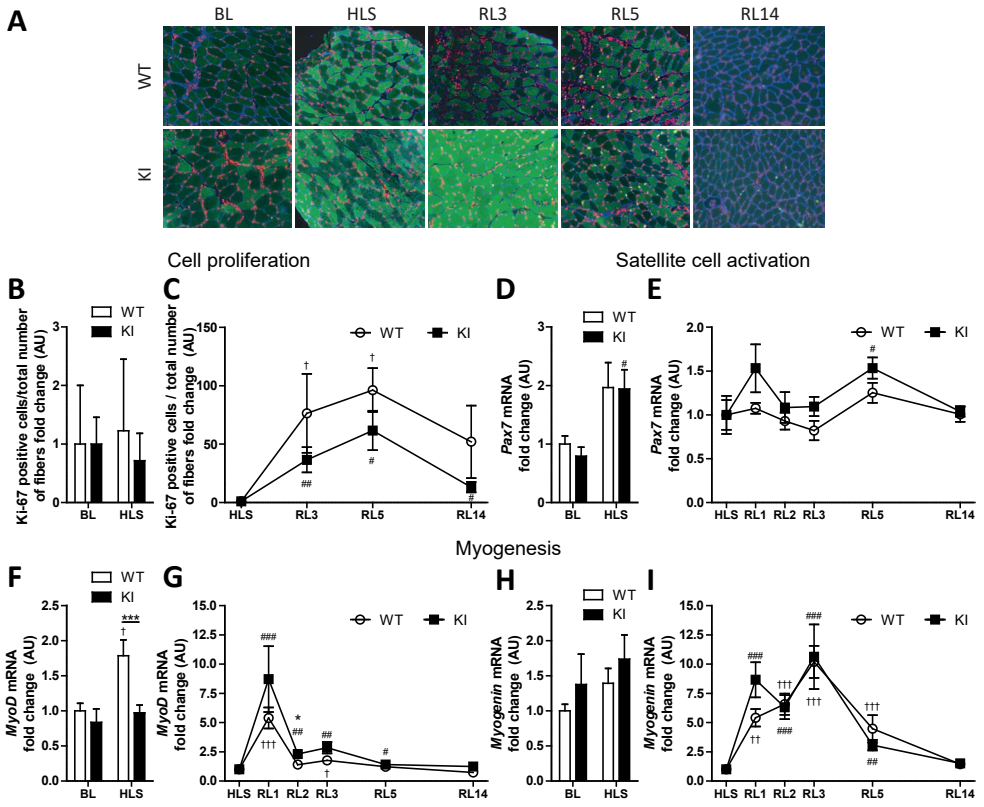


Figure 2. Rapid induction of cell proliferation and myogenesis upon muscle reloading is not dependent on GSK-3 inactivation. (A) Ki-67 positive cells in *M. soleus* at baseline (BL), after hind-limb suspension (HLS) and after 3, 5 or 14 days of reloading (RL) and (B, C) quantified as number of positive nuclei over total number of fibers in wild-type (WT) and C.A. GSK-3 knock-in (KI) mice. mRNA transcript levels of (D, E) *Pax7*, (F, G) *MyoD* and (H, I) *Myogenin* in *M. soleus*. MEAN ± SEM from n=3-8 (Ki-67 staining) or n=6-9 (mRNA) are presented. * indicates WT vs KI at a specific time-point, † indicates time effect compared to BL (bar charts) or HLS (line graphs) for WT and # indicates time effect compared to BL (bar charts) or HLS (line graphs) for KI; 1 symbol equals $p \leq 0.05$, 2 symbols equal $p \leq 0.01$, and 3 symbols equal $p \leq 0.001$.

Activation of protein synthesis signaling during muscle reloading does not require GSK-3 inactivation

Protein synthesis signaling (IGF-I/Akt/mTOR pathway) was characterized by assessment of phosphorylated over total protein abundance of well-known regulators of protein synthesis (Akt, mTOR, S6K1 and 4E-BP1) (Fig. 3A) as well as evaluation of *Redd1* mRNA expression. In general, no differences were observed at baseline between genotypes, or following unloading, with the exceptions of 4E-BP1 phosphorylation, which decreased in response to HLS in both genotypes (Fig. 3B, D, F, J), and mRNA expression of *Redd1*, which was lower in C.A. GSK-3 compared to WT at baseline and potently increased after unloading in both genotypes (Fig. 3H). In line, phosphorylation levels of proteins involved in protein synthesis signaling (Akt, mTOR, S6K1 and 4E-BP1) were increased during reloading in both WT and C.A. GSK-3 animals (Fig. 3C, E, G, K), which was more pronounced during early reloading. During the second and third day of reloading, the phosphorylation status of S6K1 was slightly higher in WT compared to C.A. GSK-3 animals (Fig. 3G). During reloading, *Redd1* gene expression rapidly decreased in both genotypes (Fig. 3I). In conclusion, inactivation of GSK-3 is not required for increased protein synthesis signaling (IGF-I/Akt/mTOR pathway) during muscle reloading.

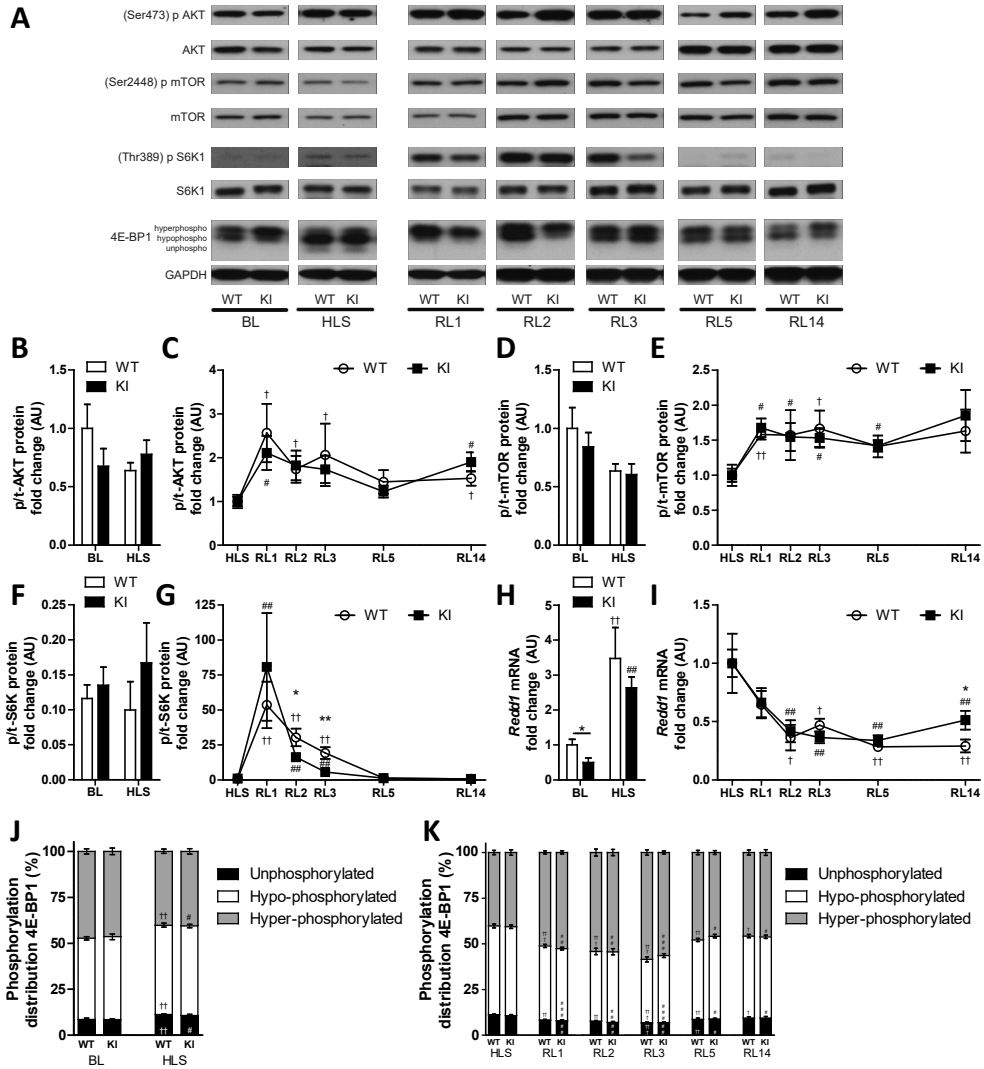


Figure 3. Activation of protein synthesis signaling during muscle reloading does not require GSK-3 inactivation. (A) Representative pictures of the Western blots of indicated phosphorylated and total proteins. Phosphorylation status of (B, C) AKT, (D, E) mTOR and (F, G) p70S6K, mRNA abundance of (H, I) *Redd1* and phosphorylation distribution of (J, K) 4E-BP1 in *M. soleus* of wild-type (WT) and C.A. GSK-3 knock-in (KI) mice. MEAN ± SEM from n=4-9 (protein) or n=7-9 (mRNA) are presented. * indicates WT vs KI at a specific time-point, † indicates time effect compared to BL (bar charts) or HLS (line graphs) for WT and # indicates time effect compared to BL (bar charts) or HLS (line graphs) for KI; 1 symbol equals $p \leq 0.05$, 2 symbols equal $p \leq 0.01$, and 3 symbols equal $p \leq 0.001$.

GSK-3 inactivation is not required for suppression of protein breakdown signaling during muscle reloading

Protein breakdown signaling was evaluated by assessment of gene expression of key constituents of the ubiquitin proteasome system (UPS) and autophagy-lysosomal pathway (ALP). At baseline, transcript levels of genes involved in the UPS (*Atrogin-1* and *MuRF1*) and the ALP (*Bnip3* and *Lc3b*) were all lower in C.A. GSK-3 animals compared to WT (Fig. 4A, C, E, G). Following unloading, mRNA expression of the E3-ligases *Atrogin-1* and *MuRF1* were increased in both genotypes (Fig. 4A, C), while transcript abundance of *Bnip3* remained unaltered (Fig. 4E) and *Lc3b* gene expression significantly increased in C.A. GSK-3 only (Fig. 4G). In response to reloading, gene expression of *Atrogin-1*, *MuRF1* and *Bnip3* decreased (Fig. 4B, D, F) which was largely unaffected by C.A. GSK-3. Despite the overall largely similar pattern, gene expression of *MuRF1* was significantly lower on reloading day 1 and 3 in WT compared to C.A. GSK-3 animals (Fig. 4D). In contrast, compared to the unloaded condition, *Lc3b* mRNA expression did not change during the entire course of reloading in both genotypes (Fig. 4H). In conclusion, C.A. GSK-3 does not affect reloading-associated decrease in protein breakdown signaling.

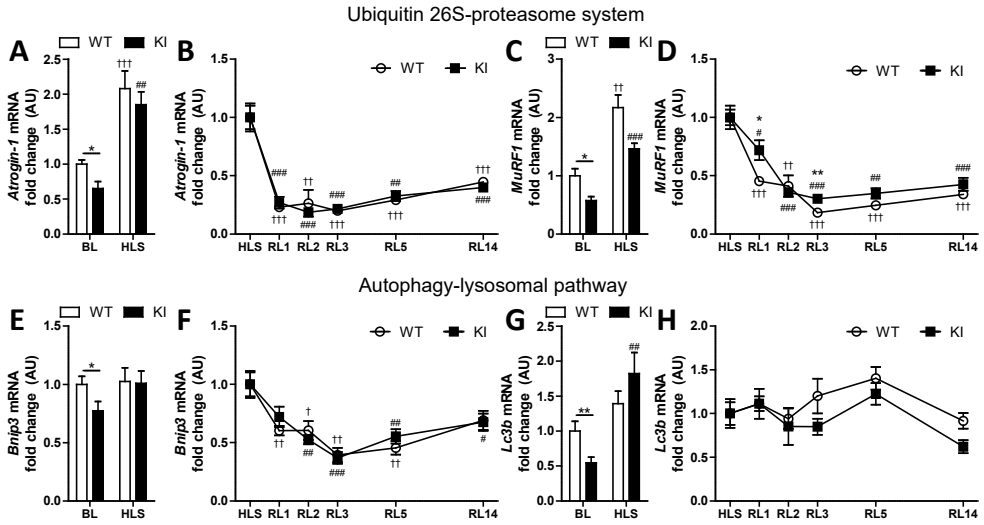


Figure 4. GSK-3 inactivation is not required for suppression of proteolysis effectors during muscle reloading. Relative mRNA abundance of (A, B) *Atrogin-1*, (C, D) *MuRF1*, (E, F) *Bnip3* and (G, H) *Lc3b* in *M. soleus* of wild-type (WT) and C.A. GSK-3 knock-in (KI) mice. MEAN ± SEM from n=7-9 are presented. * indicates WT vs KI at a specific time-point, † indicates time effect compared to BL (bar charts) or HLS (line graphs) for WT and # indicates time effect compared to BL (bar charts) or HLS (line graphs) for KI; 1 symbol equals $p \leq 0.05$, 2 symbols equal $p \leq 0.01$, and 3 symbols equal $p \leq 0.001$.

Modest unloading/reloading-associated changes in OXPHOS sub-units expression are not dependent on GSK-3 inactivation

Considering that GSK-3β has also been implicated in the regulation of oxidative substrate metabolism [24, 25], we next investigated whether indices of muscle OXPHEN (*i.e.* fiber-type composition and abundance of constituents of the mitochondrial electron transport chain) were differentially regulated in response to unloading/reloading in both genotypes. First, baseline measurements revealed that transcript levels of nuclear encoded *Sdhb* and *Cyc1* (sub-units of OXPHOS complex II and III, respectively), but not *Ndufb3* (sub-units of OXPHOS complex I, were lower in C.A. GSK-3 compared to WT *M. soleus* (Fig. 5A, C, E). Gene expression of mitochondrial-encoded *CoxII* (sub-unit of complex IV) showed a trend towards lower expression in C.A. GSK-3 animals compared to WT at baseline (Fig. 5G). Protein levels of sub-units of OXPHOS complexes however were similar between genotypes at baseline in *M. soleus* (Fig. 5I, N, L, P). As only exception, complex I protein abundance was lower in C.A. GSK-3 animals compared to WT at baseline (Fig. 5J). The fiber-type composition of *M. soleus* was similar between genotypes at baseline and was not significantly altered by unloading or reloading (Fig. S4A-D). Upon unloading, transcript levels of sub-units of OXPHOS complex I, II and IV and protein abundance of sub-units of complex II and III decreased significantly in WT and C.A. GSK-3 mice (Fig. 5A, C, G, I, L, N). In general, mRNA expression of OXPHOS sub-units increased again during reloading and the initial reloading-induced increase of *Sdhb* and *Cyc1* mRNA levels was more pronounced in C.A. GSK-3 compared to WT animals (RL1) (Fig. 5D, F). OXPHOS protein levels (Fig. 5I, K, M, O, Q) were largely unaffected by reloading in both genotypes. However, protein abundance of OXPHOS complex I was lower on day 2 of the reloading program in WT compared to C.A. GSK-3 animals (Fig. 5K). In summary, although C.A. GSK-3 animals had lower OXPHOS mRNA levels at baseline and showed enhanced reloading-induced increases in these transcripts, C.A. GSK-3 did not alter OXPHEN during reloading.

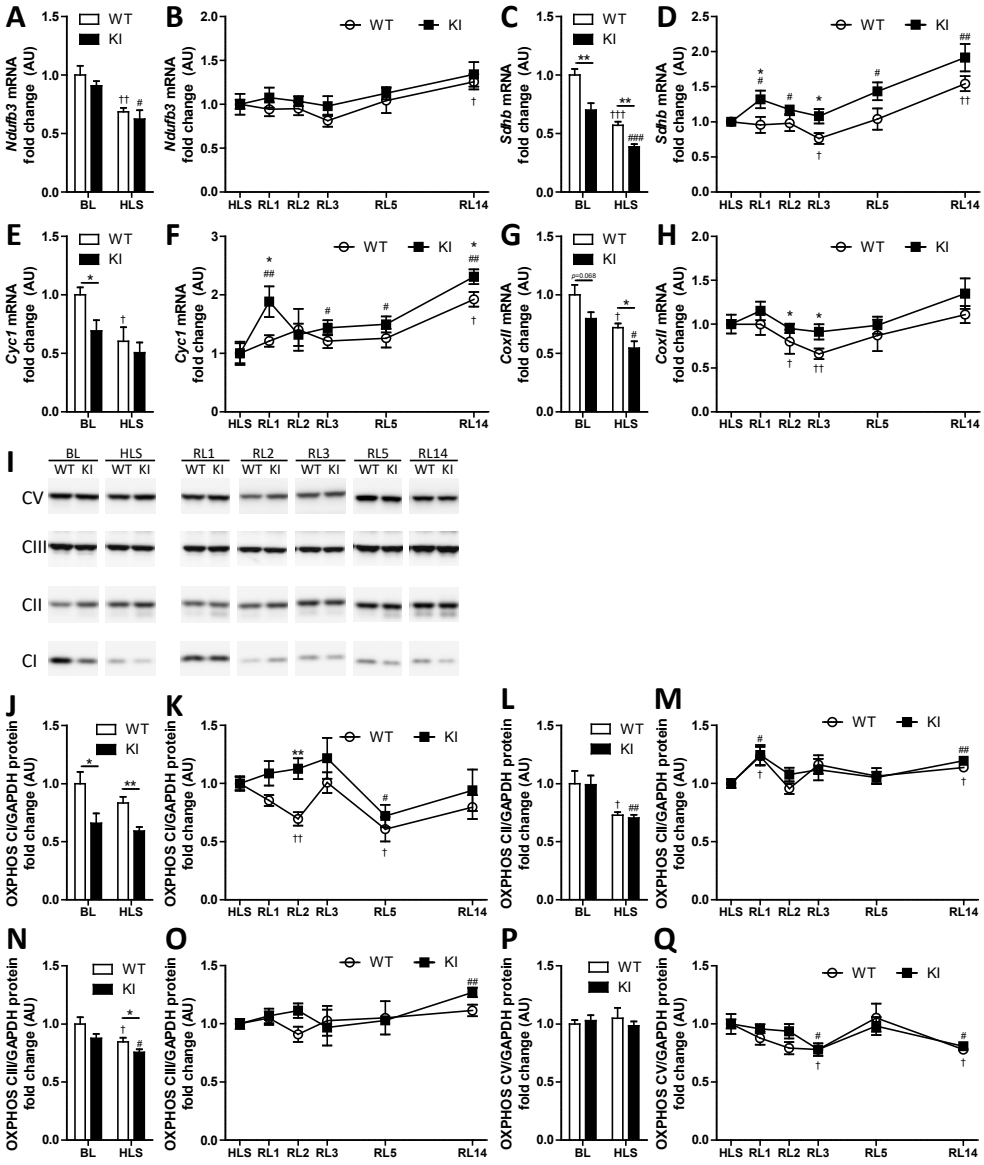


Figure 5. Modest unloading/reloading-associated changes in OXPHOS sub-units expression are not dependent on GSK-3 inactivation. Transcript abundance of (A, B) *Ndufb3*, (C, D) *Sdhb*, (E, F) *Cyc1* and (G, H) *CoxII* (sub-units of oxidative phosphorylation (OXPHOS) complex I, II, III (nuclear-encoded) and IV (mitochondrial-encoded), respectively) in *M. soleus* of wild-type (WT) and C.A. GSK-3 knock-in (KI) mice. (I) Representative pictures of the Western blots of OXPHOS proteins. OXPHOS proteins of sub-units from (J, K) Complex I, (L, M) Complex II, (N, O) Complex III and (P, Q) Complex V MEAN ± SEM from n=3-9 (protein) or n=7-9 (mRNA) are presented. * indicates WT vs KI at a specific time-point, † indicates time effect compared to BL (bar charts) or HLS (line graphs) for WT and # indicates time effect compared to BL (bar charts) or HLS (line graphs) for KI; 1 symbol equals $p \leq 0.05$, 2 symbols equal $p \leq 0.01$, and 3 symbols equal $p \leq 0.001$.

GSK-3 inactivation is dispensable for reloading-associated changes in PGC-1 levels

Given the changes in transcript levels of sub-units of the electron transport chain in response to unloading/reloading, we next investigated constituents of the PGC-1 signaling network, which are well-known to be involved in the transcriptional control of these OXPHOS sub-units. Baseline assessment revealed that the transcript level of *Pgc-1α*, but not *Pgc-1β*, was lower in C.A. GSK-3 compared to WT *M. soleus* (Fig. 6A, C). mRNA transcript levels of *Pgc-1α* and *Pgc-1β* decreased in response to HLS in both genotypes, except for *Pgc-1α* expression in C.A. GSK-3 animals (Fig. 6A, C). *Pgc-1α* gene expression remained largely unaffected during the entire course of the reloading protocol (Fig. 6B), while *Pgc-1β* mRNA expression, increased in a phasic manner during reloading, without any gross distinctions between both genotypes (Fig. 6D).

Increased PGC-1α signaling network constituents during reloading do not require GSK-3 inactivation

At baseline, with the exception of *Perm1* and *Nrf-1*, mRNA expression levels of all genes involved in the PGC-1 signaling network that we investigated were lower in C.A. GSK-3 animals compared to WT (Fig. 6E, G, I, K, M, O, Q). In response to unloading, expression levels of all genes investigated, except *Nrf-1*, *Nrf-2α* and *Pparδ*, decreased in WT animals, a response that was generally absent in C.A. GSK-3 animals (Fig. 6E, G, I, K, M, O, Q). In response to reloading, mRNA expression levels of all PGC-1 signaling network-associated genes were upregulated, with the exception of *Pparδ* (Fig. 6F, H, J, L, N, P, R). Reloading-induced increases in these markers were largely similar between genotypes with the exception of *Nrf-1*, *Nrf-2α* and *Tfam*, which were more potently induced during early reloading (RL1) in the C.A. GSK-3 animals. In conclusion, lower baseline mRNA levels of constituents of the PGC-1 signaling pathway were observed in C.A. GSK-3 animals. However, increases in constituents of the PGC-1α signaling during reloading do not require GSK-3 inactivation.

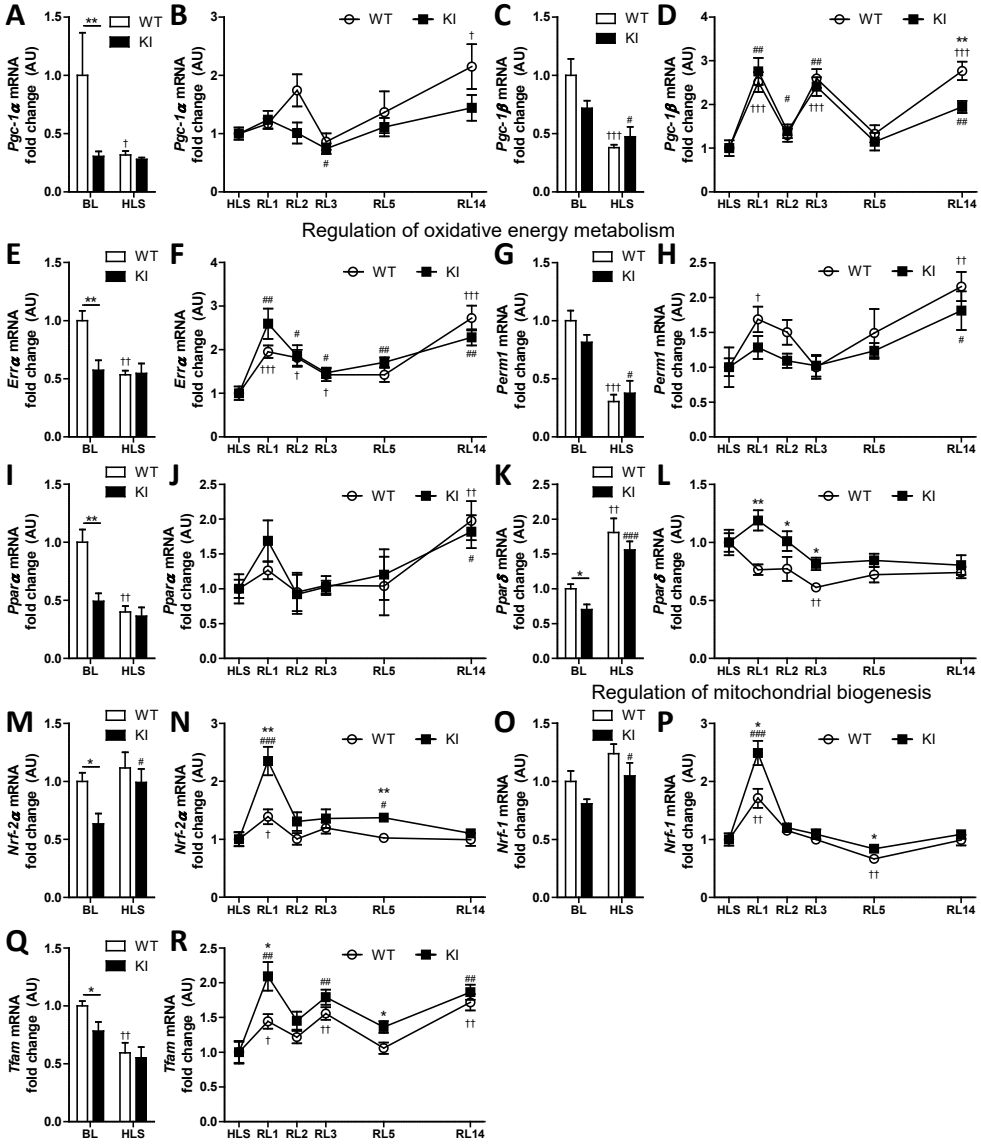


Figure 6. Activation of the PGC-1α signaling network during reloading does not require inactivation of GSK-3. mRNA transcript abundance of (A, B) *Pgc-1α*, (C, D) *Pgc-1β*, (E, F) *Errα*, (G, H) *Perm1*, (I, J) *Pparα*, (K, L) *Pparδ*, (M, N) *Nrf-2α*, (O, P) *Nrf-1* and (Q, R) *Tfam* in *M. soleus* of wild-type (WT) and C.A. GSK-3 knock-in (KI) mice. MEAN ± SEM from n=7-9 (mRNA) are presented. * indicates WT vs KI at a specific time-point, † indicates time effect compared to BL (bar charts) or HLS (line graphs) for WT and # indicates time effect compared to BL (bar charts) or HLS (line graphs) for KI; 1 symbol equals $p \leq 0.05$, 2 symbols equal $p \leq 0.01$, and 3 symbols equal $p \leq 0.001$.

Discussion

In this study, we investigated whether recovery of muscle mass and muscle OXPHEN as well as reloading-induced activation of regulatory signaling pathways are dependent on inactivation of GSK-3. WT and C.A. GSK-3 mice were subjected to a reversible disuse-induced muscle atrophy. Despite subtle but consistent differences at baseline suggesting suppression of protein turnover signaling, PGC-1α signaling and mRNA expression of several OXPHOS sub-units resulting from constitutive activation of GSK-3, no consistent or significant alterations in reloading-induced changes in muscle mass, protein turnover, post-natal myogenesis or in the regulation of muscle OXPHEN were observed.

Strikingly, baseline bodyweights of C.A. GSK-3 mice were elevated compared to their WT counterparts, which was also previously reported by Chen *et al.* [67]. In contrast, others have reported similar bodyweights of C.A. GSK-3 versus WT animals [68]. The difference in bodyweight observed in our study is not accompanied by higher muscle weights or body size and is therefore suggestive of differences in other organs or tissues in C.A. GSK-3 mice. As a possible explanation for this, C.A. GSK-3 might disrupt insulin production in β-cells [69]. As a consequence, excess glucose would likely be stored in (pre-)adipocytes, which are possibly more receptive for lipid loading in the presence of C.A. GSK-3 [70]. This would result in expansion of adipose tissue compartments contributing to a higher bodyweight in C.A. GSK-3 mice. Although we did not assess adipose tissue depots in our study, this notion is in line with a report from Brüning *et al.* showing an increased adipose compartment in muscle-specific insulin receptor KO mice [71], in which by lack of insulin signaling GSK-3 is also likely constitutively activated. Considering that IGF-I signaling controls protein synthesis [37, 42-44], downstream abrogation of the IGF-AKT signaling cascade, by introduction of inactivation-resistant GSK-3, is expected to affect protein synthesis signaling. Apart from inhibitory phosphorylation of eIF2B, GSK-3 activity can increase tuberous sclerosis complex 2 (TSC2) activity thereby inhibiting mTOR [72] and subsequent phosphorylation of the mTOR substrates p70S6K1 and 4E-BP1 [37, 42-44]. However, in our study, baseline protein synthesis signaling (IGF-I/Akt/mTOR pathway) appeared similar in C.A. GSK-3 compared to WT muscle, which is in line with the unaltered muscle weights. Conversely, lower baseline expression of genes involved in protein degradation signaling (*Atrogin-1*, *MuRF1*, *Bnip3* and *Lc3b* gene expression) are present in C.A. GSK-3 mice compared to WT animals. This is in contrast to studies showing reduced protein degradation signaling following inhibition of GSK-3 activity [23, 45]. GSK-3β can directly activate forkhead box class O (FoXO) transcription factors [73] which is well-known to increase atrogene

expression [74]. Akt phosphorylation levels and *Atrogin-1* mRNA expression normally correlate inversely because of Akt-mediated FoXO phosphorylation and subsequent suppression of *Atrogin-1* transcription [75]. However, in our study, baseline Akt phosphorylation was similar in both genotypes. This supports the involvement of alternate signaling inputs responsible for lower basal expression of genes involved in proteolysis signaling that we observed, the nature of which remain to be determined.

Besides AKT [13], several other kinases can phosphorylate GSK-3β at Ser^{9} inhibiting its activity such as protein kinase A [14], protein kinase C [15], p70 S6 kinase [16], cGMP dependent kinase [18] and mitogen-activated protein kinase [17]. The activity of these proteins is under the control of several signaling molecules including insulin, IGF-I and nitric oxide (NO) [76]. Interestingly, these signaling molecules are all responsive to altered muscle loading [11, 77-80]. More specifically, for example, NO has been shown to be reduced after muscle unloading [79] and increases in NO production during reloading can contribute to muscle regrowth [78].

Reductions in muscle mass in response to unloading were observed as expected in WT mice [11, 81], and not altered by C.A. GSK-3. Although unloading-induced loss of muscle mass was associated with significantly lower average CSA, in C.A. GSK-3 animals only, a clear leftward shift in CSA distribution in both genotypes indicates that the extent of muscle and fiber atrophy in response to unloading was not affected by constitutive GSK-3 activity. Previously, unloading-induced muscle atrophy has been associated with decreased protein synthesis signaling, *e.g.* reduced phosphorylation of Akt and S6K in rats [82, 83]. In the current study and in line with previous work from our group, protein synthesis signaling however was largely unaltered in response to unloading [11]. This may imply that a new homeostasis in muscle mass is reached, as muscle mass does not further reduce from 14 to 28 days of muscle unloading [84, 85], and that reduced anabolic signaling is likely most pronounced during the phase of active muscle loss prior to the 14-day HLS time-point. In line with this notion, muscle protein synthesis was shown to be reduced after 7 days of unloading, began to recover after 14 days and was similar to control after 21 days of muscle unloading [85]. In addition, p-AKT levels were shown to be decreased after 10 days of muscle unloading in soleus of rats, which was not associated with reductions in p-P70S6K and p-S6K levels [86]. In our study, only 4E-BP1 phosphorylation was reduced in response to unloading, which is in line with previously published data in mice [87]. In addition, we observed an unloading-induced increase in gene expression of *Redd1*, a negative regulator of mTORC1 activity and thus an inhibitor of muscle protein synthesis [37, 42], which has been reported earlier [11]. Collectively, these two observations suggest unloading-induced inhibition of muscle protein synthesis.

These unloading-induced changes were not affected by constitutive GSK-3 activity.

On the other side of the protein turnover balance, in accordance to literature [11, 88, 89], protein breakdown signaling in our study increased significantly in response to unloading which was evidenced by increased expression levels of key constituents of the UPS and ALP proteolytic pathways. Collectively these observations reflect reduced net muscle protein accretion underlying unloading-induced loss of muscle mass, which is not affected by constitutive activation of GSK-3. In addition to muscle protein turnover, myonuclear turnover is also a key determinant of muscle mass [90]. In this regard, available data on MRFs, key regulators of myogenesis, is contradicting. For example, in our study gene expression of *MyoD* and *Myf-5*, but not *Myogenin*, increased in WT animals in response to unloading. This is in line with increased levels of *MyoD* mRNA observed in rats subjected to 14 days of unloading and may serve as a compensatory mechanism to limit unloading-induced loss of muscle mass [91]. In contrast to our findings, the MYF-5 protein level decreased in response to 4-, 6- or 8 days of hind-limb casting in rats [89]. Whether or not unloading alters the abundance and activity of MRFs remains to be elucidated. As gene expression of *MyoD* increased upon unloading in *M. plantaris* but not *M. soleus* in rats [92], the characteristics of the investigated muscle may underlie these abovementioned discrepancies. In our study, subtle differences in gene expression of cell proliferation and myogenesis markers (*Cyclin D1*, *Myf-5* and *MyoD*) were observed in response to unloading in C.A. GSK-3 KI animals compared to WT, but these did not culminate in differences in unloading-induced loss of muscle mass between the two genotypes. Taken together, unloading in our study resulted in alterations in molecular signaling pathways controlling muscle mass which were largely consistent with literature. In addition, muscle mass and regulatory processes appeared overall similar in C.A. GSK-3 and WT animals during unloading, allowing assessment and comparison of the reloading response in both genotypes.

Previous reports convincingly demonstrated that GSK-3β is a negative regulator of muscle mass [62]. The presence of C.A. GSK-3 would therefore potentially limit muscle mass regain during reloading. In line with literature, phosphorylation of GSK-3β, indicative of GSK-3β inactivation, increased in response to reloading in WT animals [11, 21, 83, 93]. In addition, regain of muscle mass during the course of reloading in WT mice validated the reloading phase of our model, but was not attenuated and even increased in C.A. GSK-3 mice. In our study, regain of muscle mass was not paralleled by an increased average fiber CSA at the end of reloading period, however, a slight but clear rightward shift in CSA distribution suggested fiber size growth in both C.A. GSK-3 and WT mice. Consistent with these findings, Zhang

et al. showed that recovery of myofiber CSA in atrophied muscle takes longer than muscle mass recovery [94]. The discrepancy between changes in muscle mass and fiber CSA in unloading/reloading studies has been attributed to interstitial fluid accumulation during muscle recovery [95-97]. Indeed, muscle swelling due to post-exercise inflammation [98, 99] has previously been reported. As inflammatory clear factor-kappa light chain enhancer of activated B cells (NF-κB) signaling involves GSK-3 [100], improperly regulated inflammation and subsequent edema may underpin the uncoupled kinetics by which muscle mass and muscle CSA increase in reloading of C.A. GSK-3 muscle. Previously our group reported that muscle-specific GSK-3β KO accelerated muscle mass recovery after unloading [11]. We now report that phosphorylation of $Ser^{9/21}$-mediated GSK-3 inactivation is not required for reloading-induced muscle mass recovery. These combined findings indicate that although GSK-3 activity suppresses muscle recovery, these suppressive actions of GSK-3 are not predominantly regulated by $Ser^{9/21}$ phosphorylation. Besides the inhibitory $Ser^{9/21}$ phosphorylation, GSK-3 activity can potentially be modulated by phosphorylation of other residues (*e.g.* phosphorylation of Thr^{356} [101], Thr^{43} [102], Ser^{389} and Thr^{390} [17]) or other post-translational regulation mechanisms of GSK-3 including the binding of adapter proteins [103]. Thus, the physical presence of the GSK-3 protein regardless of its $Ser^{9/21}$ phosphorylation status could still initiate alternative signaling bypassing the effects of C.A. GSK-3 KI ($Ser^{9/21}$)-mediated signaling. Finally, although muscle mass and fiber CSA are major determinants of muscle strength, potential involvement of GSK-3 in the recovery of muscle strength independent of muscle mass cannot be ruled out. Indeed, 'high-responders' for increased muscle strength are not necessarily 'high-responders' for increased muscle mass in response to resistance training [104], indicating that the regulation of muscle mass and muscle strength could be uncoupled.

With regard to reloading, in our study, expression levels of markers for cell proliferation and myogenesis increased significantly. This is in line with the known role of these processes in reloading-induced muscle mass regeneration [11, 21, 105-109]. The reloading-induced increase in *Cyclin D1* was less potent in C.A. GSK-3 animals. In line, GSK-3β suppresses *Cyclin D1* expression in cancer cells [110]. However, as C.A. GSK-3 did not similarly affect three other markers of (satellite) cell proliferation, this suggests that GSK-3 inactivation is not required for the regulation of (satellite) cell proliferation. Previous *in vitro* studies by our group and others showed that stimulation with IGF-I or pharmacological inactivation of GSK-3 is sufficient to promote transcription of genes involved in myogenic differentiation [22, 111-113]. However, although GSK-3 was inactivated in response to reloading in WT animals in our study, the pattern of up-regulation of markers of cell proliferation and myogenesis during

early reloading appeared similar between the two genotypes. Therefore, alternative (GSK-3β independent) activation of mRNA expression of genes involved in post-natal myogenesis upon reloading is likely, for example via NF-κB- or myostatin inhibition [114, 115]. Combined, we conclude that that GSK-3 inactivation is not required for the regulation of (satellite) cell proliferation and post-natal myogenesis during muscle mass recovery from disuse-induced atrophy.

Furthermore, protein synthesis signaling was activated in response to reloading [21], and mRNA expression of *Redd1* quickly decreased upon initiation of reloading [11], which collectively is consistent with activation of muscle protein synthesis during reloading [83]. Despite increased food intake in C.A. GSK-3 animals, a potent trigger for protein synthesis [116], indices of muscle protein synthesis were not enhanced in C.A. GSK-3 animals during the course of reloading. This suggests that the extent to which protein synthesis signaling increases during reloading is mainly determined by resumed contractile activity, and does not rely on GSK-3 inactivation. Consistent with increased protein synthesis signaling, and in line with literature, muscle protein breakdown signaling (both UPS and ALP) decreased during reloading [88, 117], without distinct alterations in presence of C.A. GSK-3. Overall, our data show that reloading of atrophied muscle is accompanied by a marked down-regulation of proteolysis signaling in parallel to increased protein synthesis signaling. This is accompanied by a transient proliferative and myogenic response in conjunction with a rightward fiber size shift. During the early stages of reloading, temporally coinciding with the reloading-induced inactivation of GSK-3β, a slightly higher phosphorylation status of S6K1 and lower mRNA expression of *MuRF1* was detected in GSK-3 KI animals compared to WT animals. This is in line with the known effects of inactivation of GSK-3β on protein turnover [11] and in line with our initial hypothesis. However, despite these small changes in individual markers, our data collectively shows that recovery of muscle mass (and the molecular mechanisms controlling this) during reloading of the musculature do not require GSK-3 inactivation.

Physical inactivity-induced loss of muscle mass is often associated with a reduced muscle OXPHEN [118], whereas exercise enhances the OXPHEN of the skeletal musculature [119, 120]. Baseline mRNA expression of OXPHOS sub-units was lower in C.A. GSK-3 compared to WT mice. In line with these observations, we previously observed that GSK-3β knockdown increased mRNA expression and protein abundance of sub-units of OXPHOS complexes in cultured myotubes [25]. In line with lower baseline OXPHOS sub-unit gene expression in C.A. GSK-3 versus WT mice, transcript levels of several constituents of the PGC-1α signaling network were consistently lower in C.A. GSK-3 mice compared to WT animals. This is consistent with previously

published data of increased gene expression of *Pgc-1α* and constituents of the PGC-1α signaling network upon GSK-3β knockdown in myotubes [25].

In addition to changes in the signaling pathways controlling muscle mass and consistent with previous findings of our group, transcript levels and protein abundance of OXPHOS sub-units decreased in response to unloading in *M. soleus* [25]. Furthermore, gene expression of *Pgc-1α* decreases in *M. soleus* in response to unloading in rats, which is also similar to our own observations [121]. Whereas we previously reported that GSK-3β KO protects against unloading-induced decreases in gene expression of OXPHOS sub-units [25], the presence of C.A. GSK-3 in our current study did not impose an overall potentiation of unloading-induced decreases in gene expression and protein abundance of OXPHOS sub-units. However, gene expression of *Sdhb* and *CoxII* and protein abundance of OXPHOS complex I and III was lower in C.A. GSK-3 animals compared to WT in response to unloading. In contrast, mRNA expression of several players involved in the PGC-1 signaling decreased in response to unloading in WT animals, a response that was largely absent in C.A. GSK-3 mice. This could potentially be explained by the lower baseline expression of these constituents.

During muscle reloading, we observed a significant recovery of the mRNA expression levels of nuclear-encoded OXPHOS sub-units and key constituents of the PGC-1α signaling, which is in line with literature [24, 122]. GSK-3β KO potentiated reloading-induced gene expression of OXPHOS sub-units and GSK-3β knockdown enhanced myogenic differentiation-associated expression of OXPHOS sub-units in one of our previous studies [24]. In contrast to this data, in the present study, C.A. GSK-3 increased the gene expression of *Sdhb* and *Cyc1* at the first day of the reloading protocol, when GSK-3β was inactivated, compared to WT animals. However, in general the presence of C.A. GSK-3 failed to alter the response of OXPHOS gene expression or protein abundance during reloading in our current study. This indicates that inactivation of GSK-3β is not essential for increased gene expression of OXPHOS sub-units during muscle reloading. In contrast to our hypothesis, more pronounced reloading-induced increases in gene expression of constituents of the PGC-1α signaling were observed in C.A. GSK-3 animals compared to WT during early reloading, although gene expression of *Pgc-1α* itself was largely unaffected during reloading. However, considering the total recovery period, reloading-induced expression of constituents of the PGC-1α signaling were largely unaffected by C.A. GSK-3. Therefore, in combination with the existing literature our data indicates that inactivation of GSK-3β rather than C.A. GSK-3 modulates gene expression of OXPHOS sub-units and constituents of the PGC-1α signaling during muscle reloading.

Combined, we conclude that GSK-3β inactivation through $Ser^{9/21}$ phosphorylation is dispensable for recovery of muscle mass and muscle OXPHEN, and does not affect the underlying regulatory processes during reloading-induced recovery from disuse-induced muscle atrophy.

Acknowledgements

We would like to kindly thank Dr. Niki Ubags (MUMC+, the Netherlands), for her contribution during the animal experimental work. We thank Dr. Sakamoto for providing us with breeding pairs of the C.A. GSK-3 knock-in mice [63]. This work was supported by a grant from the Lung foundation / Netherlands Asthma Foundation (NAF 3.2.07.017), the Transnational University Limburg (tUL) and ZonMw (114024056).

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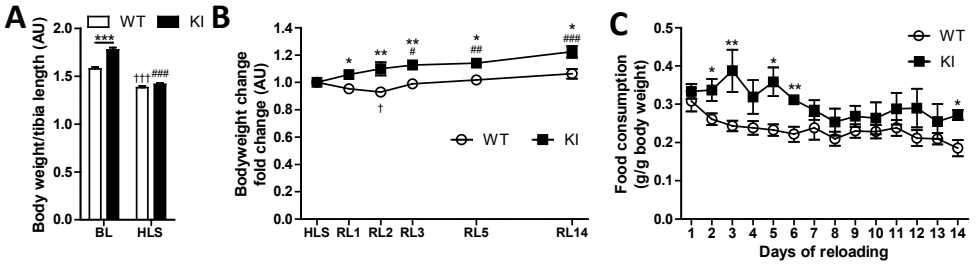
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Supplemental information

Supplemental results

Bodyweight and food intake of wild-type and C.A. GSK-3 mice

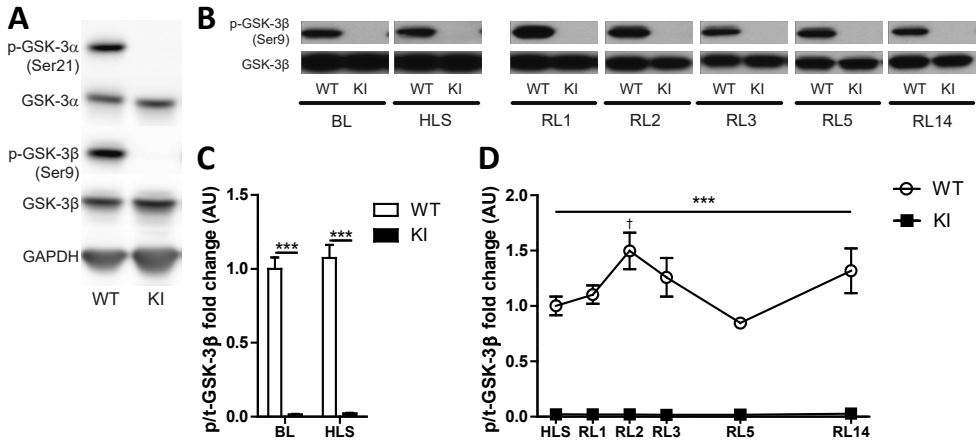
Assessment of bodyweight at baseline revealed that C.A. GSK-3 animals had a significantly higher bodyweight compared to WT mice. Correction of baseline bodyweight for tibia bone length indicated that this was not explained by an increase in body size (Fig. S1A). Unloading decreased the bodyweight of animals from both genotypes, (Fig. S1A). During reloading, compared to the unloaded condition, bodyweight only increased significantly in C.A. GSK-3 animals resulting in a higher bodyweight of C.A. GSK-3 vs WT animals during the course of the reloading protocol (Fig. S1B). During early reloading, food intake per gram of bodyweight was slightly higher in C.A. GSK-3 animals compared to WT (Fig. S1C).



Supplemental figure 1. Bodyweight and food intake of wild-type and GSK-3 knock-in mice. (A, B) Bodyweight of wild-type (WT) and C.A. GSK-3 knock-in (KI) mice at baseline (BL), following hind-limb suspension (HLS) and during reloading (RL), and (C) food intake during RL. MEAN ± SEM from n=47-57 (bodyweight and food intake). * indicates WT vs KI at a specific time-point, † indicates time effect compared to BL (bar charts) or HLS (line graphs) for WT and # indicates time effect compared to BL (bar charts) or HLS (line graphs) for KI; 1 symbol equals $p \leq 0.05$, 2 symbols equal $p \leq 0.01$, and 3 symbols equal $p \leq 0.001$.

Reloading is associated with inactivation of GSK-3β in *M. soleus*

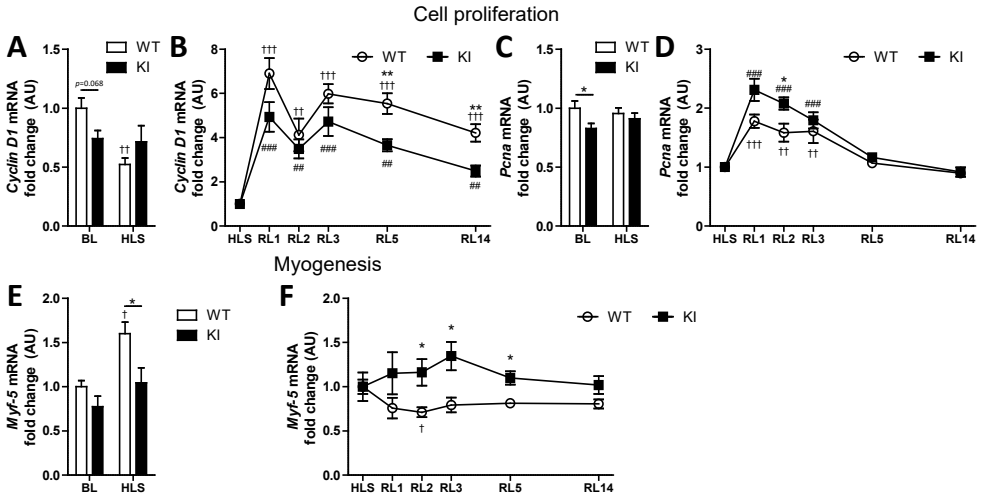
Phosphorylation of GSK-3α (Ser^{21}) and GSK-3β (Ser^{9}) was absent in C.A. GSK-3 animals, confirming the genotype (Fig. S2A). In WT animals, HLS did not alter the phosphorylation status of GSK-3β, while reloading increased the inhibitory phosphorylation of GSK-3β in *M. soleus* (Fig. S2B-D). In C.A. GSK-3 *M. soleus*, no GSK-3β phosphorylation was detected (Fig. S2B-D), validating the genotype.



Supplemental figure 2. GSK-3β is phosphorylated during reloading in M. soleus. (A) Representative pictures of the Western blots of phosphorylated and total GSK-3β and GSK-3α. (B) Representative pictures of the Western blots of Phosphorylated (Ser^{9}) and total GSK-3β. (C, D) Phosphorylation status of GSK-3β after hind-limb suspension and during reloading in *M. soleus* of wild-type (WT) and C.A. GSK-3 knock-in (KI) mice. MEAN ± SEM from GSK-3β (n=8-9) are presented. * indicates WT vs KI at a specific time-point, † indicates time effect compared to BL (bar charts) or HLS (line graphs) for WT; 1 symbol equals $p \leq 0.05$ and 3 symbols equal $p \leq 0.001$.

Expression of genes involved in cell proliferation, but not *Myf-5*, increase in response to reloading

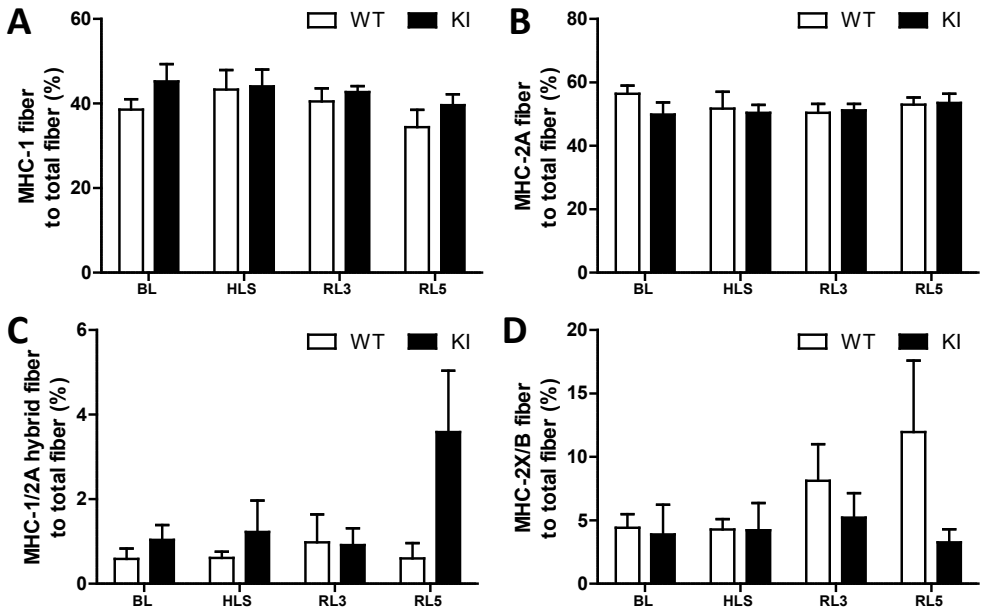
At baseline, *Cyclin D1* mRNA expression showed a trend to be lower (p = 0.068) in C.A. GSK-3 compared to WT soleus muscle, while *Pcna* mRNA expression was significantly lower (Fig. S3A, C). The expression of *Myf-5* mRNA was similar in WT and C.A. GSK-3 muscle at baseline. In response to unloading, in WT muscle only, *Cyclin D1* mRNA expression decreased while *Myf-5* increased (Fig. S3A, E). In contrast, *Pcna* mRNA levels were unaltered in both genotypes upon HLS (Fig. S3C). Transcript levels of genes involved in cell proliferation (*Cyclin D1* and *Pcna*) increased in response to reloading in both genotypes. The increase in *Cyclin D1* mRNA expression was more pronounced in WT animals (RL5), while the *Pcna* mRNA level was slightly more increased in C.A. GSK-3 animals during reloading (RL2) (Fig. S3D). *Myf-5* mRNA expression was largely unaffected by reloading (Fig. S3F).



Supplemental figure 3. Genes involved in cell proliferation, but not *Myf-5*, increase in response to reloading. mRNA transcript abundance of (A, B) *Cyclin D1*, (C, D) *Pcna* and (E, F) *Myf-5* in *M. soleus* of wild-type (WT) and C.A. GSK-3 knock-in (KI) mice at baseline (BL), after hind-limb suspension or during reloading (RL). MEAN ± SEM from n=6-9 are presented. * indicates WT vs KI at a specific time-point, † indicates time effect compared to BL (bar charts) or HLS (line graphs) for WT and # indicates time effect compared to BL (bar charts) or HLS (line graphs) for KI; 1 symbol equals $p \leq 0.05$, 2 symbols equal $p \leq 0.01$, and 3 symbols equal $p \leq 0.001$.

C.A. GSK-3 does not alter *M. soleus* fiber-type distribution

M. soleus myosin heavy chain (MHC) fiber-type distribution was not different at baseline, after HLS or during reloading between genotypes. In addition, the MHC fiber-type distribution of both genotypes did not respond to unloading or reloading (Fig. S4A-D).



Supplemental figure 4. C.A. GSK-3 does not alter skeletal muscle fiber-type distribution after unloading and during reloading. The distribution of (A) MHC-1, (B) MHC-2A, (C) MHC-1/2A and (D) MHC-2X/B fiber-types in *M. soleus* muscle at baseline (BL), after hind-limb suspension (HLS) or after 3 and 5 days of reloading (RL) of wild-type (WT) and C.A. GSK-3 knock-in (KI) mice. MEAN ± SEM from n=2-7 (MHC staining) are presented.

Chapter 3

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

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Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease. 2017 Sep 22; 1863(12):3075-3086.

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.

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, 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].

Materials and methods

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.

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.

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.

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. 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* 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.

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.

Table1. 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, *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, *Perm1*: PGC-1 and ERR-induced regulator in muscle 1, *β2M*: β-2 microglobulin, *Rpl13a*: ribosomal protein L13a, *CycloA*: cyclophilin A and *Hprt*: hypoxanthine-guanine phosphoribosyltransferase.

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 x in TE buffer and *CoxII* and *CycloA* levels were determined as described in Section 2.5.

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 x 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).

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.

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).

Results

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 (*Pdk4*) mRNA expression also increased after GSK-3β knock-down (Fig. S2).

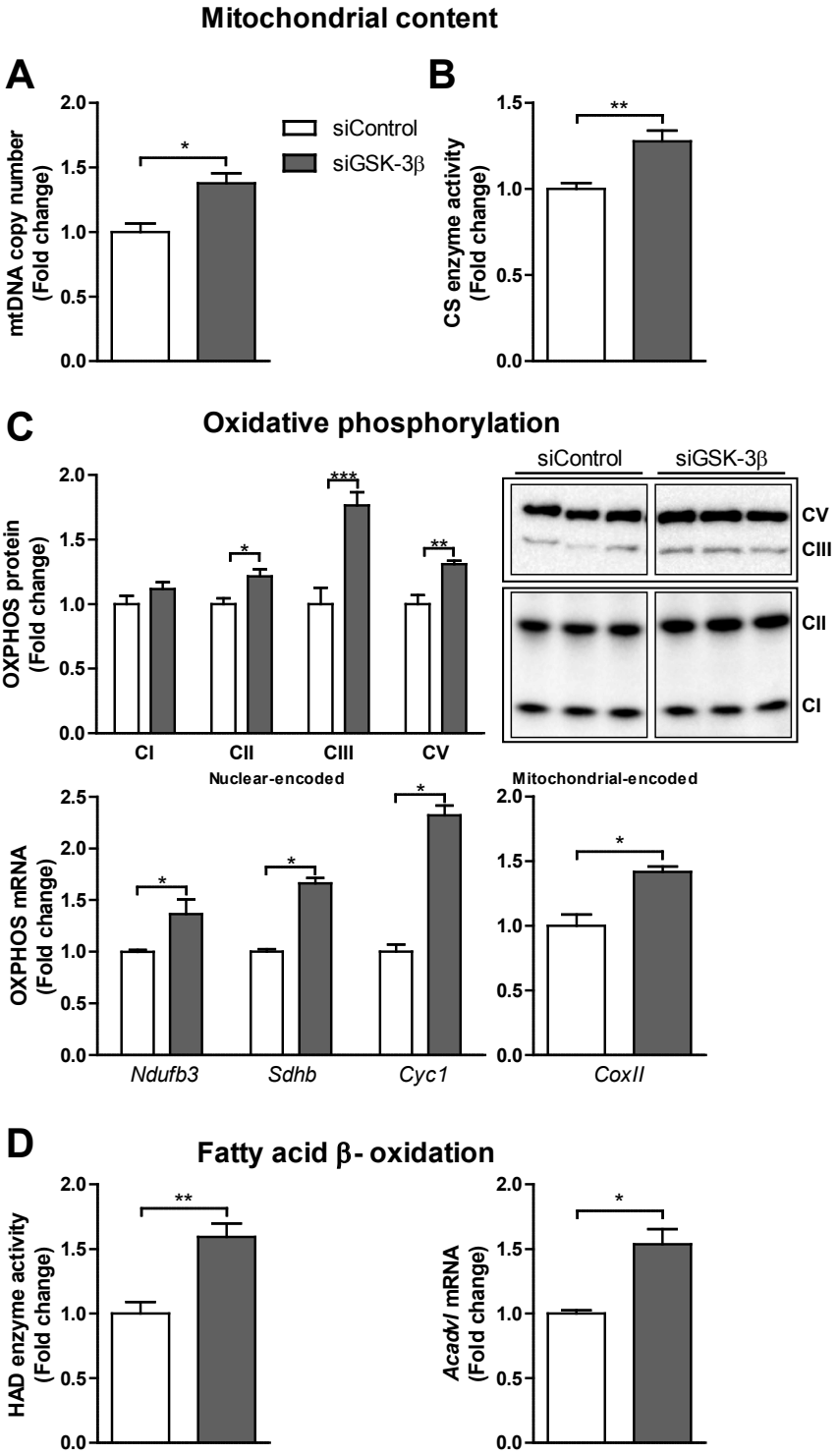

Mitochondrial content
A
B
siControl
siGSK-3β
mtDNA copy number (Fold change)
CS enzyme activity (Fold change)
C
Oxidative phosphorylation
siControl
siGSK-3β
CV
CIII
CII
CI
OXPHOS protein (Fold change)
CI
CII
CIII
CV
Nuclear-encoded
Mitochondrial-encoded
OXPHOS mRNA (Fold change)
Ndufb3
Sdhb
Cyc1
CoxII
D
Fatty acid β- oxidation
HAD enzyme activity (Fold change)
Acadvl mRNA (Fold change)

Figure 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), * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ 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.

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).

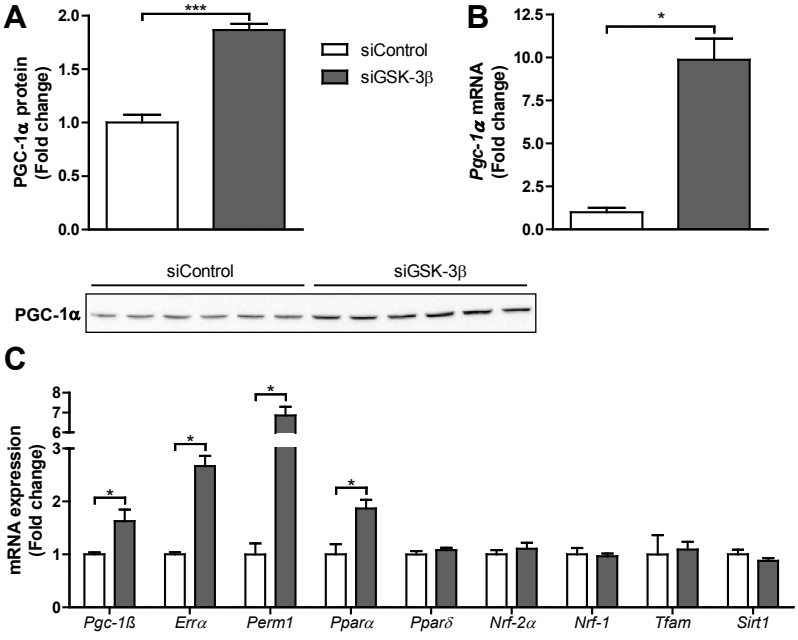


Figure 2. GSK-3β knock-down increases transcript levels of key components of the PGC-1 signaling network. C2C12 myotubes were differentiated for 5 days and transfected with siRNA constructs targeting GSK-3β (grey) or a low GC scrambled control (white). Myotubes were harvested 48 h post-transfection. (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), * $p \leq 0.05$, *** $p \leq 0.001$ vs. siControl. 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.

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-1β* or PGC-1 co-activated transcription factors (*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 energy metabolism.

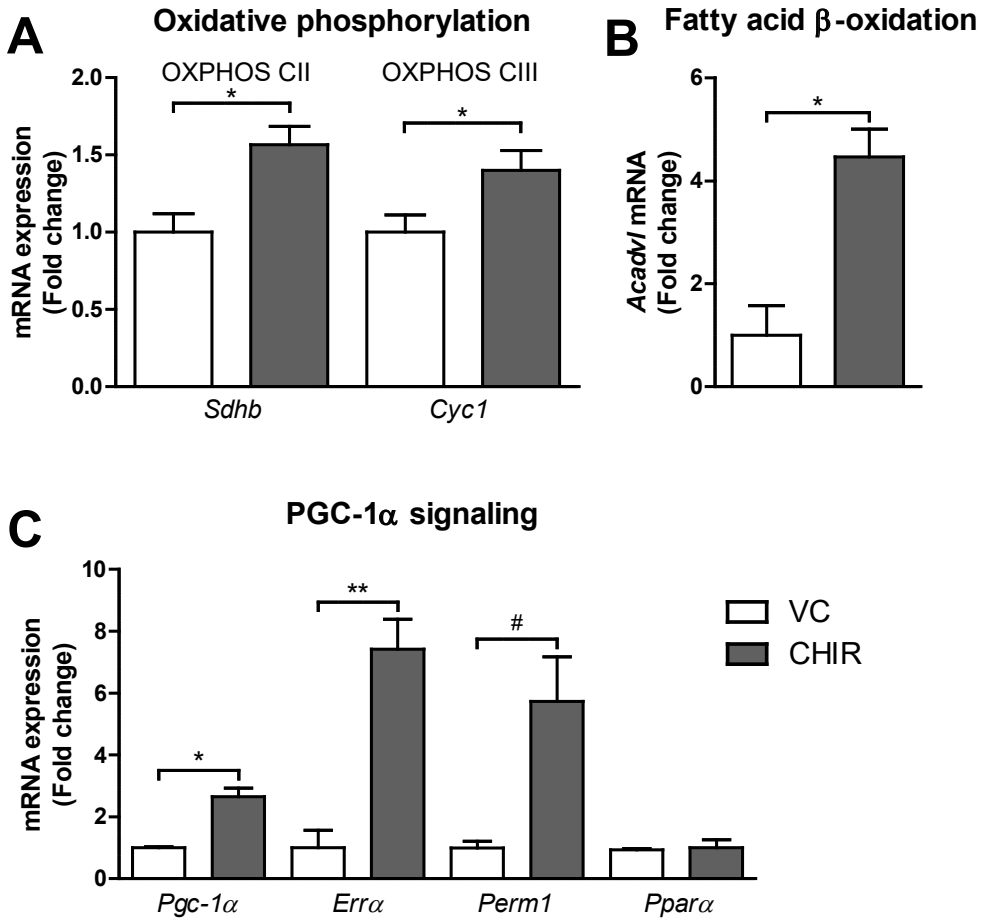


Figure 3. Pharmacological inactivation of GSK-3 increases oxidative metabolic gene expression in myotubes. C2C12 myotubes were differentiated for 5 days and treated with CHIR99021 (grey) (final concentration: 7 μM) or DMSO as vehicle (white) and transfected with a low GC scrambled control siRNA. Myotubes 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), # $p \leq 0.1$, * $p \leq 0.05$, ** $p \leq 0.01$ 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, *Pparα*: 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.

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).

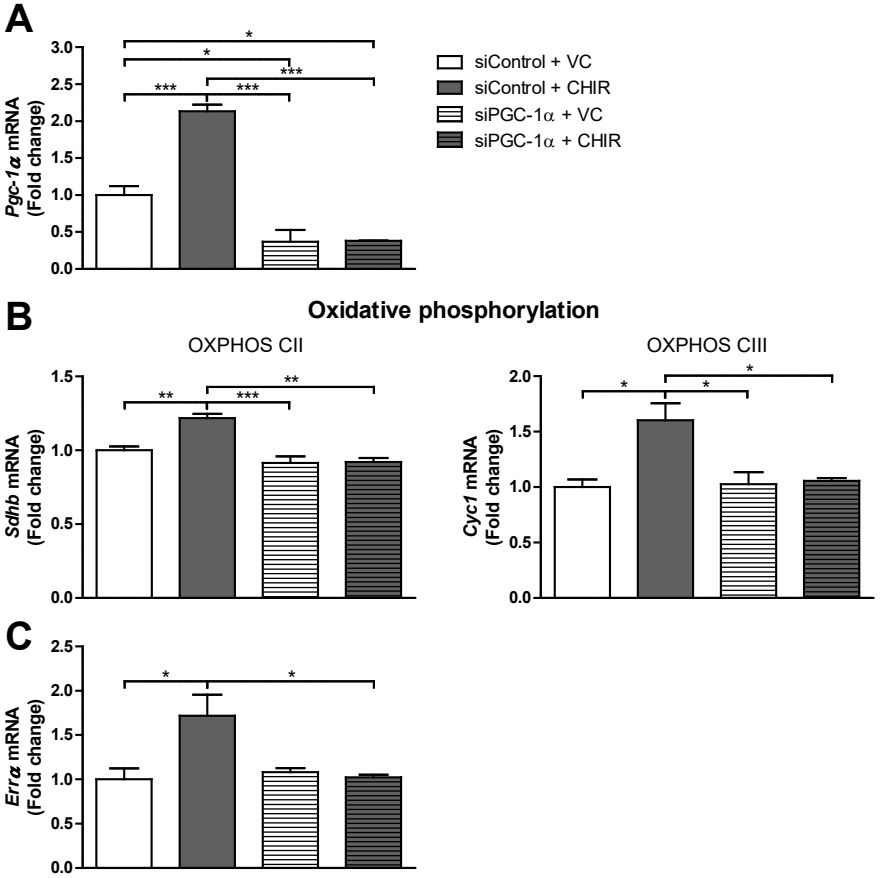


Figure 4. Pharmacological inactivation of GSK-3 increases oxidative gene expression in a *Pgc-1α*-dependent manner. C2C12 myotubes were differentiated for 5 days and treated with siRNA constructs targeting PGC-1α or a low GC scrambled control and treated with CHIR99021 (final concentration: 7 μM) or 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), * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. 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.

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. 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).

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 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* 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, *Pdk4* 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).

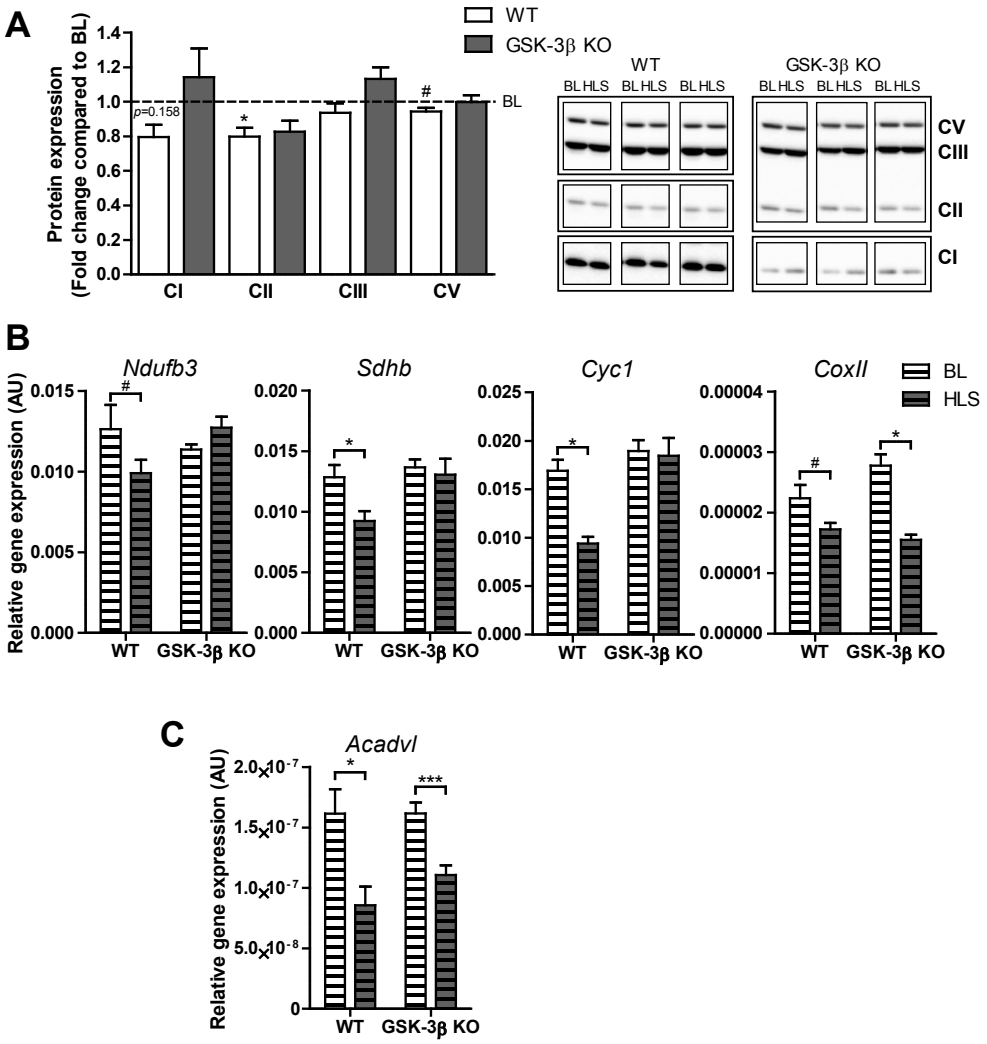


Figure 5. GSK-3β knock-out partially protects against unloading-induced decline in oxidative gene expression. Muscle-specific GSK-3β KO mice and WT littermates were subjected to HLS for 14 days. *M. gastrocnemius* was prepared for western blotting and qPCR analysis. (A) Protein abundance of OXPHOS complexes 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*. 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), # $p \leq 0.1$, * $p \leq 0.05$, *** $p \leq 0.001$. 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-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).

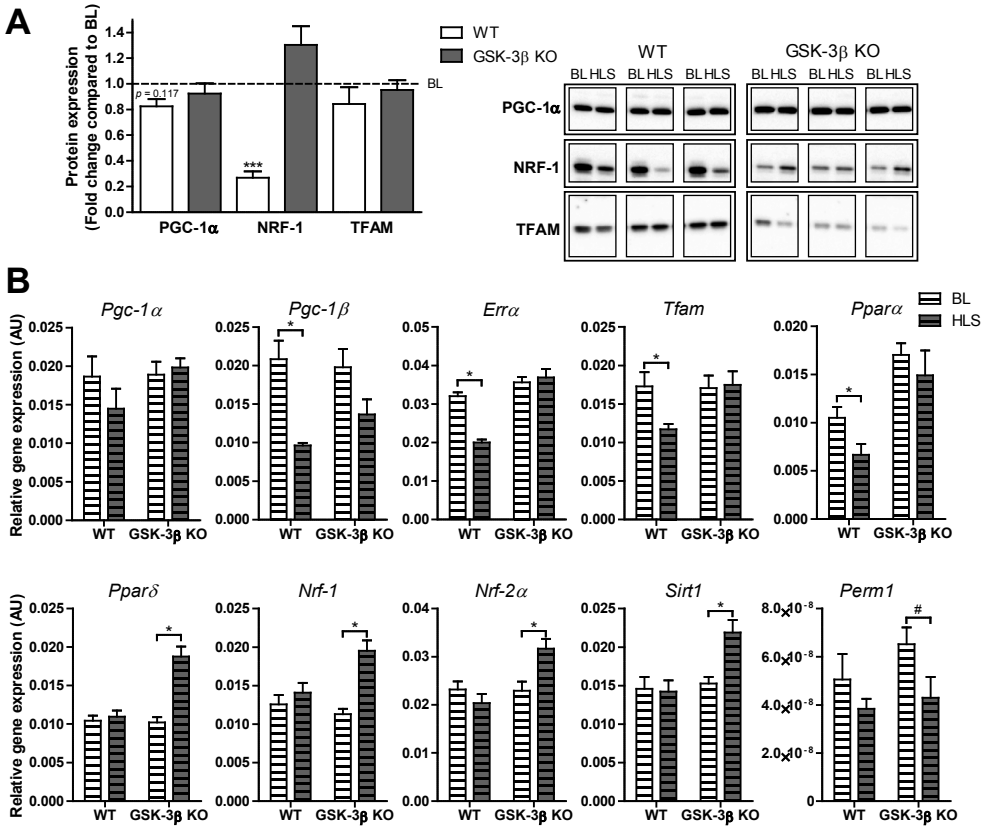


Figure 6. GSK-3β knock-out protects against unloading-induced decline in PGC-1α signaling constituents. Muscle-specific GSK-3β KO mice and WT littermates were subjected to HLS for 14 days. *M. gastrocnemius* was prepared for western blotting and qPCR analysis. (A) Protein abundance of PGC-1α, NRF-1 and 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*. 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), # $p \leq 0.1$,* $p \leq 0.05$, *** $p \leq 0.001$. 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.

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 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 *Pdk4* 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 *Pdk4* gene expression via co-activation of ERRα [48], which could explain the increase in *Pdk4* mRNA observed in our study. Increased *Pdk4* 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 *Pdk4* 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 PDK4 is mainly controlled at the transcriptional level, we did not directly measure PDK4 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.* (2016) 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 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 KO 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 lipopolysacharide (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.

Acknowledgements

We would like to thanks both Satish Patel and Jim R. Woodgett for providing us with the skeletal muscle-specific (M)GSK-3β KO C57/Bl6 mouse strain. This work was financially supported by a grant from the Dutch Lung foundation (5.2.13.067).

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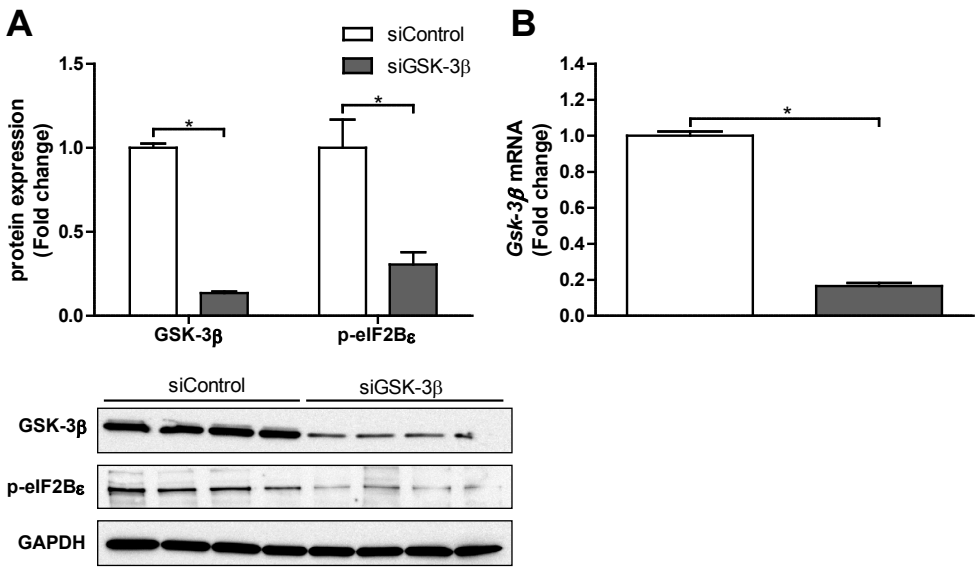
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Supplemental information

Supplemental results

GSK-3β knock-down effectively reduces GSK-3β levels and phosphorylation of eIF2Bε

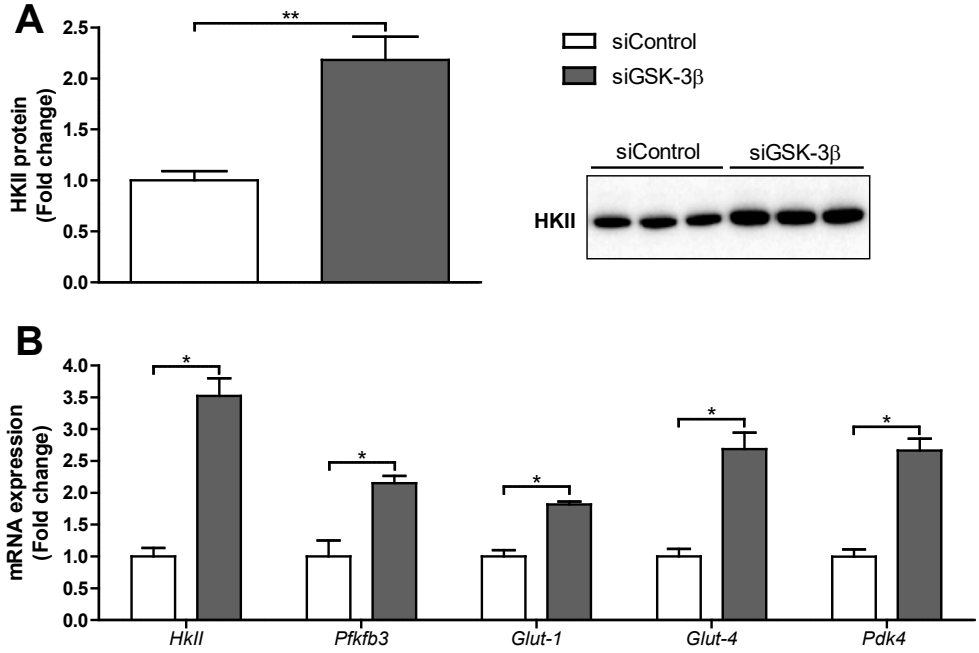
Protein levels of GSK-3β and p-eIF2Bε, a direct phosphorylation target of GSK-3β were significantly reduced after 48 h knock-down of GSK-3β in fully differentiated myotubes (day 5) compared to control (Fig. S1A). Also, GSK-3β mRNA levels were reduced by approximately 80–90% 48 h after knock-down of GSK-3β (Fig. S1B).



Supplemental figure 1. GSK-3β knock-down. 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 GSK-3β and p-eIF2Bε and (B) gene expression of *Gsk-3β*. Representative pictures of the blots are shown and Western blot data is corrected for GAPDH. 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 (n = 3 experiments), * $p \leq 0.05$ vs. siControl. GAPDH: glyceraldehyde 3-phosphate dehydrogenase, GSK-3β: glycogen synthase kinase-3β and p-eIF2Bε: phosphorylated initiation factor 2B.

GSK-3β knock-down increases expression levels of glycolytic constituents

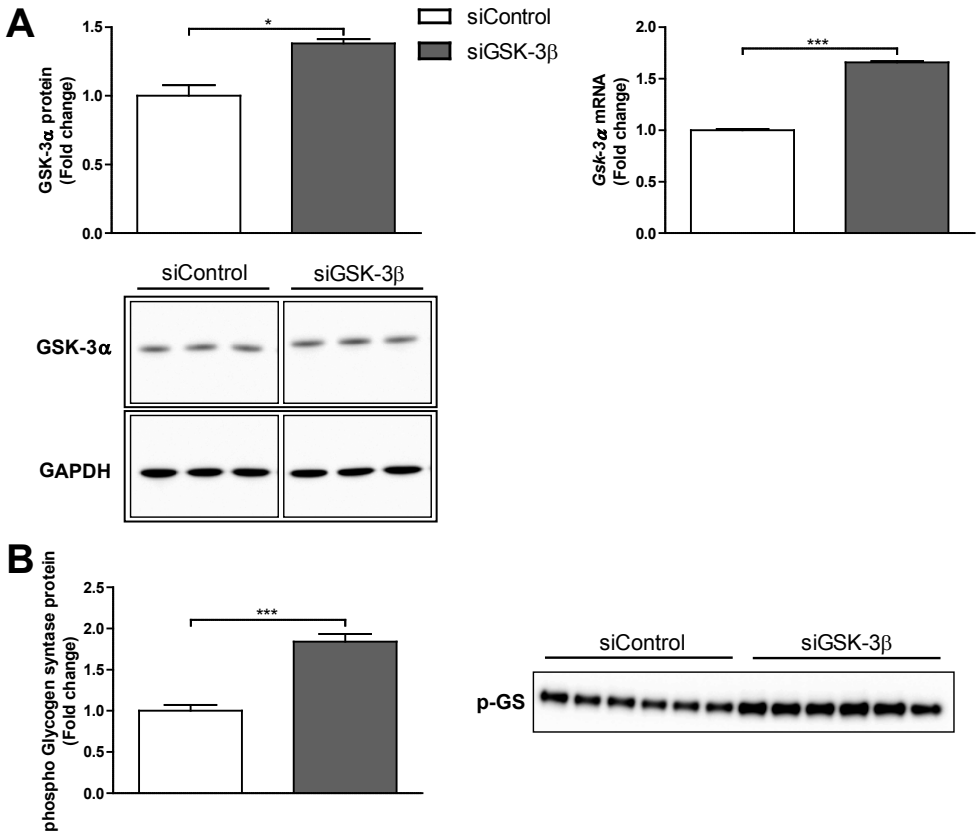
Loss of GSK-3β protein was associated with increased HKII protein abundance and increased mRNA transcript abundance of the glycolytic genes hexokinase II (*HkII*), 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (*Pfkfb3*), glucose transporter 1 (*Glut-1*) and *Glut-4* suggestive of enhanced aerobic glycolysis. Pyruvate dehydrogenase kinase 4 (*Pdk4*) mRNA expression was also increased after knock-down of the GSK-3β protein (Fig. S2).



Supplemental figure 2. GSK-3β knock-down increases expression levels of glycolytic constituents. 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 levels of HKII and (B) gene expression of glycolytic markers (*HkII*, *Pfkfb3* and *Glut-1/4*) are expressed as fold changes. A representative picture of a blot is shown and Western blot data is corrected for total protein loading assessed by Ponceau S staining. The black box around the representative picture indicates that it is cut from the same Western blot. All data are expressed as means ± SEM from n = 3/experimental condition (n = 3 experiments), * $p \leq 0.05$, ** $p \leq 0.01$ vs. siControl. GSK-3β: glycogen synthase kinase-3β, HKII: hexokinase II, *Pfkfb3*: 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3, *Glut*: glucose transporter and *Pdk4*: pyruvate dehydrogenase kinase 4.

GSK-3β knock-down increases GSK-3α abundance and p-GS levels

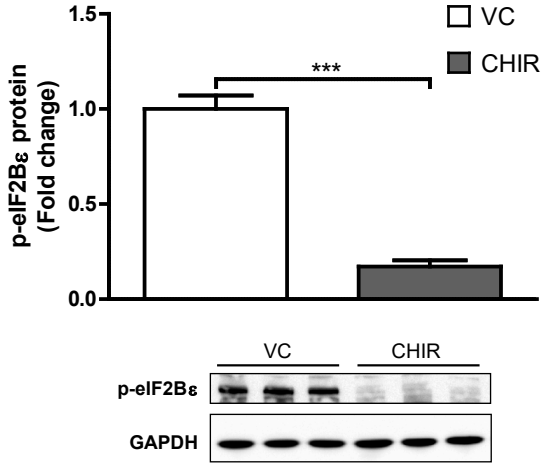
GSK-3α protein and transcript levels (Fig. S3A) were significantly higher after 48 h knock-down of GSK-3β in fully differentiated myotube cultures. In addition, phosphorylation status of the GSK-3 downstream target glycogen synthase (GS) was significantly increased upon knock-down of GSK-3β (Fig. S3B).



Supplemental figure 3. GSK-3β knock-down increases GSK-3α abundance and glycogen synthase phosphorylation. 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) Gene and protein expression of GSK-3α and (B) protein level of p-GS. Representative pictures of the blots are shown and Western blot data is corrected for total protein loading assessed by Ponceau S staining (B) or GAPDH (A). 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 = 3/experimental condition or n = 6/experimental condition for p-GS (n = 3 experiments), * $p \leq 0.05$, *** $p \leq 0.001$ vs. siControl. GSK-3α/β: glycogen synthase kinase-3α/β and GS: glycogen synthase.

Pharmacological inhibition of GSK-3 using CHIR decreases phosphorylation of p-eIF2Bε

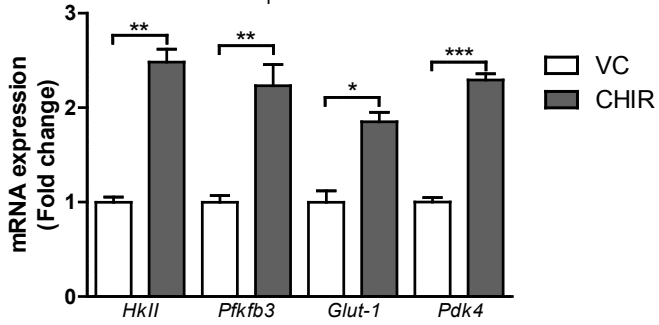
Pharmacological inhibition of the GSK-3α/β proteins using the selective GSK-3 inhibitor CHIR99021 (CHIR) resulted in a potent decrease in the phosphorylation of the direct GSK-3 target eIF2Bε (Fig. S4).



Supplemental figure 4. Pharmacological inhibition of GSK-3 with CHIR decreases the phosphorylation status of eIF2Bε. 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. Protein level of p-eIF2Bε is corrected for GAPDH and representative pictures of a blot are shown. 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 = 3/experimental condition (n = 3 experiments), *** $p \leq 0.001$ vs. siControl. VC: vehicle and p-eIF2Bε: phosphorylated initiation factor 2B.

Pharmacological inhibition of GSK-3 increases glycolytic gene expression

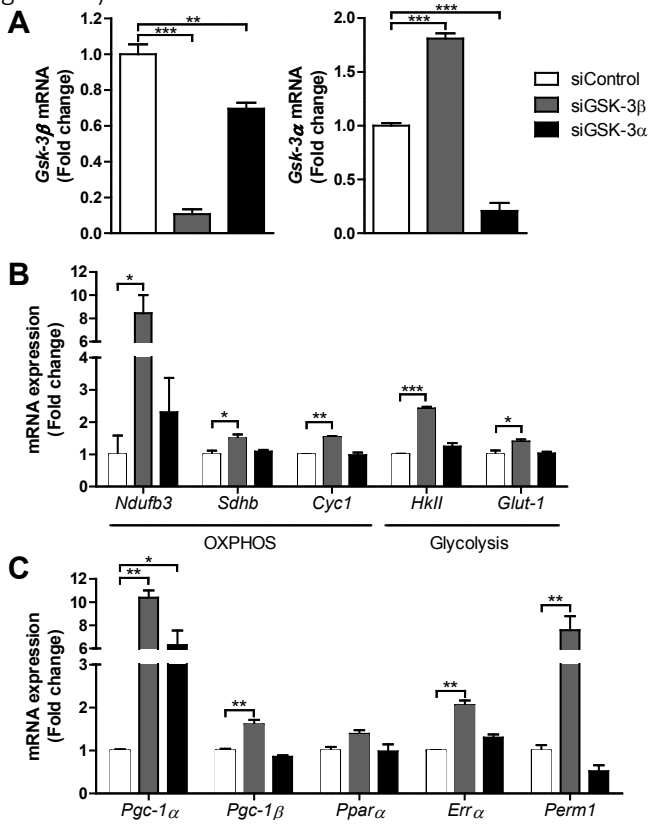
Pharmacological inhibition of the GSK-3α/β proteins using the selective GSK-3 inhibitor CHIR resulted in an increase in transcript expression of *HkII*, *Pfkfb3* and *Glut-1*, suggestive of increased aerobic glycolysis (Fig. S5). In addition, CHIR treatment increased *Pdk4* transcript levels.



Supplemental figure 5. Pharmacological inhibition of GSK-3 with CHIR increases glycolytic gene expression. 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. Transcript expression levels of glycolytic genes (*HkII*, *Pfkfb3*, *Glut-1* and *Pdk4*) are depicted. All data are expressed as means ± SEM from n = 3/experimental condition (n = 3 experiments), * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ vs. siControl. VC: vehicle, *HkII*: hexokinase II, *Pfkfb3*: 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 and *Glut-1*: glucose transporter 1 and *Pdk4*: pyruvate dehydrogenase kinase 4.

GSK-3β is the predominant GSK-3 isoform in the regulation of muscle metabolic gene expression

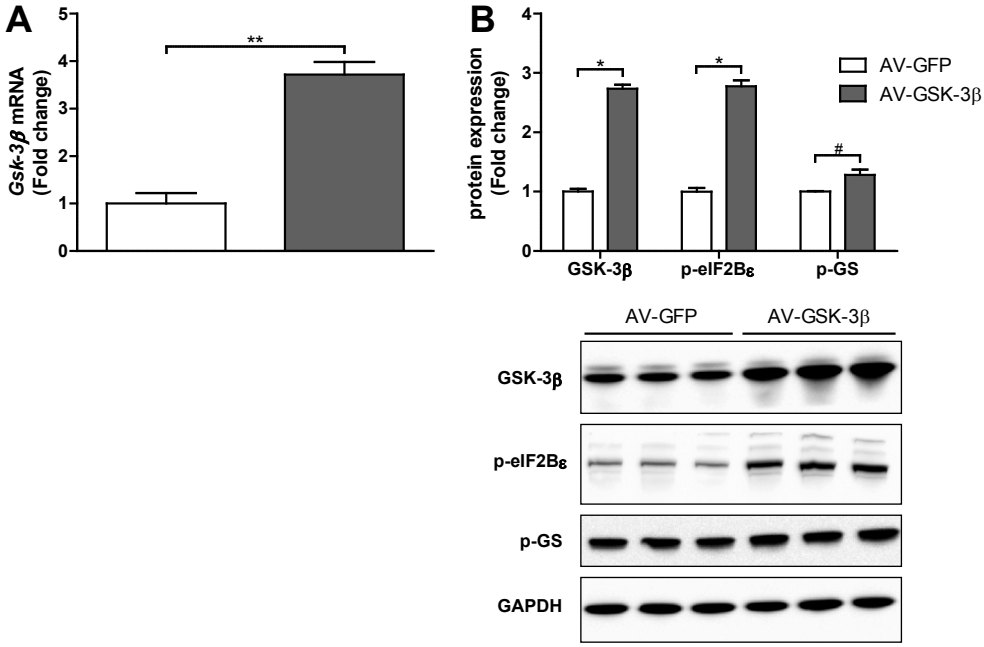
Knock-down of GSK-3β resulted in a significant increase in mRNA abundance of GSK-3α (Fig. S6A). Like knock-down of GSK-3β, GSK-3α knock-down resulted in increased transcript levels of *Pgc-1α*, however, this increase was less pronounced compared to knock-down of GSK-3β. In contrast to GSK-3β knock-down, knock-down of GSK-3α failed to increase gene expression of OXPHOS sub-units (CI, CII and CIII), *HkII*, *Glut-1* and constituents of the PGC-1 signaling network (*Errα*, *Pgc-1β*, *Pparα* and *Perm1*) (Fig. S6B-C).



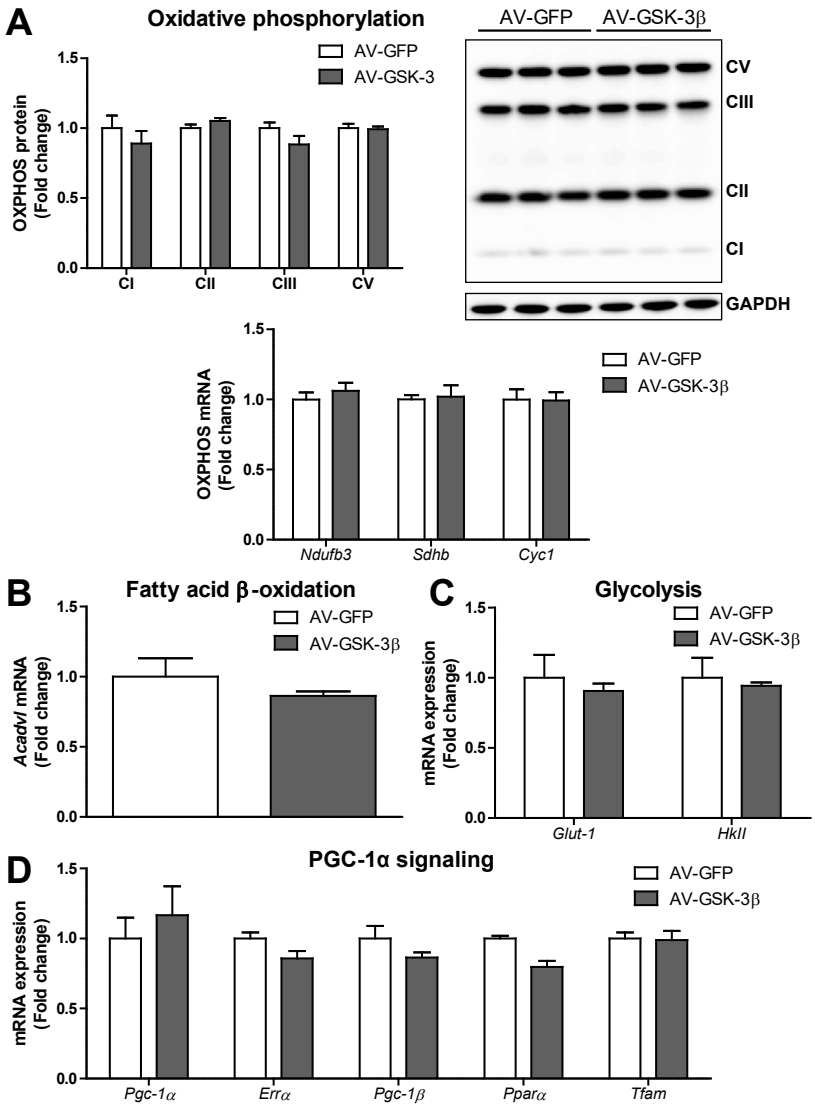
Supplemental figure 6. GSK-3β is the predominant GSK-3 isoform in the regulation of muscle metabolic gene expression. C2C12 myotubes were differentiated for 5 days and transfected with siRNA constructs targeting GSK-3β (grey), GSK-3α (black) or a low GC scrambled control (white). Myotubes were harvested 48 h post-transfection. (A) *Gsk-3β* gene expression and transcript levels of genes involved in (B) OXPHOS (subunits CI, CII and CIII), glycolysis (*HkII*, *Glut1*) and (C) the PGC-1 signaling network (*Pgc-1α/β*, *Pparα*, *Errα* and *Perm1*). All data are expressed as means ± SEM from n = 3/experimental condition (n = 3 experiments), * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ vs. siControl. *Gsk-3α/β*: glycogen synthase kinase-3α/β, OXPHOS: oxidative phosphorylation, *HkII*: hexokinase II, *Glut*: glucose transporter, *Pparα*: peroxisome proliferator-activated receptor α, *Pgc-1α/β*: PPAR gamma coactivator-1α/β, *Errα*: estrogen-related receptor α and *Perm1*: PGC-1α and ERRα regulator of muscle 1.

GSK-3β overexpression does not affect myotube metabolic gene expression or PGC-1α transcript abundance

In order to investigate whether over-expression of GSK-3β, in contrast to knock-down of the protein, would detrimentally impact expression of key mitochondrial proteins, we over-expressed GSK-3β protein in mature C2C12 myotubes. Both GSK-3β protein abundance as well as phosphorylation status of EiF2B were significantly elevated upon adenoviral over-expression of GSK-3β (Fig. S7). No significant changes were observed in mRNA and protein abundance of OXPHOS proteins or constituents of fatty acid β-oxidation, glycolysis or PGC-1 signaling (Fig. S8).



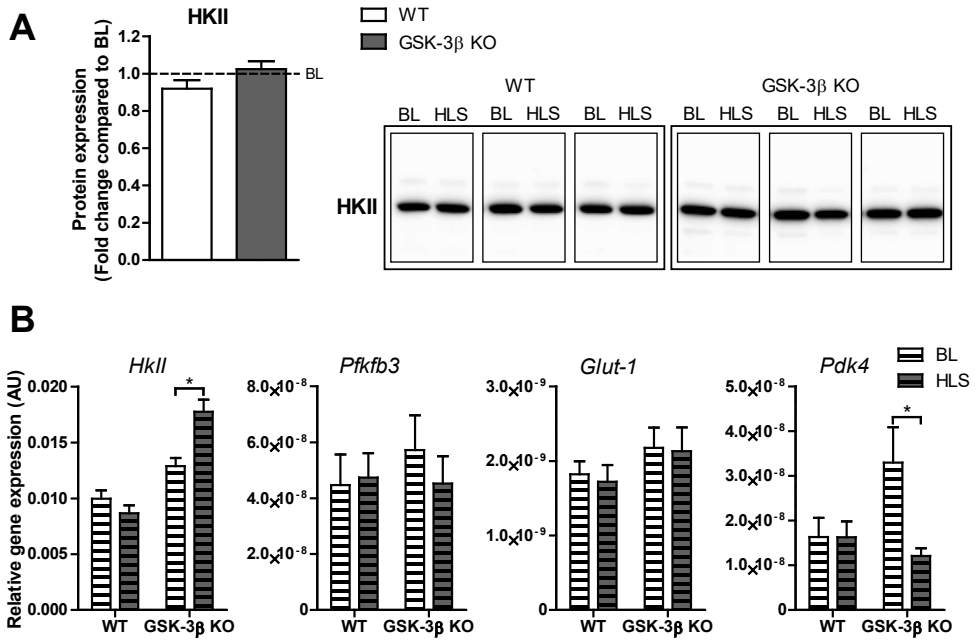
Supplemental figure 7. GSK-3β overexpression. C2C12 myotubes were differentiated for 5 days and GSK-3β (grey) or GFP (white) were over-expressed using an adenoviral-based system. Myotubes were harvested 48 h post-infection. (A) Protein expression of GSK-3β and the phosphorylated down-stream targets, p-eIF2Bε and p-GS and (B) *Gsk-3β* mRNA expression are given in fold changes compared to the control group. Representative pictures of the blots are shown and Western blot data is corrected for GAPDH. 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 = 3/experimental condition (protein) or n = 6/experimental condition (mRNA) (n = 3 experiments), # $p \leq 0.1$, * $p \leq 0.05$, ** $p \leq 0.01$ vs. siControl. GAPDH: glyceraldehyde 3-phosphate dehydrogenase, GSK-3β: glycogen synthase kinase-3β, AV: adenovirus, GFP: green fluorescent protein, p-eIF2Bε: phospho-eukaryotic initiation factor 2B and p-GS: phospho-glycogen synthase.



Supplemental figure 8. GSK-3β over-expression does not impair myotube metabolic gene expression. C2C12 myotubes were differentiated for 5 days and GSK-3β (grey) or GFP (white) were over-expressed using an adenoviral-based system. Myotubes were harvested 48 h post-infection. (A) mRNA and protein expression of OXPHOS complex sub-units and (B) transcript levels of genes involved in FAO (*Acadvl*), (C) glycolysis (*Glut-1* and *HkII*) and (D) the PGC-1 signaling network (*Pgc-1α*, *Errα*, *Pgc-1β*, *Pparα* and *Tfam*). Representative pictures of the blots are shown and Western blot data is corrected for GAPDH. 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 = 3/experimental condition or n = 6/experimental condition (mRNA FAO and glycolysis) (n = 3 experiments). GSK-3β: glycogen synthase kinase-3β, AV: adenovirus, GFP: green fluorescent protein, OXPHOS: oxidative phosphorylation, *Acadvl*: very long-chain specific acyl-CoA dehydrogenase, *HkII*: hexokinase II, *Glut*: glucose transporter, *Pparα*: peroxisome proliferator-activated receptor α, *Pgc-1α/β*: PPAR gamma coactivator-1α/β and *Errα*: estrogen-related receptor α.

Unloading does not alter glycolytic gene expression

No unloading-induced changes in HKII protein abundance was found in muscle of WT or GSK-3β KO animals (Fig. S9 A) and while *HkII* mRNA levels were unaltered in response to HLS in WT animals, these were induced by unloading in GSK-3β KO animals (Fig. S9B). Furthermore, HLS did not reduce gene expression of *Pfkfb3*, *Glut-1* and *Pdk4* in WT animals, while *Pdk4* mRNA expression was reduced by 14 days of HLS in GSK-3β KO animals (Fig. S9B).



Supplemental figure 9. Unloading-induced changes in expression of glycolytic 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 HKII of muscle-specific GSK-3β KO (grey bars) and WT (white bars) mice is given in fold change compared to BL conditions (---). (B) Gene expression of *HkII*, *Pfkfb3*, *Glut-1*, *Pdk4* 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*. 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), * $p \leq 0.05$, n=8-9. GSK-3β: glycogen synthase kinase-3β, *HkII*: hexokinase II, *Pfkfb3*: 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 and *Glut*: glucose transporter, *Pdk4*: pyruvate dehydrogenase kinase 4, BL: baseline, HLS: hind limb suspension, WT: wild type and KO: knock-out.

Chapter 4

Inactivation of glycogen synthase kinase-3β (GSK-3β) enhances mitochondrial biogenesis during myogenesis

W.F. Theeuwes, H.R. Gosker, R.C.J. Langen, N.A.M. Pansters, A.M.W.J. Schols and A.H.V. Remels.

Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease. 2018 Jun 6; 1864(9):2913-2926.

Abstract

Background

Mitochondrial biogenesis is crucial for myogenic differentiation and regeneration of skeletal muscle tissue and is tightly controlled by the peroxisome proliferator-activated receptor-γ co-activator 1 (PGC-1) signaling network. In the present study, we hypothesized that inactivation of glycogen synthase kinase (GSK)-3β, previously suggested to interfere with PGC-1 in non-muscle cells, potentiates PGC-1 signaling and the development of mitochondrial biogenesis during myogenesis, ultimately resulting in an enhanced myotube oxidative capacity.

Methods

GSK-3β was inactivated genetically or pharmacologically during myogenic differentiation of C2C12 muscle cells. In addition, *M. gastrocnemius* tissue was collected from wild-type and muscle-specific GSK-3β knock-out (KO) mice at different time-points during the reloading/regeneration phase following a 14-day hind-limb suspension period. Subsequently, expression levels of constituents of the PGC-1 signaling network as well as key parameters of mitochondrial oxidative metabolism were investigated.

Results

In vitro, both knock-down as well as pharmacological inhibition of GSK-3β not only increased expression levels of important constituents of the PGC-1 signaling network, but also potentiated myogenic differentiation-associated increases in mitochondrial respiration, mitochondrial DNA copy number, oxidative phosphorylation (OXPHOS) protein abundance and the activity of key enzymes involved in the Krebs cycle and fatty acid β-oxidation. In addition, GSK-3β KO animals showed augmented reloading-induced increases in skeletal muscle gene expression of constituents of the PGC-1 signaling network as well as sub-units of OXPHOS complexes compared to wild-type animals.

Conclusion

Inactivation of GSK-3β stimulates activation of PGC-1 signaling and mitochondrial biogenesis during myogenic differentiation and reloading of the skeletal musculature.

Introduction

Skeletal muscle tissue is characterized by a high degree of plasticity, both in terms of muscle mass regulation as in the regulation of mitochondrial oxidative capacity [1]. This malleability enables muscle tissue to adapt to different stimuli and conditions such as altered use or loading [2]. Chronic diseases such as chronic obstructive pulmonary disease (COPD), type 2 diabetes and chronic heart failure are often associated with skeletal muscle abnormalities [3-5] and reduced physical activity which contribute significantly to mortality and morbidity of the disease [6]. Besides prevention of muscle abnormalities in these patients, underlying processes involved in regeneration of the musculature need more attention. Regeneration of skeletal muscle is a vital aspect of plasticity, and insight into muscle regeneration is crucial to better understand muscle repair. During regeneration of disuse-atrophied muscle, an increase in protein synthesis [7] and recovery of mitochondrial quantity and quality [8] both occur. Regeneration or repair of skeletal muscle tissue, after an insult such as disuse atrophy, is partly dependent on myogenic precursor cells which upon activation proliferate, differentiate and fuse with existing myofibers [9, 10]. Myogenesis can be partially recapitulated *in vitro* by means of the differentiation and fusion of cultured myoblasts into myotubes [11] and *in vivo* by reloading of the musculature after disuse-induced atrophy [12]. Importantly, both reloading of disused skeletal muscle [13], as a model for *in vivo* muscle regeneration, as well as *in vitro* myogenic differentiation of muscle cells into myotubes [14] are accompanied by a potent activation of mitochondrial biogenesis which is required to accommodate altered metabolic needs of the muscle cells [15].

It has been convincingly shown that peroxisome proliferator-activated receptor (PPAR) γ co-activator-1α (PGC-1α) controls mitochondrial biogenesis and mitochondrial oxidative capacity via a wide variety of transcription factors and nuclear receptors, such as estrogen-related receptors (ERRs) [16], PGC-1- and ERR-induced regulator in muscle 1 (PERM1) [17], PPARs [18], nuclear respiratory factor 1/2α (NRF-1/2α) [19] and mitochondrial transcription factor A (TFAM) [19]. Both during myogenic differentiation *in vitro* and regeneration of muscle tissue *in vivo*, the abundance and activity of PGC-1 signaling constituents [14] increase, this is accompanied by increased mitochondrial density and oxidative capacity of the muscle cell [13]. Illustrative of its crucial importance, mitochondrial biogenesis appears to be a prerequisite for appropriate myogenic differentiation and adequate muscle regeneration as knock-down of PGC-1α blocked myotube formation *in vitro* [20] and skeletal muscle regeneration after injury was impaired by ERRα-deficiency [21].

Yet, the exact up-stream molecular mechanism controlling PGC-1-orchestrated mitochondrial biogenesis during myogenesis remains to be elucidated. Intriguingly, evidence in non-muscle cells suggests a role for glycogen synthase kinase (GSK)-3β, a ubiquitously-expressed kinase originally found to be involved in glycogen synthesis in muscle [22], in the regulation of mitochondrial metabolism. For example, inhibition of GSK-3β with lithium chloride (LiCl) potently increased expression levels of PGC-1α in several non-muscle cell types such as neuronal [23] and aortic endothelial cells [24]. Also, we previously showed that knock-down of GSK-3β as well as pharmacological inhibition of GSK-3 with CHIR99021 (CHIR) not only increased PGC-1α levels, but also resulted in an increased abundance of protein expression of several components of the mitochondrial electron transport chain post-myogenesis, *i.e.* in fully differentiated myotubes [25]. In addition, inhibition of GSK-3β has been found to accelerate the induction of the myogenic differentiation program *in vitro* [26, 27]. However, whether or not GSK-3β controls myogenesis-associated mitochondrial biogenesis has not been addressed thus far. To this end, we hypothesized that inactivation of GSK-3β increases expression of constituents of the PGC-1 signaling cascade and enhances the myogenesis-associated increase in mitochondrial biogenesis thereby improving the development of mitochondrial oxidative capacity during the myogenic differentiation program. In the present study we therefore genetically and pharmacologically modulated GSK-3β in differentiating C2C12 myoblasts and investigated the impact on myogenesis-associated activation of the PGC-1 signaling network and development of mitochondrial oxidative metabolism. 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 during muscle regeneration by analyzing muscle from wild-type (WT) and GSK-3β knock-out (KO) mice during recovery from disuse-induced muscle atrophy.

Materials and methods

Cell culture

C2C12 murine myoblasts (ATCC CRL1772, Manassas, VA, USA) 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) as previously described [28]. The myoblasts were plated at a density of $1*10^4$ cells/cm^2 in Matrigel-coated (BD Biosciences, San Jose, CA, USA) 6-well plates or at $2*10^4$ cells/cm^2 in XF96 Seahorse culture plates

(Agilent, Santa Clara, CA, USA). Cells were cultured for 24 h in GM before initiation of differentiation, by growth factor withdrawal [29] 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, 2, 4 and 6 of differentiation.

Cell processing

C2C12 cells in different phases of differentiation were washed with pre-cooled (4 °C) 1X Hank's balanced salt solution (HBSS) and harvested in whole cell lysate 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, RLT buffer (Qiagen, Hilden, Germany) containing β-mercaptoethanol (1:100) for RNA extraction, 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 extraction 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.

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−/− (wild-type (WT) control) with GSK-3βfl/fl MLC1f-Cre+/− (muscle-specific GSK-3β KO) mice [30]. Double Cre-negative litter mates served as 'WT' controls. Mice were 13 weeks old (young adults) at the initiation of the experiment. Animals had *ad libitum* access to standard chow pellets and water and were housed in a temperature-controlled room (21–22 °C) with 12:12 h light–dark cycle. Animals were randomly divided into 7 groups per genotype: baseline (BL; no intervention), 14 days of hind-limb suspension (HLS) and reloading (RL) for 1, 2, 3, 5 or 8 days. The group size for the BL-groups was n = 9 and for the HLS/RL-groups n = 8 [31]. Briefly, in order to place mice into a tail harness the 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. 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 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. After 14 days the tail harness was removed and the mice were re-introduced on their hind-limbs for 1, 2, 3, 5 or 8 days (reloading). Sodium pentobarbital was used for euthanasia and *M. gastrocnemius* were dissected using standardized 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. *M. gastrocnemius* was powdered and lysed in ice-cold RLT buffer containing β-mercaptoethanol (1:100) for RNA extraction, IP-lysis buffer (50 mM Tris, 150 mM NaCl, 10% glycerol, 0.5% Nonidet P40, 1 mM EDTA, 1 mM DTT with PhosSTOP and complete protease inhibitors, pH 7.4) for Western blotting or SET buffer (250 mM sucrose, 2 mM EDTA, 10 mM Tris, pH 7.4) for enzyme activity assays. Samples for Western blotting and enzyme assays where further homogenized with a Polytron PT 1600 E (Kinematica).

Quantitative Real-time PCR

400 ng RNA was used to synthesize cDNA with the tetro cDNA synthesis kit (Bioline, 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 (Table S1) per sample. 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 with white 384-well plates (Roche, Basel, Switzerland). 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 and checked for double peaks using the LightCycler480 software (Roche). Expression of the genes of interest was analyzed using LinRegPCR software and normalized with a correction factor calculated by GeNorm [32] based on the expression levels of a combination of at least three reference genes (*Calnexin*, *Rpl13a*, *Hprt*, *CycloA* or *β2M*).

DNA isolation

Proteinase K was added (at a final concentration of 0.2 mg/ml) and the lysates were 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 and air-dried. DNA pellets were dissolved in 10 mM Tris/HCl pH 8.0 and heated to 55 °C for 2 h followed by overnight incubation at 4 °C. When dissolved, *CoxII* and *CycloA* levels were determined as described in section 2.5 to determine mitochondrial (mt)DNA copy number.

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 10 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 until full separation as indicated by the protein ladder. The proteins on the gel were transferred to a 0.45 µm Whatman Protran Nitrocellulose Transfer membrane (Whatman, Maidstone, UK) by electroblotting (BioRad Criterion Blotter) at 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), PGC-1α (516557, Calbiochem), total OXPHOS (Mitosciences, Eugene, OR, USA), phosphorylated eukaryotic translation initiation factor 2β (p-eIF2Bε) or 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 antibodies (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).

Enzyme activity assays

After centrifugation total protein content was assessed in the supernatant with the Thermo Scientific Pierce BCA protein assay kit (Pierce Biotechnology) and used as a correction factor. Enzymatic activities of β-hydroxyacyl-CoA dehydrogenase (HAD; EC 1.1.1.35) [33], citrate synthase (CS; EC 2.3.3.1) [34], and creatine kinase (CK; EC 2.7.3.2) (Stanbio, Boerne, TX, USA) were analyzed spectrophotometrically at 340 nm, 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.

Respirometry

A Seahorse XF96 sensor cartridge with Seahorse calibrant (pH = 7.4) was incubated in a CO_2-free incubator at 37 °C 24 h prior to the measurement. Cells were washed twice with XF assay media (AM) (DMEM (Sigma) with 1× Glutamax, 1 mM sodium pyruvate, 0.9 g/l NaCl, 4.5 g/l glucose, phenol red, pH= 7.35 ± 0.05) and incubated for 1 h in assay media in a CO_2-free incubator at 37 °C. A mitochondrial stress test was performed using oligomycin (final concentration: 1 µM in AM), carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP) (final concentration: 2 µM in AM) and rotenone/antimycin-A (final concentration: both 1 µM in AM). C2C12 cells were lysed in 0.05% SDS in HBSS for protein determination, which was used to correct the oxygen consumption rate.

Statistical analysis

Data were analyzed using an unpaired student's t-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).

Results

Inactivation of GSK-3β potentiates myogenesis-associated increases in expression levels of key components of the PGC-1 signaling network

Knock-down of GSK-3β, as well as pharmacological inhibition of GSK-3 using CHIR (Fig. S1A, B), resulted in a pronounced potentiation of myogenesis-associated increases in both PGC-1α protein and *Pgc-1α* mRNA levels during C2C12 myogenic differentiation (Fig. 1A, B), in absence of profound effects on myogenic differentiation (Fig. S2A-C). Myogenesis-associated increases in *Pgc-1β* gene expression on the other hand were largely unaffected by GSK-3 inactivation (Fig. 1C). To investigate whether simultaneous treatment with CHIR and knock-down of GSK-3β has an additive effect on PGC-1α protein abundance compared to knock-down of the GSK-3β protein only, we treated cells both with siRNA's targeting GSK-3β and CHIR. The small amount of residual GSK-3β protein that remained after knock-down was successfully inhibited by CHIR, evidenced by significantly lower phosphorylation status of eIF2Bε (Fig. S3A, B). This tended to further increase PGC-1α protein levels when compared to GSK-3β knock-down only (Fig. S3C).

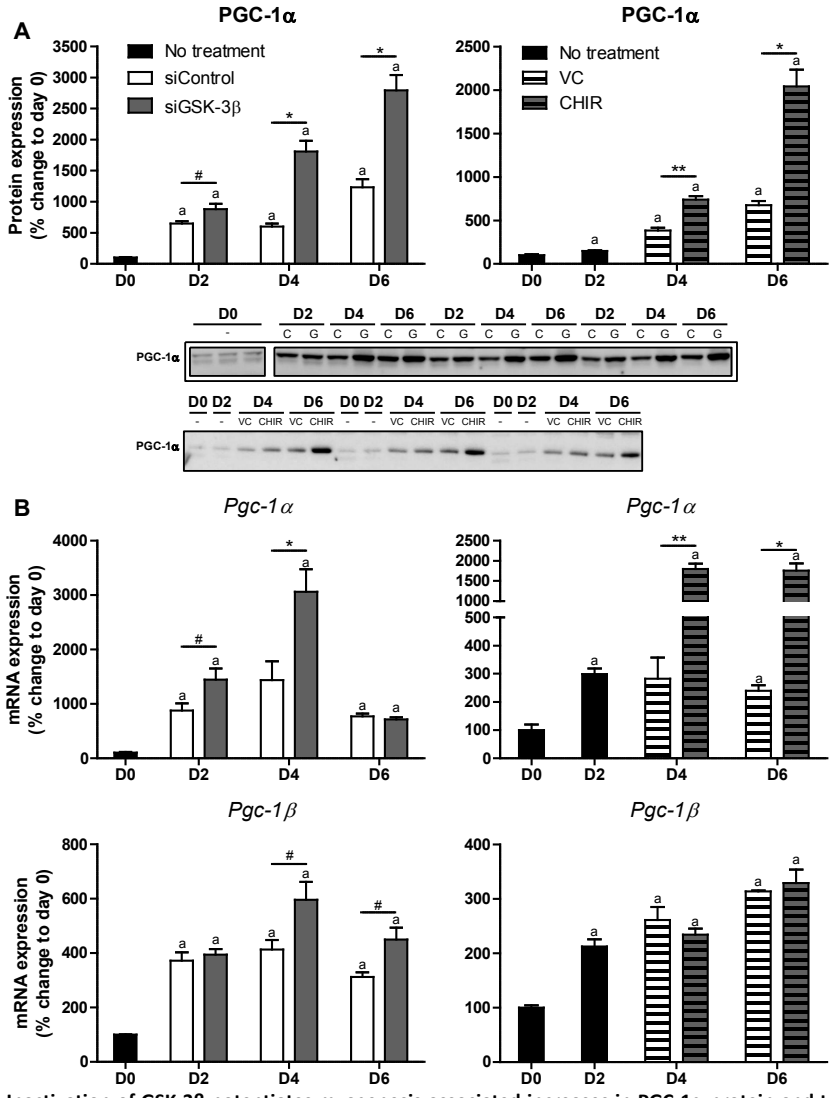


Figure 1. Inactivation of GSK-3β potentiates myogenesis-associated increases in PGC-1α protein and transcript abundance. C2C12 myoblasts were transfected with siRNA constructs targeting GSK-3β (grey) or a low GC scrambled control (white) on day 0 of differentiation or treated with CHIR99021 (striated grey) (final concentration: 7 μM) or DMSO as vehicle (striated white) on day 2 and 4 of differentiation. Cells were harvested at different time-points of differentiation (D0, D2, D4 and D6). (A) Protein levels of PGC-1α and (B) gene expression of *Pgc-1α/β* after GSK-3β knock-down (left) or pharmacological inhibition of GSK-3 (right) are expressed as percent change to day 0 (day 0/ myoblast phase is set on 100%). Representative pictures of the blots used for quantification of the graphs in this figure are shown and Western blot data is corrected for total protein loading assessed by Ponceau S staining. A black box around the representative pictures indicate that they are cut from the same Western blot. All data is expressed as means ± SEM from triplicates (n = 3 experiments). Statistical differences between treatments per time-point are tested using an unpaired Student's t-test, # $p \leq 0.1$, * $p \leq 0.05$ and ** $p \leq 0.01$. Statistical differences compared to day 0 of differentiation are tested using an unpaired Student's t-test [a] $p \leq 0.05$. D: day, C: siControl, G: siGSK-3β, GSK-3β: glycogen synthase kinase-3β and PGC-1α/β: peroxisome proliferator-activated receptor gamma coactivator-1α/β.

In addition to myogenesis-associated increases in PGC-1α abundance, expression levels of several important constituents of the PGC-1 signaling cascade (*Pparα*, *Pparδ*, *Nrf-2α* and *Tfam*) increased during the differentiation program but, in contrast to increases in PGC-1α, these inductions were not potentiated after either knock-down of GSK-3β or treatment with CHIR (Figs. 2-3). *Pparα* gene expression was below detection limit in C2C12 myoblasts, therefore % change was determined compared to day 2 of differentiation. Importantly, gene expression levels of *Errα* and *Perm1* increased after inactivation of GSK-3β, which became apparent on day 4 of the myogenic differentiation program (Figs. 2-3).

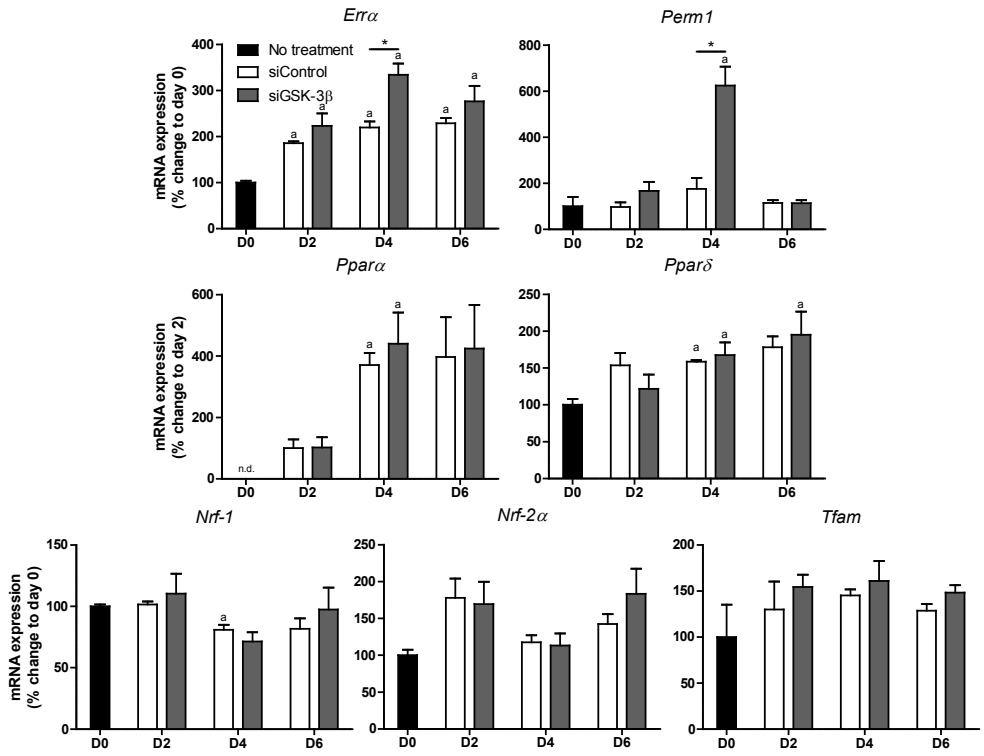


Figure 2. Knock-down of GSK-3β potentiates myogenesis-associated increases in *Errα* and *Perm1* gene expression levels. C2C12 myoblasts were transfected with siRNA constructs targeting GSK-3β (grey) or a low GC scrambled control (white) on day 0 of differentiation. Cells were harvested at different time-points of differentiation (D0, D2, D4 and D6). Gene expression of *Errα*, *Perm1*, *Pparδ*, *Nrf-1/2α* and *Tfam* after GSK-3β knock-down is expressed as percent change to day 0 (day 0/myoblast phase is set on 100%) and *Pparα* expression to day 2 (N.D: Non-detectable at D0) of differentiation as means ± SEM from triplicates (n = 3 experiments). Statistical differences between treatments per time-point are tested using an unpaired Student's t-test, * $p \leq 0.05$. Statistical differences compared to day 0 of differentiation (or day 2 for PPARα) are tested using an unpaired Student's t-test [a] $p \leq 0.05$. D: day, GSK-3β: glycogen synthase kinase-3β, *Errα*: estrogen-related receptor α, *Perm1*: PGC-1 and ERR-induced regulator in muscle 1, *Pparα/δ*: peroxisome proliferator-activated receptor α/δ, *Nrf-1/2α*: nuclear respiratory factor-1/2α and *Tfam*: mitochondrial transcription factor A.

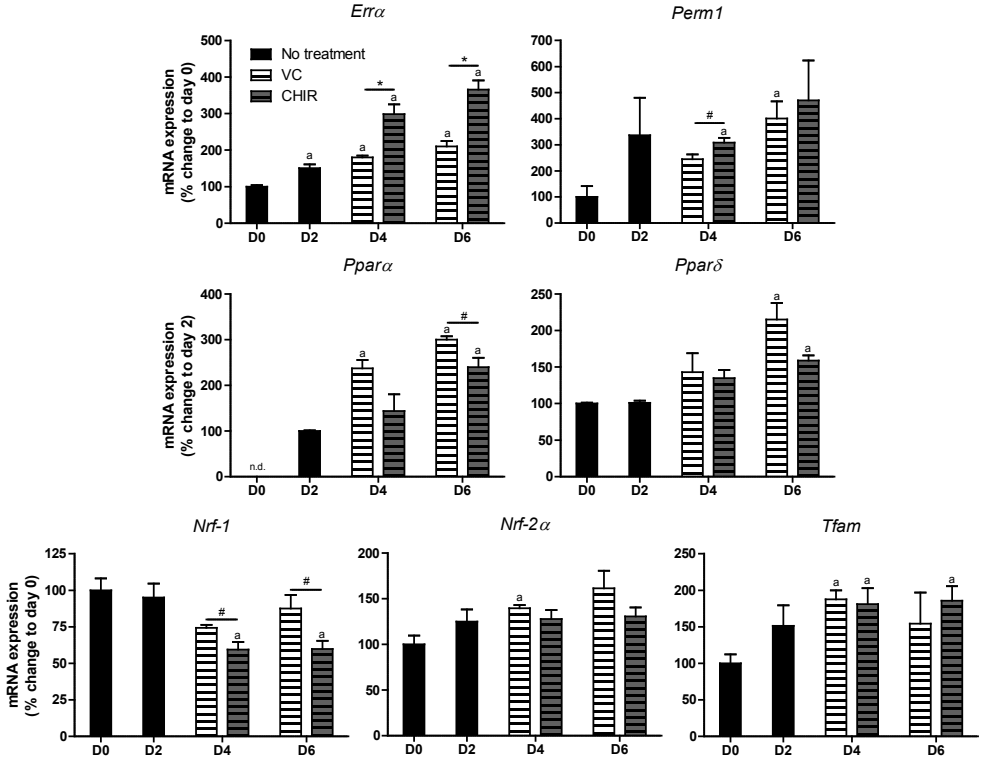


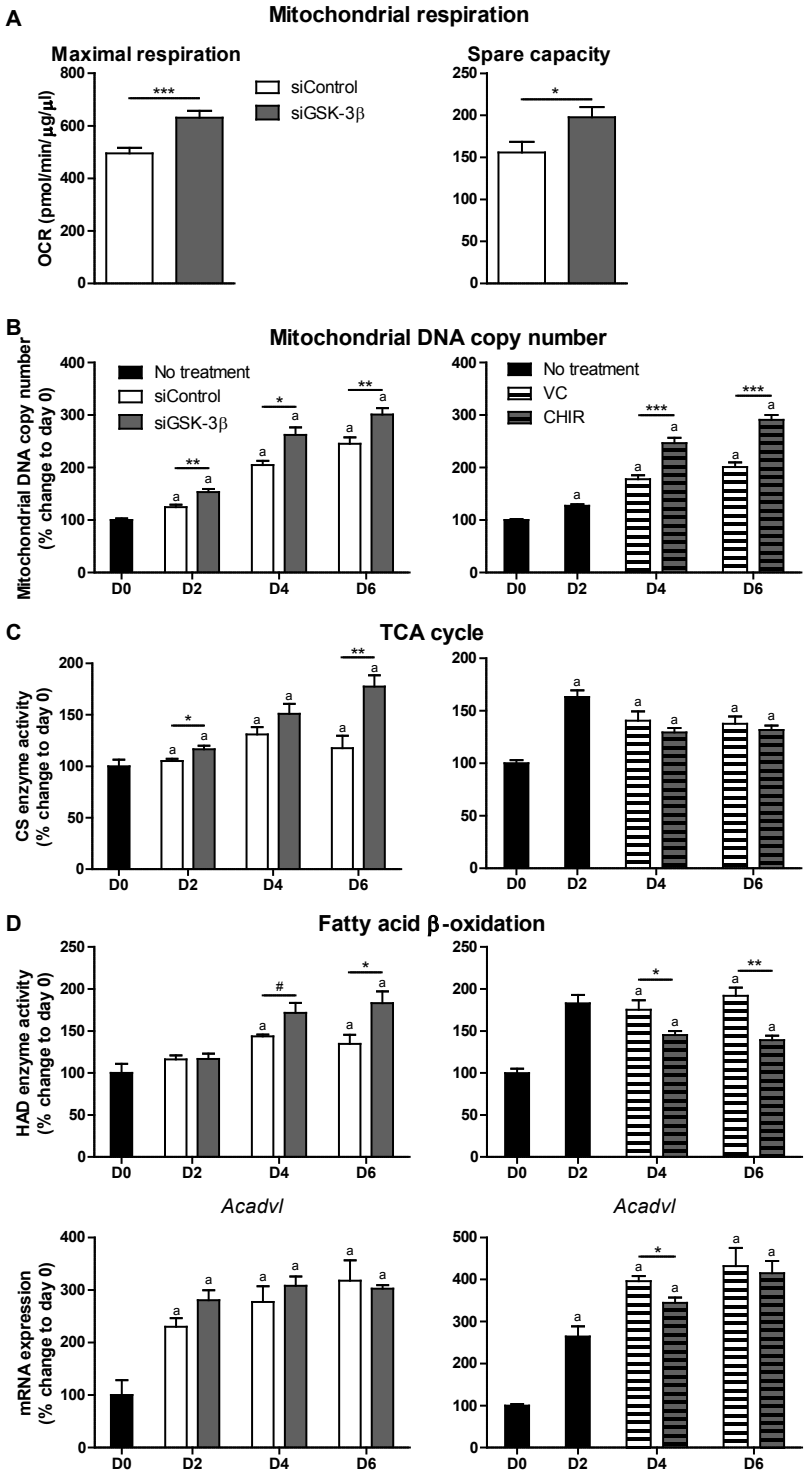
Figure 3. CHIR potentiates myogenesis-associated increases in *Errα* and *Perm1* gene expression levels. C2C12 myoblasts were treated with CHIR99021 (striated grey) (final concentration: 7 µM) or DMSO as vehicle (striated white) on day 2 and 4 of differentiation. Cells were harvested at different time-points of differentiation (D0, D2, D4 and D6). Gene expression of *Errα*, *Perm1*, *Pparδ*, *Nrf-1/2α* and *Tfam* after pharmacological inhibition of GSK-3 is expressed as percent change to day 0 (day 0/myoblast phase is set on 100%) and *Pparα* expression to day 2 (N.D: Non-detectable at D0) of differentiation as means ± SEM from triplicates (n = 3 experiments). Statistical differences between treatments per time-point are tested using an unpaired Student's t-test, # $p \leq 0.1$ and * $p \leq 0.05$. Statistical differences compared to day 0 of differentiation (or day 2 for PPARα) are tested using an unpaired Student's t-test [a] $p \leq 0.05$. D: day, GSK-3β: glycogen synthase kinase-3β, *Errα*: estrogen-related receptor α, *Perm1*: PGC-1 and ERR-induced regulator in muscle 1, *Pparα/δ*: peroxisome proliferator-activated receptor α/δ, *Nrf-1/2α*: nuclear respiratory factor-1/2α and *Tfam*: mitochondrial transcription factor A.

GSK-3β inactivation enhances the development of oxidative mitochondrial metabolism during myogenic differentiation

In line with potent inductions of both protein and mRNA levels of the master regulator of mitochondrial biogenesis (PGC-1α), inactivation of GSK-3β significantly enhanced the development of mitochondrial oxidative metabolism during C2C12 myogenic differentiation. This was evidenced by a significant increase of mitochondrial respiration after knock-down of GSK-3β indicated by elevated maximal mitochondrial O_2 consumption rate and spare capacity (Fig. 4A). Furthermore, myogenesis-associated increases in mitochondrial DNA copy number were potentiated after knock-down and inhibition of GSK-3β. Myogenesis-associated increases in enzymatic activity of key constituent of the Krebs cycle (CS) and fatty acid β-oxidation (HAD) were only increased after knock-down of GSK-3β (Fig. 4B-D). Gene expression levels of very long-chain specific acyl-CoA dehydrogenase (*Acadvl*) remained unaltered.

The potentiation of oxidative mitochondrial metabolism upon inactivation of GSK-3β was also accompanied by increased protein abundance of several key constituents of the electron transport chain (OXPHOS complexes I, II, III and V) (Fig. 5) which was found to be concurrent with moderately increased expression levels of several nuclear- (*Ndufb3*, *Sdhb* and *Cyc1*) and mitochondrial-encoded (*CoxII*) OXPHOS sub-units (Fig. S4A, B). In addition, the enhancement of cellular oxidative metabolism after inactivation of GSK-3β during myogenesis was also associated with elevated HKII protein levels (Fig. S5A). Gene expression of *HkII* was only increased after treatment with CHIR (Fig. S5B). Furthermore, the myogenesis-associated increase in gene expression of pyruvate dehydrogenase kinase 4 (*Pdk4*), indicative of increased reliance on fatty acid metabolism during myogenesis, was potentiated after pharmacological inhibition with CHIR (Fig. S5B).

Figure 4. Inactivation of GSK-3β enhances the development of oxidative mitochondrial metabolism during myogenic differentiation. C2C12 myoblasts were transfected with siRNA constructs targeting GSK-3β (grey) or a low GC scrambled control (white) on day 0 of differentiation or treated with CHIR99021 (striated grey) (final concentration: 7 μM) or DMSO as vehicle (striated white) on day 2 and 4 of differentiation. Cells were harvested at different time-points of differentiation (D0, D2, D4 and D6) or differentiated for a total of 7 days for Seahorse respirometry measurements. (A) Maximal respiration and spare capacity, (B) mtDNA copy number, (C) CS enzyme activity and (D) HAD enzyme activity and *Acadvl* gene expression. All data are expressed as means ± SEM from n = 6/experimental condition (mtDNA copy number and enzyme activity), n = 3/experimental condition (mRNA) or n = 45–46 wells (respirometry) (n = 3 experiments). mtDNA copy number, enzymatic activities and gene expression are expressed as percent change to day 0 of differentiation (day 0/myoblast phase is set on 100%). Statistical differences between treatments per time-point are tested using an unpaired Student's t-test, # $p \leq 0.1$, * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$. Statistical differences compared to day 0 of differentiation are tested using an unpaired Student's t-test [a] $p \leq 0.05$. D: day, GSK-3β: glycogen synthase kinase-3β, mtDNA: mitochondrial DNA, CS: citrate synthase, HAD: β-hydroxyacyl-CoA dehydrogenase and *Acadvl*: very long-chain specific acyl-CoA dehydrogenase. See next page ►.


A
Mitochondrial respiration
Maximal respiration
Spare capacity
OCR (pmol/min/µg/µl)
siControl
siGSK-3β
B
Mitochondrial DNA copy number
Mitochondrial DNA copy number (% change to day 0)
No treatment
siControl
siGSK-3β
VC
CHIR
C
TCA cycle
CS enzyme activity (% change to day 0)
D
Fatty acid β-oxidation
HAD enzyme activity (% change to day 0)
Acadvl
mRNA expression (% change to day 0)
D0
D2
D4
D6

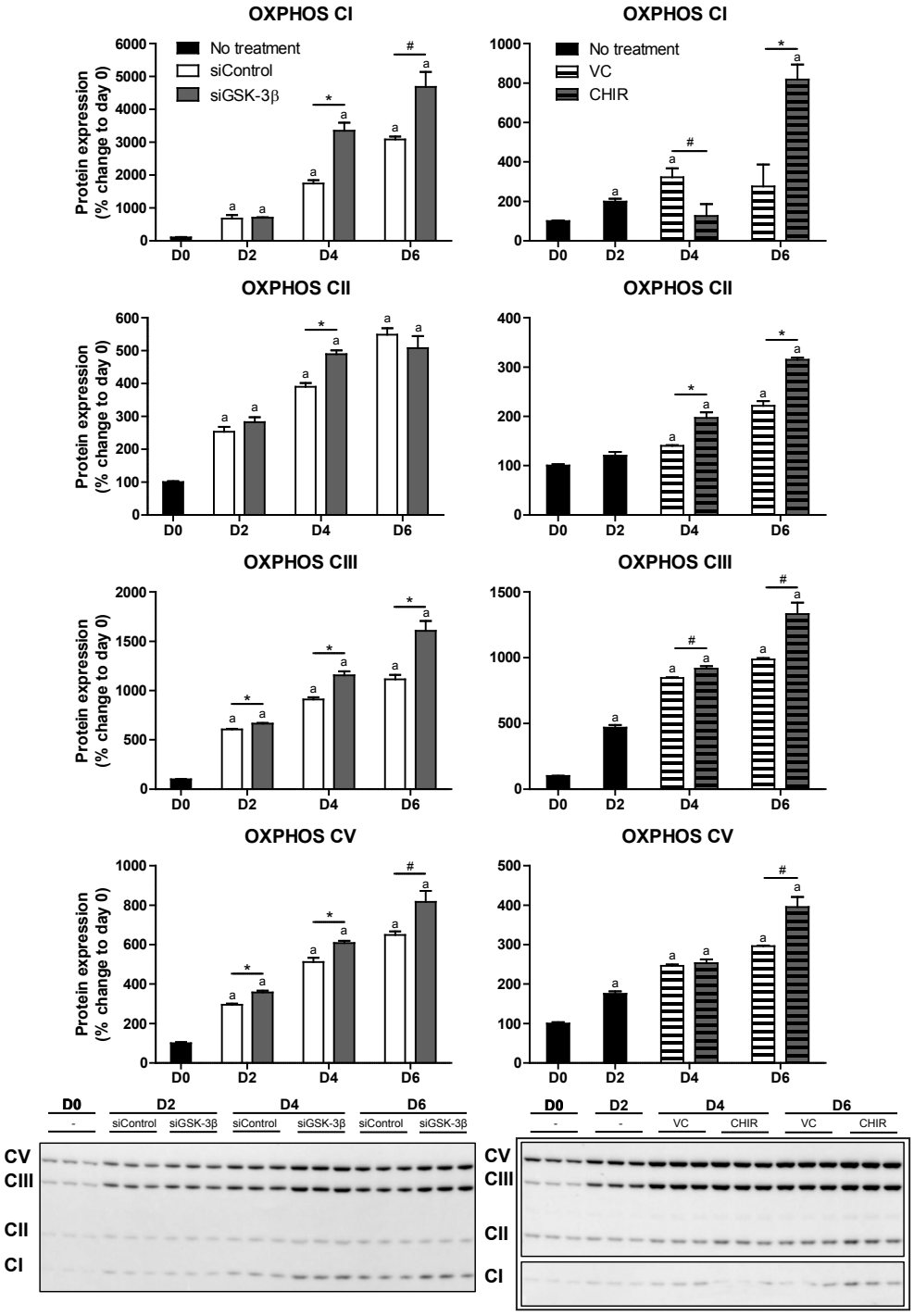

OXPHOS CI
No treatment
siControl
siGSK-3β
Protein expression (% change to day 0)
D0
D2
D4
D6
OXPHOS CI
No treatment
VC
CHIR
OXPHOS CII
OXPHOS CII
OXPHOS CIII
OXPHOS CIII
OXPHOS CV
OXPHOS CV
D0
D2
siControl
siGSK-3β
D4
D6
D0
D2
VC
CHIR
D4
D6
CV
CIII
CII
CI

Figure 5. Inactivation of GSK-3β potentiates myogenesis-associated increases in protein expression of OXPHOS sub-units. C2C12 myoblasts were transfected with siRNA constructs targeting GSK-3β (grey) or a low GC scrambled control (white) on day 0 of differentiation or treated with CHIR99021 (striated grey) (final concentration: 7 μM) or DMSO as vehicle (striated white) on day 2 and 4 of differentiation. Cells were harvested at different time-points of differentiation (D0, D2, D4 and D6). Protein levels of different complexes of the respiratory chain after GSK-3β knock-down (left) or pharmacological inhibition of GSK-3 (right) are expressed as percent change to day 0 (day 0/ myoblast phase is set on 100%). Representative pictures of the blots used for quantification of the graphs in this figure are shown and Western blot data is corrected for total protein loading assessed by Ponceau S staining. A black box around the representative pictures indicate that they are cut from the same Western blot. All data are expressed as means ± SEM from triplicates (n = 3 experiments). Statistical differences between treatments per time-point are tested using an unpaired Student's t-test, # $p \leq 0.1$ and * $p \leq 0.05$. Statistical differences compared to day 0 of differentiation are tested using an unpaired Student's t-test ^{a} $p \leq 0.05$. D: day, GSK-3β: glycogen synthase kinase-3β and OXPHOS C: oxidative phosphorylation complex.

GSK-3β knock-out potentiates reloading-induced increases in gene expression of constituents of the PGC-1 signaling network *in vivo*

Having established that inactivation of the GSK-3β protein enhanced the development of cellular mitochondrial metabolism during myogenic differentiation *in vitro*, we subsequently explored, as a proof-of-principle, 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 during myogenesis associated with muscle regeneration. To address this, we subjected WT and skeletal muscle-specific GSK-3β KO animals to a 14-day HLS protocol and subsequently studied regeneration-induced mitochondrial biogenesis at different time-points during the reloading phase. As previously described by our group, reloading of the musculature in these mice resulted in an activation of cell proliferation and induced the expression of myogenesis-associated genes, which indicates a muscle regenerative response [35].

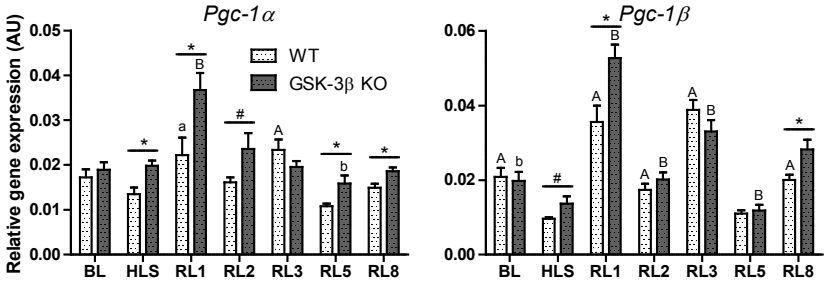


Figure 6. GSK-3β knock-out potentiates reloading-induced gene expression of *Pgc-1α/β*. Muscle-specific GSK-3β KO mice and WT littermates were subjected to HLS for 14 days, introduced back on their hind-limbs and followed during recovery (reloading). Gene expression of *Pgc-1α/β* under BL, HLS and RL conditions in GSK-3β KO (dotted grey bars) and WT (dotted white bars) mice are expressed as arbitrary units (AU) in *M. gastrocnemius*. Statistical differences are tested using an unpaired Student's t-test between genotypes per time-point, BL (n = 9/group) and HLS/RL (n = 8/group), # $p \leq 0.1$ and * $p \leq 0.05$ and within a genotype vs. HLS, for WT ^{a} $p \leq 0.1$, ^{A} $p \leq 0.05$ and for KO ^{b} $p \leq 0.1$, ^{B} $p \leq 0.05$. GSK-3β: glycogen synthase kinase-3β, *Pgc-1α/β*: peroxisome proliferator-activated receptor gamma coactivator-1α/β, BL: baseline, HLS: hind-limb suspension, RL: reloading, WT: wild-type and KO: knock-out.

Muscle-specific GSK-3β KO animals showed a potentiation of reloading-induced increases in transcript levels of *Pgc-1α* and *Pgc-1β* (Fig. 6). In addition, reloading-induced increases in gene expression of several other important constituents of the PGC-1 signaling cascade (*Errα*, *Pparα*, *Pparδ*, *Nrf-1*, *Nrf-2α* and *Tfam*) were potentiated in GSK-3β KO animals (Fig. 7) compared to WT animals. Reloading-induced increases in *Perm1* gene expression, however were not altered by knock-out of GSK-3β (Fig. 7). In contrast to these marked effects on reloading-induced alterations in gene expression, protein abundance of PGC-1α was not significantly altered by reloading of the musculature in both genotypes (Fig. S6A).

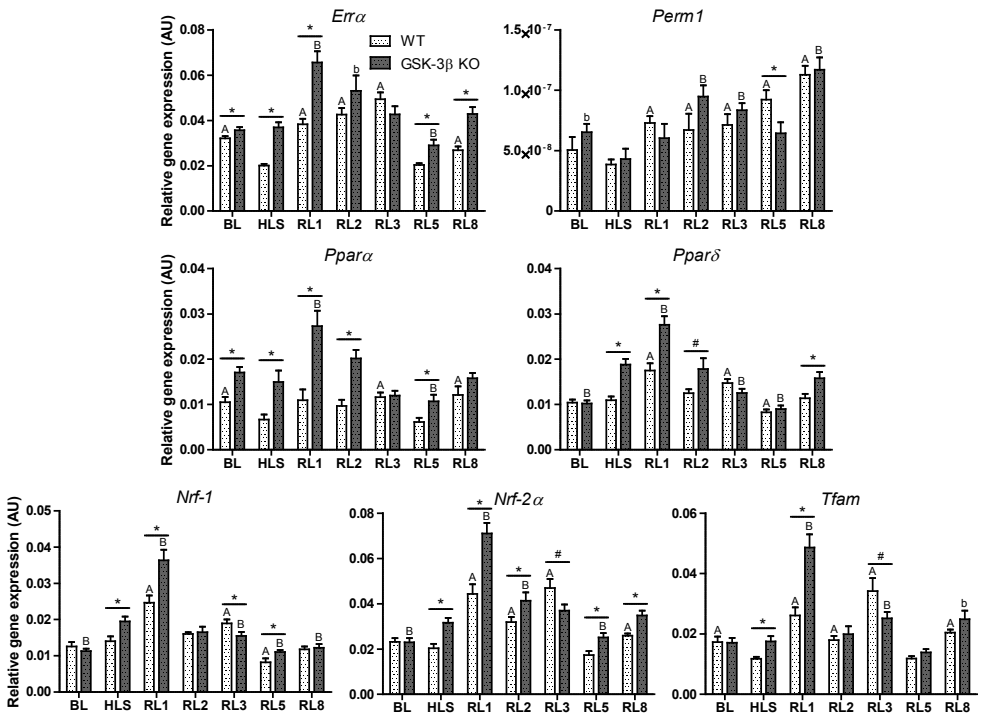


Figure 7. GSK-3β knock-out potentiates reloading-induced gene expression of PGC-1 signaling constituents. Muscle-specific GSK-3β KO mice and WT littermates were subjected to HLS for 14 days, introduced back on their hind-limbs and followed during recovery (reloading). Gene expression of genes involved in the PGC-1 signaling network regulating (A) mitochondrial biogenesis and (B) oxidative phosphorylation under BL, HLS and RL conditions in GSK-3β KO (dotted grey bars) and WT (dotted white bars) mice are expressed as arbitrary units (AU) in *M. gastrocnemius*. Statistical differences are tested using an unpaired Student's t-test between genotypes per time-point, BL (n = 9/group) and HLS/RL (n = 8/group), # $p \leq 0.1$ and * $p \leq 0.05$ and within a genotype vs. HLS, for WT ^{a} $p \leq 0.1$, ^{A} $p \leq 0.05$ and for KO ^{b} $p \leq 0.1$, ^{B} $p \leq 0.05$. GSK-3β: glycogen synthase kinase-3β, *Pparα/δ*: peroxisome proliferator-activated receptor α/δ, *Errα*: estrogen-related receptor α, *Nrf-1/2α*: nuclear respiratory factor-1/2α, *Tfam*: mitochondrial transcription factor A, OXPHOS: oxidative phosphorylation and *Perm1*: PGC-1 and ERR-induced regulator in muscle 1, BL: baseline, HLS: hind-limb suspension, RL: reloading, WT: wild-type and KO: knock-out.

GSK-3β knock-out potentiates reloading-induced increases in OXPHOS transcript levels in skeletal muscle *in vivo*

Besides the potentiating effect of GSK-3β KO on reloading-induced increases in mRNA levels of *Pgc-1α* and several constituents of the PGC-1 signaling network, muscle-specific GSK-3β KO animals also showed a potentiation of reloading-associated increases in gene expression of several nuclear-encoded OXPHOS sub-units (*Ndufb3*, *Sdhb* and *Cyc1*, respectively complex I, II and III) (Fig. 8A). However, gene expression of the mitochondrial-encoded (*CoxII*, complex IV) OXPHOS sub-unit only was increased at RL day 2 in GSK-3β KO animals compared to WT controls (Fig. 8B). Furthermore, reloading did not increase protein abundance of OXPHOS sub-units nor CS or HAD enzyme activity in either WT or muscle-specific GSK-3β KO mice (Fig. S6B, C). In addition, gene expression of key genes involved in fatty acid β-oxidation (*Acadvl*) (Fig. 8C) or glycolysis (*HkII*, *Pfkfb3*, *Glut-1* and *Pdk4*) (Fig. S7) were largely similar between both genotypes during reloading.

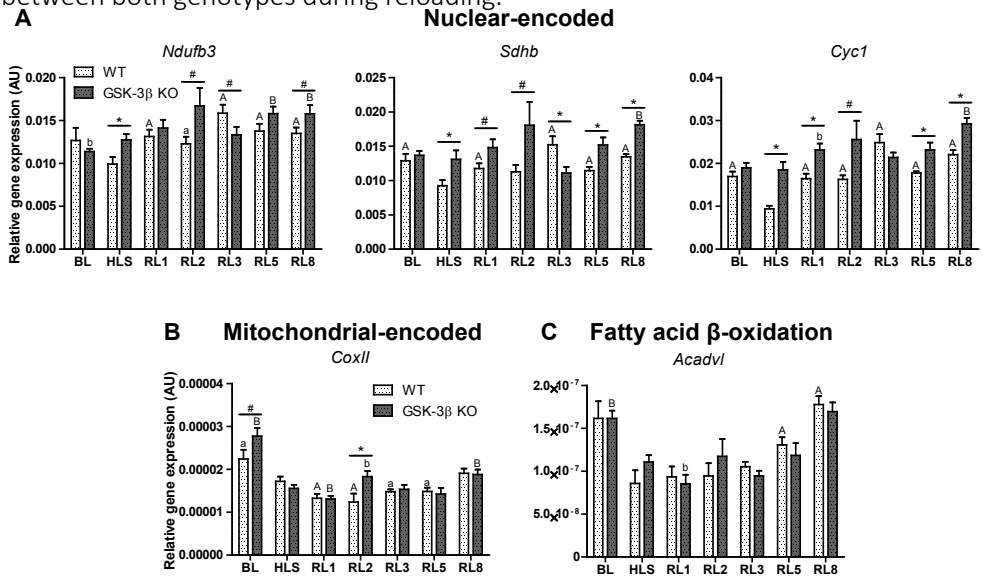


Figure 8. GSK-3β knock-out potentiates reloading-induced OXPHOS expression. Muscle-specific GSK-3β KO mice and WT littermates were subjected to HLS for 14 days, introduced back on their hind-limbs and followed during recovery (reloading). (A) Gene expression of nuclear-encoded OXPHOS sub-units (*Ndufb3*, *Sdhb* and *Cyc1* respectively Complex I, II and III) and (B) the mitochondrial-encoded OXPHOS sub-unit (*CoxII*, respectively Complex IV) and (C) *Acadvl* under BL, HLS and RL conditions in GSK-3β KO (dotted grey bars) and WT (dotted white bars) mice are expressed as arbitrary units (AU) in *M. gastrocnemius*. Statistical differences are tested using an unpaired Student's t-test between genotypes per time-point, BL (n = 9/group) and HLS/RL (n = 8/group), # $p \leq 0.1$ and * $p \leq 0.05$ and within a genotype vs. HLS, for WT ^{a} $p \leq 0.1$, ^{A} $p \leq 0.05$ and for KO ^{b} $p \leq 0.1$, ^{B} $p \leq 0.05$. 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 C1, *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.

Discussion

In skeletal muscle, GSK-3β has been appreciated for its regulatory role in glycogen metabolism and protein turnover (for review see [36]). To date, the role of GSK-3β in the regulation of mitochondrial biogenesis and oxidative energy metabolism is scarcely investigated and available studies mainly report findings in non-muscle cells. Whether GSK-3β plays a role in mitochondrial biogenesis associated with myogenic differentiation/regeneration has not been studied thus far. Here we report that both knock-down as well as pharmacological inhibition of the GSK-3β protein during the *in vitro* studied myogenic differentiation program resulted in a potentiation of myogenesis-associated increases in PGC-1α protein abundance as well as increased gene expression of *Errα* and *Perm1*. This was associated with an enhanced development of mitochondrial oxidative capacity during myogenesis as we observed elevated protein- and gene expression of OXPHOS complex sub-units, increases in activity levels of key metabolic enzymes and increased mtDNA content. Moreover, these changes culminated in a physiological relevant increase in cellular oxygen consumption induced by knock-down of GSK-3β. In addition to our *in vitro* data, muscle-specific GSK-3β KO *in vivo* potentiated reloading-associated increases in gene expression of important constituents of the PGC-1 signaling network as well as several OXPHOS sub-units.

To the best of our knowledge, this is the first study showing that inhibition of GSK-3β potentiates myogenesis-associated increases cellular oxidative capacity and expression levels of key constituents of the PGC-1 signaling network, in particular PGC-1α itself as well as *Errα* and *Perm1*. Previous studies, although scarce and in non-muscle cells, demonstrated that pharmacological inactivation of GSK-3 with LiCl resulted in activation of PGC-1 signaling and increased mitochondrial biogenesis. For example, in primary bovine aortic endothelial cells LiCl treatment resulted in increased gene expression of *Pgc-1α* and OXPHOS sub-units [24]. Furthermore, during megakaryocyte development, GSK-3β inhibition using LiCl not only increased important constituents of the PGC-1 signaling cascade, it also increased mitochondrial density and mtDNA copy number [37]. Additionally, in vascular smooth muscle, GSK-3β inhibition using LiCl resulted in increased PGC-1α protein abundance, nuclear translocation and PGC-1 signaling [38]. Although to date no studies previously investigated the effect of GSK-3β inactivation on mitochondrial biogenesis and the regulation thereof during myogenic differentiation, collectively, these observations are in line with our findings. Moreover, together with our findings in muscle cells, collectively this data suggests that the potentiation of PGC-1 signaling in response to GSK-3β inactivation may represent a cellular mechanism conserved in multiple cell

types. One important remark in this regard is that LiCl does not have a high degree of specificity for GSK-3. Indeed, LiCl not only inhibits cellular GSK-3 activity, LiCl also inhibits a number of other protein kinase and non-kinase targets. For example, LiCl selectively inhibited activation of the p38 mitogen-activated protein kinase pathway [39] and stabilizes the CCAAT/enhancer-binding protein alpha via direct proteasome inhibition [40] in a GSK-3 independent manner. In addition, enzymes required for de novo inositol synthesis are inhibited by LiCl [41, 42]. In this regard, our study adds strength to the notion that the effects of LiCl in the aforementioned studies are indeed mediated by GSK-3β as we observed these changes by using siRNA's specific for GSK-3β and a pharmacological inhibitor (CHIR) that has a much higher degree of specificity and selectivity for GSK-3 compared to LiCl [43]. Further in support of specific involvement of GSK-3 in potentiation of cellular oxidative metabolism and PGC-1 signaling is a study in cortical neuronal cells that demonstrated increased PGC-1α levels and mitochondrial content in response to SB216763, also a specific inhibitor of GSK-3 activity [23]. In contrast to these effects of GSK-3 inhibition on PGC-1α levels, no studies previously investigated the effect of GSK-3β activation on expression or activity of ERRs and PERM1. Both transcription factors are known to be highly involved in the regulation of mitochondrial oxidative function and mitochondrial biogenesis with PERM1 being one of the most recently discovered transcription factors involved in the regulation of cellular mitochondrial oxidative capacity by PGC-1. Only two studies previously reported a role for PERM1 herein, describing that PERM1 specifically controls muscle mitochondrial content and oxidative capacity [17, 44]. Collectively, our data suggests that during *in vitro* myogenesis, GSK-3β inactivation specifically activates the PGC-1/ERR/PERM1 branch of the PGC-1 transcriptional network. This is in line with a previous study from our group showing potentiation of this specific part of the PGC-1 network upon GSK-3 inactivation in mature myotubes *in vitro* [25]. During *in vivo* muscle recovery, inductions in gene expression levels of ERRα, PPAR isoforms, NRF1 and NRF2α as well as TFAM were potentiated in muscle-specific GSK-3β KO mice, compared to WT animals, showing that transcription of key regulators of different branches of the PGC-1α signaling are potentiated by GSK-3β KO during skeletal muscle reloading.

Our main finding is that knock-down or inhibition of GSK-3β potently induced PGC-1α gene expression and protein abundance during *in vitro* myogenesis. Increased expression of the *Pgc-1α* gene can result from enhanced transcription of the PGC-1α promoter. Several transcription factors have been demonstrated to be involved in transcription via binding with the PGC-1α promotor, including cyclic AMP response element binding protein (CREB) [45], myocyte enhancer factor 2 (MEF2) [46], forkhead transcription factor [47], p53 [48] and ERRα [49]. Although links between

GSK-3β and some of these proteins such as CREB, MEF2 and AMP-activated protein kinase (AMPK) have been described [50-52], the elucidation of the exact molecular mechanism underlying the impact of GSK-3β inactivation on PGC-1α transcript and protein abundance was beyond the scope of our current paper, hence further investigation is warranted. Additionally, although we did not test causality, in light of the well-known role of PGC-1 in the molecular regulation of mitochondrial oxidative metabolism and the observation that both protein levels as well as transcript levels of PGC-1α increased significantly in response to GSK-3β inactivation, potentiation of increases in cellular oxidative capacity that we observed in response to GSK-3β inactivation during myogenesis likely resulted from increased expression and activity of the PGC-1 signaling network. In support of this notion, previous research from our group showed that inactivation of GSK-3 with CHIR induced increases in transcript levels of several OXPHOS sub-units in myotubes which was prevented by knock-down of PGC-1α [25].

Previously, our group reported that pharmacological inhibition of GSK-3 with LiCl or stabile knock-down of GSK-3β enhanced C2C12 myogenic differentiation [26, 27]. More specifically, the stimulatory effects of GSK-3β inhibition in these studies were most apparent at the early stages of the myogenic differentiation program [26, 27]. Effects on mitochondrial biogenesis were not assessed in these studies. To circumvent any potential myogenesis-promoting effects of GSK-3 inactivation in our study, we applied our siRNA constructs or CHIR at time-points that would not interfere with the initial phase of myogenic differentiation. Confirming this approach, we observed that GSK-3β knock-down or pharmacological inhibition of GSK-3 with CHIR did not result in profound effects on myogenic differentiation as assessed by morphological evaluation and investigation of transcript levels of established markers of myogenesis [53, 54]. This allowed addressing the role of GSK-3β in mitochondrial biogenesis in absence of any improvement of myogenic differentiation.

During myogenic differentiation, myoblasts, mainly utilizing glucose via glycolysis as energy source, differentiate into multi-nucleated myotubes that are more dependent on oxidative phosphorylation and fatty acids for their ATP production [55]. Illustrative of this fact is that *e.g.* the abundance of OXPHOS proteins but also activity of key enzymes involved in fatty acid β-oxidation dramatically increase during myogenic differentiation [14, 15]. In addition, regeneration of the skeletal musculature was shown to be accompanied by an evident activation of mitochondrial biogenesis [56]. To accommodate these alterations in metabolic requirements of the muscle cell during myogenesis and regeneration, activation of the PGC-1 pathway is required. Illustrative of this fact is, not only that increased PGC-1 signaling and subsequent activation of

mitochondrial biogenesis has been described during myogenic differentiation as well as during muscle regeneration [56], but also that mitochondrial biogenesis as well as intact PGC-1 signaling was shown to be required for adequate muscle regeneration [57] and proper myogenic differentiation of myoblasts to myotubes *in vitro* [20]. Collectively, those observations are in line with our findings as, we observed potent increases in expression levels of key components of the PGC-1 signaling network which were paralleled with increases in multiple parameters and key regulators of mitochondrial oxidative metabolism both during myogenic differentiation *in vitro* as well as during the muscle reloading phase *in vivo*.

In line with the fact that myotubes become more dependent on OXPHOS and fatty acid utilization for ATP production during myogenic differentiation, we report a myogenic differentiation-associated increase in HAD enzyme activity and *Acadvl* expression as well as a myogenesis-associated increase in *Pdk4* gene expression. The latter appeared more pronounced after inactivation of GSK-3 by CHIR. PGC-1α promotes mitochondrial biogenesis and respiration in skeletal muscle demonstrating the ability of PGC-1α to control the utilization of oxidative metabolism over glycolytic energy production by the cell. PGC-1α is also known to regulate *Pdk4* gene expression in muscle via co-activation of ERRα [58, 59] and PPARδ [60], which could explain the increase in *Pdk4* mRNA after CHIR treatment as observed in our study. Why this was not observed after knock-down of the GSK-3β protein however remains to be established. Although PDK4 is mainly controlled at the transcriptional level, we did not directly measure PDK4 protein abundance or activity or glycolytic input in the TCA cycle precluding any hard conclusions regarding this. Moreover, although HAD activity was increased in response to knock-down of GSK-3β, the opposite was observed in response to CHIR treatment. In this regard, as we did not assess substrate metabolism (*e.g.* fatty acid oxidation rate) directly in our *in vitro* system it remains to be established whether or not inactivation of GSK-3β truly alters substrate use or specifically activates the fatty acid β-oxidation pathway during myogenesis.

Myogenic differentiation of myoblasts into multi-nucleated myotubes *in vitro* recapitulates the two final phases of postnatal myogenesis (the process where quiescent satellite cells become activated, proliferate, differentiate and fuse with existing myofibers) which are essential components of the regenerative response of the skeletal musculature after an insult such as disuse or inactivity [61, 62]. For example, important mediators of myogenesis are crucial for adequate regeneration of the skeletal musculature, more specifically *e.g.* Pax7+ satellite cells are essential for muscle regeneration [10, 63] and MyoD deficient skeletal muscle shows impaired muscle regenerative capacity [64], highlighting the requirements of myogenesis in

muscle regeneration (for review see [65]). *In vitro*, we show that potentiation of myogenic differentiation-associated increases of PGC-1α and the development of OXPHOS are likely independent of altered myogenic differentiation. *In vivo*, we report that muscle-specific GSK-3β KO results in increased gene expression of several important PGC-1 signaling constituents and nuclear-encoded OXPHOS subunits in response to reloading, but in contrast to our *in vitro* data, it remains unclear whether the effect of GSK-3β KO on the regulation of mitochondrial metabolism by PGC-1 *in vivo* represents a direct effect of GSK-3β inactivation on the regulation of cellular oxidative metabolism or whether this is a reflection of an enhanced regenerative response in these animals. Relevant in this regard is that, as previously described by our group, reloading of the musculature after disuse-induced atrophy, shown by a significant weight loss of *M. gastrocnemius* as well as *M.soleus* in both genotypes, in the same animals used for this study resulted in an activation of cell proliferation and induced the expression of myogenesis-associated genes, *i.e. MyoD* and *Myogenin*, indicative of a muscle regenerative response, which was more pronounced in GSK-3β KO animals compared to WT littermate controls [35].

Furthermore, it remains unclear why reloading in our study did not result in an increase in total protein abundance of PGC-1α and OXPHOS complexes nor in enzyme activity of CS and HAD in *M. gastrocnemius*. Potentially, in the present study, the disuse-induced loss of PGC-1α and OXPHOS protein abundance as well as HAD and CS enzyme activity levels was too mild to induce increases in these parameters during the RL-phase. Within current literature, contradicting findings have been published regarding the loss of OXPHOS and PGC-1α protein following HLS and concomitant recovery of these protein levels during reloading. For example, it was previously shown that OXPHOS and PGC-1α protein levels failed to be reduced in *M. gastrocnemius* after 3 or 7-day HLS of C57BL/6 mice [66]. In rats however 14-day HLS did result in a significant loss of PGC-1α and OXPHOS sub-unit COXIV protein content, which recovered during reloading of the hind-limbs [13] and another study confirmed these findings by showing that reloading of the skeletal musculature after 3-week HLS in rats did result in increased levels of the PGC-1α protein [8]. From this, and in light of our own data, it appears that the species of the experimental animals used influences results with findings being less pronounced or even absent in mice compared to rats.

In addition, besides contradicting data on OXPHOS and PGC-1α protein levels in response to unloading and subsequent reloading of the skeletal musculature, data on changes in activity of mitochondrial enzymes in response to unloading are also controversial. For example, activity of both CS and HAD was found to decrease in rat

M. soleus after 10 days of HLS, however, the activity of these enzymes was unchanged in *M. gastrocnemius* [67]. In addition, CS gene expression was not decreased after 14-days of HLS in mouse *M. soleus* [68] and CS activity did not change upon 3 weeks of HLS in rat *M. extensor digitorum longus* and *M. soleus* [69]. Although the lack of loss of activity of mitochondrial enzymes after HLS was surprising, even though literature is inconclusive, it might be that only a small subset of the mitochondria are affected by HLS or that specific muscles show differential responses. In this context, previously it has been shown that subsarcolemmal mitochondria, which only account for approximately 25% of the mitochondria in skeletal muscle [70], are largely lost by HLS while intermyofibrillar mitochondria are spared [71] which may explain the absence of any significant detectable changes in these parameters in whole muscle homogenates of *M. gastrocnemius* in our study.

Furthermore, an alternative explanation for the absence of increased PGC-1α protein levels, and down-stream targets, in whole muscle homogenates during the reloading phase could be that the effect of GSK-3β knock-down on PGC-1α abundance is specific for satellite cells activated in the regenerating muscle, which only account for a small proportion of the muscle regeneration response, rather than in the intact muscle fibers during regeneration. In addition, as it is known that PGC-1α can shuttle between cellular compartments including the nucleus and mitochondrion [72], affecting its function, we may have missed relocalization of PGC-1α between these compartments in response to reloading. Also, PGC-1α is subject to control by post-transcriptional modifications. For example, upon activation, AMPK can phosphorylate and activate PGC-1α [73]. Furthermore, sirtuin 1 can deacetylate and activate PGC-1α [74]. In this regard, although we did not investigate potential effects of GSK-3 inactivation on PGC-1 by post-translational modifications, GSK-3β has been shown to modulate PGC-1α protein stability. More specifically, it was found that GSK-3β can phosphorylate PGC-1α thereby marking it for ubiquitination and subsequent degradation [75, 76]. However, we did not investigate post-transcriptional modification of the PGC-1α protein (for review see [77]), known to influence its activity. Whether or not the changes we observed in reloading-induced changes in metabolic gene expression and its regulation by the PGC-1 signaling network in GSK-3 KO animals compared to WT animals culminate in a physiologically-relevant improvement of mitochondrial function or muscle contractile properties remains to be established. Investigations into *in vivo* changes in the contractile properties of the musculature were not the primary goal and therefore, we did not measure exercise capacity or other functional read-outs for muscle function, such as muscle oxygen consumption or fatigability. This, however, is an interesting aspect for future research.

In conclusion, we report an interaction between inactivation or loss of the GSK-3β protein and cellular energy metabolism during *in vitro* myogenic differentiation and regeneration of the musculature *in vivo*. In concert with the appreciated role of GSK-3β in the regulation of skeletal muscle protein turnover [28, 78], this highlights an intricate link between pathways involved in the regulation of skeletal muscle energy production and those controlling muscle mass. Nutritional or pharmacological interventions aiming to inactivate GSK-3β might prove of value during aging [79] and in chronic diseases where skeletal muscle oxidative phenotype is impaired, such as COPD [5, 80], type 2 diabetes mellitus [3] and chronic heart failure [4], in particular as impairments in the PGC-1 pathway has been described in these disorders [81-83] and likely underlie the observed abnormalities in muscle mitochondrial metabolism in these diseases. Gaining more insight in the regulation of oxidative metabolism, during regeneration/myogenesis and muscle maintenance, is crucial to improve understanding of the molecular mechanisms governing oxidative energy metabolism and is imperative to design novel treatments aimed at improving skeletal muscle oxidative metabolism and related physical functioning in chronic disease management, prevention and to improve effectiveness of training interventions.

Acknowledgements

We would like to thanks both Satish Patel and Jim R. Woodgett for providing us with the skeletal muscle-specific (M)GSK-3β KO C57/Bl6 mouse strain. This work was financially supported by a grant from the Dutch Lung foundation (5.2.13.067).

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Supplemental information

Supplemental methods

The sequences of the primer pairs used for detection of gene expression with qPCR are listed below (Table 1).

Supplemental table 1. qPCR primer sequences.

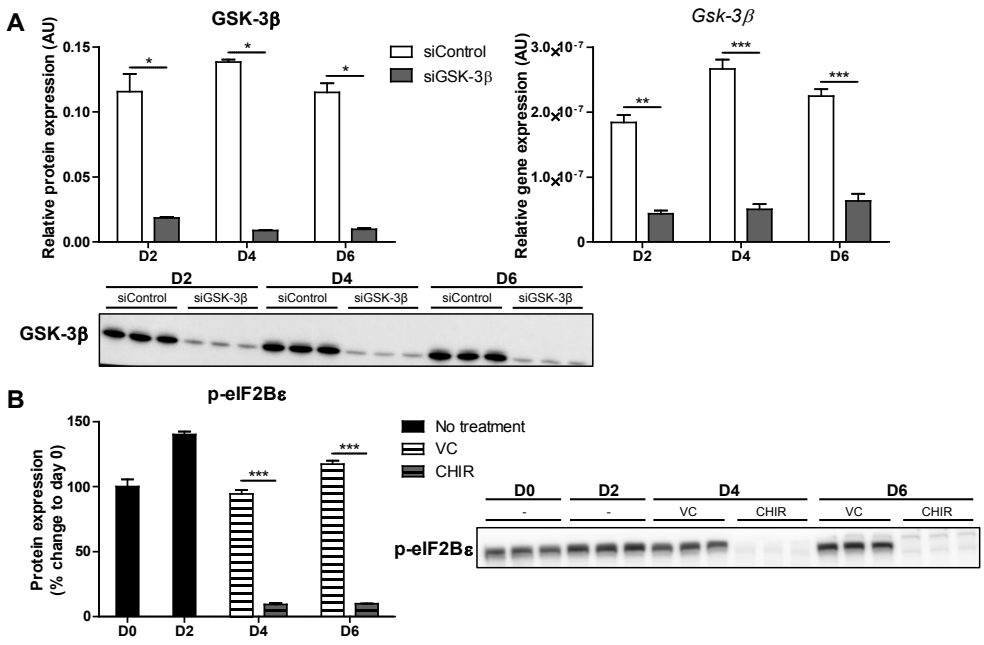
Gene	Forward primer (5'–3')	Reverse primer (5'–3')
Gsk-3β	CCACATGCTCGGATTCAGGC	TGTCCACGGTCTCCAGCATTAGTAT
Pgc-1α	CAACAATGAGCCTGCGAACA	CTTCATCCACGGGGAGACTG
Pgc-1β	ACCCTGAGAAAGCGCAATGA	CCCAGATGAGGGAAGGGACT
Errα	GGCGGACGGCAGAAGTACAAA	GCGACACCAGAGCGTTCAC
Perm1	GCTTAACAGTCAGGAAGGCAGGG	ACAGCCCAAGTGGCAAAGGCAAC
Pparα	ACTACGGAGTTCACGCATGTG	TTGTCGTACACCAGCTTCAGC
Pparδ	AGGCCCGGAGCATCCTCA	TGGATGACAAAGGGTGCGTTG
Nrf-1	AGCCACATTGGCTGATGCTT	GGTCATTTCACCGCCCTGTA
Nrf-2α	TGCTGCACTGGAAGGCTACA	TTACCCAAACCACCCAATGC
Tfam	CCGGCAGAGACGGTTAAAAA	TCATCCTTTGCCTCCTGGAA
Ndufb3	ACAGACAGTGGAAAATTGAAGGG	GCCCATGTATCTCCAAGCCT
Sdhb	AATTTGCCATTTACCGATGGGA	AGCATCCAACACCATAGGTCC
Cyc1	GCATTCGGAGGGGTTTCCAG	CCGCATGAACATCTCCCCA
CoxII	CCATCCCAGGCCGACTAA	ATTTCAGAGCATTGGCCATAGAA
HkII	AACCTCAAAGTGACGGTGGGC	AAGGACACGTCACATTTCGGAGC
Pfkfb3	AGAACTTCCACTCTCCCACCCAAA	AGGGTAGTGCCCATTGTTGAAGGA
Glut1	TGACCATCGCCCTGGCCT	GGACCAGGGCCTACTTCAAAGAAG
Pdk-4	AGGTTATGGGACAGACGCTATCATCTACT	AGACTGGGAGCTTTTCTACAGACTCAGA
Acadvl	AGACGGAGGACAGGAATCGG	ACCACGGTGGCAAATTGATC
Mck	AGGTTTTCCGCCGCTTCT	CGGTGCCCAGGTTGGA
MyoD	GGCCGTGGCAGCGAG	CGCTGTAATCCATCATGCCAT
Myogenin	CCCATGGTGCCCAGTGAA	GCAGATTGTGGGCGTCTGTA
Reference genes		
β2M	CTTTCTGGTGCTTGTCTCACTGA	GTATGTTCGGCTTCCCATTCTC
Rpl13a	CACTCTGGAGGAGAAACGGAAGG	GCAGGCATGAGGCAAACAGTC
CycloA	TTCCTCCTTTCACAGAATTATTCCA	CCGCCAGTGCCATTATGG
Hprt	TGGATATGCCCTTGACTATAATGAGTAC	AGGACTCCTCGTATTTGCAGATTC
Calnexin	GCAGCGACCTATGATTGACAACC	GCTCCAAACCAATAGCACTGAAAGG

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, *Perm1*: PGC-1 and ERR-induced regulator in muscle 1, *Gsk-3β*: glycogen synthase kinase-3β, *Ndufb3*: NADH:Ubiquinone Oxidoreductase Subunit B3, *Sdhb*: Succinate Dehydrogenase Complex Iron Sulfur Subunit B, *Cyc1*: cytochrome C1, *CoxII*: Cytochrome c oxidase II, *Acadvl*: very long-chain specific acyl-CoA dehydrogenase, *HkII*: hexokinase II, *Pfkfb3*: 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3, *Glut*: glucose transporter, *Mck*: muscle creatine kinase, *MyoD*: myogenic differentiation 1, *β2M*: β2 microglobulin, *Rpl13a*: ribosomal protein L13a, *CycloA*: cyclophilin A and *Hprt*: hypoxanthine-guanine phosphoribosyltransferase.

Supplemental results

Knock-down of GSK-3β and pharmacological inhibition of GSK-3 using CHIR99021

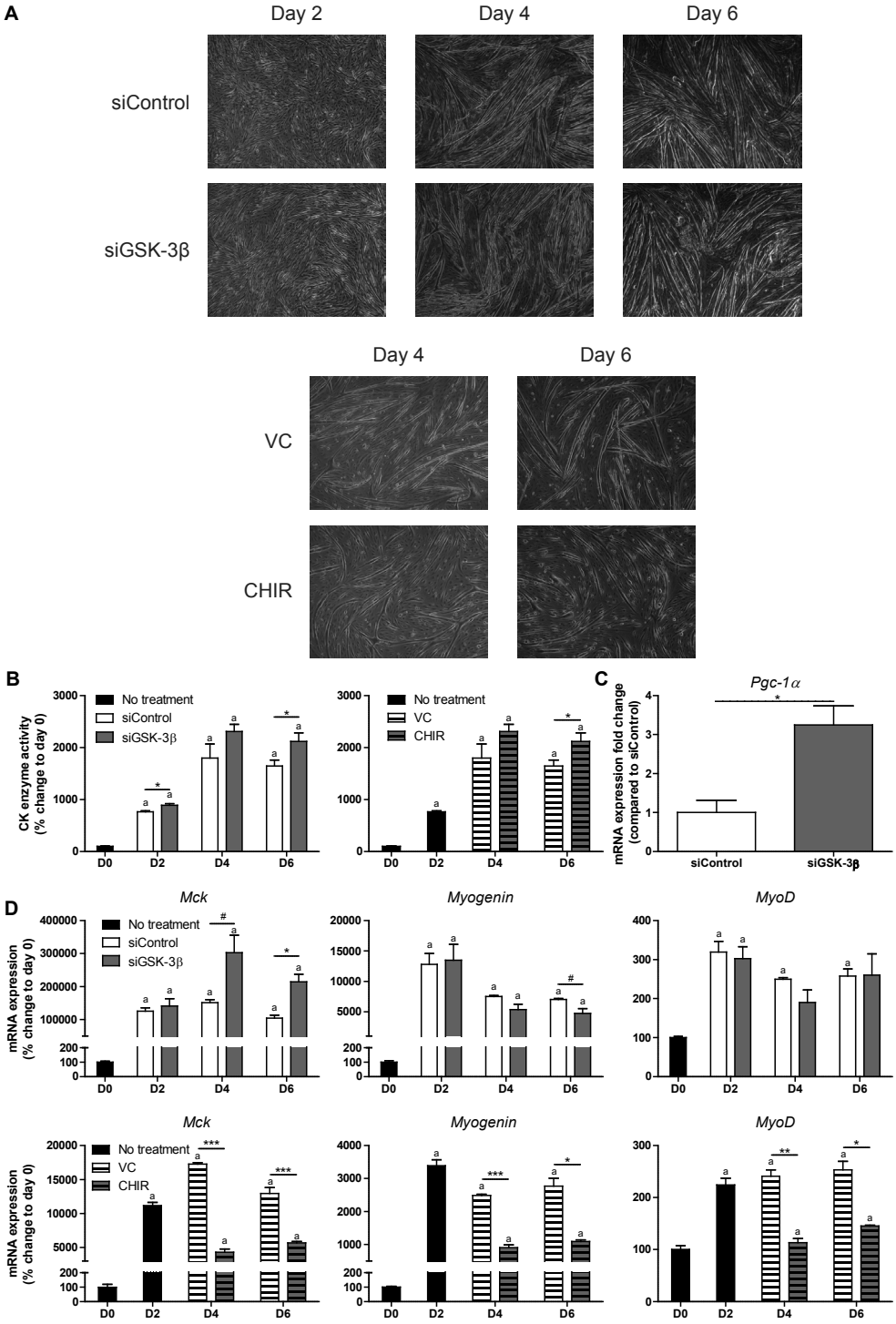
Knock-down of GSK-3β was verified by potent reductions of both protein levels and gene expression of GSK-3β during myogenic differentiation (Fig. S1A). Also, inhibition of GSK-3 using the small molecule inhibitor CHIR99021 (CHIR) reduced the phosphorylation status of a well-known direct downstream target of GSK-3, eIF2Bε (Fig. S1B).



Supplemental figure 1. GSK-3β knock-down and pharmacological inhibition of GSK-3 with CHIR99021. C2C12 myoblast were transfected with siRNA constructs targeting GSK-3β (grey) or a low GC scrambled control (white) on day 0 of differentiation or treated with CHIR99021 (striated grey) (final concentration: 7 μM) or DMSO as vehicle (striated white) on day 2 and 4 of differentiation. Cells were harvested at different time-points of differentiation (D0, D2, D4 and D6). (A) Protein and mRNA levels of GSK-3β after knock-down of GSK-3β and (B) phosphorylation status of eIF2Bε after treatment with CHIR during myogenic differentiation. Representative pictures of the blots used for quantification of the graphs in this figure are shown and Western blot data is corrected for total protein loading assessed by Ponceau S staining. All data are expressed as means ± SEM from triplicates (protein and mRNA) (n = 3 experiments). Statistical differences are tested using an unpaired Student's t-test between treatments per time-point, * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$ vs. siControl or vehicle (VC). D: day, GSK-3β: glycogen synthase kinase-3β, AU: arbitrary unit and p-eIF2Bε: phosphorylated eukaryotic translation initiation factor 2β.

Knock-down of GSK-3β or pharmacological inactivation of GSK-3 post-initiation of myogenic differentiation does not enhance *in vitro* myogenesis

With the exception of a small effect on creatine kinase (CK) gene expression and activity, evaluation of cellular morphology as well as *Myogenin* and *MyoD* mRNA transcript abundance revealed that inactivation of GSK-3 in our study did not significantly enhance the myogenic differentiation process (Fig. S2A-D). Whereas GSK-3β blockade upon initiation of myogenic differentiation enhances early myogenesis [26, 27], this data demonstrates that no profound stimulation of this process occurs when GSK-3β was inhibited at a later phase during the myogenic differentiation program. Importantly, this allowed addressing the role of GSK-3β in mitochondrial biogenesis in absence of any alterations in myogenic differentiation.

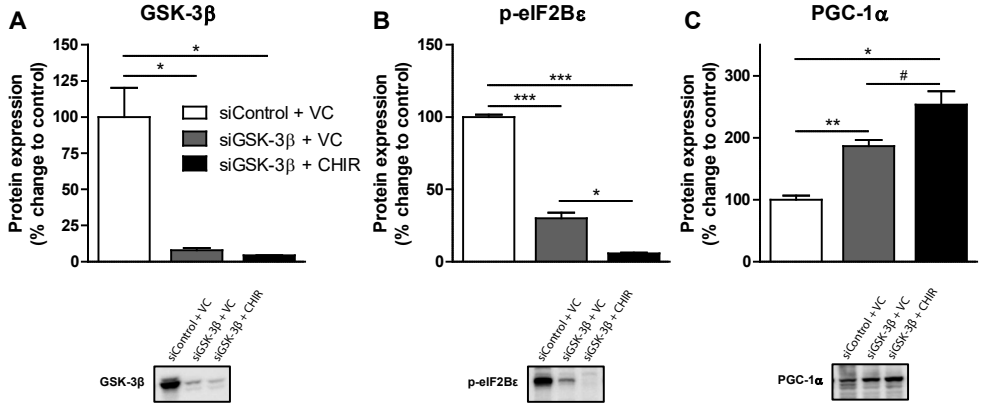

A
Day 2
Day 4
Day 6
siControl
siGSK-3β
Day 4
Day 6
VC
CHIR
B
CK enzyme activity (% change to day 0)
No treatment
siControl
siGSK-3β
No treatment
VC
CHIR
D0
D2
D4
D6
C
Pgc-1α
mRNA expression fold change (compared to siControl)
siControl
siGSK-3β
D
Mck
Myogenin
MyoD
mRNA expression (% change to day 0)
No treatment
siControl
siGSK-3β
No treatment
VC
CHIR

Supplemental figure 2. GSK-3β knock-down and pharmacological inhibition of GSK-3 with CHIR99021 does not enhance myogenic differentiation. C2C12 myoblast were transfected with siRNA constructs targeting GSK-3β (grey) or a low GC scrambled control (white) on day 0 of differentiation or treated with CHIR99021 (striated grey) (final concentration: 7 µM) or DMSO as vehicle (striated white) on day 2 and 4 of differentiation. Pictures were taken and cells were harvested at different time-points of differentiation (D0, D2, D4 and D6). (A) Pictures of C2C12 morphology during differentiation after GSK-3β knock-down or treatment with CHIR. (B) Enzymatic activity of CK and (C) gene expression of myogenic differentiation markers (*Mck*, *Myogenin* and *MyoD*) after GSK-3β knock-down or pharmacological inhibition of GSK-3 with CHIR are expressed as percent change to day 0 (day 0/myoblast phase is set on 100%). All other data are expressed as means ± SEM from sextuplicates (enzyme) or triplicates (protein and mRNA) (n = 3 experiments). Statistical differences between treatments per time-point are tested using an unpaired Student's t-test, # $p \leq 0.1$, * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$. Statistical differences compared to day 0 of differentiation are tested using an unpaired Student's t-test a $p \leq 0.05$. D: day, GSK-3β: glycogen synthase kinase-3β, CK: creatine kinase, *Mck*: muscle creatine kinase and *MyoD*: myogenic differentiation 1.

4

Pharmacological inactivation of GSK-3 enhances the *in vitro* myogenesis-associated increase of PGC-1α protein after knock-down of GSK-3β

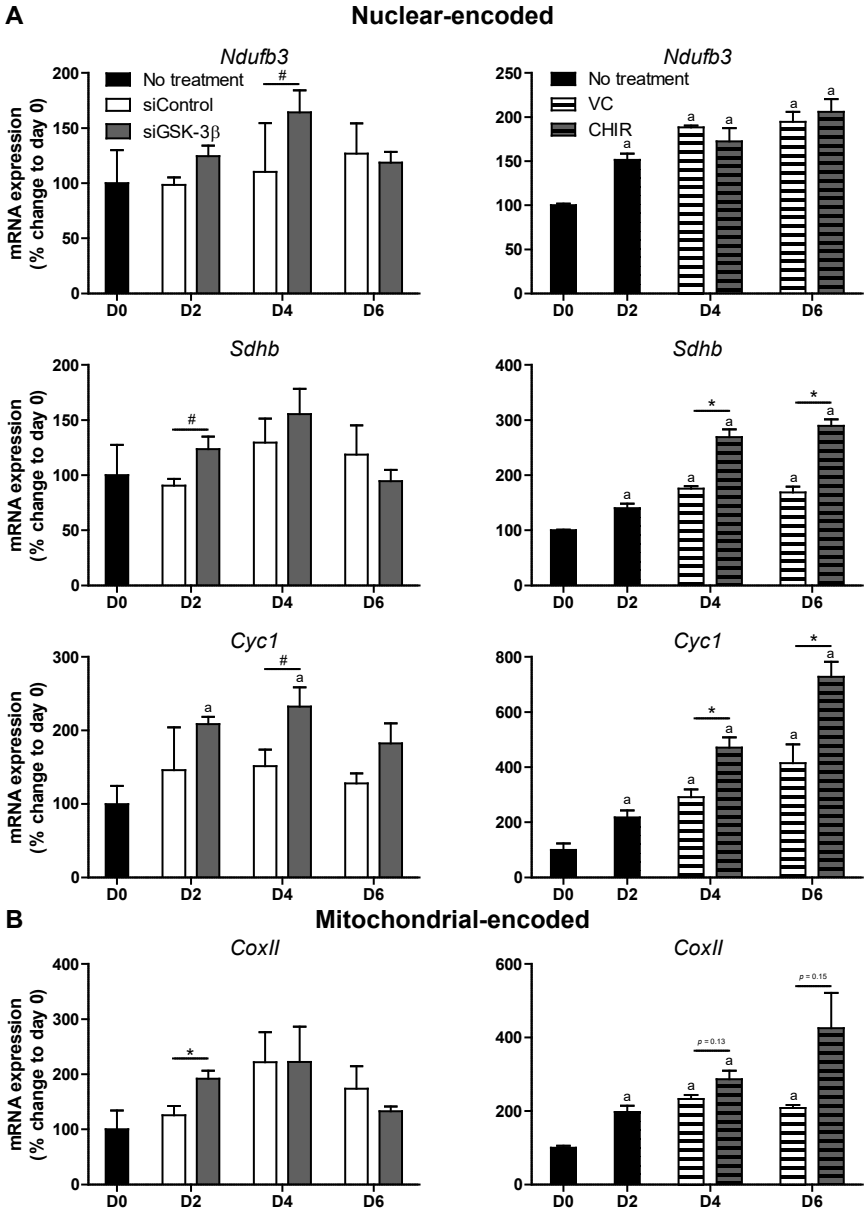
The small amount of residual GSK-3β protein left after knock-down (Fig. S3A) was inhibited by CHIR, evidenced by significantly lower phosphorylation status of eIF2Bε (Fig. S3B). This was associated with a tendency toward higher levels of PGC-1α protein (p = 0.07) when compared to GSK-3β knock-down only at day 6 of the myogenic differentiation program (Fig. S3C).



Supplemental figure 3. Pharmacological inactivation of GSK-3 enhances the *in vitro* myogenic differentiation-associated increase of PGC-1α protein after knock-down of GSK-3β. C2C12 myoblast were transfected with siRNA constructs targeting GSK-3β or a low GC scrambled control on day 0 of differentiation and treated with CHIR99021 (final concentration: 7 µM) or DMSO as vehicle on day 2 and 4 of differentiation. Cells were harvested at day 6 of differentiation (D6). (A) Protein levels of GSK-3β, (B) phosphorylation status of eIF2Bε and (C) protein levels of PGC-1α are expressed as percent change to control. Representative Western blot bands used for quantification of the graphs in this figure are shown and Western blot data is corrected for total protein loading assessed by Ponceau S staining. All data are expressed as means ± SEM from triplicates (n = 3 experiments). Statistical differences are tested using an unpaired Student's t-test between treatments, # $p \leq 0.1$, * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$ vs. siControl + vehicle (VC). GSK-3β: glycogen synthase kinase-3β, p-eIF2Bε: phosphorylated eukaryotic translation initiation factor 2β and PGC-1α: peroxisome proliferator-activated receptor gamma coactivator-1α.

Knock-down of GSK-3β or pharmacological inactivation of GSK-3 moderately potentiates myogenesis-associated increases in OXPHOS gene expression

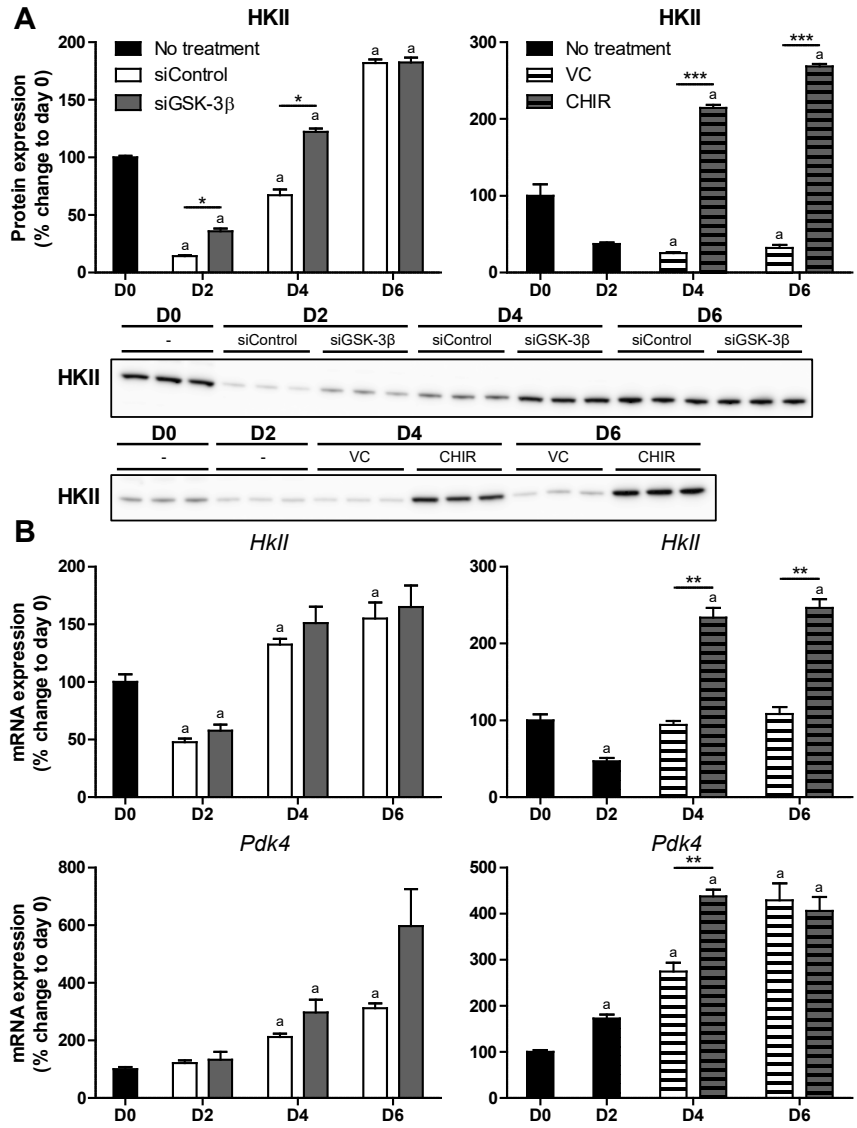
Knock-down of GSK-3β or treatment with CHIR partly potentiated myogenesis-associated increases of gene expression of several nuclear- (*Ndufb3*, *Sdhb* and *Cyc1*, respectively sub-units of CI, CII and CIII) and mitochondrial-encoded (*CoxII*, sub-unit of CIV) OXPHOS sub-units (Fig. S4A, B).



Supplemental figure 4. GSK-3β knock-down or pharmacological inhibition of GSK-3 increases gene expression of oxidative phosphorylation sub-units. C2C12 myoblasts were transfected with siRNA constructs targeting GSK-3β (grey) or a low GC scrambled control (white) on day 0 of differentiation or treated with CHIR99021 (striated grey) (final concentration: 7 μM) or DMSO as vehicle (striated white) on day 2 and 4 of differentiation. Cells were harvested at different time-points of differentiation (D0, D2, D4 and D6). Gene expression levels of sub-units of nuclear-encoded (*Ndufb3*, *Sdhb* and *Cyc1*; complex I, II and III, respectively) and mitochondrial-encoded (*CoxII*; complex IV) OXPHOS complexes after GSK-3β knock-down (left) or pharmacological inhibition of GSK-3 (right) are expressed as percent change to day 0 (day 0/myoblast phase is set on 100%). Representative pictures of the blots used for quantification of the graphs in this figure are shown and Western blot data is corrected for total protein loading assessed by Ponceau S staining. All data is expressed as means ± SEM from triplicates (n = 3 experiments). Statistical differences between treatments per time-point are tested using an unpaired Student's t-test, # $p \leq 0.1$ and * $p \leq 0.05$. Statistical differences compared to day 0 of differentiation are tested using an unpaired Student's t-test [a] $p \leq 0.05$. D: day, 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 C1 and *CoxII*: Cytochrome c oxidase II.

Knock-down of GSK-3β or pharmacological inactivation of GSK-3 potentiates myogenesis-associated increases HKII transcript and protein abundance

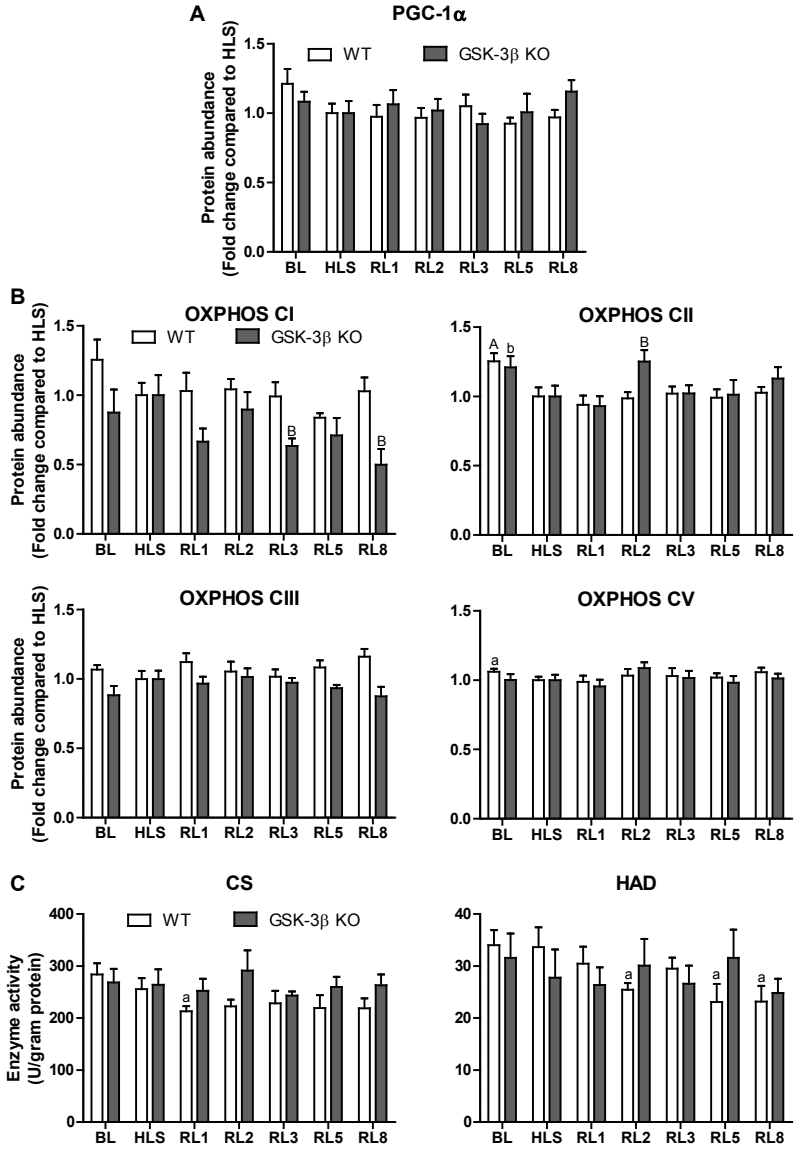
Knock-down of GSK-3β and pharmacological inhibition of GSK-3 with CHIR increased HKII protein levels during differentiation (Fig. S5A). However, gene expression of *HkII* was only increased after treatment with CHIR (Fig. S5B). In addition, the myogenesis-associated increase in gene expression of *Pdk4* was potentiated both after pharmacological inhibition of GSK-3 with CHIR and knock-down of GSK-3β (Fig. S5B).



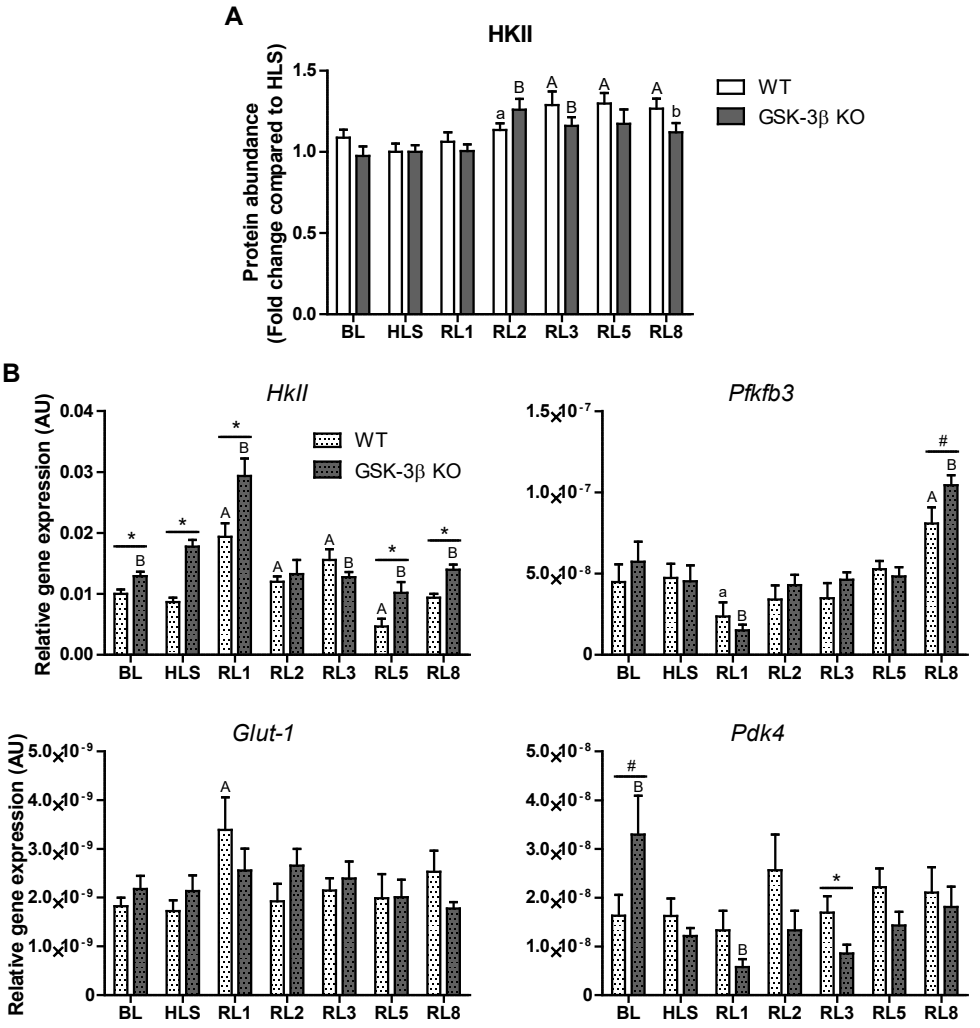
Supplemental figure 5. GSK-3β knock-down and pharmacological inhibition of GSK-3 with CHIR99021 partially increases expression levels of glycolytic constituents during myogenic differentiation. C2C12 myoblast were transfected with siRNA constructs targeting GSK-3β (grey) or a low GC scrambled control (white) on day 0 of differentiation or treated with CHIR99021 (striated grey) (final concentration: 7 μM) or DMSO as vehicle (striated white) on day 2 and 4 of differentiation. Cells were harvested at different time-points of differentiation (D0, D2, D4 and D6). (A) Protein levels of HKII and (B) gene expression of *HkII* and *Pdk4*. Representative pictures of the blots used for quantification of the graphs in this figure are shown and Western blot data is corrected for total protein loading assessed by Ponceau S staining. Gene expression is expressed as percent change to day 0 of differentiation. All data is expressed as means ± SEM from triplicates (n = 3 experiments). Statistical differences between treatments per time-point are tested using an unpaired Student's t-test, # $p \leq 0.1$, * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$. Statistical differences compared to day 0 of differentiation are tested using an unpaired Student's t-test ^{a} $p \leq 0.05$. D: day, GSK-3β: glycogen synthase kinase-3β, *HkII*: hexokinase II and *Pdk4*: pyruvate dehydrogenase kinase 4.

Reloading of disuse-induced muscle does not increase PGC-1α and OXPHOS protein levels

In both genotypes, WT and GSK-3β KO, reloading did not increase PGC-1α protein (Fig. S6A), protein expression of several OXPHOS sub-units (Fig. S6B) nor CS and HAD enzyme activity (Fig. S6C). However, protein abundance of HKII (Fig. S7A) slightly increased during reloading in both genotypes, while *HkII* gene expression was increased during reloading in GSK-3β KO animals compared to WT animals. Other genes involved in the regulation of glucose metabolism (*Glut-1*, *Pfkfb3* and *Pdk4*) where not differentially regulated during reloading of the musculature between GSK-3β KO and WT animals (Fig. S7B).



Supplemental figure 6. Reloading does not increase protein levels of PGC-1α and OXPHOS sub-units or enzyme activity of CS and HAD in WT and GSK-3β knock-out mice. Muscle-specific GSK-3β KO mice and WT littermates were subjected to HLS for 14 days, introduced back on their hind-limbs and followed during recovery (reloading). *M. gastrocnemius* was prepared for western blotting and qPCR analysis. (A) Protein abundance of PGC-1α and (B) OXPHOS sub-units and (C) enzyme activity of CS and HAD of GSK-3β KO (grey bars) and WT (white bars) mice subjected to HLS and RL. Western blot data is corrected for total protein loading assessed by Ponceau S staining. Statistical differences are tested using an unpaired Student's t-test within a genotype vs. HLS, for WT ^{a} $p \leq 0.1$, ^{A} $p \leq 0.05$ and for KO ^{b} $p \leq 0.1$, ^{B} $p \leq 0.05$, BL (n = 9/group) and HLS/RL (n = 8/group). GSK-3β: glycogen synthase kinase-3β, PGC-1α: peroxisome proliferator-activated receptor gamma coactivator-1α, OXPHOS: oxidative phosphorylation, CS: citrate synthase, HAD: β-hydroxyacyl-CoA dehydrogenase, BL: baseline, HLS: hind-limb suspension, RL: reloading, WT: wild-type and KO: knock-out.



Supplemental figure 7. Reloading does not potentiate expression of glycolytic constituents of GSK-3β knock-out mice. Muscle-specific GSK-3β KO mice and WT littermates were subjected to HLS for 14 days, introduced back on their hind-limbs and followed during recovery (reloading). *M. gastrocnemius* was prepared for western blotting and qPCR analysis. (A) Protein abundance of HKII of GSK-3β KO (grey bars) and WT (white bars) mice subjected to HLS and RL are compared to HLS. (B) Gene expression of glycolytic constituents (*HkII*, *Pfkfb3*, *Glut-1* and *Pdk4*) under BL, HLS and RL conditions in GSK-3β KO (dotted grey bars) and WT (dotted white bars) mice are expressed as arbitrary units (AU) in *M. gastrocnemius*. Western blot data is corrected for total protein loading assessed by Ponceau S staining. Statistical differences are tested using an unpaired Student's t-test between genotypes per time-point, BL (n = 9/group) and HLS/RL (n = 8/group), # $p \leq 0.1$ and * $p \leq 0.05$ and within a genotype vs. HLS, for WT ^{a} $p \leq 0.1$, ^{A} $p \leq 0.05$ and for KO ^{b} $p \leq 0.1$, ^{B} $p \leq 0.05$. GSK-3β: glycogen synthase kinase-3β, *HkII*: hexokinase II, *Pfkfb3*: 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3, *Glut-1*: glucose transporter 1, *Pdk4*: pyruvate dehydrogenase kinase 4, BL: baseline, HLS: hind-limb suspension, RL: reloading, WT: wild-type and KO: knock-out.

Supplemental references

[1] J.L. van der Velden, R.C. Langen, M.C. Kelders, E.F. Wouters, Y.M. Janssen-Heininger and A.M. Schols, Inhibition of glycogen synthase kinase-3beta activity is sufficient to stimulate myogenic differentiation, Am. J. Physiol. Cell Physiol. 290, 2006, C453–C462.
[2] J.L. van der Velden, A.M. Schols, J. Willems, M.C. Kelders and R.C. Langen, Glycogen synthase kinase 3 suppresses myogenic differentiation through negative regulation of NFATc3, J. Biol. Chem. 283, 2008, 358–366.

Chapter 5

Regulation of PGC-1α expression by a GSK-3β-TFEB signaling axis in skeletal muscle

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Biochimica et Biophysica Acta (BBA) - Molecular Cell Research. 2019 Nov 16;1867(2):118610.

Abstract

Objective

In muscle cells, the peroxisome proliferator-activated receptor γ co-activator 1 (PGC-1) signaling network, which has been shown to be disturbed in the skeletal muscle in several chronic diseases, tightly controls mitochondrial biogenesis and oxidative substrate metabolism. Previously, we showed that inactivation of glycogen synthase kinase (GSK)-3β potently increased *Pgc-1α* abundance and oxidative metabolism in skeletal muscle cells. The current study aims to unravel the molecular mechanism driving the increase in *Pgc-1α* mediated by GSK-3β inactivation.

Methods

GSK-3β was inactivated genetically or pharmacologically in C2C12 myotubes and the requirement of transcription factors known to be involved in *Pgc-1α* transcription for increases in *Pgc-1α* abundance mediated by inactivation of GSK-3β was examined.

Results

Enhanced PGC-1α promoter activation after GSK-3β inhibition suggested a transcriptionally-controlled mechanism. While myocyte enhancer factor (MEF)2 transcriptional activity was unaltered, GSK-3β inactivation increased the abundance and activity of the transcription factors estrogen-related receptor (ERR)α and ERRγ. Pharmacological inhibition or knock-down of ERRα and ERRγ however failed to prevent increases in *Pgc-1α* mRNA mediated by GSK-3β inactivation. Interestingly, GSK-3β inactivation activated transcription factor EB (TFEB), evidenced by decreased phosphorylation and enhanced nuclear localization of the TFEB protein. Moreover, knock-down of TFEB completely prevented increases in *Pgc-1α* gene expression, PGC-1α promoter activity and PGC-1α protein abundance induced by GSK-3β inactivation. Furthermore, mutation of a specific TFEB binding site on the PGC-1α promoter blocked promoter activation upon inhibition of GSK-3β.

Conclusions

In skeletal muscle, GSK-3β inactivation causes dephosphorylation and nuclear translocation of TFEB resulting in TFEB-dependent induction of *Pgc-1α* expression.

Introduction

Skeletal muscle tissue displays a high degree of plasticity both in terms of the regulation of muscle mass and in its capacity to produce energy from mitochondrial oxidative substrate metabolism [1]. Loss of skeletal muscle mass and disturbances in oxidative metabolism in (lower limb) muscle are pathophysiological aspects of several chronic diseases including chronic heart failure, type II diabetes and chronic obstructive pulmonary disease (COPD) [2-5] and even occur during normal physiological aging [6, 7]. Indicative of their clinical significance, these abnormalities culminate in reductions in skeletal muscle function leading to severe disability, reduced quality of life [8] and even increased mortality in these chronic disorders [9]. Improved understanding of the underlying molecular processes involved in the control of oxidative energy metabolism and mitochondrial biogenesis will likely accommodate the development of better treatment and prevention strategies to improve skeletal muscle function and alleviate muscle abnormalities in patients suffering from the abovementioned disorders and during aging.

Mitochondrial biogenesis and oxidative substrate metabolism in skeletal muscle are tightly controlled by the peroxisome proliferator-activated receptor γ co-activator 1 (PGC-1) signaling network [10], which has been shown to be disturbed in the musculature of COPD patients [11], type II diabetics [12, 13] and during aging [14]. Illustrative of the physiological relevance of reduced levels of PGC-1 in skeletal muscle, muscle-specific PGC-1α knock-out (KO) animals displayed a fiber-type switch towards more glycolytic muscle fibers and a reduced capacity for endurance exercise compared with wild-type animals [15]. In addition, whole-body PGC-1α KO combined with muscle-specific PGC-1β KO decreased running time till exhaustion, impaired activity of complex 1 of the electron transport chain, lowered mitochondrial (mt) DNA content and reduced fatty acid oxidative capacity [16]. *Vice versa*, muscle-specific overexpression of PGC-1α protected against muscle atrophy [17], resulted in increased mitochondrial oxidative capacity and increased the proportion of oxidative type I muscle fibers [18, 19] which was associated with increased running endurance and peak VO_2 during exercise [20, 21].

Intriguingly, evidence in non-muscle cells suggests a role for glycogen synthase kinase (GSK)-3β, originally found to regulate glycogen synthesis in muscle [22-24] and known to be involved in the control of muscle mass [25], in the regulation of mitochondrial metabolism. For example, lithium chloride, an inhibitor of GSK-3 activity, potently increased expression levels of *Pgc-1α* in neuronal [26] and aortic endothelial cells [27]. Furthermore, we recently showed that knock-down of GSK-3β as well as

pharmacological inhibition of GSK-3 with CHIR99021 (CHIR) not only increased *Pgc-1α* transcript and protein abundance, but also increased mitochondrial respiration, elevated protein abundance of several components of the mitochondrial electron transport chain and increased mtDNA copy number during myogenic differentiation [28] and in fully differentiated myotubes [29]. The molecular mechanism by which inactivation of GSK-3β mediates the increase in *Pgc-1α* mRNA transcript abundance remains to be elucidated.

In general, increased mRNA content can result from changes in mRNA stability, epigenetic DNA modifications or can be mediated by activation of specific transcription factors [30]. Transcriptional regulation of the *Pgc-1α* gene is well known to be controlled by several transcription factors able to bind the PGC-1α promoter [10], including myocyte enhancer factor (MEF)2 [31], estrogen-related receptor (ERR)α [32] and members of the microphthalmia/TFE family of transcription factors *e.g.* transcription factor binding to IGHM enhancer 3 (TFE3) [33] and transcription factor EB (TFEB) [34]. The latter have recently been described to increase *Pgc-1α* gene expression via binding to E-boxes named coordinated lysosomal expression and regulation (CLEAR)-regions on the PGC-1α promoter [33, 34]. In addition, it has been described that GSK-3β can inhibit some of these transcription factors such as MEF2 and TFEB [35, 36].

We hypothesized that one of the above mentioned transcription factors is required for increased *Pgc-1α* gene expression upon inactivation of GSK-3β. Therefore, in the present study, we genetically- and pharmacologically inactivated GSK-3β in C2C12 myotubes (*in vitro* analogues of adult skeletal muscle fibers) and subsequently investigated the molecular mechanism by which GSK-3β inactivation mediates inductions in muscle *Pgc-1α* gene expression. More specifically, we explored the association and requirement of several transcription factors with increased PGC-1α abundance mediated by inactivation of GSK-3β, focusing on MEF2, ERRα, ERRγ and TFEB.

Material and methods

Cell culture

C2C12 murine myoblasts (ATCC CRL1772, Manassas, VA, USA) were cultured as previously described [25]. In brief, myoblasts were cultured in low-glucose (1 g/l) Dulbecco's modified Eagle's medium (DMEM) (GIBCO, Carlsbad, CA, USA)

growth medium (GM) supplemented with 9% (vol/vol) fetal bovine serum (FBS) (PAA Laboratories, Pasching, Austria) and antibiotics (50 U/ml penicillin and 50 µg/ml streptomycin; both GIBCO). The myoblasts were plated in Matrigel-coated (BD Biosciences, San Jose, CA, USA) 6-well or 12-well plates (Corning life sciences, Corning, NY, USA) at a density of $1*10^4$ cells/cm^2. Cells were cultured for 24 h in GM before initiation of differentiation (day 0) by growth factor withdrawal [37] using differentiation medium (DM), consisting of low-glucose DMEM+GlutaMAX™ (GIBCO) with pyruvate, enriched with 0.5% (vol/vol) heat-inactivated (30 min at 56 °C) FBS and antibiotics (50 U/ml penicillin and 50 µg/ml streptomycin). Differentiating cells were provided with new DM on day 1, 3 and 5 of differentiation and before treatment on day 5, unless indicated otherwise.

Treatments

According to the manufacturer's guidelines, C2C12 myotubes were transfected using stealth RNAi small interfering (si)RNAs directed against GSK-3β (MSS226316), ERRα (MSS248641), ERRγ (MSS218492) or TFEB (MSS238271, all Thermo Fisher Scientific, Boston, MA, USA) to silence target gene expression with universal control siRNA (low GC scrambled control; Thermo Fisher Scientific) as control (final concentration 2–10 nM). The transfection mix was prepared in Opti-MEM reduced serum medium (Thermo Fisher Scientific) with Lipofectamine RNAiMAX (Thermo Fisher Scientific) as transfection agent. The transfection mixture 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-3 with a final concentration of 7 µM. XCT790 (X4753, Sigma-Aldrich, Saint Louis, MO, USA), a specific ERRα inverse agonist, was used to pharmacologically inhibit ERRα with a final concentration of 10 µM.

Plasmid transfections

Three hours after seeding, C2C12 myoblasts were transiently transfected with plasmids using Lipofectamine2000 (Thermo Fisher Scientific) as transfecting agent in Opti-MEM. For PGC-1α promoter activity experiments (plasmids were kindly supplied by Dr. Nancy Salma, who previously reported specific details of the promoter fragments used [33]) or MEF2 transcriptional activity experiments (pGL2-MEF2 (3×)-Luc), 200 ng of luciferase vector, 50 ng of β-galactosidase vector (pVAX1/lacZ) and 120 ng of overexpression vector (pcDNA3-MEF2A-flag, pcDNA3-MEF2C-flag or pEGFP-N1-TFEB (Addgene: #38119)) was used. The PGC-1α WT promoter contained an approximately 1000-base pair fragment of the murine PGC-1α promoter on a pGl3-basic luciferase backbone. The E-boxes located at –429 (E-box1 mutated

from 5'-CACGTG-3' to 5-'CACTTC-3') and +8 (E-box2 mutated from 5'-CACGTG-3' to 5'-GAAGTG-3') from the transcription start site were mutated [33]. For IncuCyte™ (Essen BioScience, Ltd., Welwyn Garden City, UK) microscopy experiments 500 ng of pEGFP-N1-TFEB and 500 ng of Histon 2B (H2B)-mCHERRY was used. Cells were cultured for 24 h in GM before initiation of differentiation as previously described. Cells were harvested after 24 h of differentiation unless otherwise indicated.

Cell processing

C2C12 myotubes were washed twice with pre-cooled 1× Hank's balanced salt solution (HBSS) and harvested in RLT buffer (Qiagen, Hilden, Germany) containing β-mercaptoethanol (1:100) for RNA isolation, whole cell lysate buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1% Nonidet P40) with PhosSTOP and complete protease inhibitors (both Sigma-Aldrich) for Western Blotting, whole cell lysate buffer without PhosSTOP inhibitors for phosphorylation status Western blotting, 1× reporter lysis buffer (E3971, Promega, Madison, WI, USA) for luciferase assays or Nonidet P40 lysis buffer (10 mM Tris-HCl pH 7.4, 3 mM $MgCl_2$, 10 mM NaCl and 0.5% Nonidet P40, freshly supplemented with 0.15 mM Spermine and 0.5 mM Spermidine) for the chromatin accessibility assay using cell scrapers (rubber policemen). Chromatin isolates were further processed upon harvest (see chromatin accessibility assay). All other lysates were directly stored at –80 °C.

Chromatin accessibility assay

Cell lysates were stored on ice for 5 min upon harvest and subsequently spun down for 5 min at 3000 rpm at 4 °C. The pellets were washed once with 1× nuclei wash buffer (10 mM Tris-HCl pH 7.4, 15 mM NaCl and 60 mM KCl, freshly supplemented with 0.15 mM Spermine and 0.5 mM Spermidine) and spun down for 5 min at 3000 rpm at 4 °C. The pellets were re-suspended in 200 µl MNase digestion buffer (nuclei wash buffer with 1 mM $CaCl_2$) and divided over 2 eppendorf tubes (99.5 µL/tube). MNase (50 Units) (Cat. No. 88261; Thermo Fisher Scientific) was added to one of the tubes and incubated for 5 min at RT, the other tube was used as NoMNase control. To stop the MNase digestion, a pre-warmed (37 °C) mix of 80 µl MNase digestion buffer, 20 µl MNase STOP buffer (100 mM EDTA and 10 mM EGTA), 3 µl Proteinase K (25 mg/ml) and 10 µl 20% SDS was added per sample. Samples were incubated overnight at 37 °C in a heating-block. One volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added, the samples were mixed by vigorous shaking and spun down for 15 min at maximal (max) speed at 4 °C. The aqueous phase was transferred to a new tube containing glycogen (20 µg), 0.5 volume 7.5 M NH_4OAc and 2.5 volumes of ice-cold

absolute EtOH and DNA was precipitated for 2 h at –80 °C. The samples were spun down for 15 min at max speed at 4 °C and washed twice with 70% EtOH. Air-dried pellets were dissolved in TE-buffer (1 mM Tris-HCl pH 8.0 and 0.1 mM EDTA). DNA concentration was determined using Nanodrop measurements, 100 ng of DNA was used as qRT-PCR input. Specific primers, directed to the promoter and promoter flank of the proximal PGC-1α promoter, were designed (Table S1).

RNA processing, cDNA synthesis and qRT-PCR

According to manufacturer's guidelines, RNA was isolated with the RNeasy Mini Kit (Cat. No. 74106, Qiagen) and cDNA was synthesized from 400 ng RNA with the tetro cDNA synthesis kit (Bioline, Singapore, Singapore). 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 (Table S1) per sample. 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 with white 384-well plates (Roche, Basel, Switzerland). 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 and checked for double peaks using the LightCycler480 software (Roche). Expression of the genes of interest was analyzed using LinRegPCR software and normalized with a correction factor calculated by GeNorm [38] based on the expression levels of a combination of at least four reference genes (*Calnexin*, *Rpl13a*, *Hprt*, *CycloA*, *Gusb* or *β2M*).

Lambda protein phosphatase treatment

Cell lysates were thawed on ice, spun down for 5 min at max speed at 4 °C and total protein content was assessed in the supernatant with the Pierce BCA protein assay kit (Thermo Fisher Scientific) according to manufacturer's guidelines. Samples were prepared in the presence or absence of Lambda phosphatase (LP) (P0753S, New England Biolabs, Ipswich, MA, USA) and phosphatase inhibitors (Sigma) and supplemented with MnCl, NEBuffer according to manufacturer's guidelines. LP digestion was performed for 60 min at 30 °C or without LP on ice as control. The samples 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) and boiled for 5 min at 95 °C.

Western blot

Whole cell lysates were thawed on ice, spun down for 5 min at max speed at 4 °C and total protein content was assessed in the supernatant with the Pierce BCA protein assay kit. The supernatants were diluted in Laemmli sample buffer to 1× as final concentration, adjusted to 0.5–1.5 µg/µl and boiled for 5 min at 95 °C. The samples were loaded, between 5 and 10 µg of 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 until full separation as indicated by the protein ladder. The proteins on the gel were transferred to a 0.45 µm Whatman Protran Nitrocellulose Transfer membrane (Whatman, Maidstone, UK) by electroblotting (BioRad Criterion Blotter) at 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 1h using 3% non-fat dry milk (NFDM) (FrieslandCampina, Amersfoort, the Netherlands) in Tris-buffered saline with 0.1% Tween 20 (TBST) and incubated overnight at 4 °C with primary antibodies directed to: GSK-3β (610201, BD Biosciences), PGC-1α (Cat. No. 516557 (Anti-PGC-1α, C-Terminal (777–797) Rabbit pAb), Calbiochem), TFEB (PA1–31552,Thermo Fisher Scientific), MEF2 (ab197070), ERRα (ab76228, both Abcam, Cambridge, UK) or phosphorylated eukaryotic translation initiation factor 4E-binding protein 1 (p-4E-BP1) (#9451, Cell Signaling, Danvers, MA, USA) diluted in TBST with 3% NFDM/Bovine serum albumin. The blots were probed with a peroxidase-conjugated secondary antibody (Vector Laboratories, Burlingame, CA, USA) diluted in TBST with 3% NFDM and visualized using SuperSignal West Pico or SuperSignal West Femto Chemiluminescent Substrate (both Thermo Fisher Scientific) 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, Waukesha, WI, USA). All Western blot data was corrected for total protein loading assessed by Ponceau S staining.

IncuCyte™ microscopy and image analysis

C2C12 myotubes were treated with CHIR (7 µM), Torin1 (300 nM) (S2827, Bio-Connect B.V., Huissen, The Netherlands) or DMSO as vehicle (VC) control. Pictures were made immediately after and after 1 h, 3 h, 16 h and 24 h of the treatment using the IncuCyte™ imager using the IncuCyte™ ZOOM 20× objective in the bright field, green and red channel. Nine pictures were taken across all wells on each time-point. Spectral unmixing (1.8% red removed from green) was applied using

the IncuCyte™ software. Well-differentiated myotubes were selected and all nuclei in these myotubes were counted. Nuclear abundance of TFEB-GFP, assessed by determining a clear (green) nuclear shape co-localized with H2B-mCHERRY, was semi-quantitatively determined in a blinded-controlled setting by 2 independent observers and averaged. TFEB positive nuclei (clear nuclear shape visible in the GFP channel) were counted in three independent wells for CHIR treatment and 2 independent wells for Torin1 treatment.

Statistical analysis

Data were analyzed using an unpaired Student's t-test or one-way ANOVA with a Bonferroni as post-hoc test with IBM SPSS Statistics 25 software (IBM Corp., Armonk, NY, USA). Data is expressed as mean ± SEM and significant differences are indicated as # $p \leq 0.1$, * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$ in each figure. Graphs were made using GraphPad Prism 5.0 software (GraphPad, Prism, La Jolla, CA, USA) and representative pictures of the Western blots and IncuCyte™ microscopy were added using the Adobe Illustrator CS6 software (Adobe Systems Incorporated, San Jose, CA, USA).

Results

GSK-3β inactivation results in activation of the proximal PGC-1α promoter and increases *Pgc-1α* transcript abundance and PGC-1α protein levels

Previously, we reported that knock-down of GSK-3β for 48 h resulted in a marked increase in *Pgc-1α* mRNA abundance in C2C12 myotubes [29]. Characterizing this in more detail, we now show that knock-down of GSK-3β (Fig. S1) time-dependently increased *Pgc-1α* mRNA levels with maximal upregulation observed after 48 h (Fig. 1A). In line with these results, *Pgc-1α* mRNA levels were significantly increased 48 h after pharmacological inhibition of GSK-3β with CHIR (Fig. 1B). Several *Pgc-1α* isoforms have been described which can be expressed from two promoters, namely *Pgc-1α-a* (transcribed from the proximal PGC-1α promoter) and *Pgc-1α-b* and *Pgc-1α-c* (both transcribed from the alternative PGC-1α promoter) [39]. Both knock-down as well as pharmacological inhibition of GSK-3β in our study increased *Pgc-1α-a* transcript abundance (Fig. 1C, D), but not the *Pgc-1α-b* and *Pgc-1α-c* isoforms (n.d.; non-detectable), suggesting specific activation of the proximal PGC-1α promoter. In line with this pattern in mRNA transcript levels, activity of the proximal PGC-1α promoter (luciferase activity) was increased upon inhibition of GSK-3β (Fig. 1E). Ultimately, inhibition of GSK-3 resulted in increased PGC-1α protein abundance (Fig. 1F).

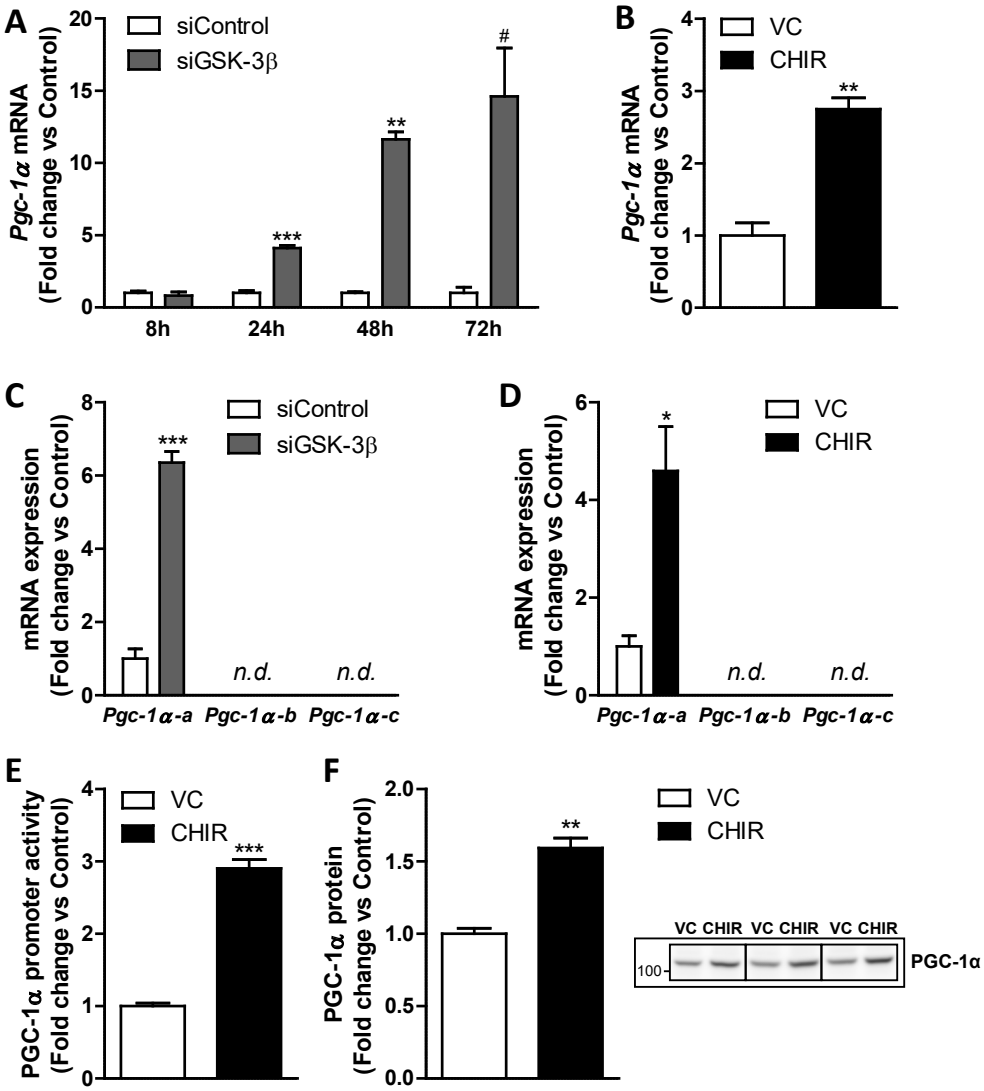


Figure 1. Inactivation of GSK-3β increases PGC-1α mRNA transcript and protein levels and enhances PGC-1α promoter activation. C2C12 myotubes were differentiated for 5 days and GSK-3β was silenced using siRNAs directed against GSK-3β (grey) or pharmacologically inhibited using CHIR (black) and compared to scrambled control or DMSO as vehicle (VC) (white bars) respectively. Gene expression of (A, B) *Pgc-1α*, (C, D) *Pgc-1α* isoforms (a, b, c) was measured 48 h after treatment if not specified otherwise. (E) PGC-1α promoter activity (luciferase activity corrected for β-galactosidase activity) was measured 24 h after CHIR treatment. (F) Protein abundance of PGC-1α was measured 48 h after treatment. Representative pictures of the Western blots are shown, the black box around the representative pictures indicates that they were cut from the same Western blot. All data is expressed as means ± SEM from n = 3/experimental condition (mRNA and protein) or n = 6/experimental condition (luciferase activity) (n = 3 experiments). Statistical differences on each time-point were tested using an unpaired Student's t-test and compared to control, # $p \leq 0.1$, * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$. n.d. = non-detectable.

The enhanced activation of the PGC-1α promoter suggested that increases in transcript and protein levels of PGC-1α upon inactivation of GSK-3 are likely regulated at the transcriptional level. These changes did not appear to rely on changes in chromatin structure, as knock-down of GSK-3β did not alter chromatin accessibility of the proximal PGC-1α promoter in C2C12 myotubes (Fig. S2). In order to investigate involvement of specific transcription factors in increased *Pgc-1α* gene expression in response to inactivation of GSK-3β, we next investigated several candidate transcription factors known to regulate *Pgc-1α* gene expression by binding to the proximal PGC-1α promoter [10, 31, 32, 34]. First, we investigated whether or not inactivation of GSK-3β was associated with activation of the respective transcription factor and, if so, we subsequently assessed whether the transcription factor was required for inductions in transcript and protein levels of PGC-1α mediated by inactivation of GSK-3β.

Inactivation of GSK-3β does not alter MEF2 transcriptional activity

We first focused on the MEF2 family of transcription factors, which are well-known to induce *Pgc-1α* gene expression by binding to specific MEF2 binding sites on the PGC-1α promoter [31]. At time-points preceding inductions of *Pgc-1α* mRNA abundance mediated by inactivation of GSK-3β (Fig. 1A), gene expression of the MEF2A, MEF2C and MEF2D isoforms were largely unaltered (data not shown). Overexpression of both the MEF2A as well as the MEF2C isoform resulted in a 50- and 160-fold induction of MEF2 transcriptional activity respectively (Fig. 2A, B), thereby confirming sensitivity and specificity of the reporter construct. Then, we assessed MEF2 transcriptional activity after inactivation of GSK-3β. Modulation of GSK-3β activity by knock-down of the GSK-3β protein or pharmacological inhibition of GSK-3 did not alter MEF2 transcriptional activity (Fig. 2C), showing that inactivation of GSK-3 is not associated with alterations in MEF2 transcriptional activity. It is therefore unlikely that increases in *Pgc-1α* gene expression upon GSK-3β inactivation are transcriptionally-regulated via MEF2.

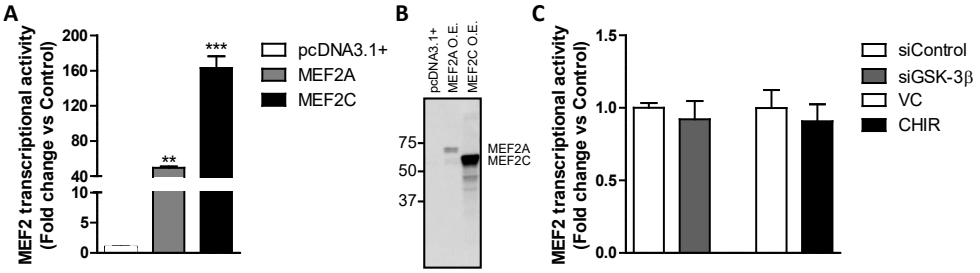


Figure 2. MEF2 transcriptional activity is unaltered upon inactivation of GSK-3β. C2C12 myoblasts were transiently transfected with a 3x-MEF2 binding domain merged to a luciferase gene. (A) MEF2 transcriptional activity was measured after overexpression of MEF2A (grey) or MEF2C (black) and compared to pcDNA3.1+ (white). (B) MEF2A and MEF2C overexpression (O.E.) was verified by Western Blotting. (C) GSK-3β was silenced using siRNAs directed against GSK-3β (grey) or pharmacologically inhibited using CHIR (black) and compared to scrambled control or DMSO as vehicle (VC) (white), respectively. Luciferase activity was corrected for β-galactosidase activity. All data is expressed as means ± SEM from n = 3/experimental condition. Statistical differences were tested using an unpaired Student's t-test and compared to control, ** $p \leq 0.01$ and *** $p \leq 0.001$.

5

ERRα and ERRγ expression levels are increased by GSK-3β inactivation, but are not required for induction of *Pgc-1α* transcript levels

Secondly, we focused on the transcription factors ERRα and ERRγ, both known to be able to bind the PGC-1α promoter and induce *Pgc-1α* expression [32, 40]. Interestingly, increases in *Pgc-1α* mRNA levels mediated by (genetic or pharmacological) inactivation of GSK-3β were accompanied by significant increases in transcript levels of *Errα* and *Errγ*, increased ERRα protein abundance and a potent induction in transcript abundance of the ERR-target gene PGC-1 and ERR-induced regulator in muscle (*Perm1*) (Fig. 3A-H). We next investigated whether ERRs were required for inductions in *Pgc-1α* expression in the context of GSK-3β inactivation. For this, we first used XCT790, a specific ERRα inverse agonist [41]. Inhibition of ERRα with XCT790 prevented upregulation of ERR target genes *Perm1* and *Pdk4* mediated by knock-down of GSK-3β, indicating effectiveness of XCT790 for inhibiting ERRα (Fig. S3A, B). However, as depicted in Fig. 3I, knock-down of GSK-3β still induced *Pgc-1α* gene expression after treatment with XCT790, although the induction of *Pgc-1α* gene expression was attenuated (Fig. 3I). As potential involvement of ERRγ was not addressed in this experiment, both ERR isoforms ERRα and ERRγ were subsequently silenced simultaneously using a genetic approach (double knock-down, Fig. S3C, D). Combined ERRα/γ knock-down did not affect the induction of *Pgc-1α* gene expression after knock-down of GSK-3β (Fig. 3J), suggesting that neither ERRα nor ERRγ are required for increases in *Pgc-1α* gene expression mediated by inactivation of GSK-3β.

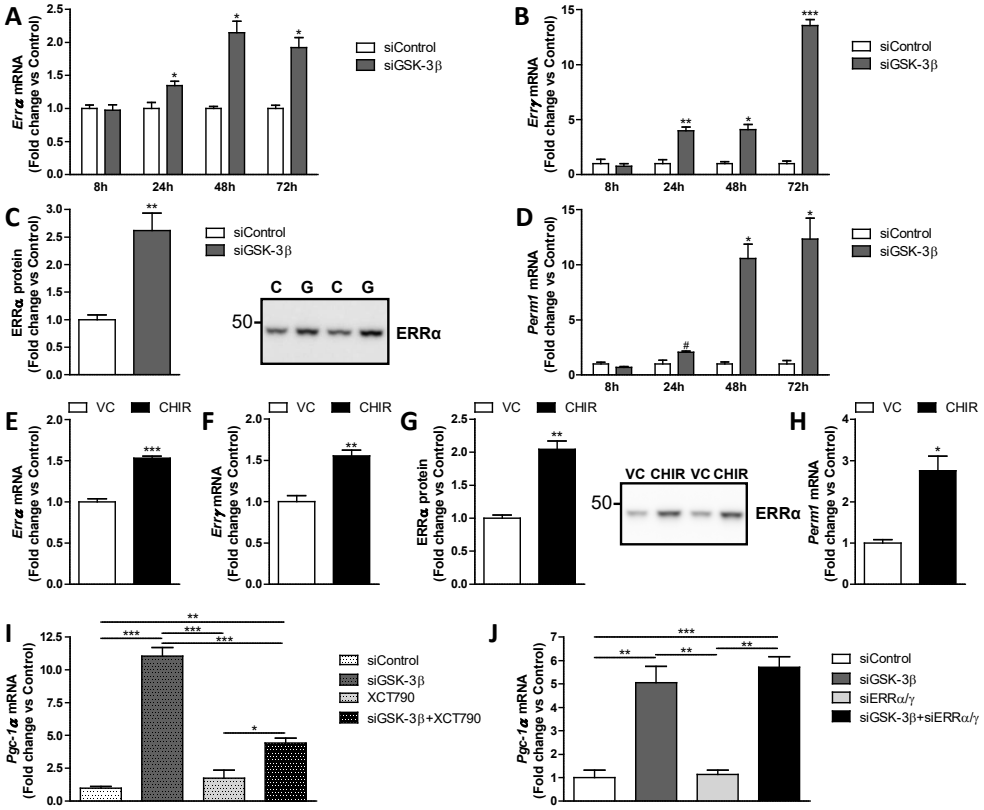


Figure 3. ERRα and ERRγ are not required for the induction of *Pgc-1α* expression mediated by inactivation of GSK-3β. C2C12 cells were differentiated for 5 days. GSK-3β was silenced using siRNAs directed against GSK-3β (grey) and compared to scrambled control (white). (A) *Errα* and (B) *Errγ* mRNA levels as well as (C) ERRα protein and (D) *Perm1* transcript levels were measured 48 h after treatment if not specified otherwise. GSK-3 was inhibited using CHIR (black) and compared to DMSO as vehicle (white). (E) *Errα* and (F) *Errγ* mRNA levels as well as (G) ERRα protein and (H) *Perm1* transcript levels were assessed 48 h after CHIR treatment. (I) After 24 h of knock-down of GSK-3β compared to scrambled control, ERRα was inhibited using XCT790 for 24 h and *Pgc-1α* gene expression was measured. (J) ERRα and ERRγ were silenced using siRNAs directed against ERRα and ERRγ (siERRα/γ) in combination or without knock-down of GSK-3β for 48 h and *Pgc-1α* gene expression was measured. Representative pictures of the blots are shown. All data is expressed as means ± SEM from n = 3/experimental condition (n = 3 experiments). Statistical differences on each time-point were tested using an unpaired Student's t-test or a One-way ANOVA with Bonferroni as post-hoc test and compared to its own control, # $p \leq 0.1$,* $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$. C= siControl, G= siGSK-3β.

Inactivation of GSK-3β results in dephosphorylation and nuclear translocation of TFEB

We next investigated TFEB, which has been recently shown to be able to activate the PGC-1α promoter [34]. Knock-down of GSK-3β tended to increase *Tfeb* mRNA levels (Fig. S4 A). Knock-down of TFEB specifically reduced *Tfeb* mRNA abundance (Fig. S4A, D) without altering *Tfe3* gene expression (Fig. S4B). Pharmacological inhibition of GSK-3β using CHIR did not increase TFEB mRNA or protein abundance (Fig. S4 D, E). However, when assessing TFEB protein abundance, we observed that pharmacological inhibition of GSK-3β using CHIR resulted in a downward mobility shift of the band corresponding to the TFEB protein (Fig. 4A). Importantly, knockdown of TFEB (Fig. S4C) confirmed specificity of the antibody, and strongly reduced the intensity of both bands, suggesting they both represent TFEB. The lower molecular weight of the TFEB protein upon CHIR treatment was suggestive of changes in post-translational modification. Of these, phosphorylation status has been convincingly shown to mediate TFEB cytosolic retention. Indeed, dephosphorylation has been implicated in TFEB nuclear translocation and subsequent transcriptional activity of TFEB [36, 42]. Interestingly, phosphatase treatment (for positive control of the phosphatase treatment; see Fig. S5) resulted in a similar mobility shift of the band corresponding to the TFEB protein of vehicle- and CHIR-treated cells (Fig. 4A), suggesting that GSK-3 inactivation may have resulted in dephosphorylation of TFEB. Secondly, we investigated if the reduced phosphorylation status of TFEB upon CHIR treatment was accompanied by changes in nuclear localization of the TFEB protein. C2C12 myotubes were transfected with TFEB-GFP and H2B-mCHERRY to assess TFEB nuclear localization. We first validated our system by inhibition of mammalian target of rapamycin (mTOR) using Torin1, which was previously shown to potently stimulate TFEB nuclear localization [43, 44]. As depicted in Fig. S6, treatment of myotubes with Torin1 resulted in enhanced nuclear localization of TFEB-GFP (Fig. S6A, B). Interestingly, pharmacological inhibition of GSK-3 with CHIR also increased nuclear localization of TFEB-GFP within 3 h after treatment with CHIR (Fig. 4B, C). Nuclear localization of TFEB-GFP remained elevated until 24 h after CHIR treatment (Representative pictures of all time-points investigated can be found in Fig. S7). Combined, these data reveal TFEB dephosphorylation and nuclear translocation upon GSK-3β inactivation, which are intracellular events that precede TFEB transcriptional activity.

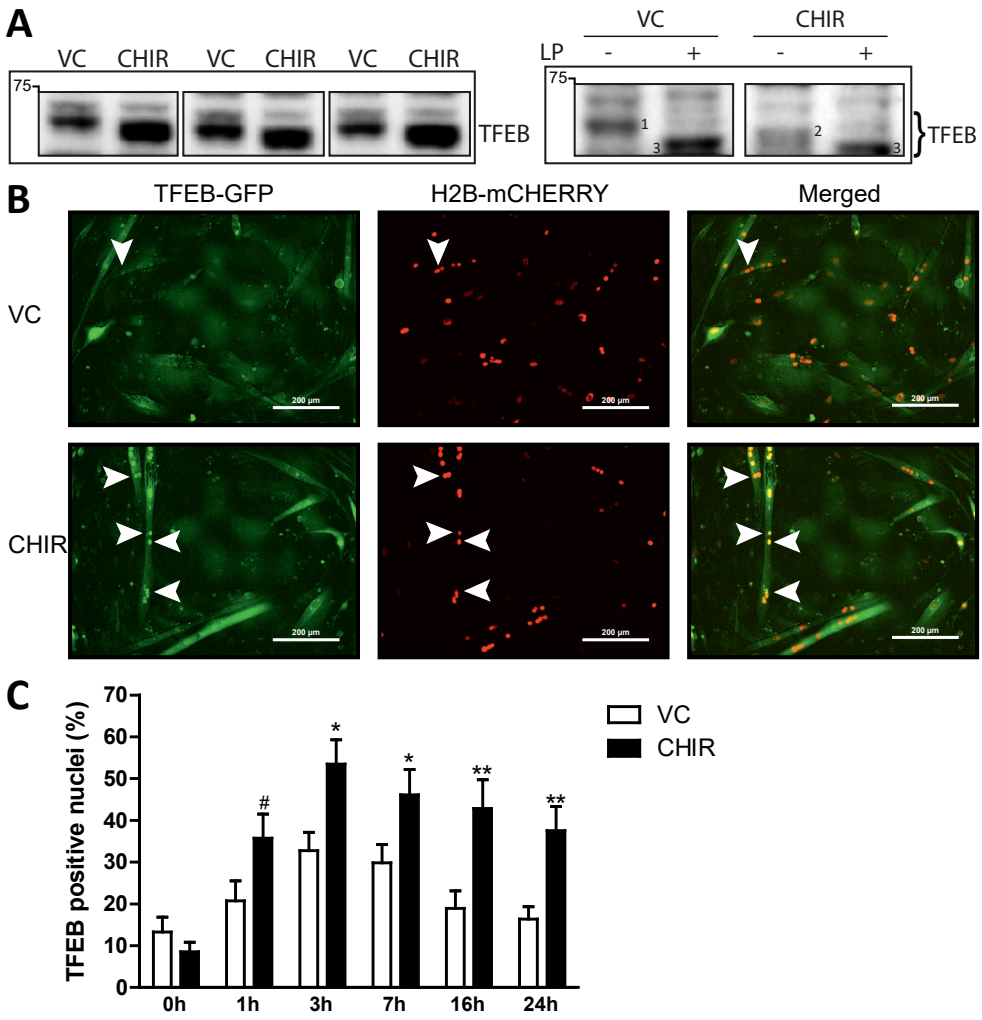


Figure 4. GSK-3 inhibition decreases phosphorylation of TFEB and results in TFEB nuclear translocation. C2C12 cells were differentiated for 5 days and GSK-3 was inhibited using CHIR for 48 h, (A) TFEB protein was assessed using Western blot and compared to DMSO as vehicle (VC). Lambda phosphatase (LP) was used to dephosphorylate proteins in cell lysates from CHIR or VC-treated myotubes. Numbers 1, 2 and 3 indicate the Western blot band of the TFEB protein on different heights of the blot. Black boxes around the representative pictures indicate that they were cut from the same blot. (B) Representative pictures after 3 h of treatment were depicted in the green channel (TFEB-GFP), the red channel (nuclei; Histon 2B (H2B)-mCHERRY) and merged channels, the IncuCyte™ ZOOM 20× objective was used. White arrows indicate examples of TFEB positive nuclei and the scale bare indicates 200 µm. (C) Quantification of the percentage of TFEB positive nuclei per time-point (n = 10-12 pictures). Subcellular localization of TFEB-GFP was analyzed in 7 day-differentiated myotubes with the IncuCyte™ imager immediately after treatment, nuclei were visualized using a H2B-mCHERRY co-transfection. In VC-treated cells 257 nuclei divided over 10 pictures originating from 3 independent wells were used. In CHIR-treated cells 293 nuclei divided over 12 pictures originating from 3 independent wells were used. Statistical differences on each time-point were tested using an unpaired Student's t-test and compared to its own control, # $p \leq 0.1$,* $p \leq 0.05$ and ** $p \leq 0.01$.

Induction in PGC-1α expression following inactivation of GSK-3β requires TFEB

After establishing that inactivation of GSK-3 activated TFEB, indicated by a reduction in TFEB phosphorylation status and enhanced TFEB nuclear translocation, we next investigated the requirement of TFEB in the induction of PGC-1α transcript and protein levels mediated by inactivation of GSK-3β. Knock-down of TFEB (Fig. S4A-C) completely abrogated increases in *Pgc-1α* mRNA levels induced by knock-down or pharmacological inhibition of GSK-3β (Fig. 5A, B). In line with changes in *Pgc-1α* transcript levels, increases in PGC-1α protein levels upon inhibition of GSK-3β were absent when TFEB was knocked-down (Fig. 5C). Furthermore, increases in PGC-1α promoter activity following GSK-3β inactivation were abrogated in absence of TFEB (Fig. 5D). In addition, site-directed mutagenesis [33] of E-boxes (specific TFEB binding region, CLEAR-element) on the PGC-1α promoter revealed that mutation of E-box1 (respectively −429 and −568 relative to the transcription start site and ATG) resulted in a small reduction of PGC-1α promoter activity while site-directed mutated of E-box2 (respectively +8 and −131 relative to the transcription start site and ATG) resulted in almost complete blockade of GSK-3β inactivation-mediated activation of the PGC-1α promoter (Fig. 5E). Interestingly, knock-down of TFEB also prevented inductions in *Errγ* and *Perm1* gene expression and blunted inductions in ERRα gene expression and protein abundance mediated by GSK-3β inactivation (Fig. S8A-C). Finally, overexpression of TFEB, verified by increased *Tfeb* mRNA levels (Fig. S9A), increased *Pgc-1α* gene expression (Fig. S9B) and PGC-1α promoter activity (Fig. S9C), indicating that overexpression of TFEB is sufficient to induce *Pgc-1α* transcription.

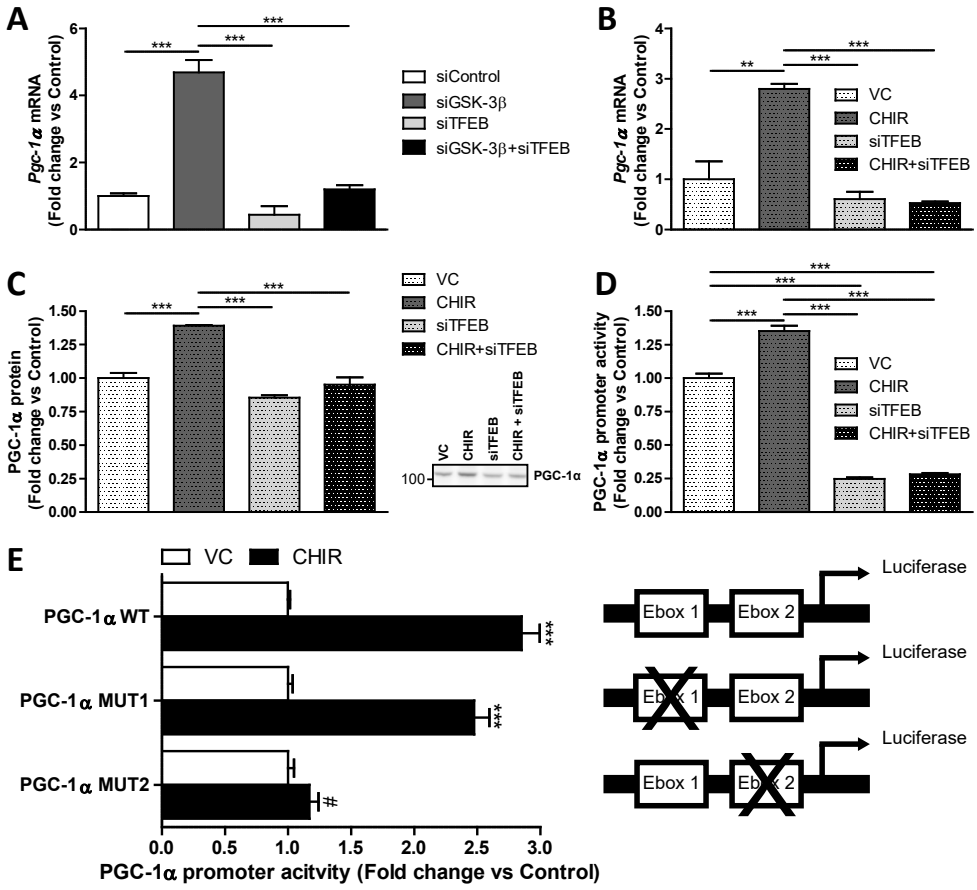


Figure 5. Knock-down of TFEB prevents PGC-1α upregulation after inactivation of GSK-3β. C2C12 were differentiated for 5 days. GSK-3β and TFEB were silenced for 48 h using siRNA directed against GSK-3β (dark grey), TFEB (light grey) or a combination (black) and compared to scrambled control (white) and (A) *Pgc-1α* was measured. Pharmacological inhibition of GSK-3 for 48 h with CHIR (dotted dark grey), siRNA directed against TFEB (dotted light grey) or combination (dotted black) were compared to DMSO as vehicle (VC) (dotted white). (B) *Pgc-1α* gene expression, (C) PGC-1α protein abundance and (D) activity of the PGC-1α promoter were measured in myotubes. (E) C2C12 myoblasts were transfected with plasmids containing promoter sequences of the PGC-1α WT or with mutated E-boxes (MUT1 and MUT2), respectively –568 and –131 from the ATG. Luciferase activity was measured after CHIR treatment (black) for 24 h and compared to DMSO as vehicle (VC) (white). A representative picture of the blot was shown. Luciferase activity was corrected for β-galactosidase activity. All data is expressed as means ± SEM from n = 3/experimental condition (mRNA and protein) or n = 6/experimental condition (luciferase activity) (n = 3 experiments). Statistical differences were tested using a One-way ANOVA with Bonferroni as post-hoc test (A-D) or an unpaired Student's t-test for each plasmid (E), # $p \leq 0.1$, ** $p \leq 0.01$ and *** $p \leq 0.001$.

Discussion

We previously showed that inactivation of GSK-3β potently increased the transcript abundance of *Pgc-1α* in myocytes [28, 29]. This was accompanied by increases in respiration [28], mtDNA copy number, activity of several enzymes involved in oxidative substrate metabolism and protein and mRNA abundance of oxidative phosphorylation (OXPHOS) complexes [29]. In the present study, we aimed to elucidate the molecular mechanism underlying this increase in *Pgc-1α* abundance. We now show that, increased *Pgc-1α* mRNA and protein levels, mediated by inactivation of GSK-3β, are associated with increased PGC-1α promoter activity indicative of a transcriptionally-controlled mechanism. After excluding potential involvement of MEF2 and ERR transcription factors, we demonstrate that GSK-3β inactivation is associated with enhanced nuclear translocation of the TFEB protein. Moreover, knock-down of TFEB completely prevented increases in *Pgc-1α* gene expression, PGC-1α promoter activity and PGC-1α protein abundance induced by inactivation of GSK-3β, convincingly demonstrating that increased *Pgc-1α* abundance mediated by inactivation of GSK-3β in skeletal muscle cells requires TFEB.

It has been well-described that epigenetic modulation of chromatin structure can regulate gene expression [45]. Our study suggests that increased *Pgc-1α* expression upon GSK-3β inactivation does not rely on epigenetic modification of chromatin structure as knock-down of GSK-3β did not alter chromatin accessibility of the proximal PGC-1α promoter in C2C12 myotubes. Transcription of the *Pgc-1α* gene is mediated by several transcription factors and is controlled by two distinct promoter regions, namely the proximal PGC-1α promoter and the alternative PGC-1α promoter (for review see [39]). Several different splice variants of PGC-1α are known to originate from activation of these 2 promotor regions [39]. More specifically, *Pgc-1α-a* is transcribed from the proximal PGC-1α promoter, while the alternative PGC-1α promoter controls transcription of *Pgc-1α-b* and *Pgc-1α-c*, additionally, truncated variants of these three isoforms are known [39]. Our data indicates that inductions in *Pgc-1α* mRNA mediated by inactivation of GSK-3β are mainly due to activation of the proximal PGC-1α promoter. Although not much is known about which transcription factors bind and activate the alternative promotor, several transcription factors have been described to bind the proximal PGC-1α promotor and thereby induce transcription of the *Pgc-1α* gene including MEF2 isoforms, ERRs, cyclic AMP response element binding protein, MyoD, TFE3 and TFEB [32-34, 46].

MEF2 isoforms are amongst the best-described transcription factors involved in transcriptional control of the proximal PGC-1α promotor [46]. More specifically,

two MEF2-binding sites, well-known sites where MEF2 proteins can bind to DNA [47], were identified on the proximal PGC-1α promoter [31] and overexpression of MEF2A, MEF2C or MEF2D activated the proximal PGC-1α promoter [31]. Although there is evidence that GSK-3β can influence MEF2 activity, likely through modulation of HDAC5 [48] (a repressor of MEF2 activity), in our study we observed that inactivation of GSK-3β did not affect MEF2 transcriptional activity or MEF2 isoform gene expression. In contrast, pharmacological inhibition of GSK-3β with AR-A014418 increases MEF2 transcriptional activity in C2C12 myoblasts [35], however, efficiency of AR-A014418 to inhibit GSK-3β was not reported. C2C12 differentiation status might also underlie this discrepancy, as we used C2C12 cells during myogenic differentiation which is known to activate MEF2 [49]. This is in contrast to Dionyssiou *et al.* who used proliferating myoblasts. Accordingly, based on our data, we conclude that there is no clear association of activity or abundance of MEF2 isoforms with increased PGC-1α abundance mediated by inactivation of GSK-3β in our study.

ERR transcription factors are well-known to be able to bind ERR response elements (ERREs) [40, 50-52] which have been identified in the proximal PGC-1α promoter region [32]. In line with these observations, overexpression of ERRα [32, 53] or ERRγ [40] increases PGC-1α promoter activity and *Pgc-1α* mRNA levels [32, 53] in cardiomyocytes and L6 myotubes. In addition, pharmacological inhibition of ERRα using XCT790 or knock-down of ERRα has been shown to decrease baseline *Pgc-1α* mRNA levels [32, 53]. Although we observed a robust induction of the abundance of both ERR isoforms in our study, ERRs were found to be dispensable for inductions in *Pgc-1α* mRNA observed after inactivation of GSK-3β. Moreover, in contrast to previous studies, XCT790 treatment failed to alter baseline *Pgc-1α* levels. Potentially, this may be explained by additional control over the PGC-1α promotor by ERRγ or even ERRβ, which were not assessed. Evidence of interactions between GSK-3β, or upstream regulators of GSK-3β activity with ERR transcription factors are limited and contradicting. For example, protein kinase B (Akt), which directly inhibits GSK-3β by phosphorylating Ser^{9} [54], was reported to phosphorylate ERRα thereby increasing its transcriptional activity in mammary gland cells [55]. In contrast, Akt-mediated phosphorylation of ERRγ suppresses ERRγ transcriptional activity in liver cells [56]. In conclusion, although the temporal profile of inductions in ERR expression and activity suggested potential involvement of the ERRs in inductions in *Pgc-1α* mediated by inactivation of GSK-3β, we show that ERRα/γ are not required for increased *Pgc-1α* gene expression upon inactivation of GSK-3β.

TFEB and its family members, TFE3, microphthalmia-associated transcription factor (MITF) and TFEC, have been shown to bind to specific E-boxes (called CLEAR-regions)

[57] and several CLEAR-regions have been identified on the proximal PGC-1α promoter [33]. Interestingly, MITF was found to bind to the proximal PGC-1α promoter but not to the alternative PGC-1α promoter [58]. Recently, the TFEB protein, and its family members, were described to transcriptionally regulate *Pgc-1α* gene expression. For example, TFEB overexpression directly induced transcription of *Pgc-1α* in liver [34]. In addition, MITF directly regulates *Pgc-1α* gene expression in melanomas thereby increasing mitochondrial respiration [58]. Moreover, overexpression of TFE3 induced PGC-1α promoter activity and increased *Pgc-1α* mRNA abundance, dependent on a CLEAR-region on the PGC-1α promoter, resulting in increased OXPHOS gene expression in C2C12 cells [33]. Data from our study suggests that the same CLEAR-region (E-box) is required for inductions in PGC-1α promoter activity mediated by inactivation of GSK-3β. In addition, overexpression of TFEB increases *Pgc-1α* mRNA levels in C2C12 myotubes [59, 60] and the skeletal musculature of mice [60], which now has been verified in our study. Muscle-specific ablation of TFEB however did not alter basal *Pgc-1α* mRNA levels in skeletal muscle and C2C12 myotubes [60], which is in line with our data after knock-down of TFEB in C2C12 myotubes. Despite the partial overlap in TFEB and TFE3 regulation of PGC-1α promoter activity [33], knock-down of TFEB completely prevented GSK-3β-mediated inductions of PGC-1α promoter activity and mRNA transcript levels, without altering *Tfe3* gene expression. This data suggests that TFEB is indispensable for increased *Pgc-1α* gene expression mediated by inactivation of GSK-3β, and reveals non-redundant roles for TFEB and TFE3 in PGC-1α regulation.

TFEB transcriptional activity is dependent on nuclear translocation, which is potentiated by dephosphorylation of the TFEB protein [61]. It has been suggested that GSK-3β can phosphorylate TFEB, resulting in cytoplasmic retention of TFEB. Indeed, Ploper *et al.* showed three putative C-terminal GSK-3 phosphorylation sites on TFEB [36] while others showed that GSK-3β can phosphorylate Ser^{134} and Ser^{138}, but not Ser^{142}, of the TFEB protein [42]. In addition, inhibition of GSK-3β resulted in TFEB nuclear translocation in human pancreatic cancer cells, HEK293T cells [43] and cultured neuroblastoma cells [62]. Our data also suggest a reduced phosphorylation status which coincides with nuclear accumulation of the TFEB protein after pharmacological inhibition of GSK-3β, in line with the inverse relationship between TFEB phosphorylation and nuclear localization reported in other cell types. Besides direct phosphorylation of TFEB by GSK-3β, inhibition of GSK-3β could also indirectly affect TFEB. Other kinases such as mTOR [44] and extracellular regulated kinases 1/2 [63] have been previously reported to phosphorylate TFEB. As GSK-3β was previously identified as a positive regulator of mTOR activity [64], GSK-3β inhibition may indirectly impact the phosphorylation status and subsequent nuclear localization of

TFEB by modulation of mTOR. Taken together, in combination with available literature our data suggests that GSK-3β phosphorylates TFEB, preventing it from nuclear localization. We confirm that TFEB is sufficient to activate *Pgc-1α* transcription as shown previously, and now show that TFEB is required inductions of *Pgc-1α* mRNA levels, PGC-1α protein abundance and activity of the proximal PGC-1α promoter in skeletal muscle cells following inactivation of GSK-3β.

In skeletal muscle, GSK-3β is constitutively active and is subject to regulatory inactivation in response to physical activity and metabolic cues. For example, GSK-3β is inactivated by an acute bout of exercise [65] or during reloading of disuse-atrophied muscle [66] in rodents but also in human muscle following cycling exercise [67]. Several kinases such as Akt [54], protein kinase A [68], protein kinase C [69], p70 S6 kinase [70] and mitogen-activated protein kinase [71] have been reported to phosphorylate GSK-3β at Ser^{9} inhibiting its activity, which are nodal points in signaling cascades activated by *e.g.* insulin-like growth factor-I and insulin. In the context of muscle plasticity, these ligands have mainly been postulated to stimulate muscle hypertrophy and regeneration through stimulation of protein synthesis [72] and myogenesis [73]. Our findings may therefore imply a coordinating role for GSK-3β in the regulation of muscle plasticity beyond control of muscle mass, as its positioning in muscle anabolic pathways allows it to act as a sensor to meet the increased energy demand of growing muscle upon its inactivation by inducing the PGC-1α and subsequent gene programs involved in oxidative energy metabolism and mitochondrial biogenesis.

In conclusion, our data shows that inactivation of GSK-3β results in dephosphorylation of TFEB, leading to nuclear translocation of the TFEB protein. In turn this induces PGC-1α promoter activity which subsequently increases *Pgc-1α* gene expression and protein abundance.

Acknowledgements

We would like to thank Dr. Nancy Salma for kindly providing us with the PGC-1α promoter plasmids (PGC-1α WT, PGC-1α MUT1 and PGC-1α MUT2). Moreover, we would like to thank Dr. Leon de Windt for providing the pcDNA3-MEF2A-flag, pcDNA3-MEF2C-flag and MEF2x3-LUC plasmid (originally described by Dr. Eric Olson). Furthermore, we would like to acknowledge Dr. Shawn Ferguson for providing the pEGFP-N1-TFEB plasmid to Addgene (Addgene plasmid #38119) and Arjan Groot for kindly providing us with the H2B-mCHERRY overexpression plasmid. In addition, we would like to thank Philippe Vangrieken for his effort with scoring TFEB nuclear localization. This work was financially supported by a grant from the Dutch Lung foundation (5.2.13.067).

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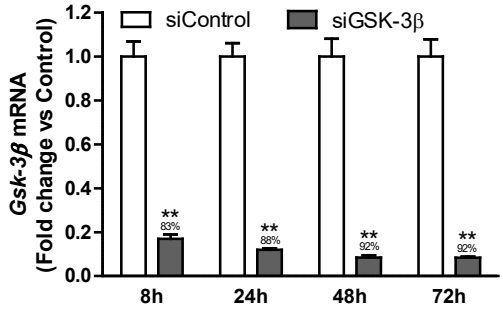
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Supplemental information

Supplemental results

Knock-down of GSK-3β effectively reduces GSK-3β mRNA

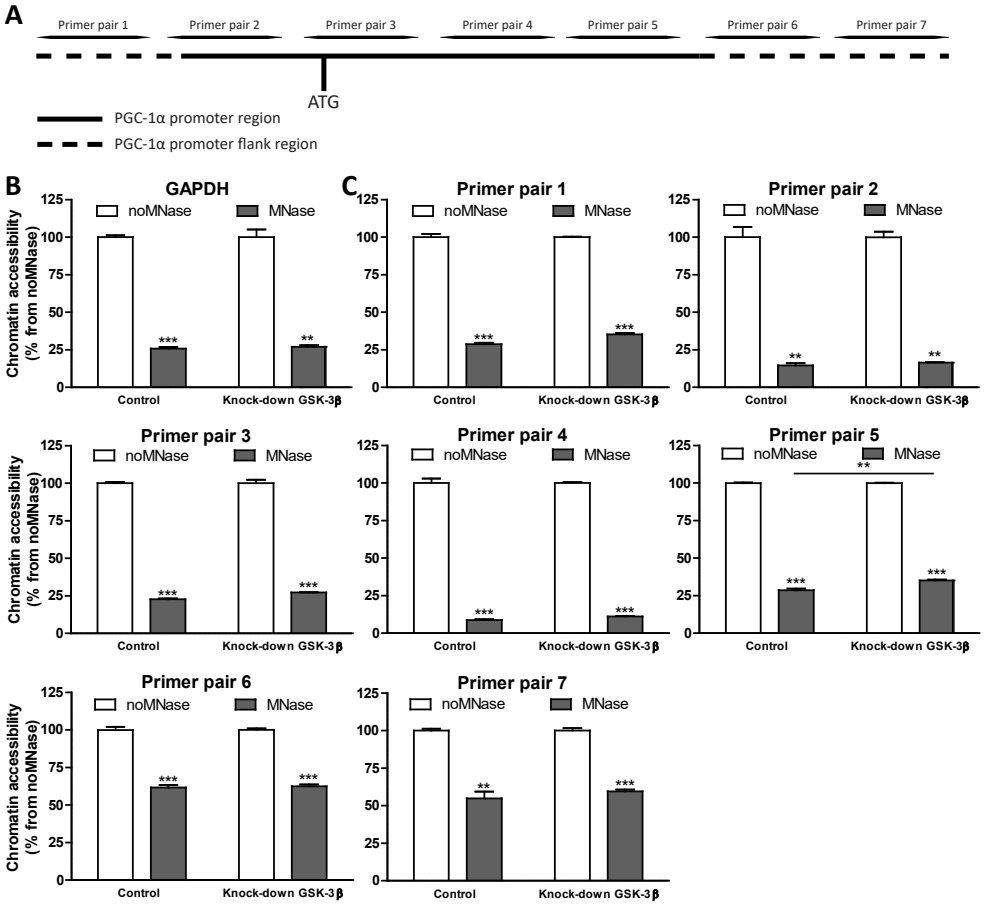
Knock-down of GSK-3β potently reduced mRNA levels of *Gsk-3β* 8, 24, 48 and 72h after knock-down (Fig. S1).



Supplemental figure 1. Knock-down of GSK-3β. C2C12 were differentiated for 5 days and GSK-3β was silenced using siRNA directed against GSK-3β (grey) and compared to scrambled control (white). Gene expression of *Gsk-3β* was measured 8, 24, 48, and 72h after knock-down and expressed as means ± SEM from n = 3/experimental condition (n = 3 experiments). Statistical differences on each time-point were tested using an unpaired Student's t-test, ** $p \leq 0.01$.

Knock-down of GSK-3β does not alter chromatin accessibility of the proximal PGC-1α promoter

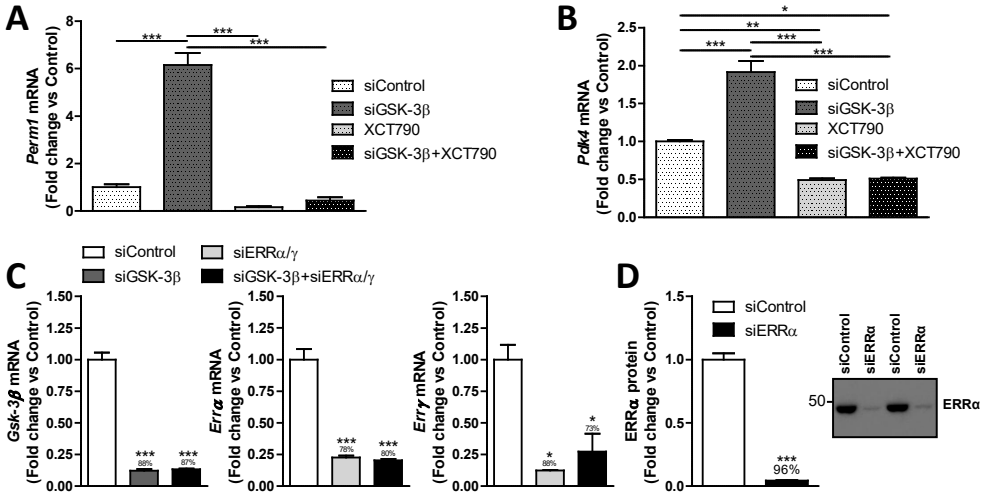
Primer locations on the proximal PGC-1α promoter are schematically indicated (Fig. S2A). MNase treatment effectively reduced RT-qPCR signal on the constitutively open GAPDH promoter, which was not altered by knock-down of GSK-3β (Fig. S2B). Knock-down of GSK-3β resulted in a small but statistically different reduction in promoter accessibility on location 5. However, overall knock-down of GSK-3β did not alter chromatin accessibility of the proximal PGC-1α promoter in C2C12 myotubes (Fig. S2C).



Supplemental figure 2. Knock-down of GSK-3β does not alter chromatin accessibility of the PGC-1α promoter region. (A) Schematic representation of the proximal PGC-1α promoter region and primer locations. C2C12 cells were fully differentiated and chromatin accessibility of the (B) GAPDH promoter and the (C) proximal PGC-1α promoter was measured 24h after knock-down of GSK-3β or scrambled control (Control). Chromatin accessibility was measured by qRT-PCR after treatment with 50 Units MNase (grey) versus a NoMNase control (white). All data is expressed as means ± SEM from n = 3/experimental condition (n = 3 experiments). Statistical differences of the MNase digestion per treatment and differences in the decrease upon MNase treatment between treatment were tested using an unpaired Student's t-test, ** $p \leq 0.01$ and *** $p \leq 0.001$.

XCT790 inhibits ERRα and knock-down of ERRα and ERRγ potently reduces ERRs

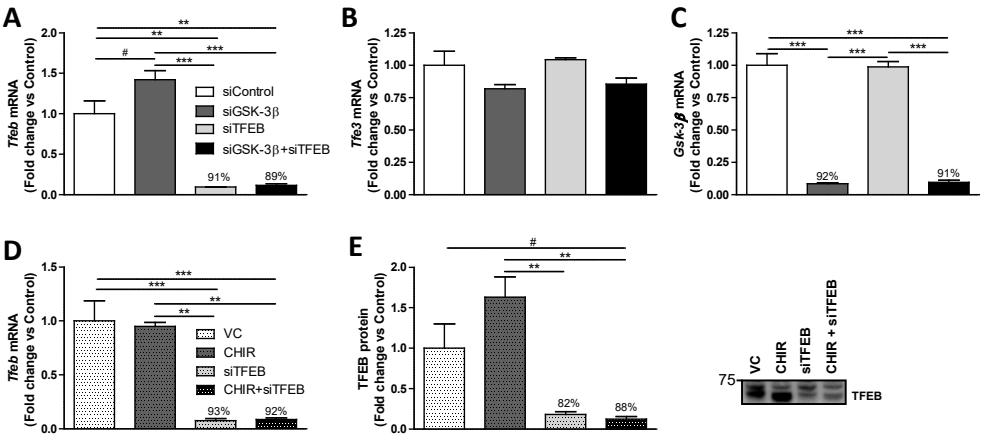
Treatment with XCT790, an inverse agonist of ERRα, effectively prevented both basal as well as GSK-3β inactivation-mediated expression of the ERRα target genes *Perm1* and *Pdk4* indicating effectiveness of XCT790 in inhibiting ERRα (Fig. S3A, B). In addition, knock-down of ERRα and ERRγ effectively reduced *Errα* and *Errγ* transcript levels as well as ERRα protein levels and did not affect knock-down efficiency of GSK-3β (Fig. S3C, D).



Supplemental figure 3. Knock-down of ERRα and ERRγ and pharmacological inhibition of ERRα using XCT790. C2C12 were differentiated for 5 days and ERRα and ERRγ were silenced using siRNAs directed against ERRα and ERRγ (grey), pharmacological inhibition of ERRα with XCT790 (dotted light grey) or in combination with siRNA directed against GSK-3β (black and dotted black) and compared to scrambled control (white) or DMSO as vehicle (VC) (dotted white). Gene expression of (A) *Perm1*, (B) *Pdk4* and (C) *Gsk-3β*, *Errα* and *Errγ* were measured by qRT-PCR. (D) ERRα protein level was measured by Western blot. A representative picture of the blots is shown. All data is expressed as means ± SEM from n = 3/experimental condition (n = 3 experiments). Statistical differences between treatments are tested using an unpaired Student's t-test or ANOVA with Bonferroni as post-hoc test, * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$.

Knock-down of TFEB efficiently decreases TFEB mRNA and protein levels

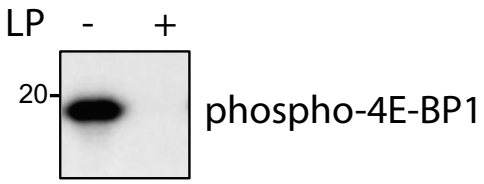
Knock-down of TFEB significantly reduced *Tfeb* mRNA (Fig. S4A) without altering *Tfe3* gene expression (Fig. S4B). Knock-down of GSK-3β was verified by a reduced *Gsk-3β* transcript level, which was reduced independently of knock-down of TFEB (Fig. S4C). Knock-down of TFEB significantly reduced TFEB mRNA and protein abundance independent of treatment with CHIR (Fig. S4D, E).



Supplemental figure 4. Knock-down of TFEB and GSK-3β. C2C12 cells were differentiated for 5 days. GSK-3β and TFEB were silenced for 48 h using siRNA directed against GSK-3β (dark grey), TFEB (light grey) or a combination (black) and compared to scrambled control (white) or treated with CHIR (dotted bars) compared to DMSO as vehicle (VC). Gene expression of (A) *Tfeb* (B) *Tfe3*, (C) *Gsk-3β* and (D) *Tfeb* and (E) protein abundance of TFEB were measured. A representative picture of the blots is shown. All data is expressed as means ± SEM from n = 3/experimental condition (n = 3 experiments). Statistical differences were tested using a One-way ANOVA with Bonferroni as post-hoc test, # $p \leq 0.1$, ** $p \leq 0.01$ and *** $p \leq 0.001$.

Lambda phosphatase treatment abolishes phosphorylation of 4E-BP1

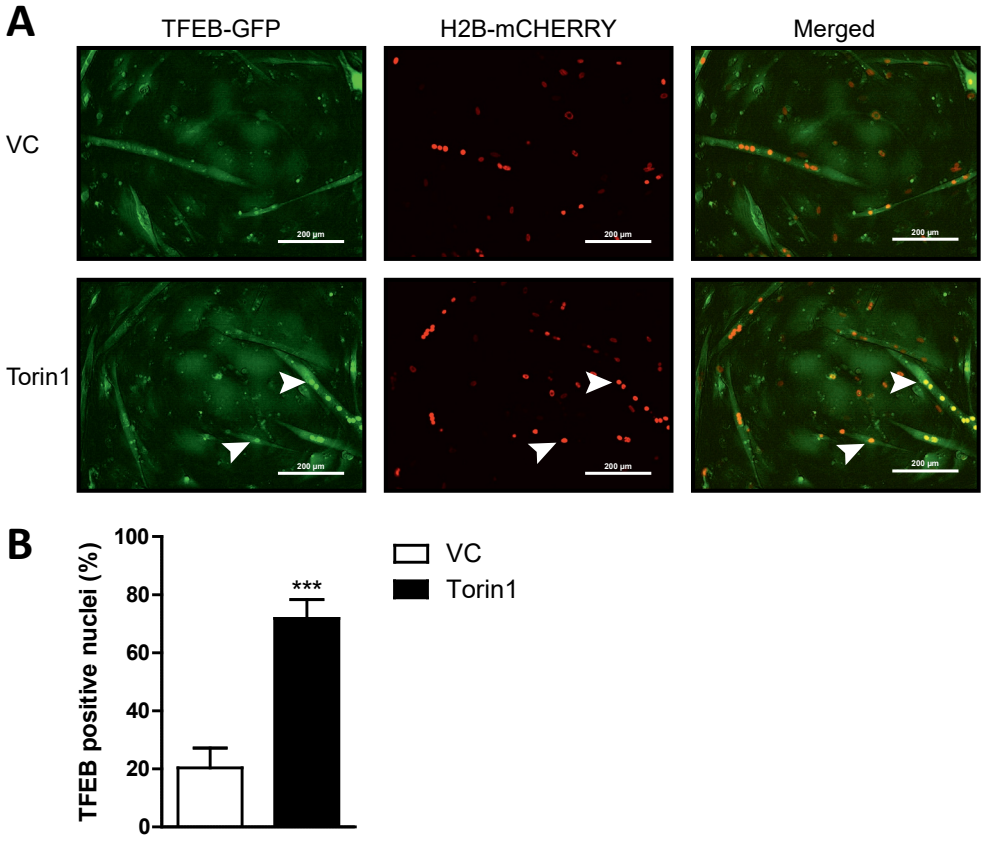
Lambda phosphatase (LP) treatment effectively reduced phosphorylation status of 4E-BP1, which was used as positive control for the LP treatment (Fig. S5).



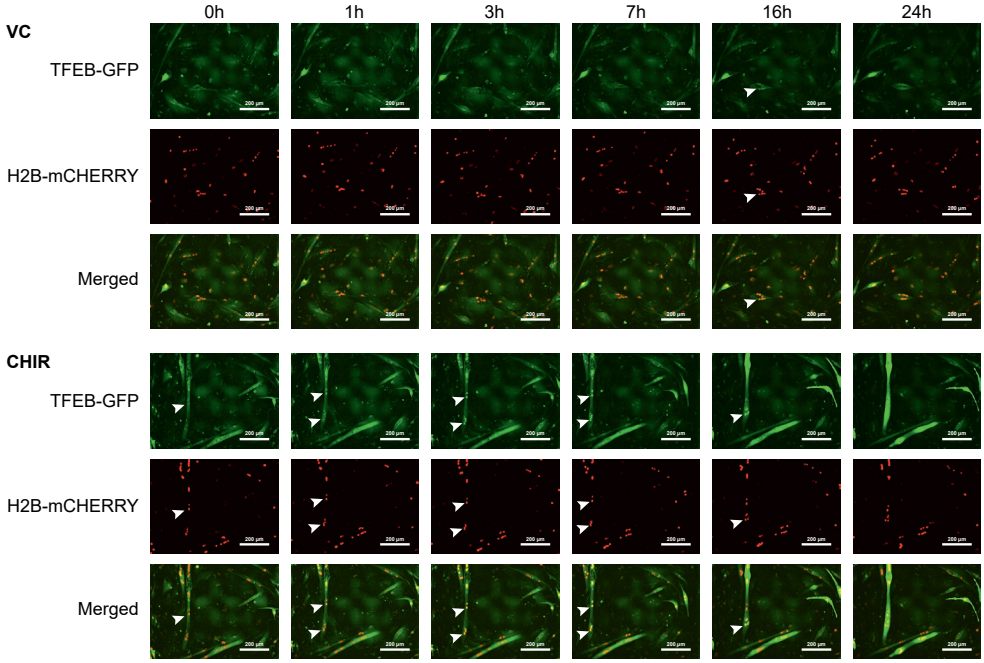
Supplemental figure 5. Lambda phosphatase treatment dephosphorylates 4E-BP1. Lysates from fully differentiated C2C12 cells were exposed to Lambda Phosphatase (LP; +) or control (-). A representative picture of the blot for phospho-4E-BP1 is shown.

mTOR and GSK-3β inhibition both result in nuclear localization of TFEB

mTOR inhibition with Torin1 resulted in nuclear translocation of TFEB-GFP (Fig. S6A, B). In addition, inactivation of GSK-3β using CHIR resulted in nuclear co-localization of TFEB-GFP with H2B-mCHERRY (Fig. 4B, C and Fig. S7).



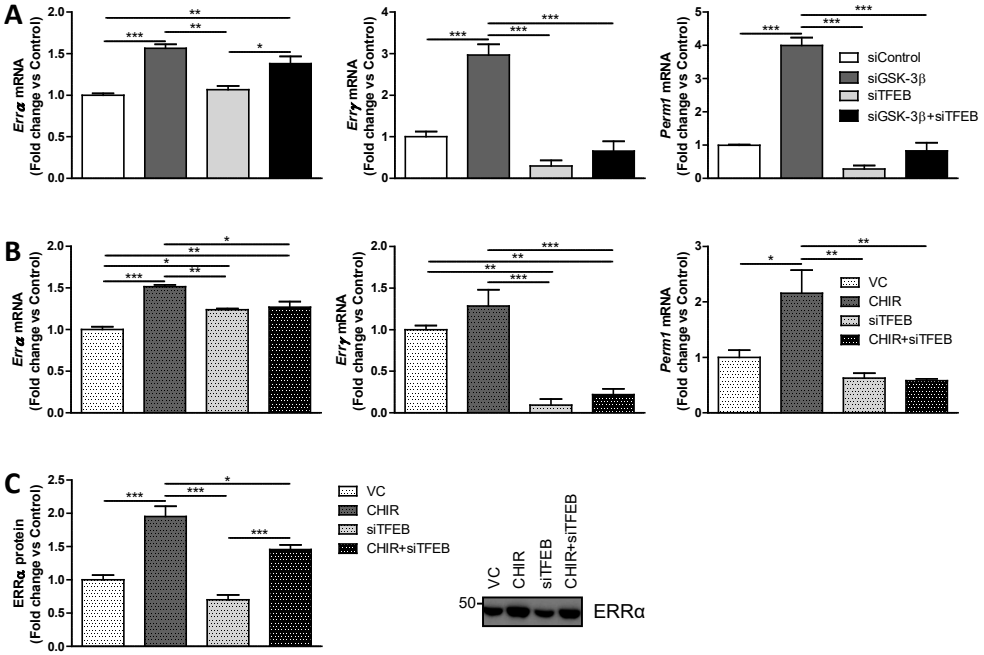
Supplemental figure 6. mTOR inhibition results in TFEB nuclear localization. Subcellular localization of TFEB-GFP was analyzed with the IncuCyte™ imager immediately after treatment, nuclei were visualized using a Histon 2B (H2B)-mCHERRY co-transfection. In vehicle (VC)-treated cells 141 nuclei divided over 5 pictures originating from 2 independent wells were used. In Torin1-treated cells 162 nuclei divided over 7 pictures originating from 2 independent wells were used. (A) Representative pictures after 1h of treatment were depicted in the green channel (TFEB-GFP), the red channel (nuclei; H2B-mCHERRY) and merged channels. (B) Quantification of the percentage of TFEB positive nuclei after 1h of treatment (n = 5-7 pictures). The IncuCyte™ ZOOM 20x objective was used. White arrows indicate examples of TFEB positive nuclei and the scale bar indicates 200 µm. Statistical differences were tested using an unpaired Student's t-test, *** $p \leq 0.001$.



Supplemental figure 7. Inhibition of GSK-3β promotes TFEB nuclear localization. Subcellular localization of TFEB-GFP was analyzed with the IncuCyte™ imager immediately after treatment, nuclei were visualized using a Histon 2B (H2B)-mCHERRY co-transfection. In vehicle (VC)-treated cells 257 nuclei divided over 10 pictures originating from 3 independent wells were used. In CHIR-treated cells 293 nuclei divided over 12 pictures originating from 3 independent wells were used. Representative pictures after 0, 1, 3, 7, 16 and 24h of treatment were depicted in the green channel (TFEB-GFP), the red channel (nuclei; H2B-mCHERRY) and merged channels, the IncuCyte™ ZOOM 20x objective was used. White arrows indicate examples of TFEB positive nuclei and the scale bar indicates 200 µm.

Knock-down of TFEB prevents the induction of *Errγ*, *Perm1* but not *Errα* mRNA mediated by inactivation of GSK-3β

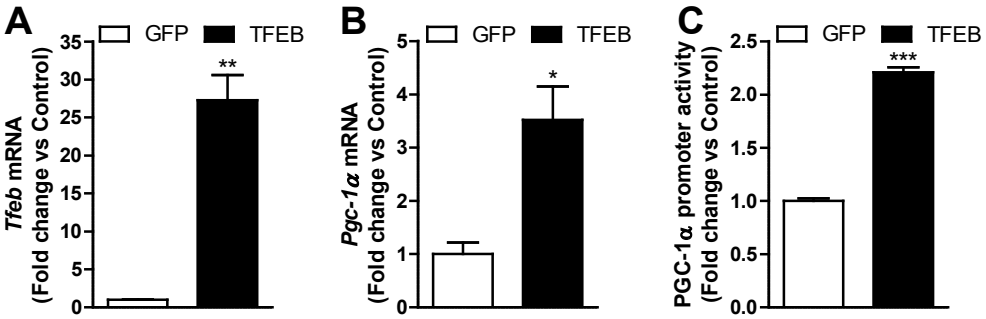
Inactivation of GSK-3β (genetic and pharmacological) increased gene expression of *Errα*, *Errγ* and *Perm1* (Fig. S8A, B) as well as ERRα protein abundance (Fig. S8C). Knock-down of TFEB prevented inductions in *Errγ* and *Perm1* mRNA levels and attenuated increased transcript and protein abundance of ERRα, mediated by inactivation of GSK-3β (Fig. S8A-C).



Supplemental figure 8. Effect of knock-down of TFEB on increased ERR expression mediated by GSK-3β inactivation. C2C12 cells were differentiated for 5 days. GSK-3β and TFEB were silenced for 48 h using siRNA directed against GSK-3β (dark grey), TFEB (light grey) or a combination (black) and compared for scrambled control (white). Pharmacological inhibition of GSK-3 for 48 h with CHIR (dotted dark grey), siRNA directed against TFEB (dotted light grey) or combination (dotted black) were compared to DMSO as vehicle (VC) control (dotted white). (A, B) *Errα*, *Errγ* and *Perm1* mRNA and (C) ERRα protein levels were measured. A representative picture of the blot is shown. All data is expressed as means ± SEM from n = 3/experimental condition (n = 3 experiments). Statistical differences were tested using a One-way ANOVA with Bonferroni as post-hoc test, * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$.

TFEB overexpression is sufficient to induce PGC-1α promoter activity

Overexpression of TFEB, verified by increased mRNA content (Fig. S9A), was sufficient to increase *Pgc-1α* mRNA levels (Fig. S9B) and PGC-1α promoter activity (Fig. S9C).



Supplemental figure 9. TFEB is sufficient to activate PGC-1α gene expression. C2C12 myoblasts were cultured and TFEB-GFP (black) was over-expressed. (A) *Tfeb*, (B) *Pgc-1α* mRNA expression and (C) PGC-1α promoter activity was measured. Luciferase activity was corrected for β-galactosidase activity. All data are expressed as means ± SEM from n = 3/experimental condition (n = 3 experiments). Statistical differences were tested using unpaired Student's t-test, * $p \leq 0.05$, ** $p \leq 0.01$ *** $p \leq 0.001$.

Supplemental methods

Table 1. Primer sequences.

Gene	Forward primer (5'–3')	Reverse primer (5'–3')
Gsk-3β	CCACATGCTCGGATTCAGGC	TGTCCACGGTCTCCAGCATTAGTAT
Pgc-1α	CAACAATGAGCCTGCGAACA	CTTCATCCACGGGGAGACTG
Pgc-1α-a	GGACATGTGCAGCCAAGACTCT	CACTTCAATCCACCCAGAAAGCT
Pgc-1α-b	GACATGGATGTTGGGATTGTCA	ACCAACCAGAGCAGCACATTT
Pgc-1α-c	AGTGACATGGATGTTGGGATTG	GAATGCCTCCGGTTACTCACTT
Errα	GGCGGACGGCAGAAGTACAAA	GCGACACCAGAGCGTTCAC
Errγ	TGTGACTTGGCTGACCGAGA	CAACGCCGAGGATCAGAATC
Perm1	GCTTAACAGTCAGGAAGGCAGGG	ACAGCCCAAGTGGCAAAGGCAAC
Tfeb	AGGTTCAACATCAATGACC	CGGATGTAATCCACAGAG
Pdk4	AGGTTATGGGACAGACGCTATCATCTACT	AGACTGGGAGCTTTTCTACAGACTCAGA
Reference genes		
β2M	CTTTCTGGTGCTTGTCTCACTGA	GTATGTTCGGCTTCCCATTCTC
Rpl13a	CACTCTGGAGGAGAAACGGAAGG	GCAGGCATGAGGCAAACAGTC
CycloA	TTCCTCCTTTCACAGAATTATTCCA	CCGCCAGTGCCATTATGG
Hprt	TGGATATGCCCTTGACTATAATGAGTAC	AGGACTCCTCGTATTTGCAGATTC
Calnexin	GCAGCGACCTATGATTGACAACC	GCTCCAAACCAATAGCACTGAAAGG
Gusb	CATTAGCAAGCTGGTCCAGAGT	GACAAAGTAACCCTTGGGATACAT
Primers on promoter sequence		
PGC-1α promoter 1	TCGGGGGTGTTGCCTTCAAACACT	AGCTTGGAACTGAAGCAAGCGCTCC
PGC-1α promoter 2	AGGCAGAGGGCTGCCTTGGAGT	ACTCATGCAGGCAACCAGCCCCT
PGC-1α promoter 3	GGCGTCATTCGGGAGCTGGATGGC	TGTCCCCTCCTCTCCCCTAGCCC
PGC-1α promoter 4	TGTGTGCGGCTGAAAGGGTTGGCT	CCCGGCTTCAGGCAGCTCTCCG
PGC-1α promoter 5	AGGGGCTCAGCGTCCAGCCTT	GCCACAGGCTCGAAGGCACGGG
PGC-1α promoter 6	TGGGGATCCTCTGGGGCCTGGT	AGGAGGCACAGGGAAAGGAGACAGGG
PGC-1α promoter 7	GCTGCTGCCTGCACAGGAACCACTA	GCCCTCCTGCCTCTGCTTCCCC
GAPDH promoter	GGGCTCTCCACCCTGCTCCCC	CAGGGCGGGCCCACCTGTGT

Gsk-3β: glycogen synthase kinase-3β, *Pgc-1α*: peroxisome proliferator-activated receptor gamma coactivator-1α, *Errα/γ*: estrogen-related receptor α/γ, *Perm1*: PGC-1 and ERR-induced regulator in muscle 1, *Tfeb*: Transcription factor EB, *Tfe3*: Transcription factor binding to IGHM enhancer 3, *Pdk4*: Pyruvate dehydrogenase kinase 4, *Gapdh*: Glyceraldehyde-3-phosphate dehydrogenase, *β2M*: β2 microglobulin, *Rpl13a*: ribosomal protein L13a, *CycloA*: cyclophilin A, *Hprt*: hypoxanthine-guanine phosphoribosyltransferase and *Gusb*: Glucuronidase Beta.

Chapter 6

General Discussion

Discussion

Skeletal muscle tissue has a remarkably high plasticity with respect to the adaptive capacity of its mass and oxidative phenotype (OXPHEN) with changes in physical activity or muscle loading being among the most potent triggers for muscle remodeling. Although this plasticity facilitates a high level of regenerative capacity to counteract degenerative triggers, a decreased muscle OXPHEN is often observed in several chronic diseases and aging [1-4]. Subsequently, muscle dysfunction and reduced exercise capacity resulting from this are associated with increased morbidity and mortality in these conditions [5-7]. In order to be able to alleviate the burden of muscle abnormalities, a better understanding of the processes regulating skeletal muscle OXPHEN is warranted. Although it was previously shown that inactivation of glycogen synthase kinase (GSK)-3β increases the expression of peroxisome proliferator-activated receptor (PPAR)γ co-activator-1α (PGC-1α) and indices of oxidative energy metabolism in non-muscle cells [8, 9], it was unknown whether GSK-3β plays a role in the regulation of oxidative substrate metabolism and mitochondrial biogenesis in skeletal muscle. In this thesis, we therefore aimed to investigate a potential role of GSK-3β in the regulation of oxidative substrate metabolism and mitochondrial biogenesis in skeletal muscle. Taken together, we convincingly showed that inactivation of GSK-3β (both genetically and pharmacologically) enhanced oxidative substrate metabolism and mitochondrial biogenesis, increased mRNA and protein abundance of PGC-1α and elevated gene expression of several constituents of the PGC-1α signaling network in fully differentiated myotubes (**Chapter 3**) as well as during myogenic differentiation (**Chapter 4**). Interestingly, with respect to the underlying molecular mechanism, we discovered that inactivation of GSK-3β is associated with dephosphorylation of transcription factor EB (TFEB) and enhanced TFEB nuclear translocation. Importantly, we demonstrated that TFEB is required for the induction of *Pgc-1α* gene expression, PGC-1α promoter activity and PGC-1α protein abundance mediated by inactivation of GSK-3β (**Chapter 5**). Although *in vivo* OXPHEN recovery during reloading did not require inactivation of GSK-3β (**Chapter 2**), muscle-specific GSK-3β knock-out (KO) protected against unloading-induced decrements in expression of oxidative phosphorylation (OXPHOS) genes and constituents of the PGC-1α signaling (**Chapter 3**) and enhanced reloading-induced expression of these genes (**Chapter 4**). In conclusion, our results indicate that inactivation of GSK-3β increases *Pgc-1α* gene expression via TFEB, subsequently enhancing skeletal muscle oxidative substrate metabolism and mitochondrial biogenesis. This chapter positions the data presented in this thesis in a broader context.

The role of GSK-3β in the regulation of skeletal muscle OXPHEN

Inactivation of GSK-3 increases PGC-1α levels

One of the main findings described in this thesis is that inactivation of GSK-3β increases mRNA and protein abundance of PGC-1α, the master regulator of mitochondrial biogenesis and oxidative substrate metabolism [10, 11], in fully differentiated myotubes and during myogenic differentiation (**Chapter 3 and 4**). This is in line with the effect of inactivation of GSK-3 described previously in several other cell types [8, 9, 12, 13]. Most of these studies used lithium chloride (LiCl) to pharmacologically inhibit GSK-3 [14]. LiCl however does not have a high degree of specificity for GSK-3 [15, 16]. To tackle this concern we used specific inhibition of GSK-3 (using the small molecule CHIR99021 [17]) and muscle-specific GSK-3β KO. These approaches increased *Pgc-1α* gene expression in myotubes and during muscle reloading respectively. This suggest that inactivation of GSK-3 results in enhanced *Pgc-1α* gene expression in skeletal muscle and other cell-types.

Distinct roles for GSK-3α and GSK-3β in the regulation of muscle OXPHEN

Previously, distinct non-redundant roles for both GSK-3 isoforms have been reported. Hence, we investigated GSK-3 isoform specific involvement in the regulation of *Pgc-1α* gene expression in muscle. Despite the close structural homology between the two GSK-3 isoforms, they are not functionally redundant. For example, while mice with specific deletion of GSK-3α are viable [18] GSK-3β KO mice die during embryonic development [19]. This indicates that during embryonic development specific functions of GSK-3β cannot be compensated by GSK-3α. Recently, genome-wide transcriptome analysis also revealed distinct roles for GSK-3α and GSK-3β in regulating embryonic stem cell fate [20]. In adulthood it seems that GSK-3α has a more critical role compared with GSK-3β in regulating hepatic glucose metabolism [18], while GSK-3β is the predominant regulator of glycogen synthase in skeletal muscle [21]. To investigate the role of both GSK-3 isoforms in the regulation of skeletal muscle OXPHEN we specifically knocked-down GSK-3α or GSK-3β, which allowed us to discriminate between the two isoforms in their involvement in the regulation of skeletal muscle OXPHEN. Knock-down of GSK-3α resulted in a much less potent induction of *Pgc-1α* gene expression compared with knock-down of GSK-3β. Moreover, GSK-3α knock-down did not result in increased gene expression of OXPHOS sub-units and constituents of the PGC-1α signaling network, while knock-down of GSK-3β did. Therefore, we conclude that GSK-3β is the predominant GSK-3 isoform involved in the regulation of oxidative substrate metabolism and mitochondrial biogenesis in skeletal muscle. In addition to the effect of GSK-3β on *Pgc-1α* gene expression, GSK-3β can phosphorylate the PGC-1α protein predisposing

it for intra-nuclear proteasomal degradation, which decreases the overall activity of PGC-1α [22, 23]. This suggests that besides transcriptional regulation, GSK-3β is also involved in post-transcriptional regulation of PGC-1α. Altogether, inactivation of GSK-3β thus potently induced PGC-1α levels.

Specific activation of the ERR-branch of the PGC-1α signaling

In addition to a potent upregulation of PGC-1α, we also reported that inactivation of GSK-3β is associated with specific activation of the estrogen-related receptor (ERR)-branch of the PGC-1α signaling network, known to be involved in the regulation of genes involved in oxidative substrate metabolism and OXPHOS in muscle [24]. Indicative of this is the increased gene expression of ERR-target genes (pyruvate dehydrogenase kinase 4 (*Pdk4*) and PGC-1 and ERR-induced regulator in muscle 1 (*Perm1*)) [25, 26] in response to inactivation of GSK-3β. PDK4 plays an important role in metabolic flexibility as an increased PDK4 level is indicative for favoring fatty acid β-oxidation over oxidative carbohydrate metabolism for adenosine triphosphate (ATP) production [27]. Our data is in line with increased *Pdk4* gene expression in glia and neuronal cells in response to LiCl treatment [12]. PERM1 is a muscle-specific regulator of oxidative capacity and known to regulate mitochondrial biogenesis and OXPHOS gene expression. Intriguingly, the downstream effect of overexpression of PERM1 in muscle [26, 28] largely overlaps the effect of inactivation of GSK-3β reported in this thesis. In contrast to our *in vitro* results using specific inhibition of GSK-3 or GSK-3β knock-down, studies in non-muscle cells revealed that inhibition of GSK-3 using LiCl also increased gene expression and protein abundance of nuclear respiratory factors (NRFs) and mitochondrial transcription factor A (TFAM) [8, 9], another branch of the PGC-1α signaling network. However, off-target effects of LiCl or tissue-specific effects of inactivation of GSK-3β could potentially explain this. Nonetheless, muscle-specific GSK-3β KO mice were protected against unloading-induced loss and subsequent enhanced reloading-associated regain in gene expression of all constituents of the PGC-1α signaling network that we assessed. Interestingly, gene expression of OXPHOS genes known to be under the control of ERRα are decreased [29, 30], while GSK-3β activity is increased in the skeletal muscle of type II diabetics [31]. This suggests, in concert with specific activation of the ERR-branch of the PGC-1α signaling upon inactivation of GSK-3β, that inactivation of GSK-3β might have pharmacological potential in reversing the decreased OXPHOS gene expression in skeletal muscle in conditions associated with reduced muscle OXPHEN. In conclusion, besides the upregulation of PGC-1α expression, it seems that inactivation of GSK-3β in skeletal muscle cells specifically activates the ERR-branch of the PGC-1α signaling network.

Inactivation of GSK-3β enhances mitochondrial biogenesis and the regulation of oxidative energy metabolism in skeletal muscle

Interestingly and in line with the well-known role of PGC-1α in the transcriptional control of OXPHOS genes [32], increased gene expression of OXPHOS sub-units mediated by inactivation of GSK-3β in skeletal muscle cells required PGC-1α (**Chapter 3**). In addition, muscle-specific GSK-3β KO mice were protected against unloading-induced decrements in nuclear-encoded OXPHOS gene expression (**Chapter 3**). In line with this protective effect, reloading of the hind-limbs of these disuse-atrophied mice even potentiated the gene expression of OXPHOS sub-units (**Chapter 4**). In contrast, the phosphorylation of $Ser^{21/9}$-mediated inactivation of GSK-3α/β is not required for reloading-induced OXPHOS gene expression (**Chapter 2**), which indicates that GSK-3 inactivation is dispensable for reloading-associated changes in the regulation of muscle OXPHEN. However, other post-translational modifications of GSK-3β (*e.g.* phosphorylation of Thr^{356} [33], Thr^{43} [34], Ser^{389} and Thr^{390} [35]) have been reported to decrease its activity, which were not assessed in our study. Since GSK-3β phosphorylation at Ser^{9} is the best-characterized inhibitory phosphorylation of GSK-3β (and increased during reloading of disused muscle), we focused on $Ser^{21/9}$-mediated inactivation of GSK-3α/β. In line with our results, inhibition of GSK-3β increased gene expression and protein abundance of OXPHOS sub-units in primary cortical neurons and bovine aortic endothelial cells [8, 9]. In contrast, Martin *et al.* reported that LiCl treatment did not result in higher abundance of OXPHOS sub-units in glia and neuronal cells, while increasing basal and maximal mitochondrial respiration [12]. The latter confirmed increased mitochondrial respiration at the end of myogenic differentiation following knock-down of GSK-3β that we observed (**Chapter 3**). In addition and in concert with our data, inactivation of GSK-3β increased mitochondrial DNA (mtDNA) copy number in bovine aortic endothelial cells and neuronal cells [8, 9]. Increased activity of citrate synthase, an important enzyme involved in the tricarboxylic acid (TCA) cycle, was previously reported after LiCl treatment in neuronal cells [9] and is in line with our data (**Chapter 3 and 4**). Taken together, the robust induction of PGC-1α and subsequent downstream effects on oxidative energy metabolism mediated by inactivation of GSK-3β in myotubes (**Chapter 3**) and several non-muscle cells suggest that GSK-3β controls PGC-1α and mitochondrial biogenesis through a conserved pathway present in multiple tissues and organs.

Inactivation of GSK-3β does not alter skeletal muscle fiber type distribution

Although we observed that inactivation of GSK-3β potently upregulated OXPHOS gene expression and mitochondrial respiration it seems that GSK-3β does not play a role in regulating the proportion of oxidative fiber types, despite also being

under the control of PGC-1α [36]. Previously, data of our group revealed that the *M. soleus* fiber type distribution of muscle-specific GSK-3β KO mice was unaltered [37]. In addition, the fiber type distribution in *M. soleus* was similar in constitutively active GSK-3 knock-in mice compared to wild-type animals (**Chapter 2**). PGC-1α and PGC-1β both regulate gene expression of structural proteins of slow-twitch oxidative fibers via amongst others, myocyte enhancer factor 2 (MEF2) [36, 38] and PPARδ [39, 40]. Although there is evidence that GSK-3β can influence MEF2 activity, likely through modulation of histone deacetylase 5 (a repressor of MEF2 activity) [41], we observed that inactivation of GSK-3β did not affect MEF2 transcriptional activity or MEF2 isoform gene expression (**Chapter 5**). Moreover, knock-down of GSK-3β did not increases gene expression of PPARδ (**Chapter 3**). This suggests that inhibition of GSK-3β does not alter MEF2 and PPARδ-mediated slow-twitch myosin heavy chain transcription, which is in line with the unaltered fiber type distribution in mice with altered activity of GSK-3β in skeletal muscle. Taken together, this indicates a role for GSK-3β in the regulation of skeletal muscle OXPHEN independent of regulating the oxidative fiber type proportion. This is in line with specific activation of the ERR-branch of the PGC-1α signaling network.

Identification of a GSK-3β/TFEB/PGC-1α signaling axis in skeletal muscle

Intriguingly, in-depth analysis of the molecular regulation of *Pgc-1α* gene expression activated by inactivation of GSK-3β revealed that TFEB is indispensable for increased *Pgc-1α* expression upon inactivation of GSK-3β (**Chapter 5**).

The MiT family of transcription factors

TFEB is a member of the microphthalmia/TFE (MiT) family of basic helix-loop-helix leucine zipper (bHLH-Zip) transcription factors that are highly conserved in vertebrates [42]. An identical and essential basic region for DNA binding and largely similar HLH and Zip regions highlight their close structural homology [43]. Other members of this class of transcription factors are microphthalmia-associated transcription factor (MITF), transcription factor binding to IGHM enhancer 3 (TFE3) and transcription factor EC (macrophage-specific [44]). All MiT transcription factors recognize and bind specific E-boxes (5'-(GT)CACGTG(AC)-3'), also known as coordinated lysosomal expression and regulation (CLEAR)-regions. As this name implies, CLEAR-regions are present on the promoter sequence of several lysosomal biogenesis-related genes [45]. In addition, several autophagy-related genes are under the control of MiT proteins [46, 47]. Furthermore, CLEAR-regions are present on the TFEB promoter indicative of a self-regulatory feedback loop [48]. Interestingly, recent reports suggest

a role for TFEB and other MiT family members in the regulation of multiple metabolic processes [43] such as mitochondrial biogenesis and oxidative substrate metabolism in skeletal muscle.

The regulation of TFEB activity

The activity of TFEB is controlled by several means such as protein-protein interactions and post-translational modifications that influence the cellular localization of TFEB. Phosphorylation is the main process by which TFEB activity can be modulated [43]. Indeed, the cellular location of TFEB depends largely on its phosphorylation status. Recently, several proteins able to phosphorylate TFEB or other MiT transcription factors were identified including protein kinase B (AKT) [49], mammalian target of rapamycin (mTOR) [50], GSK-3β [51], mitogen-activated protein kinase [52] and extracellular signal-regulated kinase [47]. Interestingly, the above-mentioned kinases that have been described as regulators of TFEB activity have also been implicated in the regulation of GSK-3β activity suggesting a potential role of GSK-3β in regulating TFEB. Indeed, we report that inactivation of GSK-3β resulted in a reduced phosphorylation status of the TFEB protein, which suggests that GSK-3β can phosphorylate TFEB (**Chapter 5**). In general, phosphorylation of TFEB is associated with cytosolic retention. In line with this notion is that mTOR-mediated phosphorylation of Ser^{211} on TFEB is associated with binding to 14-3-3 scaffold proteins [53-55], a phospho-binding protein well-known for its role in cytosolic retention of transcription factors [56, 57]. Additionally, Marchand *et al.* showed that both GSK-3β and mTOR inhibitors promote dissociation of TFEB from 14-3-3 indicating a functional role for the phosphorylation sites of TFEB targeted by GSK-3β in cytosolic retention [58].

GSK-3β and mTOR are thus both important proteins regulating the activity of TFEB. In response to exercise circulating levels of insulin-like growth factor-I (IGF-I) rise [59] and stimulate the AKT signaling cascade [60]. AKT is well-known to phosphorylate both mTOR as well as GSK-3β in response to activation. While GSK-3β is inactivated upon AKT-mediated phosphorylation, mTOR activity increases. In this situation the mTOR-mediated TFEB phosphorylation (Ser^{142} and Ser^{211}) [53, 55, 61] likely increases while phosphorylation of TFEB mediated by GSK-3β (Ser^{138} and Ser^{134}) [51] diminishes. In contrast, GSK-3β was previously identified as a positive regulator of mTOR activity [62]. GSK-3β inhibition may therefore decrease mTOR activity thereby also indirectly activating TFEB. However, the interplay between mTOR and GSK-3β in the regulation of TFEB activity needs to be revealed in more detail. To date, it is not fully understood which phosphorylation site(s) on TFEB determine(s) the ability for nuclear translocation. Our data confirms that GSK-3β regulates TFEB activity because

dephosphorylation of TFEB mediated by inactivation of GSK-3β was associated with nuclear translocation (**Chapter 5**). Hence, pharmacological inhibition of GSK-3β reveals a window of opportunity where dephosphorylation of TFEB by phosphatases results in its nuclear translocation. It has been proposed that calcineurin is an important phosphatase controlling TFEB dephosphorylation [63]. Interestingly, calcineurin is activated through intra-cellular calcium release initiated by physical exercise [64] and starvation [63]. However, whether calcineurin is essential for TFEB nuclear translocation in response to inhibition of GSK-3β in our study remains to be investigated.

Potential involvement of post-translational modifications other than phosphorylation in the regulation of TFEB activity is less studied. Recently, acetylation of TFEB in the brain has been reported [65, 66]. The scarce data on the functional relevance of TFEB acetylation however is contradicting. One study indicates that TFEB acetylation results in nuclear translocation and increased DNA binding capability of the TFEB protein [65]. In contrast, another report showed that sirtuin 1 (SIRT1) overexpression increased the gene expression of several downstream targets of TFEB, indicating that TFEB deacetylation increased its transcriptional activity [67]. In line, inhibition of SIRT1 decreased TFEB regulated gene transcription but did not affect TFEB nuclear translocation [66]. This highlights that the effect of TFEB (de)acetylation on subcellular localization or transcriptional activity of the TFEB protein in skeletal muscle remains to be elucidated. Our data suggest that the main post-translational modification of TFEB is phosphorylation as phosphatase treatment resulted in a similar mobility shift of the band corresponding to the TFEB protein of vehicle- and CHIR99021-treated myotubes (**Chapter 5**). In conclusion, phosphorylation of the TFEB protein appears to be the main process that controls TFEB activity in skeletal muscle, which amongst others is influenced by GSK-3β.

TFEB and PGC-1α promoter activity

Several previous studies reported functional CLEAR-regions on the proximal PGC-1α promoter [43, 48, 68], indicating that TFEB is a potential regulator of *Pgc-1α* gene transcription. Indeed, we reported that overexpression of TFEB or TFE3 is sufficient to increase *Pgc-1α* gene expression in skeletal muscle cells (**Chapter 5**), which is in line with previously published data [68-70]. Specific mutation of a CLEAR-region located on the proximal PGC-1α promoter prevented its activation mediated by TFE3 overexpression [68] showing functionality of these CLEAR-regions on the PGC-1α promoter. Interestingly, the same CLEAR-region is required for activation of the proximal PGC-1α promoter mediated by inactivation of GSK-3β (**Chapter 5**). In addition, muscle-specific ablation of TFEB did not alter basal *Pgc-1α* mRNA levels in skeletal muscle and C2C12 myotubes [70], which is in line with our data after

knock-down of TFEB in C2C12 myotubes. This implies that TFEB is not required for basal *Pgc-1α* gene expression. Importantly, TFEB was found to be indispensable for increased PGC-1α transcription mediated by inactivation of GSK-3β. Thus, upon nuclear translocation TFEB can bind to CLEAR-regions located on the proximal PGC-1α promoter, increasing the PGC-1α promoter activity and initiating *Pgc-1α* gene expression.

Next to directly inducing *Pgc-1α* gene expression, TFEB might also be involved in indirect activation of *Pgc-1α* transcription. Recently it has been shown that TFEB overexpression activates vascular endothelial growth factor-stimulated AMP-activated protein kinase (AMPK) signaling via Ca^{2+}/calmodulin-dependent protein kinase kinase-β in vascular endothelial cells [71]. AMPK has previously been reported to activate SIRT1, which is able to activate *Pgc-1α* gene expression [72]. Whether TFEB activation in response to inactivation of GSK-3β also activates AMPK in skeletal muscle is currently unknown. Taken together and in concert with literature, our data reveals that inactivation of GSK-3β is associated with activation of the PGC-1α promoter via TFEB.

The role of TFEB in the regulation of mitochondrial biogenesis and oxidative capacity
As described above, increased TFEB activity in skeletal muscle is associated with the induction of *Pgc-1α* mRNA transcription. Hence, it is not unlikely that TFEB is involved in the regulation of mitochondrial biogenesis and oxidative capacity, processes known to be controlled by PGC-1α. Indeed, Mansueto *et al.* showed that TFEB plays an important role in the regulation of several metabolic pathways by using muscle-specific TFEB overexpression and KO mice. Gene ontology enrichment analysis revealed that TFEB overexpression increased gene expression of several genes involved in lipid and glucose homeostasis, while TFEB KO downregulated the expression of these genes in skeletal muscle [70]. Interestingly and consistent with these results, we showed that knock-down of GSK-3β increased the gene expression of several genes involved in glucose as well as lipid metabolism (**Chapter 3**), indicating a role for GSK-3β in coordinating several aspects of cellular energy production potentially via TFEB. In addition, increased mtDNA copy number, OXPHOS abundance and gene expression of several genes involved in mitochondrial biogenesis and oxidative energy metabolism mediated by inactivation of GSK-3β largely overlap with previously reported effects of overexpression of TFEB or TFE3 on these processes [68, 70]. As stated previously in this thesis, GSK-3β might selectively enhance muscle OXPHEN without altering myofiber types. Accordingly, TFEB overexpression did not alter muscle fiber type distribution [70]. The absence of TFEB in skeletal muscle increased the number of morphologically abnormal mitochondria.

This abnormal morphology was associated with mitochondrial dysfunction leading to reduced ATP production, resulting in reduced metabolic flexibility during physical exercise [70]. This renders TFEB KO mice unable to exploit the beneficial effects of exercise on muscle function. Taken together, the effects of TFEB overexpression on mitochondrial biogenesis and oxidative energy metabolism largely overlap with the effects of inactivation of GSK-3β that we observed, suggesting that GSK-3β might regulate these processes through TFEB. Interestingly and next to initiating *Pgc-1α* gene expression, TFEB has been shown to regulate metabolic flexibility in skeletal muscle independent from PGC-1α [70]. However, we show that the inactivation of GSK-3β-mediated induction of OXPHOS gene expression requires PGC-1α which underscores that TFEB is primarily positioned upstream of PGC-1α.

TFE3 and TFEB have partially functional overlap in the regulation of mitochondrial biogenesis and oxidative energy metabolism. Indicative of this functional overlap, whole-body TFE3 KO mice display cellular and metabolic phenotypes that are similar to tissue-specific TFEB KO mice. TFE3 KO mice display dysregulated systemic glucose and lipid metabolism resulting in enhanced diet-induced obesity and diabetes [73]. Impaired lipid catabolism was also reported in liver-specific TFEB KO mice while overexpression of TFEB in several obesity models rescues the obese phenotype [48]. Interestingly, TFEB overexpression rescues diet-induced obesity in TFE3 KO mice while TFE3 overexpression rescues diet-induced obesity in liver-specific TFEB KO mice [73]. This indicates that TFE3 and TFEB might play a cooperative role in metabolic adaptation in response to external signals. Both TFE3 KO and muscle-specific TFEB KO mice have reduced endurance capacity [70, 73], while overexpression of TFEB in the skeletal muscle increases endurance capacity [70]. The reduced exercise capacity of these animals also indicates that TFEB function is non-commutable by TFE3 and *vice versa*. Despite the partial overlap in TFEB and TFE3 regulation of PGC-1α promoter activity [68], knock-down of TFEB completely prevented GSK-3β-mediated inductions of PGC-1α promoter activity and mRNA transcript levels, without altering *Tfe3* gene expression (**Chapter 5**). This suggests that TFEB is indispensable for increased *Pgc-1α* gene expression mediated by inactivation of GSK-3β and thus reveals non-redundant roles for TFEB and TFE3 in PGC-1α regulation. Of note, a putative co-requirement of TFE3 in the TFEB-mediated regulation of *Pgc-1α* gene expression cannot be ruled out as TFEB-TFE3 heterodimer formation is common prior to DNA binding [43, 74, 75].

Next to enhancing *Pgc-1α* gene expression and mitochondrial biogenesis, TFE3 can also bind to CLEAR-regions on the fission1 (*Fis1*) promoter [73] indicating that MiT transcription factors directly regulate mitochondrial fission. Previously, TFEB and TFE3 have been reported to also directly exert transcriptional control over several autophagy related genes [45, 47], suggesting that members of the MiT family of

transcription factors mediate mitochondrial turnover. Although not investigated in our study, several previous reports indeed indicate that GSK-3β is involved in the regulation of autophagy [76]. Interestingly, it has been suggested that this is regulated via TFEB [58]. Hence, GSK-3β and TFEB might therefore act as central regulators of mitochondrial quality.

Sequence of events: the emerging significance of the GSK-3β/TFEB/PGC-1α signaling network

Our data shows that inactivation of GSK-3β induces *Pgc-1α* gene expression via nuclear translocation of TFEB. In addition, PGC-1α is required for the inactivation of GSK-3β-mediated increase in muscle OXPHEN (**Chapter 3**). This in combination with existing literature supports the proposed sequence of events depicted in Fig. 1. In short, inactivation of GSK-3β results in dephosphorylation of TFEB facilitating nuclear translocation of the TFEB protein. Upon binding to CLEAR-regions located on the proximal PGC-1α promoter TFEB initiates promoter activation increasing *Pgc-1α* gene expression and protein abundance (**Chapter 5**). Subsequently, PGC-1α enhances skeletal muscle mitochondrial biogenesis and oxidative substrate metabolism (**Chapter 3 and 4**).

Physical (in)activity and TFEB

As described above, TFEB is involved in the regulation of processes known to be involved in physical activity-associated remodeling of skeletal muscle, *i.e.* initiation of mitochondrial biogenesis and the molecular regulation of muscle oxidative capacity via PGC-1α. Hence, it is not unlikely that physical activity increases the activity of TFEB. Illustrative of this is the observation that exercise indeed increased TFEB nuclear localization in skeletal muscle of mice [69, 70]. In addition, electrically-stimulated chronic muscle contractile activity increased the total abundance of TFEB, however TFEB nuclear localization was not assessed [77]. This was associated with increased protein abundance of PGC-1α and the OXPHOS sub-unit cytochrome c oxidase [77]. Furthermore, Medina *et al.* reported that the physical exercise-mediated increase in TFEB nuclear translocation depended on calcineurin in skeletal muscle [63]. It appears that nuclear translocation of TFEB depends on the severity of the exercise stimulus because acute exhaustive contraction led to nuclear translocation of TFEB whereas mild exercise did not [70]. Whether TFEB also moves to the nucleus during reloading of disused muscle however remains unknown. Although speculatively in nature, the protective effect of muscle-specific GSK-3β KO on *Pgc-1α* gene expression upon unloading and enhanced *Pgc-1α* gene expression in response to reloading could be due to increased TFEB nuclear content in these mice. Taken together, literature convincingly shows that TFEB translocates to the nucleus in response to physical activity and that this is associated with exercise-induced mitochondrial adaptations.

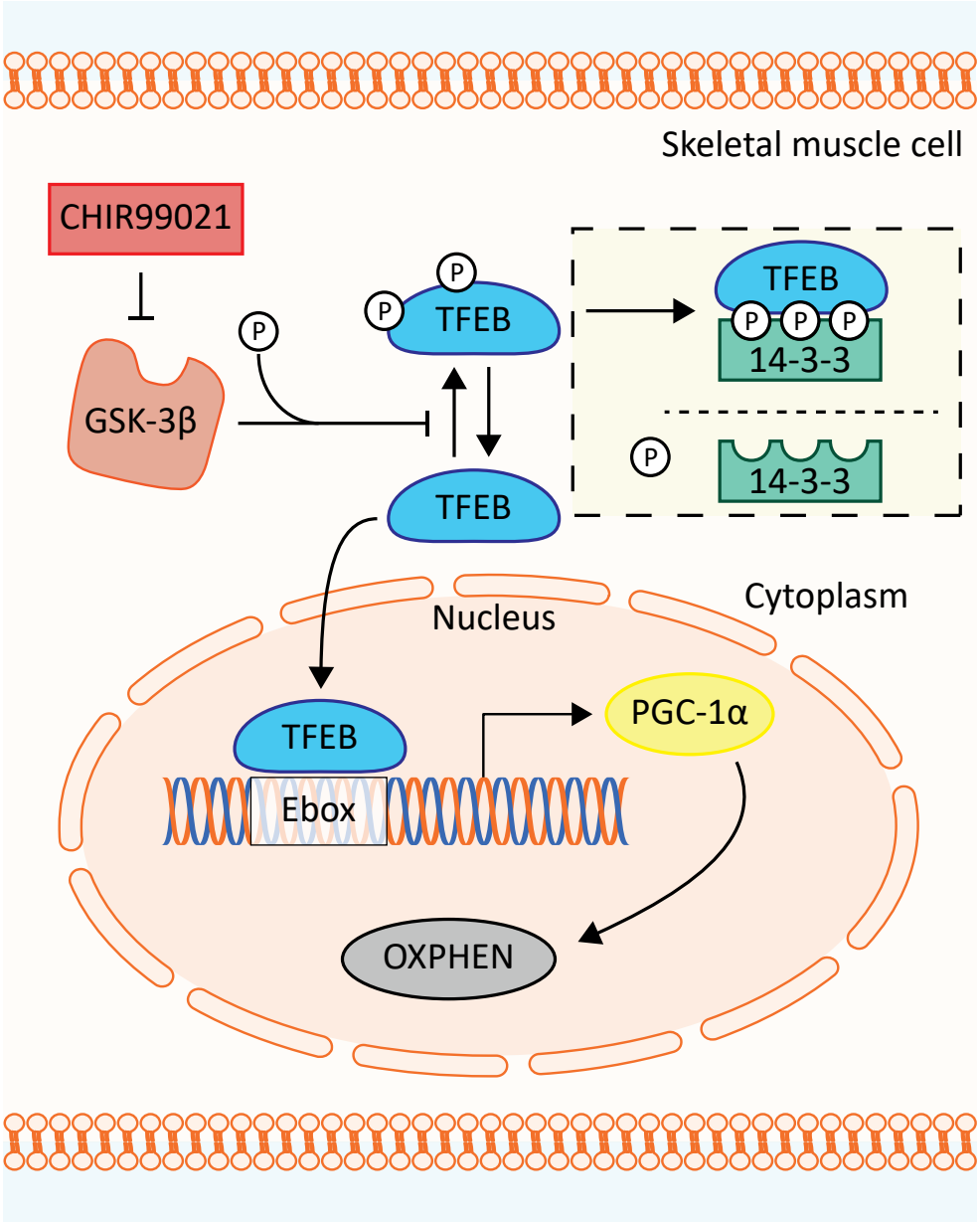


Figure 1. Schematic representation of the proposed sequence of events based on the main findings of this thesis. Inactivation of GSK-3β using CHIR99021 results in dephosphorylation of TFEB. While phosphorylated TFEB is known to bind to 14-3-3 proteins resulting in cytosolic retention, TFEB dephosphorylation results in nuclear translocation. Inside the nucleus TFEB binds to E-boxes called CLEAR-regions increasing *Pgc-1α* gene expression. Increased PGC-1α levels induce skeletal muscle OXPHOS abundance and mitochondrial biogenesis. Glycogen synthase kinase-3β (GSK-3β), transcription factor EB (TFEB), coordinated lysosomal expression and regulation (CLEAR), peroxisome proliferator-activated receptor-γ co-activator-1α (PGC-1α) and oxidative phosphorylation (OXPHOS).

Physical activity decreases GSK-3β activity in skeletal muscle

Exercise is the best-known trigger inducing skeletal muscle PGC-1α expression. This has been well established both in rodents [78, 79] as well as in humans [80]. In addition, skeletal muscle reloading after unloading-induced muscle atrophy is associated with increased PGC-1α abundance in rodents (**Chapter 2 and 4**) [81, 82]. *Vice versa*, reduced *Pgc-1α* gene expression has been described in human skeletal muscle after physical inactivity, *e.g.* after leg casting [83] or bed rest [84]. Intriguingly, in rodents, exercise [85-87] and reloading of disused muscle [37] is also associated with an increased level of the inhibitory phosphorylation of GSK-3β (Ser^9) (**Chapter 2**). This together with the findings in this thesis describing that GSK-3β inactivation upregulates PGC-1α suggests that exercise-induced inactivation of GSK-3β may underlie increases in PGC-1α and subsequent beneficial adaptation of the muscle to exercise. However, reports describing the effect of exercise on the phosphorylation status (and hence activation) of GSK-3 in human skeletal muscle vary in their findings. Sakamoto *et al.* reported that exercise increased the inhibitory phosphorylation of GSK-3α and thus decreased GSK-3α activity in human muscle, while GSK-3β activity was unaffected [88]. Furthermore, low and high-intensity cycling exercise did not decrease GSK-3 activity in skeletal muscle [89]. In contrast, Mascher *et al.* reported increased phosphorylation of both GSK-3 isoforms in human muscle directly after endurance exercise [90] and resistance training increased GSK-3β (Ser^9) phosphorylation in human skeletal muscle [91]. The latter is consistent with increased gene expression of *Pgc-1α* in *M. soleus* and *M. gastrocnemius* of rats subjected to resistance training [92]. Mascher *et al.* reported that the phosphorylation of both GSK-3 isoforms remained elevated during a 3-hour post-exercise recovery period [90]. This is interesting because the exercise-induced expression of *Pgc-1α* mRNA in skeletal muscle peaks within this timeframe in humans [80] and in mice [79]. Several kinases such as AKT [93], protein kinase A [94], protein kinase C [95], p70 S6 kinase [96] and mitogen-activated protein kinase [35] have been reported to phosphorylate GSK-3β at Ser^9, inhibiting its activity. These are all nodal points in signaling cascades activated by for example IGF-I and insulin. In the context of muscle plasticity, these ligands stimulate muscle hypertrophy and regeneration through stimulation of protein synthesis [97] and myogenesis [98]. In summary, although not all literature is consistent, physical activity appears to be able to increase the inhibitory Ser^9 phosphorylation of GSK-3β thereby decreasing its activity. Physical activity-mediated inactivation of GSK-3β thus may contribute to activity-induced upregulation of PGC-1α in skeletal muscle.

Disease-related factors, other than inactivity, associated with reduced skeletal muscle mitochondrial biogenesis and oxidative capacity

Reduced muscle OXPHEN in humans

As described above, GSK-3β inactivation may mediate exercise-induced activation of the PGC-1α signaling cascade, but whether or not abnormalities in the activation status of GSK-3β underlie or contribute to decreased muscle PGC-1α levels and its downstream mitochondrial aberrations in human skeletal muscle is unclear. Several diseases or conditions are associated with abnormalities in skeletal muscle mitochondrial biogenesis and oxidative substrate metabolism. Aging and chronic diseases are well-known examples. More specifically, mitochondrial abundance is decreased in skeletal muscle of patients with chronic obstructive pulmonary disease (COPD) [99], type II diabetes [100], chronic heart failure (CHF) [101] and chronic kidney disease (CKD) [102] compared with healthy controls. In line with these findings, reduced abundance and activity of key mitochondrial enzymes involved in the TCA cycle and fatty acid β-oxidation have been observed in the skeletal musculature of patients with COPD [103, 104], CHF [105, 106], CKD [107] and type II diabetes [100]. Next to reduced abundance and functionality of skeletal muscle mitochondria, the expression level of PGC-1α has been shown to be reduced in the musculature of COPD patients [2], type II diabetics [1, 108], CHF patients [109] and during normal physiological aging [110]. In these conditions, reduced levels of PGC-1α in skeletal muscle often coincide with lower abundance of sub-units of OXPHOS complexes involved in the electron transport chain [30, 111, 112]. The data presented in this thesis underscores that GSK-3β is a negative regulator of PGC-1α and that inactivation of GSK-3β is associated with increased PGC-1α abundance and enhanced mitochondrial biogenesis in muscle. Hence, GSK-3β may serve as an interesting pharmacological target for treatment aiming to increase PGC-1α and subsequently enhancing muscle mitochondrial biogenesis and oxidative energy metabolism in these conditions. Besides physical inactivity (see previous section), several other disease-related external stimuli such as chronic inflammation, malnutrition and hypoxia could contribute to the development of mitochondrial dysfunction potentially via modulation of GSK-3β activity. The impact of specific disease-related factors on GSK-3β activity and the PGC-1α level in muscle is described in the following section.

Chronic inflammation

Chronic low-grade systemic inflammation often accompanies the above-mentioned chronic diseases [113]. An increased level of the pro-inflammatory cytokine tumor necrosis factor (TNF)α in the circulation is a well-known hallmark of chronic inflammation [114]. TNFα reduces *Pgc-1α* gene expression in cultured myocytes

and cigarette smoke exposure-induced TNFα is negatively associated with skeletal muscle *Pgc-1α* mRNA abundance [115], indicating that inflammatory mediators exert a negative effect on mitochondrial biogenesis and the molecular regulation thereof by the PGC-1α signaling network. Elevated TNFα levels have been associated with reduced gene expression of *Pgc-1α* in COPD patients [116]. In addition, increased levels of TNFα reduced OXPHOS protein abundance, mitochondrial respiration and mitochondrial biogenesis in cultured skeletal muscle cells [115-117]. Lipopolysaccharide (LPS) exposure is often used to induce inflammation in experimental models and is known to increase circulating levels of TNFα [118]. It was previously shown that LPS reduced mitochondrial respiration of *in vitro* cultured myotubes, dissected mouse myofibers [119] and isolated porcine skeletal muscle mitochondria [120]. LPS exposure also reduced gene expression of *Pgc-1α* in several other cell types or tissues [121, 122], indicating the widespread consequences of (chronic) inflammation on the reduction of mitochondrial biogenesis. With regard to the impact of inflammatory mediators on the activation status of GSK-3β, TNFα did not alter total GSK-3 protein levels during myogenic differentiation [123]. Furthermore, TNFα failed to change insulin-stimulated inactivation of GSK-3 in cultured human muscle cells [123]. In addition, LPS treatment did not alter the phosphorylation status of GSK-3β [124], indicating that chronic inflammation likely does not alter GSK-3β activity in skeletal muscle. Therefore, it seems unlikely that inflammation affects muscle OXPHEN via alterations in GSK-3β activity. However, as muscle-specific GSK-3β KO prevents inflammation-induced muscle atrophy [124] GSK-3β inhibitors could still be beneficial for muscle mass maintenance in chronic inflammation-induced muscle atrophy.

Malnutrition

Chronic diseases are often associated with malnutrition (defined as an insufficient energy or nutrient intake) [125], which evidently can contribute to reductions in muscle mass [126]. Malnutrition is also associated with increased muscle fatigability [127], decreased OXPHOS complexes activity and lower mtDNA content in skeletal muscle of rats [128, 129]. Undernutrition (inadequate nutritional intake resulting in any deficiency in macro- or micronutrients) is associated with weakening of the respiratory muscles in COPD patients, which in response to refeeding is reversible [130]. Moreover, undernutrition resulted in respiratory muscle atrophy and is associated with impaired mitochondrial respiratory capacity of diaphragm muscle in rats [131, 132]. In contrast, undernutrition-associated hind-limb skeletal muscle atrophy was not associated with reduced mitochondrial respiration [133]. Furthermore, caloric restriction (reduced caloric intake without a nutritional deficiency) has been repetitively shown to increase skeletal muscle mitochondrial

biogenesis in rodents [134, 135]. Malnutrition can result in deficiencies in essential macronutrients, minerals or vitamins amongst others iron and vitamin D [136]. Vitamin D deficiency has been associated with muscle atrophy and loss of muscle OXPHEN [137]. Interestingly, improving the vitamin D status of vitamin D deficient adults is associated with improved mitochondrial oxidative function in skeletal muscle [138]. Iron deficiency as a result of malnutrition has been associated with impaired exercise capacity in COPD and CHF patients [139, 140]. In addition, iron is an essential component for multiple protein complexes involved in the OXPHOS system [141]. Indeed, several *in vivo* studies revealed that iron deficiency reduced mitochondrial abundance or respiration in skeletal muscle [142-146]. Interestingly, iron therapy increased the exercise capacity of iron-deficient CHF patients [147]. Illustrative of a potential role for GSK-3β in malnutrition-induced muscle abnormalities, muscle-specific GSK-3β KO mice are protected against starvation-induced muscle atrophy [148]. With regard to the impact of malnutrition on the activation status of GSK-3β, malnutrition-associated diaphragm atrophy was accompanied by reduced phosphorylation of GSK-3β (*i.e.* GSK-3β activation) [149]. In addition, acute energy deprivation decreased the phosphorylation status of AKT and 4E-BP1 indicating reduced activity of AKT-signaling [150]. This implies that acute energy deprivation potentially activates GSK-3β because the AKT-mediated inhibitory phosphorylation of GSK-3β might be impaired. Furthermore, amino-acid exposure is associated with inactivation of GSK-3β in skeletal muscle myoblasts [151], indicating that amino-acid deprivation might increase GSK-3β activity in muscle. Also, supplementation of an active form of vitamin D previously increased the phosphorylation of AKT [152] and the inhibitory Ser^{9} phosphorylation of GSK-3β [153] in human leukemia cells. In line, plasma levels of the active form of vitamin D were positively associated with the inhibitory phosphorylation of GSK-3β in adipose tissue but not in skeletal muscle [154]. Although malnutrition involves many aspects of nutrition, several cases of malnutrition or energy deprivation may thus be associated with increased activity of skeletal muscle GSK-3β.

Hypoxia

End-stage COPD and CHF patients may suffer from chronic or intermittent hypoxia [155-157]. In these conditions hypoxia is associated with a lower muscle aerobic capacity compared with healthy controls [158] resulting in impaired endurance capacity [159, 160]. Surprisingly, there is no evidence that oxygen therapy structurally improves skeletal muscle OXPHEN in these patients. Environmental hypoxia is associated with a loss of skeletal muscle OXPHEN (for review see [161]). Hypoxia-inducible factor-1α (HIF-1α) facilitates adaptive responses to reduced oxygen availability. An essential adaptive response to chronic hypoxia controlled by HIF-1α is a shift from oxidative towards anaerobic glycolytic metabolism [162-164].

However, the effect of experimentally-induced hypoxia on skeletal muscle PGC-1α mRNA and protein abundance described in literature varies, which could partly be explained by the different duration and severity of the hypoxia trigger that was applied. Previous studies reported decreased [165] and unaltered [166] PGC-1α protein abundance upon hypoxia in rodent skeletal muscle. Furthermore, hypoxia reduced [164] and increased [167] *Pgc-1α* gene expression in murine *in vitro* muscle cultures. In addition, hypoxia decreased *Pgc-1α* gene expression in murine skeletal muscle [166]. The hypoxia-induced inhibition of the PGC-1α signaling cascade was found to be HIF-1α-dependent in cultured myofibers [164]. Slot *et al.* reported that reduced *Pgc-1α* gene expression in murine skeletal muscle and *in vitro* muscle cultures in response to hypoxia was associated with reduced gene expression of several OXPHOS sub-units [164, 166]. In general, it thus appears that hypoxia per se results in a loss of mitochondrial mass and OXPHEN in skeletal muscle [161]. Regarding effects of hypoxia on GSK-3β activity in muscle, literature is scarce and inconclusive. Chronic hypobaric hypoxia increased the total abundance of GSK-3β in rat skeletal muscle but GSK-3β activity or phosphorylation status was not assessed [168]. In addition, hypoxia exposure reduced the phosphorylation of GSK-3β in C2C12 myoblasts suggesting enhanced GSK-3β activity [169]. Moreover, hypoxia decreased the phosphorylation of GSK-3β increasing its activity in human liver cancer cells [170] and vascular smooth muscle [171]. Chrinic exposure of hypoxia for 4 weeks did not result in altered phosphorylation of GSK-3β in skeletal muscle of mice [172]. Taken together, due to lack of convincing evidence a conclusive remark regarding a potential causal involvement of GSK-3β in hypoxia-induced loss of muscle OXPHEN cannot be stated.

GSK-3β is a key node in the regulation of skeletal muscle mass and OXPHEN

The data presented in this thesis and work in non-muscle cells [8, 9] convincingly demonstrate that GSK-3β negatively controls the regulation of mitochondrial biogenesis and cellular OXPHEN. This highlights a link between the signaling pathways controlling muscle mass on one hand and muscle oxidative capacity on the other since (as outlined in **Chapter 1** of this thesis) GSK-3β can also suppress protein synthesis and contributes to protein degradation pathways in skeletal muscle [148, 173-179]. Illustrative of its importance in the regulation of muscle mass, GSK-3β inactivation decreased myofibril loss and muscle atrophy in response to denervation and starvation [148]. Furthermore, muscle-specific GSK-3β KO prevents against inflammation-induced muscle atrophy [123].

Our data shows a role for TFEB in the regulation of PGC-1α (**Chapter 5**). Whether TFEB is involved in the protective effects of GSK-3β inhibition on muscle mass is not known. However, TFEB has previously been described to regulate lysosomal biogenesis and autophagy [47], both processes being essential in autophagy-lysosomal pathway-mediated protein breakdown. Furthermore, TFEB overexpression activates mTOR complex1 (mTORC1) activity [180], which is a main regulatory complex in the control of muscle protein synthesis. Besides a potential direct involvement of TFEB in the regulation of muscle protein synthesis, TFEB activation mediated by inactivation of GSK-3β may also influence muscle mass regulation through PGC-1α. Recently it has been shown that overexpression of PGC-1α or PGC-1β increased protein synthesis in C2C12 myotubes, which was dependent on ERRα [181]. Moreover, exercise preconditioning (increasing PGC-1α) or muscle-specific overexpression of PGC-1α protected against denervation-, starvation- and hind-limb suspension-induced muscle atrophy [182-185]. Also, muscle-specific overexpression of PGC-1α4, an isoform expressed from the alternative promoter that has undergone post-transcriptional splicing, resulted in hypertrophy of muscle and cultured myotubes without increasing OXPHOS gene expression [186]. Summarized, our data highlights an intricate link between pathways involved in the regulation of skeletal muscle energy production and those controlling muscle mass with a central role for GSK-3β (Fig. 2). Our findings may therefore imply a coordinative role for GSK-3β in the regulation of muscle plasticity beyond control of muscle mass as its positioning in muscle anabolic pathways allows GSK-3β to act as a sensor to meet the increased energy demand of growing muscle upon its inactivation by inducing the PGC-1α and subsequent gene programs involved in oxidative energy metabolism and mitochondrial biogenesis.

Conclusion

This thesis shows that inactivation of GSK-3β potentiates skeletal muscle oxidative substrate metabolism and mitochondrial biogenesis via PGC-1α through activation of TFEB. Although the fundamental insight gained by research conducted in this thesis needs to be validated, in general this data contributes to the understanding of the regulation of mitochondrial biogenesis and OXPHEN in skeletal muscle. This ultimately may have therapeutic implications for alleviation of muscle abnormalities in chronic diseases such as COPD [2], type II diabetes mellitus [1, 108], CKD [187], CHF [188] and during aging [110], that are often characterized by both loss of muscle mass and deterioration of muscle oxidative capacity. The therapeutic potential of specific GSK-3β inhibitors, TFEB agonists and intervention strategies aiming to increase skeletal muscle PGC-1α signaling needs to be investigated.

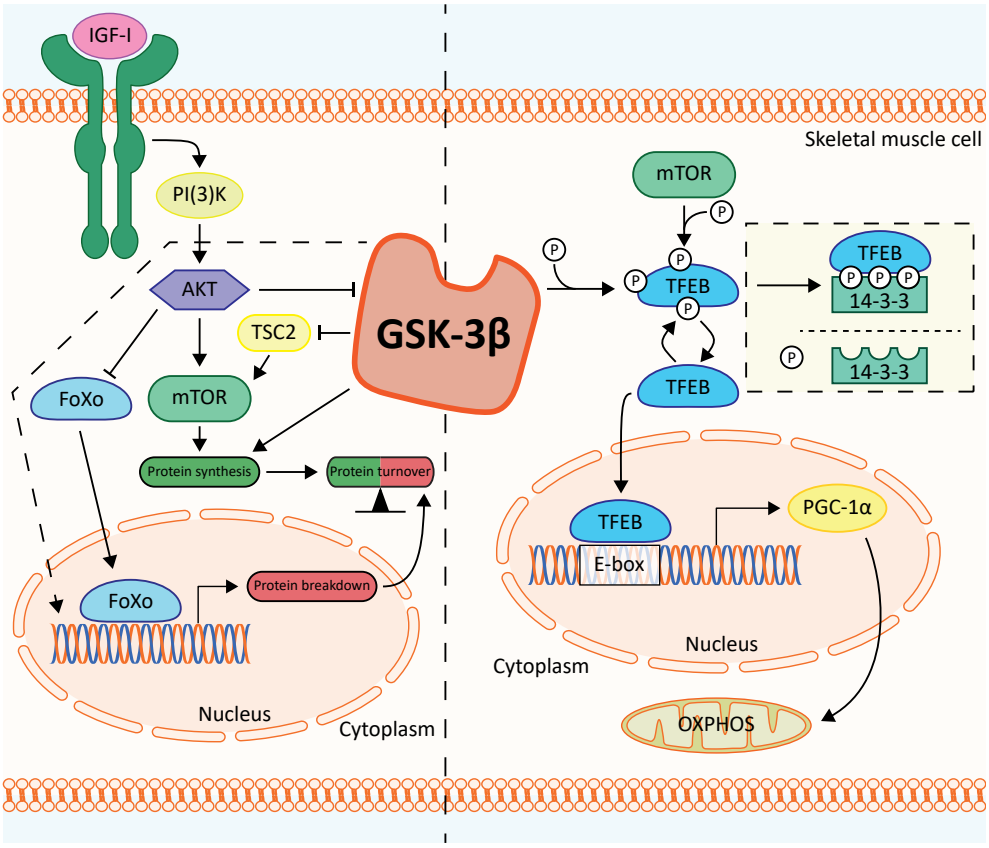


Figure 2. GSK-3β is an important central hub in the regulation of skeletal muscle mass as well as skeletal muscle OXPHEN. Left: IGF-I activates PI3K. In turn, AKT is activated by PI3K. AKT stimulates protein synthesis via activation of mTOR and inhibition of GSK-3β. Furthermore, AKT inhibits FoXO-mediated expression of genes involved in protein breakdown. Note that AKT stimulates protein synthesis and inhibits proteolysis indirectly via inactivation of GSK-3β. A dashed arrow indicates a not fully understood path. **Right**: GSK-3β and mTOR phosphorylate TFEB, which then detaches from 14-3-3 proteins and translocates into the nucleus. Within the nucleus TFEB binds to E-boxes called CLEAR-regions increasing *Pgc-1α* gene expression. Increased PGC-1α levels increases skeletal muscle mitochondrial biogenesis and OXPHOS abundance. Insulin-like growth factor-I (IGF-I), phosphoinositide 3-kinase (PI3K), protein kinase B (AKT), mammalian target of rapamycin (mTOR), glycogen synthase kinase-3β (GSK-3β), forkhead box class O FoXO, tuberous sclerosis complex 2 (TSC2), transcription factor EB (TFEB), coordinated lysosomal expression and regulation (CLEAR), peroxisome proliferator-activated receptor γ co-activator-1α (PGC-1α) and oxidative phosphorylation (OXPHOS).

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Summary

Skeletal muscle tissue has a remarkably high plasticity. This is reflected by its large capacity to adapt its mass and oxidative phenotype (OXPHEN; defined as the proportion of oxidative muscle fibers and mitochondrial oxidative capacity) in response to, amongst others, changes in physical activity or muscle loading. In **Chapter 1**, the molecular pathways regulating skeletal muscle OXPHEN are introduced. This primarily encompasses the peroxisome proliferator-activated receptor-γ co-activator-1 (PGC-1)α signaling network which has been convincingly shown to be a pivotal pathway in the regulation of mitochondrial biogenesis not only in skeletal muscle but in a wide variety of cell types. In addition, this chapter introduces glycogen synthase kinase (GSK)-3β and its role in the regulation of PGC-1α in non-muscle cells. This led to the main objective of this thesis: 'To investigate the role of GSK-3β in oxidative substrate metabolism and mitochondrial biogenesis in skeletal muscle'. The models that were used to investigate this are presented and physical (in)activity is introduced as one of the most potent external triggers affecting muscle OXPHEN. Furthermore, **Chapter 1** introduces the involvement of GSK-3β in the regulation of skeletal muscle mass, *e.g.* muscle protein turnover and post-natal myogenesis.

In **Chapter 2**, we investigated if GSK-3β inactivation during muscle reloading (after a period of muscle unloading) is essential for improvements in myogenesis, protein turnover signaling, PGC-1α signaling and subsequent expression of oxidative phosphorylation (OXPHOS) sub-units. We address this hypothesis using whole-body constitutively active (C.A.) GSK-3α/β knock-in and wild-type mice and investigated the soleus muscle of the hind-limb under baseline conditions, directly after 14-days of hind-limb suspension (HLS; a disuse-induced atrophy model) and during reloading of the hind-limbs. No consistent or significant alterations in reloading-induced changes in muscle mass, protein turnover, post-natal myogenesis nor in the regulation of muscle OXPHEN were observed between the two genotypes. In this chapter, we conclude that GSK-3 inactivation is dispensable for the above-mentioned processes during muscle reloading after a period of inactivity. However, subtle but consistent differences are observed at baseline between the two genotypes suggesting suppression of protein turnover signaling, PGC-1α signaling and mRNA expression of several OXPHOS sub-units resulting from constitutive activation of GSK-3.

These findings led to the hypothesis that inactivation of GSK-3β increases gene expression of PGC-1α, which subsequently induces mitochondrial biogenesis and gene expression of OXPHOS sub-units in adult muscle (**Chapter 3**). To address this hypothesis, we used pharmacological and genetic approaches to inhibit GSK-3β in fully differentiated C2C12 murine myotubes, as an *in vitro* analogue of myofibers. In addition, we used muscle-specific GSK-3β knock-out (KO) mice at baseline and

directly after 14-days of HLS. Largely in line with our findings in the C.A. GSK-3β knock-in mice (**Chapter 2**), overexpression of GSK-3β did not alter the expression of PGC-1α or OXPHOS sub-units in C2C12 myotubes. However, pharmacologic and genetic inhibition of GSK-3β potently increases mRNA and protein content of PGC-1α and OXPHOS sub-units in C2C12 myotubes. This is accompanied by increased levels of mitochondrial (mt)DNA. Furthermore, we reveal that increased gene expression of OXPHOS sub-units mediated by inhibition of GSK-3β requires PGC-1α by deploying double knock-down experiments. In addition, muscle-specific GSK-3β KO protects against unloading-induced loss of gene expression of components of the PGC-1α signaling cascade and OXPHOS sub-units. In conclusion, in this chapter we identified that GSK-3β inactivation increases PGC-1α levels in muscle cells and subsequently induces mitochondrial biogenesis and expression of sub-units of OXPHOS complexes.

Several molecular mechanisms control the oxidative capacity of the muscle during both maintenance as well as regeneration of adult skeletal muscle. Impairments in these regulatory mechanisms contribute to the development of skeletal muscle abnormalities. In **Chapter 4**, we therefore investigated if inactivation of GSK-3β also potentiates the PGC-1α signaling cascade during myogenic differentiation and during recovery of inactivity-induced muscle atrophy. We report that GSK-3β inhibition increases the abundance of key constituents of the PGC-1α signaling pathway and increases expression of OXPHOS sub-units during myogenic differentiation of C2C12 myoblast into myotubes, which is in line with the effects of inhibition of GSK-3β in fully differentiated myotubes that we describe in **Chapter 3**. Ultimately, knock-down of GSK-3β during myogenic differentiation results in enhanced mitochondrial respiration at the end of the myogenic differentiation program. In addition to these findings, *in vivo* experiments reveals that muscle-specific GSK-3β KO potentiates reloading-induced inductions in gene expression of components of the PGC-1α signaling and OXPHOS sub-units after a period of inactivity. Overall, we conclude that inactivation of GSK-3β potentiates the PGC-1α signaling, resulting in mitochondrial biogenesis and mitochondrial respiration during myogenic differentiation and muscle recovery after a period of physical inactivity.

In **Chapter 5**, we aimed to elucidate the molecular mechanism by which inactivation of GSK-3β increases *Pgc-1α* mRNA abundance in skeletal muscle cells. We used fully differentiated C2C12 myotubes to fundamentally underpin this underlying molecular mechanism. PGC-1α promoter activity enhances following inactivation of GSK-3β while chromatin accessibility of the PGC-1α promoter remained unaltered, indicating a transcriptionally-controlled mechanism. Subsequently, several transcription factors known to be involved in the transcriptional control of PGC-1α were pharmacologically

or genetically inhibited in C2C12 myotubes in order to investigate their possible involvement in transcriptional regulation of PGC-1α mediated by inactivation of GSK-3β. Inhibition of GSK-3β did not alter myocyte enhancer factor 2 (MEF2) transcriptional activity, indicating that MEF2 isoforms are likely not involved in enhanced *Pgc-1α* transcription mediated by inactivation of GSK-3β. Furthermore, while knock-down of GSK-3β increased estrogen-related receptor (ERR) expression levels and potentiated transcription of its down-stream targets, ERR transcription factors are not essential for increased *Pgc-1α* levels mediated by inhibition of GSK-3β. Interestingly, inhibition of GSK-3 activity results in nuclear translocation of transcription factor EB (TFEB). Furthermore, increased *Pgc-1α* gene expression and activation of the PGC-1α promoter mediated by inactivation of GSK-3β require TFEB. In addition, mutation of a TFEB binding sequence located on the proximal PGC-1α promoter blocked PGC-1α promoter activation induced by inactivation of GSK-3. Overall, we report that inactivation of GSK-3β causes nuclear translocation of TFEB, which is required for induction of PGC-1α promoter activation and subsequent transcription of the *Pgc-1α* gene.

Finally, in **Chapter 6**, the overall results of this thesis are discussed and placed in a broader perspective. We review the potential role of GSK-3β as key node in the regulation of skeletal muscle mass and OXPHEN in the context of disease-related factors that might be associated with increased activity of GSK-3β in skeletal muscle including physical inactivity, malnutrition and hypoxia. From a clinical perspective, the therapeutic potential of GSK-3β inhibitors or TFEB agonists is highlighted to treat or prevent muscle abnormalities during ageing and in chronic diseases such as chronic obstructive pulmonary disease, chronic heart failure, chronic kidney disease and type II diabetes (diseases that are often characterized by both loss of muscle mass and deterioration of muscle OXPHEN). Furthermore, we highlight our novel finding that inactivation of GSK-3β phosphorylates TFEB, resulting in its nuclear translocation, which results in increased promoter activation and gene expression of PGC-1α. Ultimately, inactivation of GSK-3β potentiates skeletal muscle oxidative substrate metabolism and mitochondrial biogenesis via this process.

Nederlandse Samenvatting

Onze spieren bezitten een opmerkelijk vermogen om zich aan te passen aan veranderende omstandigheden. Dit betekent dat zowel spiermassa als spiermetabolisme zich normaliter goed kunnen aanpassen aan veranderingen in onder andere fysieke activiteit of spierbelasting. Weefsels, waaronder ook de skeletspieren, bestaan uit cellen. Cellen bevatten op hun beurt verschillende celorganellen waaronder mitochondriën. Mitochondriën zijn erg belangrijk voor energieproductie door middel van substraatoxidatie, ofwel de verbranding van voedingsstoffen met behulp van zuurstof ook wel energiemetabolisme genoemd. **Hoofdstuk 1** introduceert de moleculaire processen die mitochondriële biogenese (de aanmaak van nieuwe mitochondriën) reguleren. De peroxisome proliferator-activated receptor-γ co-activator-1 (PGC-1)α signalering is essentieel voor dit proces en is onder andere betrokken bij het aansturen van de aanmaak van de zogenaamde oxidatieve fosforylering (OXPHOS) complexen. Deze OXPHOS-complexen zijn belangrijk voor de energieproductie van de cel en bevinden zich in de mitochondriën. Dit hoofdstuk zet ook de betrokkenheid van het eiwit glycogeen synthase kinase (GSK)-3β in de regulatie van PGC-1α in andere celtypen uiteen en het daaruit voortvloeiende algemene doel van dit proefschrift: 'het onderzoeken van de rol van GSK-3β in de regulatie van oxidatief energiemetabolisme en mitochondriële biogenese in de spier'. De betrokkenheid van GSK-3β in de moleculaire regulatie van spiermassa (eiwitaanmaak,-afbraak en postnatale myogenese (dat laatste is de vorming en regeneratie van spierweefsel)) wordt eveneens in **Hoofdstuk 1** besproken. Daarnaast introduceren we de experimentele modellen die we gebruikt hebben om de rol van GSK-3β in mitochondriële biogenese te onderzoeken. Fysieke (in)activiteit wordt daarbij gepositioneerd als belangrijke factor die energiemetabolisme in de spier kan beïnvloeden.

In **Hoofdstuk 2**, hebben we vervolgens onderzocht of uitschakelen van GSK-3β essentieel is voor de processen van myogenese, de aansturing van eiwitaanmaak en -afbraak, PGC-1α signalering en de aanmaak van OXPHOS-complexen tijdens spierherstel na inactiviteit. We hebben dit onderzocht door gebruik te maken van transgene muizen waarbij een permanent actieve vorm van GSK-3 door middel van genetische modificatie is aangebracht (in het hoofdstuk aangeduid met 'C.A. GSK-3 KI' muizen). Deze muizen hebben vervolgens een protocol doorlopen waarbij fysieke inactiviteit werd gesimuleerd door 14 dagen de achterpoten te ontlasten door muizen op te hangen aan hun staart. Om spierherstel te stimuleren zijn deze muizen vervolgens teruggeplaatst op hun achterpoten. We vonden geen uitgesproken verschillen tussen de gewone en transgene muizen tijdens de herstelfase. Hieruit hebben we geconcludeerd dat uitschakelen van GSK-3 niet noodzakelijk is voor boven-genoemde processen tijdens spierherstel na een periode van fysieke

inactiviteit. Daarentegen vonden we wel dat de aanmaak van onderdelen van de PGC-1α signalering en OXPHOS-complexen verminderd is in de spieren van transgene muizen ten opzichte van de gewone dieren onder basale condities.

De bovenstaande bevindingen hebben geleid tot de volgende hypothese: 'het uitschakelen van GSK-3β in de skeletspier verhoogt PGC-1α signalering, hetgeen mitochondriële biogenese en verhoogde aanmaak van de OXPHOS-complexen tot gevolg heeft'. Om deze hypothese te toetsen hebben we in **Hoofdstuk 3** gebruik gemaakt van een celkweek model van volledig gedifferentieerde spiercellen. Om de rol van GSK-3β in mitochondriële biogenese in de spiercel te onderzoeken hebben we de activiteit van GSK-3β farmacologisch geremd of het GSK-3β eiwit verwijderd. Ook hebben we transgene muizen gebruikt waarbij het GSK-3β eiwit door middel van genetische modificatie is verwijderd uit de skeletspier (in het hoofdstuk aangeduid met 'GSK-3 KO') en deze spieren geanalyseerd vóór en ná 14 dagen inactiviteit (zoals hierboven beschreven). Farmacologische remming en genetische verwijdering van GSK-3β verhoogden de aanmaak van zowel PGC-1α als de OXPHOS-complexen in deze spiercellen. Ook was de hoeveelheid mitochondrieel DNA (mtDNA; een veelgebruikte maat voor de hoeveelheid mitochondriën in de cel) verhoogd. Deze verhoogde aanmaak van OXPHOS-complexen na het uitschakelen van GSK-3β was afhankelijk van de aanwezigheid van PGC-1α. Tevens waren de dieren zonder het GSK-3β eiwit in de spier beschermd tegen een door fysieke inactiviteit geïnduceerd verlies van belangrijke spelers in de PGC-1α signalering en de OXPHOS-complexen. Hierdoor hebben we geconcludeerd dat uitschakelen van GSK-3β leidt tot een verhoogde aanmaak van het PGC-1α in de spier, hetgeen vervolgens mitochondriële biogenese en daarmee een vermeerdering van de hoeveelheid OXPHOS-complexen tot gevolg heeft.

Het onderhouden van de spieren en spierherstel zijn beide belangrijk voor het intact houden van gezonde skeletspieren. Daarom hebben we in **Hoofdstuk 4** onderzocht of het uitschakelen van GSK-3β tijdens myogene differentiatie (als onderdeel van spierontwikkeling) en spierherstel na een periode van inactiviteit ook leidt tot verhoging van PGC-1α signalering, mitochondriële biogenese en aanmaak OXPHOS-complexen. Hiervoor hebben we gebruik gemaakt van gekweekte spiercellen tijdens het proces van myogene differentiatie en hebben we de muizen zonder het GSK-3β eiwit in de spier gebruikt die na 14 dagen inactiviteit teruggeplaatst zijn op hun achterpoten om spierherstel te stimuleren. Net als in de gedifferentieerde spiercellen leidde uitschakelen van GSK-3β tijdens myogene differentiatie tot een verhoging van belangrijke spelers van de PGC-1α signalering alsook de aanmaak van OXPHOS-complexen. Aan het einde van de myogene differentiatie resulteerde

uitschakelen van GSK-3β tot een verhoogd mitochondrieel zuurstofverbruik wat duidt op een verbeterde functie van de mitochondriën. Tijdens de herstelfase (dus bij het weer belasten van de achterpoten) namen de PGC-1α signalering en de aanmaak van OXPHOS-complexen sterker toe in de muizen zonder GSK-3β in de spier vergeleken met de controledieren. Hierdoor concluderen we dat uitschakelen van GSK-3β tijdens spierherstel leidt tot een verhoogde mitochondriële biogenese en een verbeterde mitochondriële functie.

In **Hoofdstuk 5** hebben we getracht te ontrafelen hoe het uitschakelen van GSK-3β nu precies leidt tot een verhoging van de aanmaak van PGC-1α in de spiercellen. Hiervoor hebben we wederom gebruik gemaakt van volledig gedifferentieerde spiercellen. Naast een verhoogde aanmaak van PGC-1α leidde het uitschakelen van GSK-3β ook tot een verhoogde activiteit van de PGC-1α promoter (een stukje DNA dat de aanmaak van PGC-1α reguleert). Om dit verder te onderzoeken hebben we de betrokkenheid van verschillende transcriptiefactoren, waarvan bekend was dat deze aan de PGC-1α promoter kunnen binden en vervolgens diens aanmaak kunnen beïnvloeden, onder de loep genomen. Dit is gedaan door middel van farmacologische remming en genetische verwijdering van deze transcriptiefactoren. Hierdoor hebben we de betrokkenheid van verschillende transcriptiefactoren (myocyte enhancer factor 2 (MEF2) en estrogen-related receptor (ERR)) uitgesloten. Het uitschakelen van GSK-3β zorgt er wel voor dat een andere transcriptiefactor (transcriptie factor EB (TFEB)) geactiveerd wordt. Door het uitschakelen van GSK-3β kan TFEB namelijk naar de celkern (de plaats in de cel waar de aanmaak van genen gereguleerd wordt) migreren. Hierdoor kan TFEB aan de PGC-1α promoter binden, wat de aanmaak van PGC-1α tot gevolg heeft. Hieruit concluderen we dat het uitschakelen van GSK-3β resulteert in de translocatie van TFEB naar de celkern. Dit is noodzakelijk voor de activatie van de PGC-1α promoter en de aanmaak van PGC-1α.

In **Hoofdstuk 6** worden de resultaten van dit proefschrift besproken aan de hand van de meest recente wetenschappelijke literatuur en worden de resultaten in breder perspectief geplaatst. In dit hoofdstuk wordt GSK-3β als sleutelspeler in de regulatie van spiermassa en spiermetabolisme gepresenteerd. Verschillende aspecten van chronische ziekten die mogelijk geassocieerd zijn met een verandering van de activiteit van GSK-3β in de skeletspieren worden belicht. Vanuit een klinisch perspectief geeft dit het therapeutische potentieel aan van farmaceutica die GSK-3β kunnen remmen of TFEB kunnen activeren om spierafwijkingen (zoals verlies van spiermassa en/of -metabolisme) te verhelpen of te voorkomen bij patiënten waarbij de spiermassa is verminderd en/of de spierstofwisseling is aangedaan zoals patiënten met een chronische longziekte, chronisch hartfalen en bij ouderen. De

algemene conclusie van dit proefschrift is dat het uitschakelen van GSK-3β leidt tot aanmaak van PGC-1α via TFEB in spiercellen. De verhoogde aanmaak van PGC-1α na het uitschakelen van GSK-3β gaat gepaard met een verhoging van het oxidatieve energiemetabolisme en de mitochondriële biogenese.

Valorization

Societal relevance

Prevalence and burden of skeletal muscle dysfunction

Aging as well as several chronic diseases, such as chronic obstructive pulmonary disease (COPD), chronic kidney disease, type II diabetes and chronic heart failure, are characterized by a decline in peripheral muscle mass and a reduced number and impaired functionality of skeletal muscle mitochondria, primarily in the lower limbs [1-4]. The latter is associated with a reduced capacity of the muscle to produce energy. These skeletal muscle abnormalities ultimately dramatically affect the quality of life of these patients and are associated with increased morbidity and even higher mortality [5-7]. Indeed, good physical performance and muscle health strongly correlate with the ability to perform daily life activities, which is positively associated with quality of life and represses healthcare costs [8]. Indicative of the colossal impact of the abovementioned muscle abnormalities, sarcopenia defined as the loss of muscle mass and muscle function as a result of ageing, is highly prevalent in healthy aged individuals (10% [9]). Alarmingly, sarcopenia is even more prevalent in patients suffering from chronic diseases, such as type II diabetes and COPD [10-12] and numbers are predicted to rise further in the near future [13]. Sarcopenia is thus highly prevalent and, as stated above, correlates with an inability to adequately perform daily life tasks which ultimately leads to a reduced social status and increased hospitalization [14]. In 2014, the costs of sarcopenia-related hospitalization in the United States was estimated to be $40.4 billion [15]. Although Bruyère *et al.* could not make definitive conclusions regarding the economic burden of sarcopenia, because the studies used were heterogeneous in the assessment of sarcopenia and the type of costs evaluated, their systematic review gives a recent overview of several papers indicating elevated healthcare-cost in sarcopenic individuals. In addition, already in 2000, Janssen *et al.* estimated that a 10% reduction in the prevalence of sarcopenia in the United States alone would save $1.1 billion in healthcare-related expenses per year [16]. Treatment or even prevention of skeletal muscle impairments can therefore contribute significantly to increasing quality of life of large groups of patients and can help with decreasing the massive healthcare costs associated with (the management of) chronic diseases [17, 18]. Besides the high prevalence of skeletal muscle abnormalities, the above-mentioned diseases are also often associated with a high cardiometabolic risk. For example, COPD patients have an increased risk for the development of cardiovascular disease [19]. Interestingly, reduced skeletal muscle oxidative phenotype (OXPHEN) has been suggested as a major driver of increased cardiometabolic risk in COPD [20] and type II diabetes [21, 22]. This further stresses the need to minimize skeletal muscle abnormalities and

thereby reduce cardiometabolic disease-related healthcare costs in these patient populations [23].

Future perspective for GSK-3β and TFEB to alleviate muscle abnormalities

In this thesis, we describe a previously unknown role for glycogen synthase kinase (GSK)-3β in the molecular regulation of mitochondrial biogenesis and oxidative energy metabolism in skeletal muscle. We unraveled that inactivation of GSK-3β activated peroxisome proliferator-activated receptor-γ co-activator-1 (PGC-1)α gene expression via transcription factor EB (TFEB), which resulted in enhanced mitochondrial biogenesis and oxidative substrate use in skeletal muscle. This indicates that both GSK-3β as well as TFEB may serve as potential targets for therapeutic intervention to alleviate abnormalities at the level of the mitochondrion in skeletal muscle during ageing or in chronic disease. However, the relevance or efficacy of specific GSK-3β inhibitors or TFEB agonists in this context remains to be explored in more detail. Moreover, it has been reported that the inhibitory phosphorylation of GSK-3β and nuclear abundance of TFEB increase during physical activity. Combined with the data presented in this thesis, this indicates that improving muscle OXPHEN via modulation of the GSK-3β-TFEB-PGC-1α pathway can be achieved by adopting a healthier and more active lifestyle. As a long-term perspective, the knowledge gained by research described in this thesis could aid in the development of GSK-3β inhibitors, TFEB agonists or lifestyle interventions aiming to alleviate muscle abnormalities in the above-mentioned conditions.

Treatment strategies for increasing muscle oxidative phenotype

Exercise and exercise mimetics

Physical exercise is the most common and effective method to increase skeletal muscle health. However, exercise training is not always feasible in humans due to disease-related symptoms or age-related frailty. In addition, lifestyle changes are particularly hard to accomplish in elderly [24]. Nonetheless, exercise training or increased loading of skeletal muscle are highly effective evidence-based intervention methods for improving skeletal muscle function in several chronic conditions, including COPD [25]. However, the anabolic and mitochondrial response to exercise training is often blunted in COPD patients [26] as well as during aging [27, 28]. Alterations in the abundance or activity of TFEB in these conditions might underlie this

mal-adaptability, because TFEB knock-out mice have been shown to have a reduced ability to increase mitochondrial function and maintain the same exercise capacity compared to control animals. This makes them unable to fully benefit from exercise in terms mitochondrial regeneration [29], which speculatively could result in a blunted anabolic response. In this context, pharmacological activation of the pathways controlling muscle OXPHEN and muscle mass (so called 'exercise-mimetics') may prove beneficial to alleviate skeletal muscle abnormalities in the above-mentioned disorders and the aging population [30]. The mechanistic insight in the regulation of skeletal muscle PGC-1α expression gained in this thesis suggests a potential role for specific GSK-3β inhibitors or TFEB agonists as exercise-mimetics. Although the effects of inactivation of GSK-3β on mitochondrial biogenesis and oxidative energy metabolism and the involvement of TFEB herein needs to be studied in humans, the promising potential of GSK-3β inhibitors for clinical implication is clear.

Pharmacological potential of GSK-3β inhibitors

In order to verify the importance of GSK-3β in the (dys)regulation of skeletal muscle mitochondrial biogenesis and energy metabolism, it needs to be elucidated whether or not GSK-3β abundance or activity is altered in skeletal muscle in the above-mentioned human conditions associated with loss of muscle OXPHEN. In this context, it has been shown that the abundance and activity of GSK-3β is higher in skeletal muscle of obese type II diabetic patients compared with lean and weight-matched non-diabetic subjects [31]. In addition, GSK-3β phosphorylation was found to be reduced (indicating increased GSK-3β activity) in skeletal muscle of pregnancy-associated diabetes mellitus patients [32]. If GSK-3β activity is also increased in skeletal muscle of COPD patients is unclear. Several studies reported contradicting evidence on the phosphorylation status of GSK-3β in skeletal muscle of COPD patients. Indeed, unaltered [33, 34] and increased [35] levels of phosphorylated/inactivated (Ser^{9}) GSK-3β have both been reported in skeletal muscle of COPD patients. Literature on the activity of GSK-3β in skeletal muscle in chronic human diseases is thus scarce and contradictory and needs to be investigated in closer detail. Furthermore, it needs to be elucidated if structural absence of GSK-3β (in ablation modalities) or short-term acute inactivation of GSK-3β exert similar effects on TFEB nuclear translocation. To date, several experimental studies reveal that inactivation of GSK-3β in skeletal muscle is sufficient for nuclear translocation and activation of TFEB. Besides the effect of inactivation of GSK-3β on TFEB nuclear translocation in healthy conditions, these effects have not been verified in experimental models of chronic disease associated with reduced mitochondrial biogenesis. In order to fully exploit the benefits of exercise on muscle function, specific inhibition of GSK-3β

(or activation of TFEB) combined with an exercise protocol may be a potential novel treatment strategy [29]. Indicative of the therapeutic potential of GSK-3β inhibition in augmenting muscle functionality, lithium chloride (LiCl; an inhibitor of GSK-3) improved muscle strength and muscle mass in a murine muscle dystrophy model [36]. However, whether or not pharmacological inhibition of GSK-3β can rescue mitochondrial abnormalities in skeletal muscle remain to be investigated. Therefore, new studies should focus on the effect of exercise and pharmacological inhibition of GSK-3β on TFEB nuclear translocation in skeletal muscle in health and disease. In order to accomplish this, placebo-controlled pilot experiments aiming to investigate the effect of GSK-3β inhibitors in combination with or without exercise training on skeletal muscle mitochondrial biogenesis should reveal the clinical potential of GSK-3β inhibitors. Interestingly, lithium is clinically approved and often used to treat bipolar disorder [37]. However, some minor side effects of lithium treatment have been reported [38]. The clinical use of lithium indicates that lithium as well as more specific GSK-3β inhibitor are likely safe for human application. In this thesis, we report that GSK-3β inactivation enhanced TFEB nuclear translocation. In addition, TFEB was required for the inactivation of GSK-3β-mediated induction of PGC-1α, hence TFEB agonist might also be a promising pharmacological intervention aiming to induce muscle PGC-1α levels and thereby enhance muscle mitochondrial biogenesis.

Pharmacological potential for TFEB agonists

The role of TFEB in the regulation of PGC-1α and the subsequent control over mitochondrial biogenesis and oxidative energy metabolism was only recently discovered [29]. Despite this, several studies using multiple cell-types now support that nuclear translocation of TFEB, or other microphthalmia/TFE (MiT) family members, increases the expression of PGC-1α and enhance mitochondrial biogenesis [29, 39, 40]. Interestingly, TFEB agonists that stimulate nuclear translocation are being developed and tested [41]. However, if pharmacological activation of TFEB can rescue mitochondrial abnormalities in skeletal muscle remains to be investigated. In order to accomplish this, placebo-controlled pilot experiments aiming to investigate the effect of these compounds on mitochondrial function can be the first step to elucidate potential pharmacological potential of TFEB agonist in humans. Interestingly, digoxin (chemical TFEB agonist) is clinically approved and thus found to be safe for human application. The above-mentioned trials could increase our understanding of mitochondrial abnormalities in skeletal muscle within human disease and ultimately could reverse aging- or chronic disease-associated skeletal muscle mitochondrial deficits.

A potential role for GSK-3β and TFEB in neurodegenerative disorders

Next to skeletal muscle, mitochondria are highly important for the function of a myriad of other tissues, for example the brain. Growing lines of evidence suggest that mitochondrial dysfunction is involved in neurodegenerative diseases such as Parkinson's, Alzheimer's and Huntington's disease. Interestingly, it has been proposed that inactivation of GSK-3β, aiming to prevent mitochondrial dysfunction, is a potential treatment of neurodegenerative disorders. Pre-clinical data showed that pharmacological inhibition of GSK-3β restored mitochondrial biogenesis in a mouse model of Parkinson's disease [42]. Interestingly, Alzheimer's disease is linked to increased activity of GSK-3β in the cortex. Furthermore, reduced levels or decreased activity of PGC-1α and TFEB have been associated with Alzheimer's disease progression [43, 44]. This suggest that GSK-3β inhibitors or TFEB agonists might also be beneficial for patients with Alzheimer's disease. Intriguingly, pre-clinical data revealed that inhibition of GSK-3β using LiCl reduced both β-amyloid as well as tau protein (the two major protein aggregates involved in Alzheimer's disease progression) formation [45, 46]. LiCl treatment was previously reported to reduce cognitive decline in Alzheimer's disease [47]. This indicates that inhibition of GSK-3β indeed might be suitable to alleviate the burden of Alzheimer's disease [48].

Conclusion

The data in this thesis convincingly shows that inactivation of GSK-3β in skeletal muscle enhances PGC-1α-mediated mitochondrial biogenesis and oxidative substrate metabolism via TFEB. This suggests that both GSK-3β inhibitors as well as TFEB agonists are potential therapeutic strategies to treat or even prevent deficits in skeletal muscle OXPHEN associated with chronic diseases and aging. In addition, as outlined in the general discussion (**Chapter 6**), GSK-3β is involved in both the regulation of muscle mass as well as the regulation of muscle mitochondrial biogenesis and oxidative energy metabolism. Thus, specific GSK-3β inhibitors might be beneficial in alleviating both abnormalities in muscle mass and muscle OXPHEN that often coincide in chronic diseases. To date however it remains unclear if pharmacological inhibition of GSK-3β or activation of TFEB can induce skeletal muscle mitochondrial biogenesis or enhance oxidative substrate metabolism in a clinical setting. In the future, new randomized placebo-controlled human trials should shed light on these remaining questions. In addition, particular care should be given to confirm safety of novel pharmaceuticals for human application. Furthermore, in order to minimize side

effects, the possibility to use specific drug-delivery techniques should be explored to transport the medication to the targeted tissue, for example the skeletal muscle [49]. Moreover, the beneficial effects of inhibition of GSK-3β on the brain, as discussed above, indicates a broader perspective for the pharmacological inhibition of GSK-3β in human diseases. Although speculative in nature, the data presented in this thesis thus may be applied in several research disciplines beyond skeletal muscle pathology.

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Dankwoord

Het proefschrift is klaar en ik ben trots op mezelf over de totstandkoming ervan. Tijdens mijn promotietraject heb ik als onderzoeker en als mens veel geleerd, waarvoor ik collega's, vrienden en familie dankbaar ben. Daarom wil ik tot slot in dit hoofdstuk iedereen bedanken die vakinhoudelijk of op persoonlijk vlak heeft bijgedragen aan de totstandkoming van mijn proefschrift.

Natuurlijk wil ik mijn dank uitspreken aan mijn promotieteam. Allereerst wil ik mijn promotor **Annemie** bedanken. Annemie, bedankt dat ik de kans heb gekregen om mezelf binnen de onderzoeksgroep Longziekten te ontwikkelen, waardoor ik nu zoveel ervaringen rijker ben. Ik waardeer de tijd die je in mijn leerproces hebt gestoken. Van onze vergaderingen heb ik geleerd om de hoofdboodschap nooit uit het oog te verliezen. Ik heb me de afgelopen 4 jaar erg thuis gevoeld binnen de afdeling Longziekten. Ondanks dat onze vergaderingen niet heel frequent waren, heb je me altijd het gevoel gegeven dat, mocht er iets zijn, ik altijd bij je zou kunnen aankloppen.

Uiteraard wil ik ook graag mijn copromotoren bedanken. **Alex**, jou wil ik bedanken voor je begeleiding tijdens mijn promotietraject. Door je intensieve feedback op mijn stukken heb ik de afgelopen jaren veel geleerd over wetenschappelijk schrijven. Ook voel ik me, mede dankzij jou, veel zekerder tijdens presentaties. Ik heb onze bijna wekelijkse vergaderingen altijd als erg leerzaam ervaren. Je deur stond altijd open en het was vaak gemakkelijk om tussen de bedrijven door snel afspraken te maken. Die vrije en vaak effectieve manier van werken heb ik altijd erg fijn gevonden. Onze samenwerking tijdens mijn promotietraject hebben me gevormd tot onafhankelijk onderzoeker, waarvoor ik je zeer dankbaar ben. Ik ben er trots op om de eerste promovendus te zijn die onder jouw begeleiding promoveert. Hopelijk volgen er nog vele na mij. Ook wil ik je bedanken voor de gezellige tijd naast het werk, onder andere de Duvel's/gemberbiertjes op het terras.

Harry, bedankt voor al de tijd die je in mijn promotietraject hebt gestoken en de constructieve feedback die je hebt gegeven. Van je kwaliteit om het overzicht van het gehele plaatje te bewaren heb ik veel geleerd. Ook jouw deur stond altijd letterlijk open waardoor het makkelijk was om snel tussendoor het een en ander te bespreken of af te spreken. Het vrijgeven van jullie tuin voor verschillende PUL-activiteiten helpen enorm om de vakgroep longziekten tot een hechte groep te vormen. In die hechte groep heb ik me erg thuis gevoeld.

Ook wil ik mijn collega's bedanken voor de prachttijd bij longziekten. **Wouter**, bedankt voor de ongeveer drie jaar als kantoorgenoot. Vanaf de eerste dag heb ik het super

gevonden om met jou een kantoor te delen. Ik denk dat we altijd een goede balans tussen gezelligheid en hard werken hebben gehad. Van onze werkdiscussies over laboratoriumtechnieken tot gesprekken over de layout van onze posters en figuren, tot verscheidene disco-kwartiertjes. Kortom altijd één groot feest. Ik bewonder je doorzettingsvermogen en samenwerkvaardigheden. Verder wens ik je een goede afronding van je eigen promotietraject. Ik ben ervan overtuigd dat dat wel gaat lukken! Naast een fijne collega, ben je ook een goede vriend geworden. 'Dikke vrienden voor altijd!'. Ik wil je bedanken voor alle gezellige lunchpauzes, koffiebreaks, borrels, spelletjesavonden, fietsrondjes in de zon en de keren dat we samen gegeten hebben. **Rosanne**, ik heb altijd erg veel lol met je gehad, zowel tijdens werktijd als daarbuiten. Bedankt voor alle gezellige koffiepauzes, de voetbal- en handbalavonden en alle borrels. Als onderzoeker heb ik je hoog in het vaandel staan en wens je in het vervolg van je wetenschappelijke carrière veel succes. Maar eerst, veel succes en plezier in Barcelona en Manchester! **Pieter**, bedankt voor de veelvoudige discussies in het lab over mijn en jouw experimenten. Je kritische kijk maakt je in mijn ogen een gedegen wetenschapper. Naast de gezellige tijd op de universiteit wil ik je ook bedanken voor je gastvrijheid, hiervoor wil ik natuurlijk **Judith** ook bedanken. Meerdere malen hebben we bij jullie thuis de BBQ ontstoken, spelletjes gespeeld (onder het mom van: 'al het goud is voor Wessel') en geborreld, waarvan ik altijd erg genoten heb. Ook wil ik je bedanken voor de introducerende fietsrondjes in het Zuid-Limburgse heuvelland, toevallig was het altijd goed weer als ik op de fiets zat. **Anita**, ook jou wil ik bedanken voor de fijne samenwerking en gezelligheid bij pulmonologie, onder andere bij alle leuke koffiepauzes, lunchpauzes, borrels en diners. Jij stond altijd paraat om met experimenten te helpen. **Karin**, bedankt voor de gezellige fietstochtjes die we samen gemaakt hebben en ik wil je succes wensen in je opleiding tot longarts. **Caspar** en **Cheryl** bedankt voor jullie vriendschap en openheid toen ik naar Maastricht verhuisde. Mede door jullie ben ik vanaf het begin van mijn PhD nooit met tegenzin naar de universiteit gegaan. Ook wil ik jullie bedanken voor de gezellige spelletjesavonden waarop we vele malen Saboteur gespeeld hebben. Wel baal ik nog steeds van die sneeuw! Ik wens jullie samen veel succes in het vervolg van jullie wetenschappelijke carrière waar die ook zal zijn. Door jullie allemaal als vrienden op de werkvloer heb ik altijd met veel plezier gewerkt.

Verder wil ik de rest van mijn **collega's van longziekten** (Rianne, Viviënne, Bregtje, Chiel, Marco, Wouter G., Lisanne, Martijn, Jules, Niki U., Sara, Sarah, Niki R., Mieke, Juliette, Justine, Juanita, Lieke en Vasili) bedanken voor de wetenschappelijke discussies, de gezellige gezamenlijke lunchpauzes en de altijd leuke labuitjes. Ook wil ik mijn eerste kantoorgenoot **Stephanie** bedanken voor het beantwoorden van al mijn vragen omtrent de logistiek van de universiteit tijdens het begin van mijn PhD

traject. **Ramon**, naast het bovengenoemde wil ik jou ook bedanken voor het delen van je expertise in de regulatie van spiermassa en je kritische vragen bij labmeetings en journal clubs. Naast input van mijn promotieteam heb ik vaak nuttige input van jou gekregen waar ik tijdens het schrijven van verscheidenen hoofdstukken van dit proefschrift veel aan heb gehad.

Naast directe collega's van longziekten wil ik iedereen bedanken waarmee ik heb samengewerkt voor experimenten en het schrijven van artikelen. **Nicky** en **Koen** bedankt voor de samenwerking en dat ik gebruik kon maken van het spiermateriaal van door jullie voorheen uitgevoerde experimenten. Ook wil ik de **analisten van het Maastro lab** bedanken voor de introductie en het gebruik van de Seahorse. Daarnaast wil ik **Rianne** en **Merel** bedanken voor de introductie en uitleg over de IncuCyte. De afgelopen jaren heb ik veel mede-PhD studenten ontmoet die ik wil bedanken voor een gezellige tijd en/of samenwerking, onder andere **Caitlin** en **Philippe**.

Voor de gezonde mix tussen ontspanning en werk in Maastricht wil ik ook mijn **handbalvrienden van Manos** bedanken, in het speciaal **Lieke**, **JC**, **Anna**, **Anniko**, **Joep**, **Florian**, **Vianney**, **Theresa** en **Sonja** waar ik vaak voor of na de training samen mee gegeten heb. Ook wil ik mijn teamgenoten van diverse **Ha-Stu HS2** teams bedanken. Op de vrijdagen samen trainen en in het weekend samen winnen of verliezen. Samen handballen en het teamgevoel wat ik de afgelopen jaren gevoeld heb, hebben me altijd veel energie gegeven. In heb bijzonder wil ik **Roger** en **Willem** bedanken omdat we jarenlang samen hebben gespeeld en gefeest en omdat jullie altijd voor me klaar staan.

Hannah, **Jiri**, **Lise** en **Michelle** ik heb erg veel plezier gehad tijdens de gezelschapsspellen die we samen in het weekend speelden. Ik waardeer jullie gastvrijheid en ons gedeelde enthousiasme over gezelschapspellen. Naast al het spelplezier wil ik jullie ook bedanken voor onder andere de gezellige feestjes, zoals de trip naar het konijnenhol en oud op nieuw! **Jiri**, jou wil ik extra bedanken voor alle gezelligheid, als teamgenoot en als beste vriend uit Nijmegen. Jij staat op de momenten dat ik je nodig heb altijd voor me klaar waardoor ik altijd op je kan rekenen.

Ik wil graag ook mijn familie bedanken omdat jullie altijd voor me klaar staan. **Astrid** en **Frank**, jullie hebben me van jongs af aan gestimuleerd om op eigen benen te staan, kritisch te zijn, te geloven in mezelf en altijd mezelf te blijven. Hiervoor ben ik jullie zeer dankbaar. Mijn tweelingbroer **Jakko** en broertje **Timo**, jullie staan ook altijd voor me klaar en geven me nog altijd een gevoel van thuis. **Jakko**, opgroeien

met een tweelingbroer betekent een levenslange vriendschap! We hebben samen geleerd om als je door anderen vergeleken wordt er niet altijd teveel van aan te trekken. Samen staan we nog altijd sterk! **Timo**, van jouw vrije kijk op het leven kan iedereen lering trekken. Ik bewonder je creativiteit en doorzettingsvermogen.

Anne natuurlijk wil ik jou bedanken omdat ik bij jou helemaal mezelf kan zijn. Jij stond elk weekend voor me klaar in Nijmegen en laat me realiseren dat er veel belangrijkere dingen zijn dan werk. Jij bent mijn rots in de branding als het allemaal net niet lekker liep. Ook wil ik jou bedanken voor elke taartje dat ik per publicatie verdiend heb, een super beloning voor het harde werken! Ik bewonder het geduld dat jij hebt gehad de afgelopen 4 jaar alsmede je positieve kijk en invloed op je omgeving. Ik kijk er ontzettend naar uit om voortaan niet elk weekend heen en weer te hoeven reizen en vooral om samen met jou te kunnen zijn. Al zal ik mijn Maastrichtse vrienden en collega's zeker gaan missen.

List of Publications

W.F. Theeuwes[$], N.A.M. Pansters[$], A.M.W.J. Schols, K.J.P. Verhees, H.R. Gosker, C.C. de Theije, R.H.P. van Gorp, M.C.J.M. Kelders, O. Ronda, A. Haegens, A.H.V. Remels[$], R.C.J. Langen[$]. Recovery of muscle mass and muscle oxidative phenotype following disuse does not require GSK-3 inactivation. *Biochim Biophys Acta Mol Basis Dis. 2020 Feb; Epub ahead of print*. [$]Contributed equally.

W.F. Theeuwes, H.R. Gosker, A.M.W.J. Schols, R.C.J. Langen[$], A.H.V. Remels[$]. Regulation of PGC-1α expression by a GSK-3β-TFEB signaling axis in skeletal muscle. *Biochim Biophys Acta Mol Cell Res. 2019 Nov 16;1867(2):118610*. [$]Contributed equally.

W.F. Theeuwes, H.R. Gosker, N.A.M. Pansters, R.C.J. Langen, A.M.W.J. Schols, A.H.V. Remels. Inactivation of glycogen synthase kinase 3β (GSK-3β) enhances skeletal muscle oxidative metabolism during myogenesis. *Biochim Biophys Acta Mol Basis Dis. 2018 Sep;1864(9 Pt B):2913-2926.*

W.F. Theeuwes, H.R. Gosker, R.C.J. Langen, K.J.P. Verhees, N.A.M. Pansters, A.M.W.J. Schols, and A.H.V. Remels. Inactivation of glycogen synthase kinase 3β (GSK-3β) enhances skeletal muscle oxidative metabolism. *Biochim Biophys Acta Mol Basis Dis. 2017 Dec;1863(12):3075-3086*.

H. Schellekens, P.N. De Francesco, D. Kandil, **W.F. Theeuwes**, T. McCarthy, W.E. van Oeffelen, M. Perello, L. Giblin, T.G. Dinan, J.F. Cryan. Ghrelin's Orexigenic Effect Is Modulated via a Serotonin 2C Receptor Interaction. *ACS Chem Neurosci. 2015 Jul 15;6(7):1186-97*.

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Curriculum Vitae

Wessel Frank Theeuwes was born in Breda, The Netherlands on January 6th, 1992. In 2009, he graduated from secondary school Frencken College in Oosterhout. In the same year, he started the laboratory scientist study at Avans Hogeschool in Breda with minors in laboratory animal science and biotechnology. In 2013, he graduated for his bachelor's degree after completing a research internship at Food for Health Ireland and the Bioscience institute at University College Cork. During this internship, he investigated the G-protein coupled receptor interactions and modulations in satiety, appetite and food reward signaling. In 2013, he started the master Animal Science at Wageningen University. During this study, Wessel completed an internship at the Behavioural Neuroscience department of the Donders institute and the department of Neurophysiology at Radboud University in Nijmegen. During this internship, he investigate the stress hormone distribution in the primary somatosensory cortex in rat. In addition, he finished a thesis at the department of Human and Animal Physiology at Wageningen University. During this thesis, Wessel investigated the effect of dietary vitamins on mitochondrial flexibility and density in white adipose tissue. In 2015, he obtained his master's degree diploma from Wageningen University.

After obtaining his MSc title, Wessel started his PhD trajectory at the department of Respiratory Medicine at Maastricht University Medical Center+ under the supervision of Prof. dr. Annemie Schols, Dr. Alexander Remels and Dr. Harry Gosker. The main focus of his PhD research was to investigate the involvement of GSK-3β in the regulation of skeletal muscle mitochondrial biogenesis and oxidative energy metabolism. Wessel will continue his academic career as postdoctoral fellow in the department of Experimental Rheumatology at Raboud UMC in Nijmegen. He will investigate the molecular mechanisms underlying the effective adipose stem cell treatment in osteoarthritis.


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