Regulation of skeletal muscle plasticity by the transcriptional coregulators PGC-1α and NCoR1

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ABSTRACT

Skeletal muscle plasticity is regulated by a wide range of factors, among which environmental stimuli such as exercise and nutrition play a central role. Importantly, changes in skeletal muscle phenotype exert a direct impact on health and risk to premature death. In fact, physical inactivity promotes the development of diseases like cancer, heart diseases, skeletal muscle wasting and type 2 diabetes. In contrast, exercise training has been extensively shown to lower the risk of these pathologies, mainly by enhancing the metabolic fitness, mass and function of skeletal muscle tissue. Skeletal muscle remodelling is highly regulated at the transcriptional level through the coordinated interplay between transcription factors and coregulators. The transcription factors estrogen-related receptor α (ERRα) and proliferator-activated receptor β/δ (PPARβ/δ) play a key regulatory function of skeletal muscle metabolism, while their coactivator PPARγ coactivator 1α (PGC-1α) and corepressor nuclear receptor corepressor 1 (NCoR1) have emerged as potential modulators of skeletal muscle plasticity. However, the physiological role and the mechanisms by which PGC-1α and NCoR1 regulates skeletal muscle phenotype and function are not fully understood.

In order to define the role of NCoR1 in skeletal muscle plasticity and to identify its potential interplay with PGC-1α, we initially characterized NCoR1 muscle-specific knockout (mKO) mice. We observed that the deletion of NCoR1 in skeletal muscle resulted in enhanced oxygen consumption (VO_2) during treadmill running, lower maximal force and increased ex vivo fatigue resistance under maximal stimulation. Interestingly, microarray analysis of NCoR1 mKO and PGC-1α muscle-specific transgenic (mTg) mice skeletal muscle revealed an up-regulation of genes related to oxidative metabolism in both mouse models. Consistently, we found that PGC-1α knockdown in cultured myotubes inhibited the up-regulation of mitochondrial enzymes induced by NCoR1 knockdown. Moreover, ERRα and PPARβ/δ were identified as direct targets of both NCoR1 and PGC-1α. However, only the inhibition of ERRα was able to block the effects of NCoR1 knockdown in C_2C_{12} myotubes. Subsequently, during the second study of this thesis, the functional interplay between PGC-1α and PPARβ/δ was further determined by using different genetic mouse models. Surprisingly, our data demonstrated that the PGC-1α-PPARβ/δ axis does not control whole body metabolism under basal conditions. Actually, PPARβ/δ was found to be dispensable for

the positive effects of PGC-1α on whole body (e.g. VO_2) and skeletal muscle oxidative metabolism. Altogether, these studies demonstrate that, under basal conditions, NCoR1 and PGC-1α modulate skeletal muscle oxidative metabolism specifically by controlling ERRα-mediated gene expression.

Finally, skeletal muscle remodelling induced by chronic overload was studied by using the experimental model of synergist ablation (SA). Interestingly, SA has been shown to induce skeletal muscle hypertrophy through the activation of the mammalian target of rapamycin complex 1 (mTORC1), while mTORC1 can enhance skeletal muscle oxidative metabolism by regulating the PGC-1α-Ying Yang 1 transcriptional complex. Accordingly, in the last study of this thesis the potential function of the mTORC-1-PGC-1α axis in SA-induced skeletal muscle remodelling was defined by using PGC-1α mTg and mKO mice. As expected, SA strongly induced mTORC1 activation and skeletal muscle hypertrophy, though these effects were independent of PGC-1α. Moreover, SA down-regulated PGC-1α mRNA levels, consistent thus with the global repression of glycolytic and oxidative metabolism. Functional analyses further demonstrated that, SA promoted a switch toward a slow-contractile phenotype characterized by lower peak force and higher fatigue resistance, which was not altered in PGC-1α mTg mice. However, genetic ablation of PGC-1α preserved peak force after SA, an effect that seems to be related to the regulation of myosin heavy chain 2B, myosin regulatory light chain (MLC) and MLC kinase 2 by PGC-1α. Hence, we have found that PGC-1α is not involved in skeletal muscle hypertrophy and metabolic remodelling induced by SA, while this coactivator seem to be partially involved in the functional adaptations to SA. However, SA did not fully resemble the effects of resistance exercise in human skeletal muscle, thus the relevance of PGC-1α as a therapeutic target aiming at promoting skeletal muscle growth remains to be further explored under different conditions.

Therefore, the studies performed during this thesis have revealed new molecular mechanisms by which coregulators mediate skeletal muscle plasticity, especially related with the control of oxidative metabolism. Considering the relevance of skeletal muscle metabolic fitness in the development and prevention of metabolic diseases, these data has direct biomedical relevance. However, the therapeutic potential of the mechanisms here described remain to be defined in future studies.

ABBREVIATIONS

1RM	1 repetition maximum
AMPK	AMP-activated protein kinase
ATF2	activating transcription factor 2
CBP	CREB binding protein
CREB	cAMP-response element binding protein
CSA	fibre cross sectional-area
DAD	deacetylase activation domain
EDL	extensor digitorum longus
ERR	estrogen-related receptor
ETC	electron transport chain
FOXO3	fork-head transcription factor O3
GCN5	general control of amino-acid synthesis 5
GO	gene ontology
GPS2	G protein pathway suppressor 2
HAT	histone acetyltransferase
HDAC	histone deacetylase
HID	histone interaction domain
IGF-1	insulin-like growth factor 1
MAFbx	muscle atrophy F-box
MAPK	mitogen-activated protein kinase
MEF2	myocyte enhancer factor 2
MLC	myosin regulatory light chain
MLCK2	MLC kinase 2
mTORC1	mammalian target of rapamycin complex 1
MuRF1	muscle RING-finger protein-1
MyHC	myosin heavy chain
NCoR1	nuclear receptor corepressor 1
NCoR2	nuclear receptor corepressor 2
NFκB	nuclear factor-kappaB
NRF	nuclear respiratory factor
PGC-1	PPARγ coactivator-1

ABBREVIATIONS

PI3K	phosphatidylinositol 3-kinase
PKD	protein kinase D
PPAR	peroxisome proliferator-activated receptor
PRC	PGC-1-related coactivator
RD	repression domains
RER	respiratory exchange ratio
RID	receptor interaction domains
RS	serine/arginine rich domain
SIRT1	sirtuin 1
SMRT	silencing mediator of retinoic acid and thyroid hormone receptor
SRC	steroid receptor coactivator
TBL1	transducin β-like 1
TBLR1	TBL-related 1
TFAM	mitochondrial transcription factor A
VO_2	oxygen consumption
YY1	Yin Yang 1

CHAPTER 1: Introduction

Skeletal muscle remodelling in health and disease

Skeletal muscle is one of the most abundant tissues in mammals, with a central function in the modulation of whole body homeostasis and health status. This tissue plays an important metabolic function since it represents the main place of energy expenditure, glycogen storage and insulin-stimulated glucose uptake (Egan and Zierath, 2013). Skeletal muscle is formed by a heterogeneous population of fibres, exhibiting different metabolic and functional properties. Accordingly, skeletal muscle fibres are classified as slow-oxidative (type 1) and fast-glycolytic (type 2) fibres, with type 2 fibres further distinguished as 2A, 2B and 2X. Type 1 fibres show the highest mitochondrial content, oxidative capacity and fatigue resistance, while they predominantly express myosin heavy chain 1 (MyHC-1) and slow-twitch properties (Schiaffino and Reggiani, 2011). On the other hand, type 2B and 2X fibres exhibit high glycolytic metabolism, low mitochondrial content, low fatigue resistance and fast-twitch properties, while type 2A fibres have intermediate characteristics between type 1 and 2B/X fibres (Schiaffino and Reggiani, 2011). Moreover, type 2A, 2B and 2X fibres specifically express MyHC-2A, MyHC-2B and MyHC-2X, respectively, though MyHC-2B is not detected in human skeletal muscle (Schiaffino and Reggiani, 2011). These divergent phenotypes are regulated by a highly specific subset of genes that differentially lead to expression of proteins involved in both skeletal muscle metabolism and contractility (Drexler et al., 2012; Wu et al., 2003). For instance, proteomic analysis of the slow-oxidative muscle soleus shows an overrepresentation of gene ontology (GO) terms related to mitochondria and oxidative metabolism, whereas overrepresented GO terms in the fast-glycolytic muscle extensor digitorum longus (EDL) are related to sarcoplasmic reticulum and carbohydrate metabolism (Drexler et al., 2012). Skeletal muscle phenotype is therefore regulated at the transcriptional level, with different transcription factors and coregulators modulating the expression or repression of fibre type-specific genes (Baar, 2010; Coffey and Hawley, 2007; Egan and Zierath, 2013; Gundersen, 2011).

CHAPTER 1: Introduction

Skeletal muscle remodelling by physical inactivity

Different stimuli have been shown to modify skeletal muscle phenotype, with environmental factors like nutrients and physical activity playing a major role in the regulation of this process. A sedentary life style has been recognized as the main pathogenic factor of non-communicable diseases (e.g. obesity and type 2 diabetes), which ultimately lead to premature death worldwide (Booth et al., 2008; Lee et al., 2012; Thyfault and Booth, 2011). Physical inactivity has been reported to increase the risk of different types of cancer, heart diseases and type 2 diabetes among other diseases (Booth and Laye, 2009). The increase in morbidity and mortality induced by a sedentary life style is the consequence of the maladaptation of a wide spectrum of tissues and organs, which ultimately lead to impaired whole body homeostasis (Figure 1). Importantly, among the different tissues involved in the development of metabolic diseases, skeletal muscle seems to play a mayor pathogenic role. Insulin resistance in skeletal muscle has been shown to drive the development of whole body metabolic syndrome (Kim et al., 2000; Petersen et al., 2007), a process that has been directly linked to lower levels of physical activity (Alibegovic et al., 2009; Mikines et al., 1991; Richter et al., 1989). In fact, skeletal muscle metabolic function is severely impaired by physical inactivity, which is characterized by a lower mRNA, protein and activity levels of different key regulatory proteins of both glycolytic and oxidative metabolism (Alibegovic et al., 2010; Bienso et al., 2012; Brocca et al., 2012; Chen et al., 2007; Ringholm et al., 2011). Importantly, impaired skeletal muscle energy metabolism has been extensively shown to be a hallmark of a number of non-communicable diseases. The metabolic impairment induced by physical inactivity has been linked to the repression of a specific subset of genes involved in aerobic metabolism and substrate turnover. Skeletal muscle from type 2 diabetic patients exhibit a gene expression signature characterized by the down-regulation of genes involved in oxidative metabolism (Barres et al., 2013; Barres et al., 2009; Mootha et al., 2003; Patti et al., 2003), implying that transcription factors and coregulators are deregulated in the context of metabolic diseases.

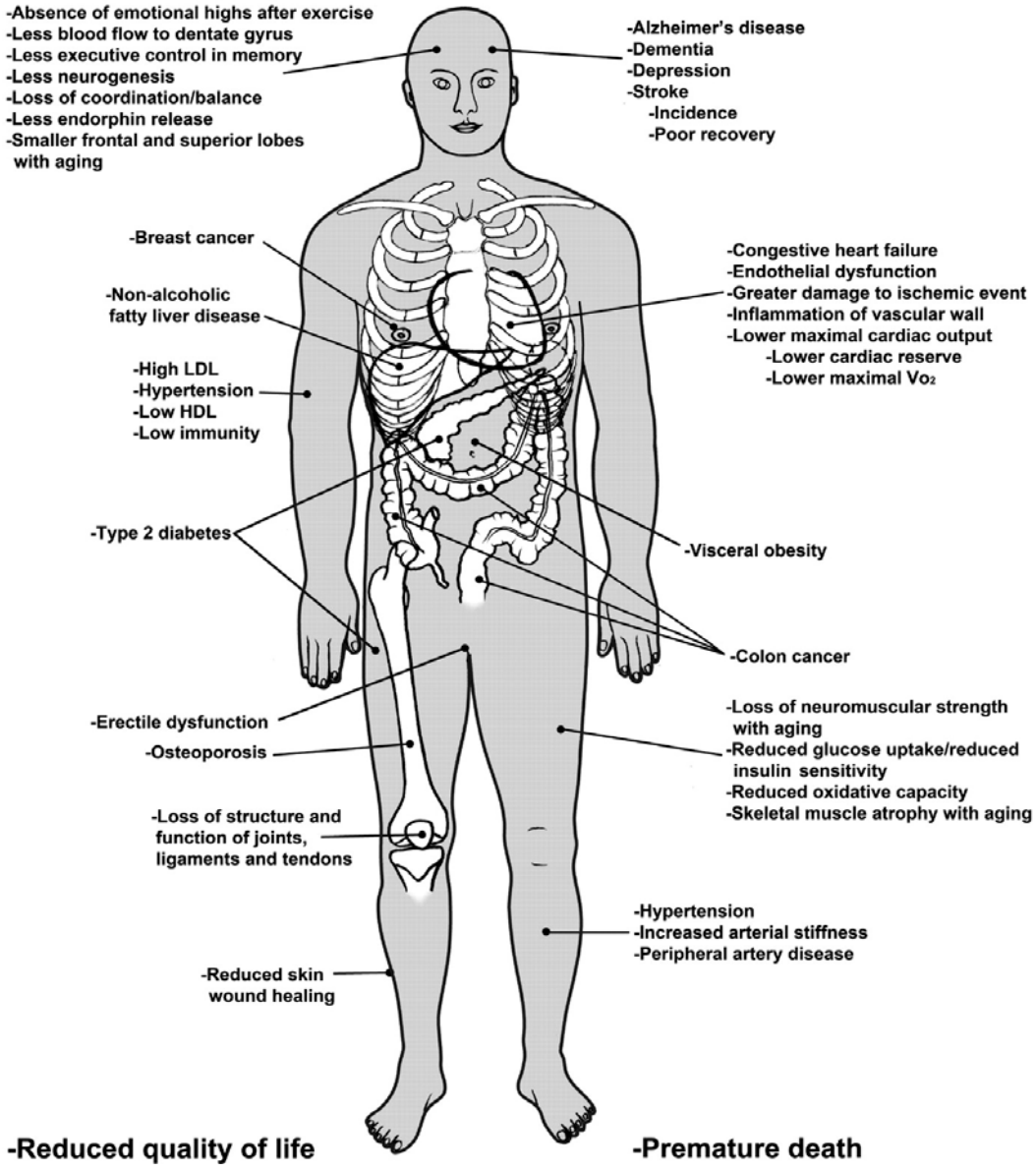

Figure 1 Pathological effects of physical inactivity at the whole body level (from (Booth and Laye, 2009)).

Furthermore, physical inactivity as well as several pathologies have been shown to promote a decrease in skeletal muscle mass, fibre cross sectional-area (CSA) and force generation (Figure 1). Importantly, skeletal muscle mass loss contributes to the progression and mortality associated with diseases such as cancer, heart failure, chronic obstructive pulmonary disease and renal failure (Glass and Roubenoff, 2010). It should be noted that skeletal muscle atrophy can also take

place in response to non-pathological conditions, including aging and caloric restriction (Glass and Roubenoff, 2010; Parr et al., 2013), indicating thus that environmental stimuli also play an important role in the modulation of skeletal muscle mass. Indeed, although a wide spectrum of pharmacological approaches have been undertaken to combat skeletal muscle wasting, resistance exercise remains the most efficient strategy to maintain or enhance muscle mass (Glass and Roubenoff, 2010; von Haehling et al., 2012). Skeletal muscle atrophy induced by physical inactivity is directly associated to a decrease in the rate of skeletal muscle protein synthesis, while the rate of protein degradation exhibit an opposite response (Bodine, 2013). These changes in protein turnover following skeletal muscle unloading have been suggested to be highly regulated at the transcriptions levels. Actually, the gene expressions signature linked to skeletal muscle atrophy shows a clear association to biological process regulating protein degradation, mainly linked to the ubiquitin proteasome pathway (Bodine, 2013; Schiaffino et al., 2013). Therefore, the skeletal muscle phenotype undergoes extensive remodelling following physical inactivity and during pathological conditions, which is mainly reflected by a global repression of different metabolic-related genes, lower oxidative capacity, skeletal muscle atrophy and lower maximal force. Collectively, these changes in skeletal muscle phenotype are thought to affect whole body function and to increase the prevalence and development of non-communicable diseases.

Skeletal muscle remodelling by physical activity

In stark contrast to physical inactivity, a higher exercise performance and metabolic fitness are strongly associated to a decreased prevalence of metabolic diseases and premature death (Booth and Roberts, 2008). Exercise training can accordingly lower the risk of a number of diseases, including type 2 diabetes, hypertension, cancer and skeletal muscle wasting (Booth and Roberts, 2008). Consequently, irrespectively of the type of exercise, physical activity is currently considered as one of the most efficient therapeutic approaches to treat metabolic diseases (Egan and Zierath, 2013), whereas resistance exercise is thought to be more

effective for the treatment of muscle wasting-related diseases (Bodine, 2013; Glass and Roubenoff, 2010). However, whole body and skeletal muscle adaptations to exercise are very specific, with endurance and resistance exercise representing two extremes in the spectrum of exercise modes and functional adaptations.

Endurance or aerobic exercise is characterized by exercise performed against a low load for a long period of time, in which most of the energetic demands are provided by the aerobic oxidation of both carbohydrate and fatty acids. Endurance training induces a wide range of beneficial adaptations, such as an improved body composition, higher insulin sensitivity, lower blood pressure and improved cardiovascular function (Table 1). This kind of exercise also increases maximal oxygen consumption (VO_{2max}), which is associated to an increased fatty acid oxidation and skeletal muscle glycogen sparing at submaximal exercise. Collectively, these adaptations contribute to the improved endurance performance and metabolic health observed after endurance training, though skeletal muscle-specific play a key role in the regulation of these adaptations (Holloszy and Coyle, 1984). In fact, besides increasing skeletal muscle vascularization, endurance training boosts mitochondrial content and metabolic function of skeletal muscle fibres. The effects of endurance exercise on mitochondrial function was first described in rat skeletal muscle, where endurance training was shown to increase mitochondrial content, the activity of mitochondrial enzymes and mitochondrial respiration (Holloszy, 1967). Importantly, similar results have been reported both in rodent and human skeletal muscle, strongly supporting the positive effects of endurance exercise on skeletal muscle oxidative metabolism (Egan and Zierath, 2013; Holloszy and Coyle, 1984). Skeletal muscle remodelling induced by endurance exercise is regulated by a wide range of pathways and molecules, but gene transcription is thought to play a key regulatory mechanism (Coffey and Hawley, 2007; Egan and Zierath, 2013; Gundersen, 2011). In fact, it has been demonstrated that endurance trained athletes exhibit a highly specific gene expression signature in skeletal muscle tissue, in which GO terms related to mitochondrial structure and function are significantly overrepresented (Stepto et al., 2009). Furthermore, exercise training enhances skeletal muscle oxidative metabolism by inducing transient changes in the mRNA

levels of metabolic-related genes that in the long term result in a progressive increase of their protein content (Coffey and Hawley, 2007; Egan and Zierath, 2013; Perry et al., 2010), indicating that gene transcription is an early process in the control of skeletal muscle remodelling.

On the other hand, resistance or strength training is characterized by exercise performed against a high load (e.g. 80% of 1 repetition maximum (1RM)), which is normally above the aerobic capacity of the active muscles. At the whole body level, unlike endurance exercise, resistance exercise induces a mild impact on the cardiovascular system and oxidative capacity, whereas it significantly increases lean mass, bone mineral density and basal metabolic rate (Table 1). Moreover, resistance exercise also produce specific adaptations in skeletal muscle tissue, among which the increase in fibre size, myofibrillar protein synthesis and force generation are the most characteristic changes (Table 1). Improvements in force generation however are initially due to neural adaptations and thus in the absence of skeletal muscle hypertrophy, with skeletal muscle-specific adaptations playing a major role following chronic resistance exercise (Fry, 2004). Interestingly, most of the adaptations observed in skeletal muscle following resistance training are thought to be directly related to the intensity at which exercise is performed, with resistance exercise performed at 80-90% of 1RM resulting in maximal increases in both fibre CSA and maximal force (Fry, 2004). Resistance training has been proposed to induce a fibre type switch toward type 2A fibres, which is consistent with the high recruitment of this fibre type during this kind of exercise (Egan and Zierath, 2013; Fry, 2004). Furthermore, mechanical overload of mouse skeletal muscle significantly increases protein synthesis, with type 2A fibres showing the highest increase in CSA (Goodman et al., 2012). Consistent with the higher rate of protein synthesis induced by resistance exercise, it has been demonstrated that mechanical overload also induces a significant increase in ribosomal content, an effect that is highly regulated at the transcriptional level (Goodman et al., 2011; von Walden et al., 2012). Importantly, gene transcription seems to also be a key process in the modulation of skeletal muscle remodelling induced by resistance exercise, since strength trained athletes show a very specific gene expression signature in which genes related to protein

synthesis are significantly up-regulated in skeletal muscle (Stepto et al., 2009). Therefore, even though endurance and resistance training induce divergent effects on skeletal muscle remodelling, it appears that adaptations to both of these types of exercise require the regulation of specific gene expression signatures.

Table 1 Whole body and skeletal muscle-specific adaptations to endurance and resistance exercise (from (Egan and Zierath, 2013)).

	Aerobic (Endurance)	Resistance (Strength)
Skeletal Muscle Morphology and Exercise Performance		
Muscle hypertrophy	↔	↑ ↑ ↑
Muscle strength and power	↔ ↓	↑ ↑ ↑
Muscle fiber size	↔ ↑	↑ ↑ ↑
Neural adaptations	↔ ↑	↑ ↑ ↑
Anaerobic capacity	↑	↑ ↑
Myofibrillar protein synthesis	↔ ↑	↑ ↑ ↑
Mitochondrial protein synthesis	↑ ↑	↔ ↑
Lactate tolerance	↑ ↑	↔ ↑
Capillarisation	↑ ↑	↔
Mitochondrial density and oxidative function	↑ ↑ ↑	↔ ↑
Endurance capacity	↑ ↑ ↑	↔ ↑
Whole-Body and Metabolic Health		
Bone mineral density	↑ ↑	↑ ↑
Body composition		
Percent body fat	↓ ↓	↓
Lean body mass	↔	↑ ↑
Glucose metabolism		
Resting insulin levels	↓	↓
Insulin response to glucose challenge	↓ ↓	↓ ↓
Insulin sensitivity	↑ ↑	↑ ↑
Inflammatory markers	↓ ↓	↓
Resting heart rate	↓ ↓	↔
Stroke volume, resting and maximal	↑ ↑	↔
Blood pressure at rest		
Systolic	↔ ↓	↔
Diastolic	↔ ↓	↔ ↓
Cardiovascular risk profile	↓ ↓ ↓	↓
Basal metabolic rate	↑	↑ ↑
Flexibility	↑	↑
Posture	↔	↑
Ability in activities of daily living	↔ ↑	↑ ↑

Aerobic exercise training generally encompasses exercise durations of several minutes up to several hours at various exercise intensities, incorporating repetitive, low-resistance exercise such as cycling, running, and swimming. Resistance training generally encompasses short-duration activity at high or maximal exercise intensities, and increases the capacity to perform high-intensity, high-resistance exercise of a single or relatively few repetitions such as Olympic weightlifting, bodybuilding, and throwing events. ↑, values increase; ↓, values decrease; ↔, values remain unchanged; ↑ or ↓, small effect; ↑↑ or ↓↑, medium effect; ↑↑↑ or ↓↓↓, large effect; ↔↑ or ↔↓, no change or slight change.

Transcriptional coregulators and skeletal muscle remodelling

Gene transcription is highly regulated by the coordinated interaction between transcription factors and coregulators. Transcription factors are a family of proteins able to interact with specific binding sites along the DNA known as response elements or *cis*-regulatory elements, which are mainly located at the promoter region of target genes. In contrast, coregulators are not able to directly interact with the DNA, but instead they form multi-protein complexes that interact with transcription factors and thus regulate their activity. Importantly, coregulators can be classified as coactivators or corepressors if they promote or repress gene transcription, respectively. The main mechanism by which coregulators modulate gene transcription is by recruiting different proteins with histone acetyltransferase (HAT) and deacetylase (HDAC) activity. Consequently, gene transcription is mainly activated when transcription factors recruit coactivator complexes containing HAT activity that induce an open conformation of the chromatin, whereas corepressors promote the opposite effect through HDAC-induced chromatin condensation (Figure 2). The role of coregulators in skeletal muscle physiology has not been fully elucidated, but the peroxisome proliferator-activated receptor γ (PPARγ) coactivator-1α (PGC-1α) and the nuclear receptor corepressor 1 (NCoR1) have emerged as potential regulators of skeletal muscle plasticity.

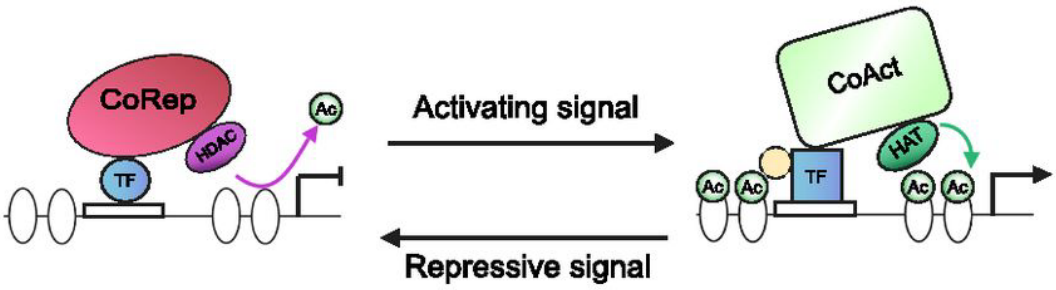

Figure 2 Main mechanism by which transcriptional corepressors (CoRep) and coactivators (CoAct) modulate gene transcription (from (Mottis et al., 2013)). TF: transcription factor, HAT: histone acetyltransferase, HDAC: histone deacetylase, Ac: acetylation.

The coactivator PGC-1α

The transcriptional coactivator PGC-1α was initially described as a coactivator of the nuclear receptor PPARγ in brown adipose tissue (Puigserver et al., 1998), while subsequent studies identified PGC-1β (Kressler et al., 2002; Lin et al., 2002a) and PGC-1-related coactivator (PRC) (Andersson and Scarpulla, 2001) as additional members of the PGC-1 family of coactivators. The PGC-1α protein can interact with its target transcription factors through three LXXLL motifs located in the N-terminal transcriptional activation domain (Figure 3). In addition, the PGC-1α protein contains a central regulatory domain involved in transcriptional repression, while the C-terminus domain contain a serine/arginine rich domain (RS) and an RNA binding domain that have been involved in protein-protein interaction and the regulation of mRNA splicing, respectively (Figure 3). However, despite the similarities between the member of the PGC-1 family of coactivators, PGC-1β and PRC do not recapitulate all of the effects induced by PGC-1α (Lin et al., 2005; Scarpulla et al., 2012). PGC-1α lacks enzymatic activity and its positive effects on gene transcription have been shown to be significantly enhanced by the recruitment of HAT proteins, including cAMP-response element binding protein (CREB) binding protein (CBP), p300 and steroid receptor coactivator-1 (SRC-1) (Puigserver et al., 1999; Wallberg et al., 2003). Interestingly, PGC-1α has been proposed to modulate gene transcription by recruiting the TRAP/Mediator complex and mRNA processing through its C-terminal domain (Monsalve et al., 2000; Wallberg et al., 2003). Finally, also in the C-terminal domain, PGC-1α has been shown to interact with BRG1-associated factor 60a that consequently allows the recruitment of the switch/sucrose non-fermentable chromatin-remodelling complex (Li et al., 2008). The multi-protein complex formed by PGC-1α hence contain proteins able to couple chromatin remodelling and mRNA processing, allowing thus the efficient regulation of gene transcriptions (Figure 3).

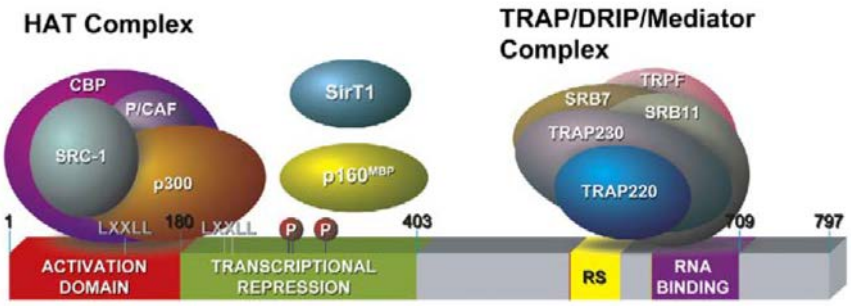

Figure 3 Protein structure and main components of the PGC-1α multi-protein complex (from (Lin et al., 2005)).

PGC-1α and skeletal muscle remodelling

The PGC-1α coactivator complex interacts with and regulates the activity of a number of transcription factors involved in a wide range of biological functions, among which energy metabolism, protein degradation and angiogenesis are particularly relevant for skeletal muscle function (Figure 4). Actually, PGC-1α has been shown to positively regulate the expression of different nuclear and mitochondrial encoded genes involved in metabolic pathways such as the TCA cycle, fatty acid β-oxidation and the electron transport chain (ETC) (Scarpulla et al., 2012). Moreover, PGC-1α overexpression specifically in skeletal muscle promotes a fibre type switch toward a slow-oxidative phenotype through the regulation of myocyte enhancer factor 2 (MEF2) (Lin et al., 2002b; Summermatter et al., 2012). Importantly, these transcriptional changes directly improve VO_{2peak}, endurance performance and ex vivo fatigue resistance (Calvo et al., 2008; Lin et al., 2002b; Summermatter et al., 2012), demonstrating the physiological relevance of this coactivator. The mechanism by which PGC-1α enhances oxidative metabolism involves the activation of a specific subset of transcription factors, among which the nuclear respiratory factor 1 (NRF1), NRF2, estrogen-related receptor α (ERRα), PPARα and PPARβ/δ have been proposed to play key regulatory functions (Ehrenborg and Krook, 2009; Lin et al., 2005). Importantly, PGC-1α indirectly modulates the expression of mitochondrial encoded genes by inducing the

expression of mitochondrial transcription factor A (TFAM) via NRF1 and NRF2 (Lin et al., 2005). PGC-1α has also been shown to increase the transcriptional activity of Yin Yang 1 (YY1) in a mammalian target of rapamycin complex 1 (mTORC1) dependent manner, thus boosting skeletal muscle oxidative metabolism (Blattler et al., 2012; Cunningham et al., 2007). Interestingly, in addition to enhance substrate utilisation via aerobic metabolism, PGC-1α also promotes lipid storage in skeletal muscle fibres by activating the liver X receptor α (Summermatter et al., 2010), resembling the metabolic adaptations induced by exercise in human skeletal muscle (van Loon and Goodpaster, 2006). Furthermore, the activation of ERRα by PGC-1α induces the expression of the vascular endothelial growth factor and, consequently, promotes angiogenesis in skeletal muscle (Arany et al., 2008; Chinsomboon et al., 2009). These data indicate that PGC-1α overexpression in skeletal muscle is sufficient to control most of the metabolic and functional adaptation induced by endurance exercise.

Interestingly, even though PGC-1α acts primarily as a transcriptional coactivator, it has also been shown that PGC-1α can repress the expression of genes involved in skeletal muscle atrophy. However, the mechanism by which PGC-1α repress gene expression is currently unknown. Reporter gene assay experiments have demonstrated that PGC-1α can significantly decrease the transcriptional activity of the transcription factors fork-head transcription factor O3 (FOXO3) and nuclear factor-kappaB (NFκB) (Brault et al., 2010; Sandri et al., 2006). The repression of these transcription factors by PGC-1α is highly relevant, since it prevents skeletal muscle mass loss during both aging and following denervation (Brault et al., 2010; Sandri et al., 2006; Wenz et al., 2009). Actually, FOXO3 induce the expression of the muscle-specific E3 ubiquitin ligases muscle RING-finger protein-1 (MuRF1; also known as Trim63) and muscle atrophy F-box (MAFbx; also known as atrogene 1 and Fbxo32), which are key regulators of skeletal muscle unloading-induced atrophy (Bodine et al., 2001). Both of these E3 ubiquitin ligases are up-regulated in response to skeletal muscle unloading and their genetic ablation decreases skeletal muscle mass loss following unloading (Bodine et al., 2001). Consistent with the effects of PGC-1α on muscle mass and FOXO3 activity, the overexpression of this coactivator attenuates MuRF1 and MAFbx up-regulation observed during skeletal muscle

atrophy (Sandri et al., 2006), effect that has been linked to lower levels of protein degradation (Brault et al., 2010). Moreover, it has been recently shown that a new identified transcript variant of PGC-1α called PGC-1α4 is able to promote skeletal muscle hypertrophy and protect against atrophy when overexpressed in skeletal muscle (Ruas et al., 2012). PGC-1α thus seems to be a versatile coregulator, regulating energy metabolism and protein degradation through both transcriptional activation and repression.

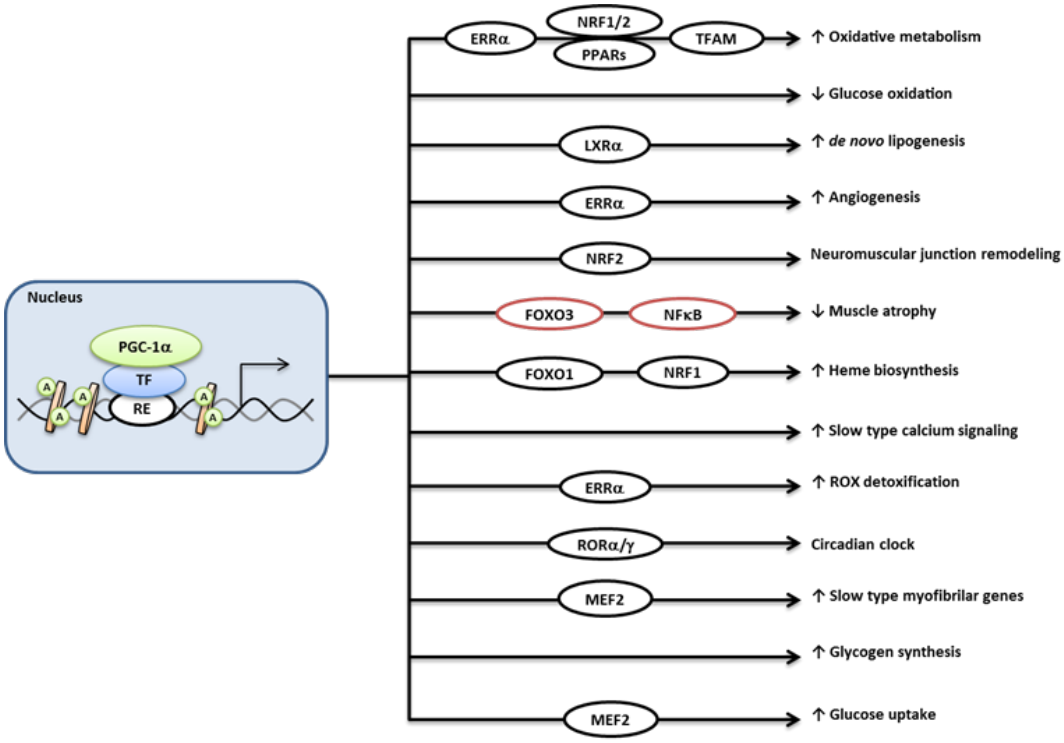

Figure 4 Main transcription factors and biological process regulated by PGC-1α in skeletal muscle (from (Pérez-Schindler and Handschin, 2013)). ERRα: estrogen-related receptor α, NRF1/2: nuclear respiratory factor 1 and 2, FoxO1/3: forkhead box O1/3, TFAM: mitochondrial transcription factor A, MEF2: myocyte enhancer factor 2, PPAR: peroxisome proliferator-activated receptor, RORα/γ: retinoic acid receptor-related orphan receptor α/γ, LXRα: liver X receptor α, NFκB: Nuclear factor-kappaB, RE: transcription factor response elements, A: histone acetylation.

Regulation of PGC-1α expression and activity in skeletal muscle

Among the different stimuli promoting skeletal muscle PGC-1α expression and activity, exercise exerts the strongest effects (Egan and Zierath, 2013; Pérez-Schindler and Handschin, 2013). In fact, skeletal muscle PGC-1α expression levels are higher in trained subjects, while it is significantly down-regulated by physical inactivity (Alibegovic et al., 2010; Brocca et al., 2012; Kramer et al., 2006). The effects of skeletal muscle contraction on PGC-1α expression were first described in rodent skeletal muscle, where both acute and chronic exercise was shown to significantly up-regulate the mRNA and protein levels of this coactivator in skeletal muscle (Baar et al., 2002; Goto et al., 2000; Terada et al., 2002; Terada et al., 2005; Terada and Tabata, 2004). Importantly, a single session of either endurance or high-intensity interval training have been shown to strongly induce a transient increase of PGC-1α mRNA in human skeletal muscle (Bartlett et al., 2012; Coffey et al., 2006; Gibala et al., 2009; Pilegaard et al., 2003; Russell et al., 2005), which when repeated over time increases PGC-1α protein levels (Burgomaster et al., 2008; Hood et al., 2011; Little et al., 2011; Little et al., 2010; Mathai et al., 2008; Perry et al., 2010; Russell et al., 2003). Interestingly, changes in PGC-1α expression induced by exercise are thought to be sensitive to the intensity at which exercise is performed, with higher intensities resulting in bigger effects (Egan et al., 2010; Nordsborg et al., 2010; Tadaishi et al., 2011).

Exercise-induced PGC-1α expression involves a wide number of signal pathways, though only few proteins seem to be essential (Figure 5). In fact, skeletal muscle contraction mediates the transcription of this coactivator by inducing the recruitment of the transcription factors MEF2 and activating transcription factor 2 (ATF2) at the promoter region of the PGC-1α gene (Akimoto et al., 2004). The up-regulation of PGC-1α mRNA induced by MEF2 and ATF2 following skeletal muscle contraction has been reported to require the activation of protein kinase D (PKD) and p38 mitogen-activated protein kinase (MAPK) (Akimoto et al., 2008; Pogozelski et al., 2009). In addition to the activation and recruitment of these essential transcription factors to the PGC-1α promoter, exercise has been also shown to

reduces DNA methylation levels, decreasing thus the transcriptional repression of this gene (Barres et al., 2012). Importantly, PGC-1α activity is also regulated at the post-translational level by different modification, such as phosphorylation, acetylation and methylation among others (Fernandez-Marcos and Auwerx, 2011). Exercise however seems to mainly modulate PGC-1α activity through phosphorylation and acetylation. On one hand, skeletal muscle contraction can efficiently increase the activity of AMP-activated protein kinase (AMPK) and p38 MAPK (Egan and Zierath, 2013), both of which have been shown to directly phosphorylate and activate PGC-1α (Jager et al., 2007; Puigserver et al., 2001). These data suggest that skeletal muscle contraction-mediated PGC-1α activation might be dependent of its phosphorylation by AMPK and p38 MAPK. On the other hand, PGC-1α can be acetylated and deacetylase by sirtuin 1 (SIRT1) and general control of amino-acid synthesis 5 (GCN5), respectively (Lerin et al., 2006; Rodgers et al., 2005). Exercise has been also suggested to activate PGC-1α by promoting its deacetylation, a process that is thought to be regulated by the interplay between AMPK and SIRT1 (Canto et al., 2010). In addition, it has been recently reported that exercise decrease PGC-1α acetylation levels mainly by decreasing its interaction with the acetyl transferase GCN5 rather than through SIRT1-mediated deacetylation (Philp et al., 2011). The effects of exercise on PGC-1α activity seem to involve a complex interplay between phosphorylation and acetylation, but the precise mechanism and proteins regulating these processes need to be further studied.

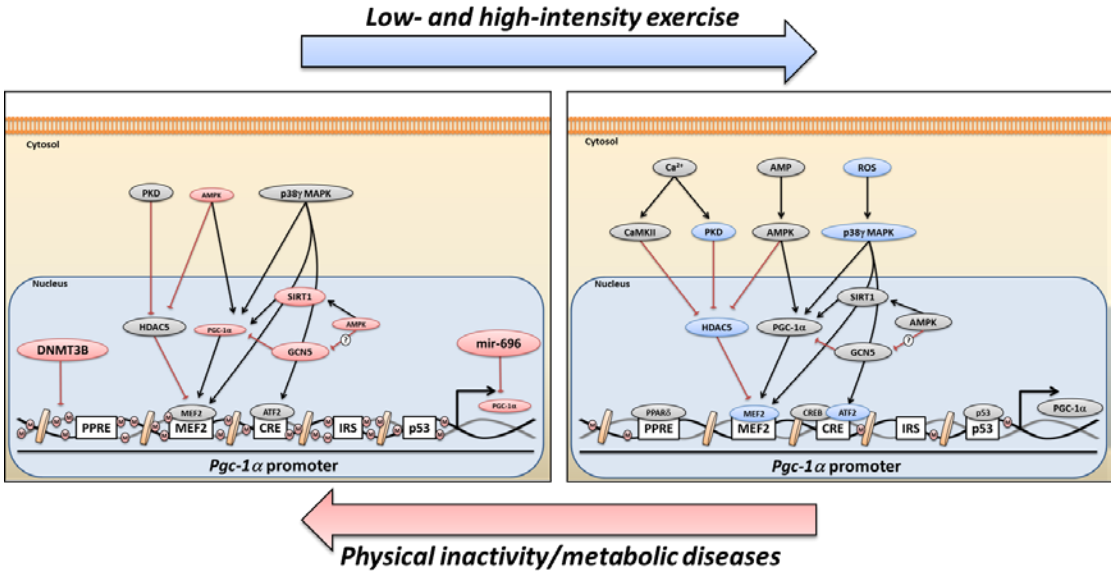

Figure 5 Schematic representation of the signal pathways regulating PGC-1α expression and activity in skeletal muscle (from (Pérez-Schindler and Handschin, 2013)).

The corepressor NCoR1

Similar to PGC-1α, the transcriptional corepressor NCoR1 does not exhibit enzymatic activity and it regulates gene transcription by forming a large multi-protein complex. While NCoR1 and its homolog protein NCoR2 (also known as silencing mediator of retinoic acid and thyroid hormone receptor, SMRT) were first found to mediate ligand-independent repression of the thyroid hormone and the retinoic acid receptor (Chen and Evans, 1995; Horlein et al., 1995), these corepressor have been shown to interact with and repress a wide range of transcription factors (McKenna and O'Malley, 2010; Mottis et al., 2013). The protein structure of these corepressors is highly similar (Figure 6), mainly characterized by the presence of three repression domains (RD) that allow the recruitment of proteins able to inhibit gene transcription. Interestingly, NCoR1 is able directly interact with histones through their histone interaction domain (HID), while it can also interact and enhance HDAC3 activity through their deacetylase activation domain (DAD) (Ishizuka and Lazar, 2005). Moreover, the NCoR1 protein also contains three receptor

interaction domains (RID) located in the C-terminal domain that allow the direct interaction with different target transcription factors (Webb et al., 2000). The NCoR1 complex can be formed by different proteins in a context dependent manner (Mottis et al., 2013), but G protein pathway suppressor 2 (GPS2), transducin β-like 1 (TBL1), TBL-related 1 (TBLR1) and HDAC3 represent the core subunits (Yoon et al., 2003). Collectively, the NCoR1 protein contains key functional domains that allow the interaction with specific transcription factors and the regulation of chromatin condensation, which consequently leads to transcriptional repression.

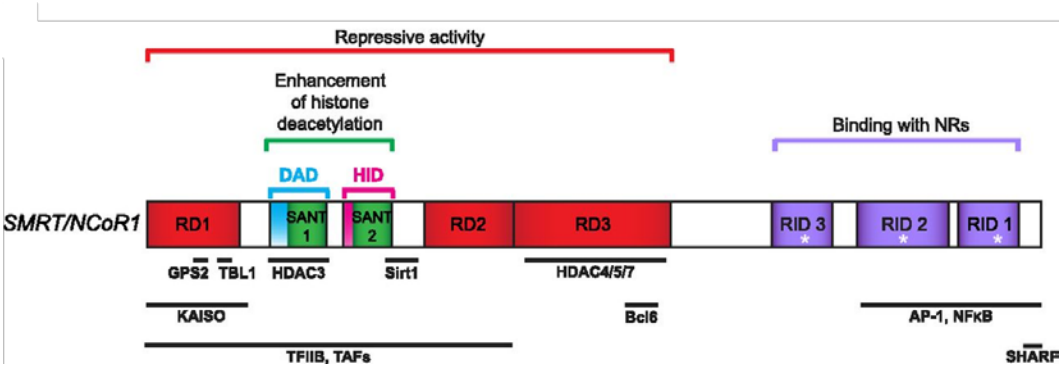

Figure 6 Protein structure of NCoR1 and NCoR2 (also known as SMRT; from (Mottis et al., 2013)).

NCoR1 and skeletal muscle remodelling

In stark contrast to PGC-1α, the role of NCoR1 in the control of skeletal muscle phenotype and function remains poorly understood. This corepressor however has been implicated in the control of skeletal muscle cell differentiation by modulating the expression of key regulatory proteins of this process such as MyoD, TRα1 and Csl (Bailey et al., 1999; Busson et al., 2005; Kitamura et al., 2007). Importantly, NCoR1 has been demonstrated to directly regulate proteins that play a relevant function in skeletal muscle plasticity, including HDAC3, PPARs and the regulatory subunit of phosphatidylinositol 3-kinase (PI3K) p85α among others (Furuya et al., 2007; Ishizuka and Lazar, 2005; McKenna and O'Malley, 2010). The

study of NCoR1 in vivo has been mainly limited by the embryonic lethality exhibited by NCoR1 global knockout mice (Jepsen et al., 2000), but conditional knockout mouse model have recently been generated and revealed the involvement of NCoR1 in the control of energy metabolism (Li et al., 2011; Yamamoto et al., 2011). Further supporting the metabolic function of NCoR1, it has been shown that disruption of NCoR1-HDAC3 interaction enhances whole body oxidative metabolism, improves insulin sensitivity and alters circadian behaviour (Alenghat et al., 2008). The effects of NCoR1 on the circadian clock depend on the regulation of the transcription factor Rev-erbα (Feng et al., 2011), which has recently been proposed to be a pivotal modulator of skeletal muscle oxidative metabolism (Woldt et al., 2013). Further suggesting a role of NCoR1 in skeletal muscle plasticity, the NCoR1-HDAC3 complex has been reported to be an essential regulator of class IIa HDACs (Fischle et al., 2002), which are negative regulators of slow oxidative muscle fibre formation (Potthoff et al., 2007). These data indicate that NCoR1 is in fact able to modulate skeletal muscle metabolism and regulate the activity of a wide spectrum of proteins with known function in skeletal muscle remodelling, but future studies are required to fully elucidate its physiological relevance and molecular mechanism of action.

Regulation of NCoR1 expression and activity in skeletal muscle

Under basal conditions, NCoR1 seems to be equally expressed in oxidative and glycolytic muscles (Schuler et al., 1999; Schuler and Pette, 1998). Interestingly, NCoR1 expression has been proposed to be sensitive to skeletal muscle contraction. In fact, rat skeletal muscle shows lower levels of NCoR1 mRNA following chronic low-frequency electrical stimulation (Schuler et al., 1999). Similarly, acute treadmill running has also been shown to decrease NCoR1 transcript levels in mouse skeletal muscle (Yamamoto et al., 2011). Consistent with the metabolic function of this corepressor, NCoR1 expression has been also proposed to be regulated by nutrients, which is reflected by the negative effects of low-glucose and high-fatty acids on NCoR1 mRNA levels (Yamamoto et al., 2011). Conversely, both high-glucose and insulin induce the up-regulation of NCoR1 in skeletal muscle (Yamamoto et al.,

2011). Post-translational modifications can also control the effects of NCoR1 on gene transcription, mainly by modulating its cellular localization. The regulation of NCoR1 activity by post-translational modifications in skeletal muscle is not well understood, but data from non-muscle cells has revealed that NCoR1 can be phosphorylated by Akt and MEKK1 (Baek et al., 2002; Hermanson et al., 2002). Phosphorylation of NCoR1 by these protein kinases promotes gene transcription by inducing its translocation from the nucleus to the cytosol (Baek et al., 2002; Hermanson et al., 2002). Interestingly, mTORC1 activation of S6K2 has been suggested to promote NCoR1 nuclear localization and repression of PPARα in the liver (Kim et al., 2012; Sengupta et al., 2010). Importantly, most of the signal pathways promoting NCoR1 phosphorylation are highly regulated by exercise (Egan and Zierath, 2013), strongly suggesting a role of NCoR1 in exercise-mediated skeletal muscle remodelling.

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CHAPTER 2: Aims of the thesis

The current knowledge shows that transcriptional coregulators drive several aspects of skeletal muscle remodelling, mainly in the context of physical activity/inactivity and metabolic diseases. Of particular interest, activation of the coactivator PGC-1α has been extensively shown to improve skeletal muscle oxidative capacity, while the corepressor NCoR1 has emerged as a potential regulator of skeletal muscle plasticity. Importantly, among the wide spectrum of transcription factors that PGC-1α and NCoR1 modulate, ERRα and PPARβ/δ are the most prominent candidates to play a role in skeletal muscle. Here, by using different genetic mouse models, bioinformatics and cell culture approaches we aimed to identify the potential role and molecular mechanisms by which PGC-1α and NCoR1 modulate skeletal muscle phenotype. Accordingly, this thesis has five specific aims:

1. Define the role of NCoR1 in the regulation of skeletal muscle phenotype and function under basal conditions (study 1).

2. Elucidate the potential interplay between NCoR1 and PGC-1α in the regulation of skeletal muscle oxidative metabolism (study 1).

3. Determine the functional interplay between PGC-1α and PPARβ/δ in the regulation of skeletal muscle oxidative metabolism in vivo (study 2).

4. Define whether PGC-1α regulates skeletal muscle hypertrophy induced by chronic mechanical overload (study 3).

5. Determine the function of PGC-1α in the regulation of the metabolic and functional adaptations to chronic overload of skeletal muscle (study 3).

CHAPTER 3: The corepressor NCoR1 antagonizes PGC-1α and ERRα in the regulation of skeletal muscle function and oxidative metabolism

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The Corepressor NCoR1 Antagonizes PGC-1α and Estrogen-Related Receptor α in the Regulation of Skeletal Muscle Function and Oxidative Metabolism

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Skeletal muscle exhibits a high plasticity and accordingly can quickly adapt to different physiological and pathological stimuli by changing its phenotype largely through diverse epigenetic mechanisms. The nuclear receptor corepressor 1 (NCoR1) has the ability to mediate gene repression; however, its role in regulating biological programs in skeletal muscle is still poorly understood. We therefore studied the mechanistic and functional aspects of NCoR1 function in this tissue. NCoR1 muscle-specific knockout mice exhibited a 7.2% higher peak oxygen consumption (VO_{2peak}), a 11% reduction in maximal isometric force, and increased *ex vivo* fatigue resistance during maximal stimulation. Interestingly, global gene expression analysis revealed a high overlap between the effects of NCoR1 deletion and peroxisome proliferator-activated receptor gamma (PPARγ) coactivator 1α (PGC-1α) overexpression on oxidative metabolism in muscle. Importantly, PPARβ/δ and estrogen-related receptor α (ERRα) were identified as common targets of NCoR1 and PGC-1α with opposing effects on the transcriptional activity of these nuclear receptors. In fact, the repressive effect of NCoR1 on oxidative phosphorylation gene expression specifically antagonizes PGC-1α-mediated coactivation of ERRα. We therefore delineated the molecular mechanism by which a transcriptional network controlled by corepressor and coactivator proteins determines the metabolic properties of skeletal muscle, thus representing a potential therapeutic target for metabolic diseases.

Improved muscle performance is directly linked to a lower prevalence of metabolic diseases (9, 50). In fact, while physical exercise and training can lower morbidity and mortality, physical inactivity has been recognized as one of the main risk factors for these pathologies (8). Lower whole-body aerobic capacity, muscle mitochondrial content, and oxidative activity, which all correlate with a sedentary lifestyle, contribute to the development of metabolic disorders (9, 25, 34, 38). Therefore, maintenance or improvement of skeletal muscle function, especially its oxidative metabolism, should be considered among the first interventions in the treatment and prevention of metabolic diseases.

Skeletal muscle is a highly plastic tissue that can quickly adapt to different physiological (e.g., exercise) and pathological (e.g., overnutrition) stimuli. In fact, muscle fibers can change their gene expression profile and phenotype to a great extent through diverse epigenetic mechanisms (3, 6, 31). Accordingly, muscle remodeling is highly regulated by different transcription factors and coregulator complexes, which are able to modify chromatin structure and thereby regulate gene transcription (27, 41). The nuclear receptor corepressor 1 (NCoR1) is a ubiquitously expressed corepressor, originally identified as the mediator of ligand-independent transcriptional repression of the thyroid hormone and the retinoic acid receptor (22). NCoR1 interacts with several transcription factors through its receptor interaction domains located in the C terminus (48). However, because NCoR1 lacks intrinsic histone deacetylase (HDAC) activity, it regulates gene transcription by forming a large protein complex in which G protein pathway suppressor 2 (GPS2), transducin β-like 1 (TBL1), TBL-related 1 (TBLR1), and HDAC3 represent the core subunits (52). In fact, the NCoR1-HDAC3 interaction plays an essential role in the control of gene transcription, since HDAC3 is directly activated by the deacetylase activation domain (DAD) of NCoR1 (23).

NCoR1 interacts with different proteins that play an important role in muscle physiology, such as peroxisome proliferator-activated receptors (PPAR) and p85α (15, 32), although its role in skeletal muscle remains largely enigmatic. Cell culture experiments implied that NCoR1 modulates myoblast differentiation through the regulation of the expression and transcriptional activity of several transcription factors, e.g., MyoD, TRα1, and Csl (5, 10, 26). The role of NCoR1 *in vivo* is not well understood because *Ncor1*$^{-/-}$ mice embryos die during gestation (24). Recently, conditional knockout models revealed that NCoR1 is an important player in skeletal muscle and adipose tissue energy metabolism (28, 51). In skeletal muscle, NCoR1 deletion enhances oxidative metabolism and slightly improves insulin tolerance under a high-fat diet (51). Specific genetic ablation of NCoR1 in white adipose tissue lowers inflammation and improves whole-body insulin sensitivity (28). However, the mechanism by which NCoR1 deletion results in these effects is not well understood.

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NCoR1 is expressed in adult glycolytic and oxidative muscles at equal levels (42, 43). Chronic low-frequency stimulation of rat hind limb and acute endurance exercise in mice repress the expression of NCoR1 in this tissue (42, 51). Interestingly, by using knockout mice, it has been demonstrated that the formation of slow oxidative muscle fibers is negatively regulated by class IIa HDACs (39), which depend on interaction with the NCoR1-HDAC3 complex in order to induce protein deacetylation (14). Consistent with this, the global disruption of the NCoR1-HDAC3 complex in mice results in improved energy metabolism (e.g., higher insulin sensitivity and oxygen consumption) and altered circadian behavior (1). The aim of this study was to further investigate the role of NCoR1 in different aspects of skeletal muscle function (e.g., force generation) and, importantly, to elucidate the elusive mechanism by which NCoR1 regulates oxidative metabolism in this tissue.

MATERIALS AND METHODS

Animal housing and NCoR1 MKO mouse generation. Mice were housed in a conventional facility with a 12-h night/12-h day cycle with free access to food and water. All experiments were performed on adult male mice with the approval of the Swiss authorities. NCoR1 muscle-specific knockout (MKO) animals were generated as previously described (51). Briefly, *Ncor1*$^{loxP/loxP}$ mice were crossed with *HSA-Cre* transgenic mice to generate NCoR1 MKO mice. *Ncor1*$^{loxP/loxP}$ animals without *Cre* expression were used as control (CON) mice. No overt phenotypic differences between CON and wild-type (WT) mice were observed. Genotyping was performed from tail biopsy specimens by PCR using specific primer pairs to detect the presence of the 5′ and 3′ *loxP* sites. The presence of the 5′ *loxP* site resulted in an amplicon of 450 bp (WT allele, 403 bp), while the presence of the 3′ *loxP* site resulted in an amplicon of 346 bp (WT allele, 207 bp) (see Fig. S1A in the supplemental material). Specific primer pairs to detect *Cre* recombinase resulted in an amplicon of 320 bp in NCoR1 MKO animals (see Fig. S1A). In addition, using muscle samples, recombination was confirmed by PCR using the forward and reverse primers used to detect the 5′ and 3′ *loxP* sites, respectively. Consequently, a 246-bp band was detected exclusively in NCoR1 MKO animals (see Fig. S1B). The recombination of the *Ncor1* floxed allele decreased its mRNA specifically in skeletal and, to a lesser extent, cardiac muscle compared to that in CON mice (see Fig. S1C). Importantly, previous work has indicated that the slight decrease of NCoR1 mRNA in the heart of MKO mice does not affect cardiac morphology and function (51).

The generation and characterization of transgenic mice expressing peroxisome proliferator-activated receptor gamma (PPARγ) coactivator 1α (PGC-1α) under the control of the MCK promoter has been published (30). These PGC-1α muscle-specific transgenic (mTg) mice exhibit a 9.5-fold increase in skeletal muscle PGC-1α mRNA (see Fig. S3A in the supplemental material), higher oxidative metabolism, and improved exercise performance (11, 30).

Exercise performance assessment. Animals were acclimatized to treadmill running for 2 days. On the first day, mice ran in a closed treadmill (Columbus Instruments) for 5 min at 10 m/min with a 0° slope, followed by 5 min at 14 m/min with a 5° incline. On the second day, animals ran for 5 min at 10 m/min with a 5° incline, followed by 5 min at 14 m/min. To determine maximal exercise performance and peak oxygen consumption (VO_{2peak}), indirect calorimetry was performed during a maximal exercise test. Thus, 2 days after the acclimatization, mice were placed in a closed treadmill for 6 min at 0 m/min with a 5° incline. Subsequently, the test started at 8 m/min for 3 min with a 5° incline and the speed was increased 2 m/min every 3 min until exhaustion.

To determine endurance performance, mice were placed in an open treadmill (Columbus Instruments) for 5 min at 0 m/min with a 5° incline, followed by 5 min at 8 m/min. Mice ran for 20 min at 60, 70, 80, and 90% of the maximal speed reached in the maximal exercise test (average of the group), and then the speed increased to 100% of the maximal speed until exhaustion. The endurance test was performed at least 3 days after the maximal test.

Blood lactate analysis. Blood lactate was measured from the tail vein of mice fasted overnight (16 h) or fed animals before and after different time points after treadmill running (see the maximal exercise test) using a lactate meter (Nova Biomedical).

***In vivo* measurement of muscle contractility.** Grip strength of the fore and hind limbs was measured with a grip strength meter (Chatillon). To determine the maximal strength, three measurements were performed with at least 60 s of recovery between each repetition, and the maximum value obtained was used for the analysis. To assess isometric fatigue resistance, mice were placed on top of an elevated grid, and the maximum time that they could remain on the inverted grid was recorded.

***Ex vivo* determination of muscle contractility.** Maximal force and fatigue resistance of extensor digitorum longus (EDL) and soleus were measured with a muscle testing setup (Heidelberg Scientific Instruments). Contraction amplitude was digitalized at 4 kHz with an AD Instruments converter. After the determination of the optimal length, force generation of EDL and soleus during a single twitch was measured in response to a 15-V pulse for 0.5 ms. Tetanic contraction was assessed in response to a 15-V pulse at 150 Hz for 400 ms for EDL and 1,100 ms for soleus. Maximal force and kinetics of the single twitch and tetanus were recorded and analyzed.

After 10 min of recovery following the tetanic stimulation, fatigue resistance of EDL and soleus was assessed under two different protocols. First, a long-interval protocol was performed by stimulating the muscles with a 15-V pulse at 150 Hz for 350 ms at 3.6-s intervals during 6 min for EDL and 10 min for soleus, followed by 10 min of recovery. Subsequently, a short-interval protocol was performed by stimulating the muscles with a 15-V pulse at 150 Hz for 350 ms at 1-s intervals during 2 min for EDL and 3 min for soleus. Changes in force generation are expressed as a percentage of initial force (first tetanus) (18).

Cell culture experiments. C_2C_{12} myoblasts were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (growth medium). To induce differentiation, growth medium of 90 to 95% confluent myoblasts was changed to DMEM supplemented with 2% horse serum and 1% penicillin-streptomycin (differentiation medium). Cells were maintained at 37°C, 95% O_2, and 5% CO_2.

Experiments using C_2C_{12} cells were performed on fully differentiated myotubes 4 days after differentiation was induced. For knockdown experiments, different adenoviruses containing specific short hairpin RNAs (shRNAs) against NCoR1, PGC-1α, PGC-1β, and LacZ (control) were used. Cells were incubated with the corresponding adenovirus for 24 h, and then adenovirus-containing medium was exchanged for fresh differentiation medium for another 24 h. In addition, cells were treated for 48 h with 0.2% dimethylsulfoxide (DMSO) (as a control), 10 μM XCT790 (Sigma-Aldrich) to inhibit estrogen-related receptor α (ERRα), or 1 μM GSK0660 (Sigma-Aldrich) to inhibit PPARβ/δ, together with the corresponding adenovirus. Three independent experiments each were performed in triplicate.

Luciferase assays were performed on 12-well plates using COS-7 cells (African green monkey kidney fibroblast-like cells) grown in growth medium without antibiotics. COS-7 cells were used in our experiments as a heterologous experimental system because of their high transfection efficiency and suitability for expressing constructs containing simian virus 40 (SV40) promoters. Cells were transfected using Lipofectamine 2000 (Invitrogen) with 0.1 μg pRL-SV40 (E2231; Promega), 0.3 μg pPPRE X3-TK-luc (1015; Bruce Spiegelman; Addgene), 0.3 μg pERRE-luc (gift of Junichi Sadoshima) (36), 0.4 μg pBABE puro PPAR delta (8891; Bruce Spiegelman; Addgene), 0.4 μg pERRα (gift of Vincent Giguère), 0.4 μg pFlag-NCoR (gift of Christopher K. Glass), and 0.4 μg pAd-Track HA PGC-1α (14427; Pere Puigserver; Addgene). The total amount of plasmid DNA was kept constant at 1.6 μg per well by using the control plasmid

pAdtrack-CMV (ATCC). Twenty-four hours after transfection, cells were lysed with 250 μl of 1× passive lysis buffer (Promega), and luciferase activity was measured in 75 μl of lysate in a 96-well plate using the Dual-Glo luciferase assay system (Promega). *Renilla* (pRL-SV40) luciferase activity was used for normalization. Five independent experiments each were performed in triplicate.

RNA isolation and real-time PCR. Total RNA was isolated from C_2C_{12} myotubes, liver, kidney, white adipose tissue, heart, gastrocnemius (GAS), soleus, plantaris, tibialis anterior, extensor digitorum longus, and quadriceps from NCoR1 MKO mice or gastrocnemius from PGC-1α mTg animals using lysing matrix tubes (MP Biomedicals) and TRI reagent (Sigma-Aldrich) according to the manufacturer's instructions. RNA concentration was measured with a NanoDrop 1000 spectrophotometer (Thermo Scientific). One microgram of RNA was treated with DNase I (Invitrogen) and then reversed transcribed using hexanucleotide mix (Roche) and SuperScript II reverse transcriptase (Invitrogen). The level of relative mRNA was quantified by real-time PCR on a StepOnePlus system (Applied Biosystems) using Power SYBR green PCR master mix (Applied Biosystems). The analysis of the mRNA was performed by the $\Delta\Delta C_T$ method using TATA binding protein (TBP) as the endogenous control. Primers used for target genes and TBP had the same PCR efficiency. TBP transcript levels were not different between genotypes or different experimental conditions. Primer sequences can be found in Table S3 in the supplemental material.

Mitochondrial DNA measurement. DNA was isolated from gastrocnemius using a NucleoSpin tissue kit (Macherey-Nagel). Real-time PCR analysis was performed to measure COX2 (mitochondrial DNA) and β-globin (nuclear DNA) levels.

Skeletal muscle staining. Oxidative fibers were detected by NADH staining of 12-μm cross-sections from the tibialis anterior. Staining was performed by exposing the sections to 0.8 mg/ml NADH in the presence of 1 mg/ml nitroblue tetrazolium. Periodic acid-Schiff (PAS) staining was performed with a PAS kit by following the manufacturer's instructions (Sigma-Aldrich).

Protein isolation. Tissue samples were powdered on dry ice and homogenized with a polytron device in 300 μl of ice-cold lysis buffer (50 mM Tris-HCl, pH 7.5, 250 mM sucrose, 1 mM EDTA, 1 mM EGTA, 0.25% NP-40 substitute, 50 mM NaF, 5 mM $Na_4P_2O_7$, 0.1% dithiothreitol [DTT], and fresh protease and phosphatase inhibitor cocktail). Samples then were shaken at 1,300 rpm for 30 min at 4°C. Samples were subsequently centrifuged at 13,000 × *g* for 10 min at 4°C, and the protein concentration of the supernatant was determined by the Bradford assay (Bio-Rad). Equal aliquots of protein were boiled for 5 min in Laemmli sample buffer (250 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.01% bromophenol blue, and 5% β-mercaptoethanol).

Western blotting. Samples were separated on SDS-polyacrylamide gels and then transferred to nitrocellulose membranes for 60 min. Membranes were blocked for 1 h in 3% milk, Tris-buffered saline, and 0.1% Tween 20 (TBST) before overnight incubation at 4°C with the appropriate primary antibody in TBST (1:1,000 dilution). Proteins were detected with a primary antibody to p-AMPKα^{T172} (2535; Cell Signaling) and AMPKα (2603; Cell Signaling). As a loading control, eEF2 (2332; Cell Signaling) was used. Following incubation, membranes were washed 3 times with TBST before incubation with an appropriate peroxidase-conjugated secondary antibody in TBST (1:10,000 dilution). Antibody binding was detected using the enhanced chemiluminescence horseradish peroxidase (HRP) substrate detection kit (32106; Pierce).

Microarray and bioinformatic analysis. RNA from gastrocnemius was isolated with an miRNeasy minikit (Qiagen), and microarray was performed using the GeneChip Gene 1.0 ST Array System (Affymetrix). In addition, gene ontology (GO) analysis was performed with the online tool FatiGO (http://babelomics.bioinfo.cipf.es/index.html) (2). Finally, microarray data were analyzed using Motif Activity Response Analysis (MARA; http://www.mara.unibas.ch/cgi/mara) (47).

Statistical analysis. Values are expressed as means ± standard errors of the means (SEM). Statistical significance was determined with unpaired two-tailed *t* tests or one-way analysis of variance (ANOVA) with Tukey's *post hoc* test. A P value <0.05 was considered significant.

Microarray data accession number. Microarray data can be found at the Gene Expression Omnibus (GEO) under accession number GSE40439.

RESULTS

Muscle NCoR1 deletion enhances VO_2 during maximal exercise and decreases muscle contractility. The role of NCoR1 in skeletal muscle was studied using NCoR1 MKO mice. A full description of this animal model has been recently published elsewhere (51). First, we assessed maximal oxidative capacity and treadmill running performance. Both genotypes exhibited the same exercise performance during maximal and endurance exercise tests, as reflected by equal speed, distance, time, work, and power (see Table S1 in the supplemental material). However, NCoR1 MKO mice reached a significantly higher (7.2%) VO_{2peak} during the maximal exercise test (CON mice, 125 ± 1.2 ml/kg of body weight/min; MKO mice, 134 ± 1.5 ml/kg/min; $P < 0.001$). In fact, NCoR1 MKO animals showed a higher VO_2 at 50, 80, 90, and 100% of maximal speed (Fig. 1A). Blood lactate measurement before and after maximal exercise, or during fasting, was not different in NCoR1 MKO mice (see Fig. S2A and B in the supplemental material). Consistent with this, respiratory exchange ratio (RER) measurement during the maximal exercise test did not show differences between CON and NCoR1 MKO mice (see Fig. S2C), indicating that energy substrate utilization was not altered by NCoR1 deletion. We then performed NADH and PAS staining of skeletal muscle to determine the proportion of oxidative fibers and glycogen content, respectively. NCoR1 MKO animals showed a 10% increase in oxidative fibers compared to CON mice (Fig. 1B), but glycogen content was not significantly affected (Fig. 1C). Furthermore, we have found higher levels of AMP-activated protein kinase (AMPK) phosphorylation in skeletal muscle of NCoR1 MKO mice (Fig. 1D). Altogether, these data show that NCoR1 deletion in striated muscle results in an enhanced oxidative metabolism, particularly during high-intensity exercise.

An important aspect of muscle function besides endurance is the ability to generate force. To assess muscle contractility *in vivo*, we measured maximal grip strength of the fore and hind limbs. We observed a trend for lower peak isometric force generated by the fore limbs ($P = 0.078$) and a significant reduction by 11% in the hind limbs of NCoR1 MKO animals (Fig. 1E). We then quantified muscle fatigue resistance during predominantly isometric muscle contraction by measuring the maximal time that mice could remain on an inverted grid. Consistent with the lower maximal isometric force, isometric fatigue resistance was also decreased in NCoR1 MKO animals (Fig. 1F). To exclude systemic and neural factors that could affect force generation *in vivo*, we also determined muscle contractility in isolated muscles. Absolute ($P < 0.05$) and specific ($P = 0.056$) muscle contractility in response to a single twitch was decreased in the glycolytic muscle extensor digitorum longus (EDL) but not in the oxidative muscle soleus (Fig. 2A and B). Conversely, when EDL and soleus were subjected to maximal tetanic stimulation, no differences between genotypes were observed (Fig. 2C and D). Moreover, the contractile kinetics of EDL and soleus in response to a single twitch or a maximal tetanic stimulation were not significantly different be-

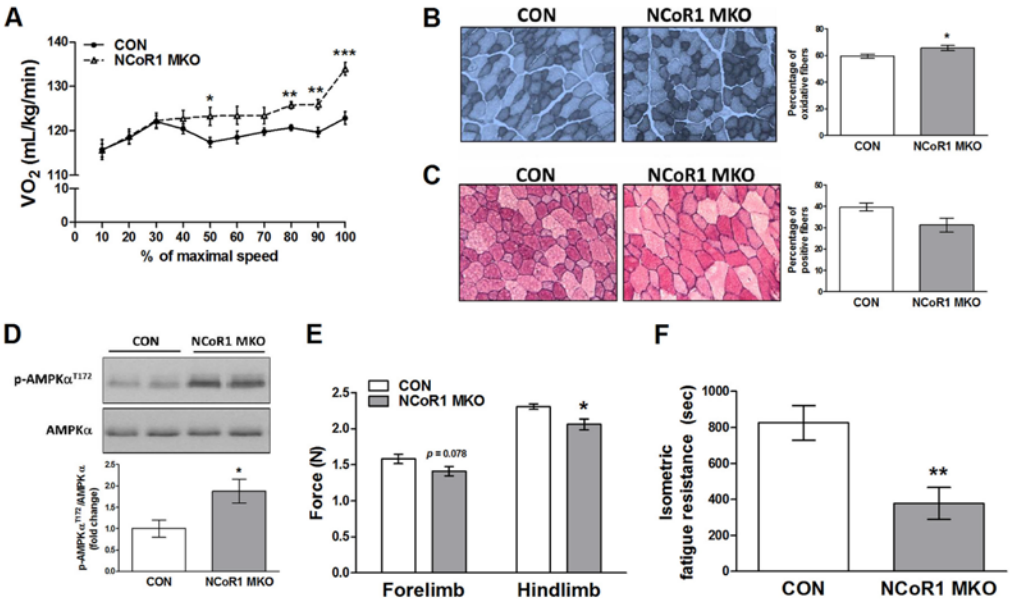


FIG 1 Exercise performance and *in vivo* contractile properties of NCoR1 MKO mouse skeletal muscle. (A) VO_2 during the maximal exercise test ($n = 8$ CON and $n = 7$ NCoR1 MKO mice). (B and C) Representative pictures and quantification of NADH (B) and PAS (C) staining of tibialis anterior ($n = 3$ to 4 CON and $n = 3$ to 4 NCoR1 MKO mice). (D) Representative blots and quantification of gastrocnemius AMPK phosphorylation levels ($n = 7$ CON and $n = 4$ NCoR1 MKO mice). (E and F) *In vivo* assessment of maximal grip strength and isometric muscle fatigue resistance ($n = 13$ CON and $n = 12$ NCoR1 MKO mice). Values represent means ± SEM. $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (**) for CON versus NCoR1 MKO mice.

tween CON and NCoR1 MKO mice (see Table S1 in the supplemental material).

Finally, muscle fatigue resistance was determined *ex vivo* by a long- and short-interval protocol. As expected, soleus exhibited a lower decrease in force than EDL in the long-interval protocol, but no differences between genotypes were found (Fig. 2E). Similarly, soleus had higher fatigue resistance than EDL during the short-interval protocol. However, in this protocol, EDL from NCoR1 MKO animals exhibited a lower decrease in force generation, while soleus of NCoR1 MKO and CON mice were identical (Fig. 2F). In fact, in the short-interval protocol, EDL from NCoR1 MKO mice generated ~43% more force from tetanic stimulation no. 35 to 100 (Fig. 2F, inset), indicating a higher muscle fatigue resistance. Therefore, it seems that glycolytic muscles are more susceptible to the effect of NCoR1 deletion on muscle contractility, resulting in a decreased maximal isometric force and increased fatigue resistance during maximal stimulation.

NCoR1 and PGC-1α target a common subset of genes involved in oxidative metabolism. The phenotype exhibited by NCoR1 MKO animals implies a direct link between NCoR1 and oxidative metabolism. At the transcriptional level, mitochondrial function is mainly regulated by the PPARγ coactivator 1 (PGC-1) family of coactivators, which are able to activate different transcription factors, such as ERRα and NRF-1 (29). Interestingly, NCoR1 MKO mice mirror some aspects of the phenotype exhibited by PGC-1α mTg mice, such as the enhanced oxidative metabolism and decreased maximal force (11, 30, 45). To explore the idea of an NCoR1-PGC-1α cross talk and to further characterize the oxidative phenotype of NCoR1 MKO mice, microarray analyses of gene expression patterns in NCoR1 MKO and PGC-1α mTg skeletal muscles were compared. GO enrichment analysis revealed overrepresentation of transcripts related to metabolic pathways, such as oxidative phosphorylation and the citrate cycle (tricarboxylic acid [TCA] cycle), in both NCoR1 MKO and PGC-1α mTg animals (Fig. 3A and B; also see Fig. S4A to F in the supplemental material). Interestingly, when both microarray data sets were compared, we observed that 188 of the genes affected by NCoR1 deletion were also found in the PGC-1α mTg data set, suggesting that ~50% of the genes regulated by NCoR1 are also targets of PGC-1α (Fig. 3C). When only the genes found with the GO terms oxidative phosphorylation and citrate cycle (TCA cycle) were compared, we found that all of the genes present in the NCoR1 MKO data set were also targets of PGC-1α (Fig. 3C). Interestingly, we observed that in both microarrays all of these common genes were actually upregulated (Fig. 3D and E). Several of these transcripts were validated by real-time PCR analysis of GAS, EDL, and soleus (see Fig. S3B and C in the supplemental material), confirming the results obtained with the microarrays. However, no fiber type-specific (oxidative versus glycolytic) differences in terms of regulation of gene expression were observed in NCoR1 MKO mice (see Fig. S3C). Furthermore, skeletal muscle from NCoR1 MKO and PGC-1α mTg mice had higher transcript levels of mitochondrial genes involved in oxidative metabolism, such as cytochrome *c* oxidase subunit I (*COX1*), ATP synthase F0 subunit 6 (*ATP6*), and NADH dehydrogenase subunit 1 (*ND1*) (Fig. 3F; also see Fig. S3B). However, no differences in transcription factor A, mitochondrial (*TFAM*) mRNA levels, and mitochondrial DNA content were found as a consequence of NCoR1 deletion (Fig. 3G; also see Fig. 6A). These results further demonstrate the role of NCoR1 in the transcriptional control of different mitochondrial

CHAPTER 3: The corepressor NCoR1 antagonizes PGC-1α and ERRα in the regulation of skeletal muscle function and oxidative metabolism

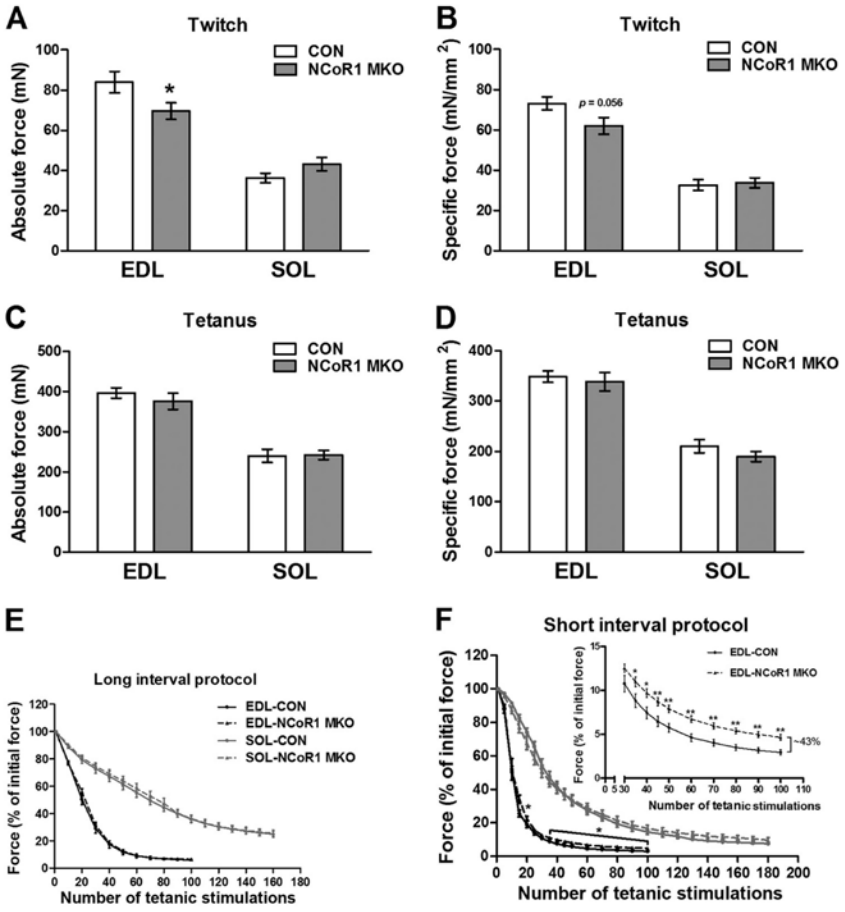
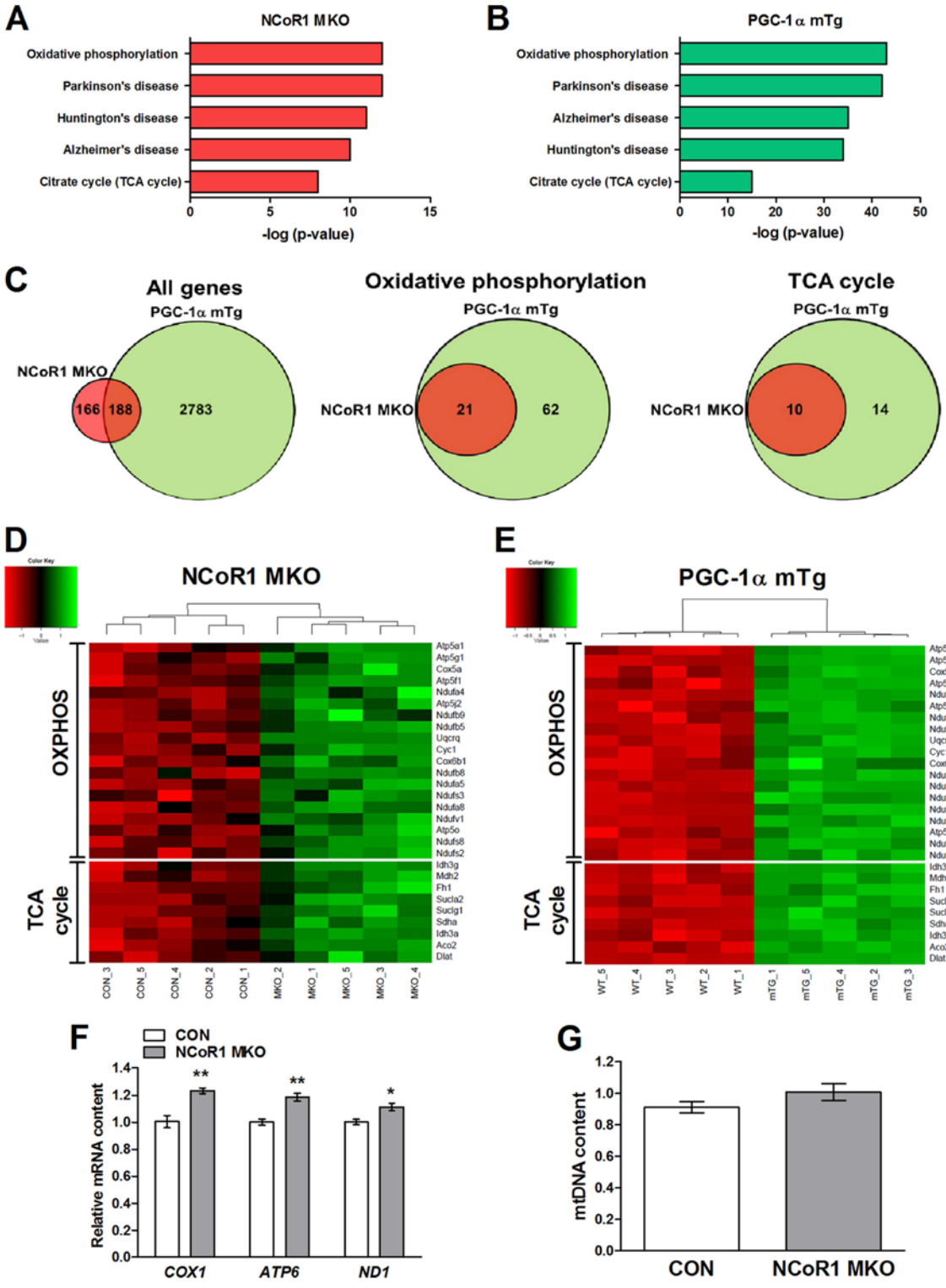


FIG 2 *Ex vivo* contractile properties of NCoR1 MKO mouse skeletal muscle. (A to D) *Ex vivo* assessment of extensor digitorum longus (EDL) and soleus (SOL) contractility in response to a single twitch or a maximal tetanic stimulation ($n = 10$ CON and $n = 14$ NCoR1 MKO muscles per group). (E and F) *Ex vivo* assessment of EDL and SOL fatigue resistance in response to repeated tetanic stimulation through a long- and short-interval protocol. The inset in panel F shows the section with significant differences ($n = 10$ CON and $n = 14$ MKO muscles per group). Values represent means ± SEM. $P < 0.05$ (*) and $P < 0.01$ (**) for CON versus MKO mice.

pathways and strongly suggest a role of PGC-1α in the control of the oxidative phenotype of the NCoR1 MKO animals.

Interestingly, the mRNA level of PGC-1α was not different between NCoR1 MKO and CON mice (Fig. 4A; also see Fig. S5A in the supplemental material). In contrast, we found an increase in PGC-1β and a slight decrease in PGC-1-related coactivator (PRC) transcript levels in gastrocnemius and soleus from NCoR1 MKO mice (Fig. 4A; also see Fig. S5A). To dissect the contribution of the PGC-1 family members to the effects induced by NCoR1 knockdown, we studied C_2C_{12} myotubes in culture that were virally transfected with two different shRNAs specifically targeting NCoR1 (Fig. 4B; also see Fig. S5B and C and S6A and B in the supplemental material). We then selectively induced the knockdown of PGC-1α or PGC-1β with specific shRNA constructs (Fig. 4B; also see Fig. S5B). Similar to the NCoR1 MKO animals, NCoR1 knockdown in C_2C_{12} myotubes also induced an upregulation of succinate dehydrogenase complex, subunit A, flavoprotein (*SDHa*), NADH dehydrogenase 1α (ubiquinone) subcomplex 5 (*NDUFA5*), NADH dehydrogenase 1β (ubiquinone) subcomplex 5 (*NDUFB5*), and fumarate hydratase 1 (*FH1*) (Fig. 4C), in addition to the mitochondrial genes *COX1* and *ATP6* (Fig. 4C). Surprisingly, NCoR1 knockdown in C_2C_{12} myotubes induced a big increase in PGC-1α mRNA (Fig. 4B). Importantly, however, although the simultaneous knockdown of PGC-1α partially prevented its upregulation, shRNA-mediated reduction of PGC-1α was sufficient to strongly inhibit the upregulation of *SDHa*, *NDUFA5*, *NDUFB5*, *FH1*, *COX1*, and *ATP6* mRNA induced by NCoR1 knockdown (Fig. 4C). In the cultured muscle cells, NCoR1 knockdown did not alter PGC-1β gene expression (see Fig. S5B), while the opposite was observed in the NCoR1 MKO animals (Fig. 4A; also see Fig. S5A). In stark contrast to the knockdown of PGC-1α, the upregulation of the nuclear and mitochondrial genes induced by NCoR1 knockdown was not significantly affected by the simultaneous knockdown of PGC-1β in the cultured myotubes (Fig. 4D). Consistent with the microarray analysis, these results indicate



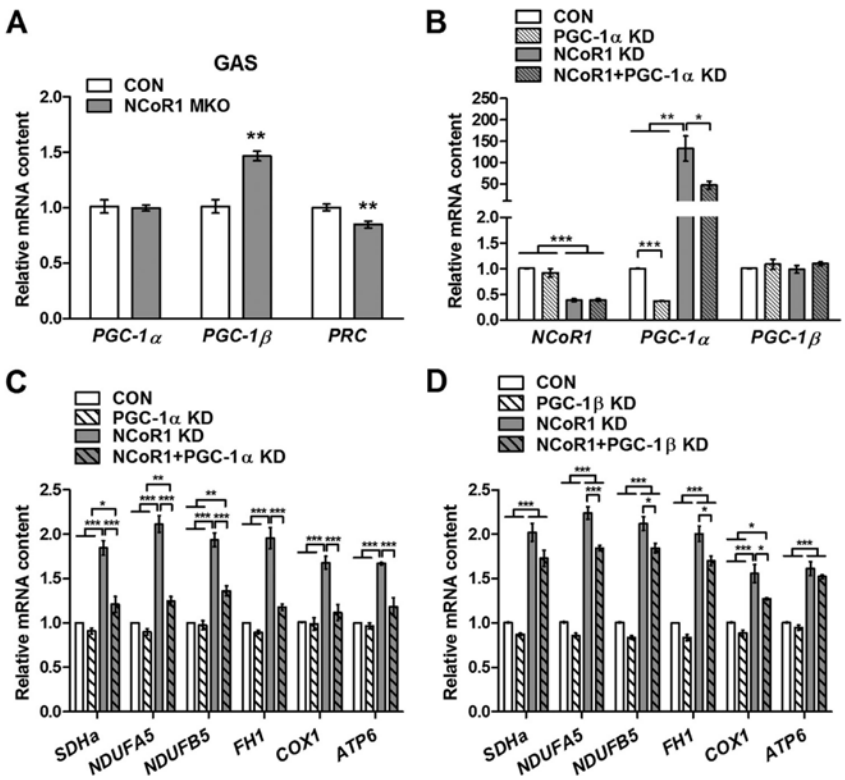


FIG 4 Role of PGC-1α/β in NCoR1 regulation of oxidative metabolism. (A) Gastrocnemius (GAS) mRNA levels of the PGC-1 family of coactivators ($n = 7$ CON and $n = 5$ NCoR1 MKO mice). (B to D) Adenoviral knockdown (KD) of LacZ (CON) or NCoR1 alone or in combination with PGC-1α or PGC-1β KD for 48 h in C_2C_{12} myotubes ($n = 3$ independent experiments each performed in triplicate). Values represent means ± SEM. $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***) for CON versus MKO mice or as indicated.

that NCoR1 deletion enhances PGC-1α action on oxidative metabolism, probably due to a reduced competition between NCoR1 and PGC-1α for the regulation of common target genes.

NCoR1 and PGC-1α regulate oxidative metabolism through opposite modulation of ERRα. Our data suggest that NCoR1 and PGC-1α target the same subset of transcription factors involved in the regulation of oxidative metabolism with opposite effects on transcriptional activation. In order to elucidate potential common transcription factors of NCoR1 and PGC-1α in skeletal muscle, we performed motif activity response analysis (MARA) (47) of our microarray data to predict the core set of transcription factors which significantly change their activity in response to NCoR1 deletion or PGC-1α overexpression. Consistent with the oxidative phenotype of these two mouse models, retinoid X receptor (RXR) and ERRα (also known as *Esrra*) were among the top 4 most significant motifs (z value, ≥1.5) (Fig. 5A and B; also see Table S2 in the supplemental material) and exhibited some of the highest levels of activity in response to NCoR1 deletion and PGC-1α overexpression (Fig. 5C to F). Given that RXRs are the main partners of PPARs (13), this suggests possible PPARβ/δ activation in MKO animals. In fact, PPARβ/δ or ERRα in complex with PGC-1α and PGC-1β are known to be key players in the regulation of muscle metabolism (13, 16, 29). In contrast, the role of the transcription factors with significantly decreased activity (z value, ≤−1.5) in NCoR1 MKO and PGC-1α mTg mice (Fig. 5A and B; also see Table S2) is not well known. Similar to the microarray analysis, the comparison of both MARA performances showed that 44% of the transcription factors found in the NCoR1 MKO data set were also predicted in PGC-1α mTg MARA; there is actually a larger overlap among motifs with increased activity (5 out of 9) than among ones with decreased activity (2 out of 7) (Fig. 5G). In summary, MARA-

FIG 3 Similarities between NCoR1 MKO and PGC-1α mTg mice on oxidative metabolism. (A and B) Top 5 KEGG pathways from GO analysis of the up- and downregulated genes from the NCoR1 MKO and PGC-1α mTg microarray data sets ($n = 5$ per group). (C) Venn diagrams showing the overlap between NCoR1 MKO and PGC-1α mTg microarray data sets. (D and E) Heat maps generated using probe set intensities of the overlapping transcripts between NCoR1 MKO and PGC-1α mTg related to the GO terms oxidative phosphorylation (OXHPOS) and citrate cycle (TCA cycle). (F) Real-Time PCR analysis of mRNA levels of mitochondrial genes of gastrocnemius from CON ($n = 7$) and NCoR1 MKO ($n = 5$) animals. (G) Measurement of mitochondrial DNA (mtDNA) content of gastrocnemius from CON ($n = 7$) and NCoR1 MKO ($n = 5$) animals. Values represent means ± SEM. $P < 0.05$ (*) and $P < 0.01$ (**) for CON versus NCoR1 MKO mice.

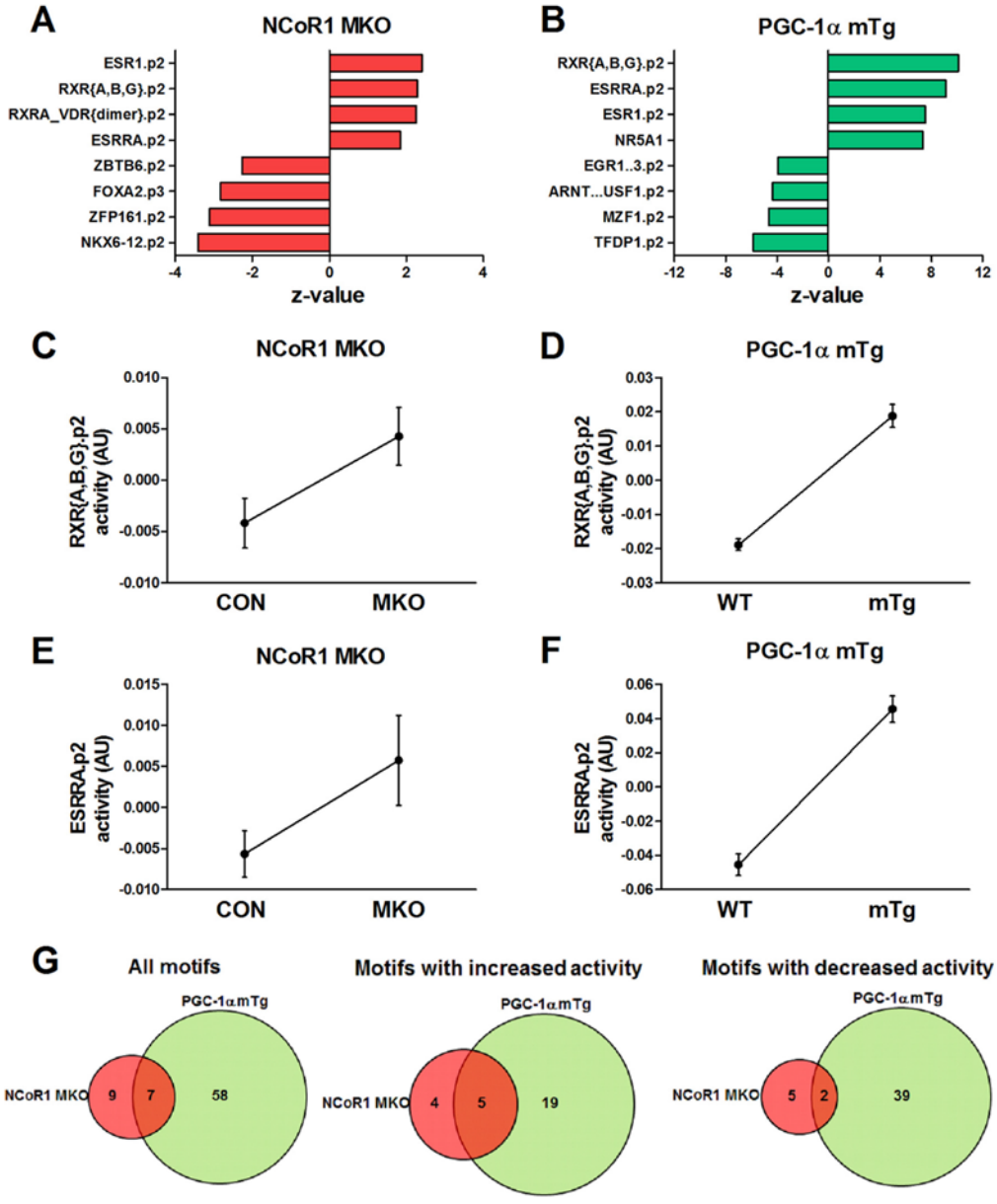


FIG 5 Identification of common transcription factor binding partners of NCoR1 and PGC-1α. (A and B) The top 4 transcription factor motifs exhibiting increased and decreased activity in MARA of the microarray data performed on gastrocnemius from NCoR1 MKO and PGC-1α mTg mice ($n = 5$ per group). (C to F) Changes in the activity of RXRs and ERRα (ESRRA) in NCoR1 MKO and PGC-1α mTg skeletal muscle predicted by MARA analysis of microarray data ($n = 5$ per group). (G) Venn diagrams showing the overlap between NCoR1 MKO and PGC-1α mTg MARA analysis.

based biocomputational prediction strongly suggests ERRα and possibly the RXR heterodimerization partner PPARβ/δ as common targets of NCoR1 and PGC-1α.

The mRNA levels of PPARβ/δ, ERRα, and several transcription factors revealed by MARA were not increased in NCoR1 MKO mice (Fig. 6A), indicating that increased activity is the consequence of lower repression rather than of higher expression. In order to explore the potential repressive effect of NCoR1 on ERRα and PPARβ/δ transcriptional activity, we transfected COS-7 cells with a reporter plasmid containing PPAR response elements (PPRE-luc) or ERR response elements (ERRE-luc) together with expression plasmids for PPARβ/δ and ERRα, respectively. We

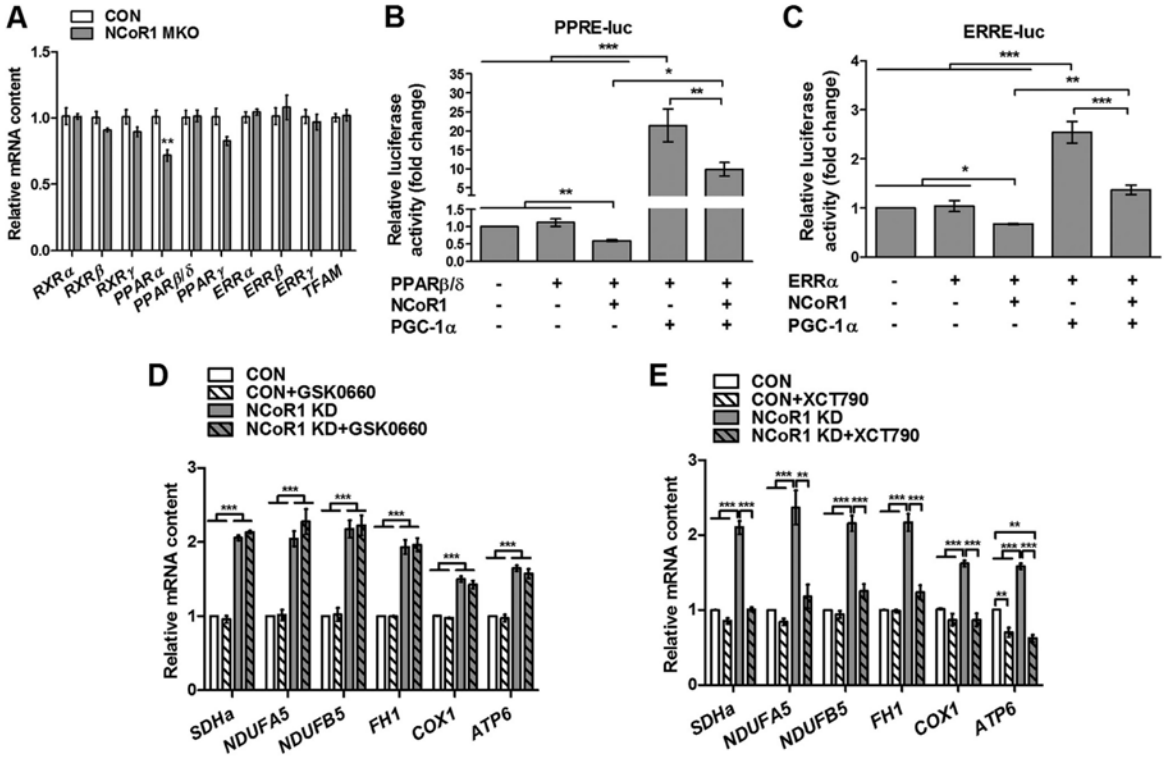


FIG 6 Role of PPARβ/δ and ERRα in NCoR1 regulation of oxidative metabolism. (A) Gastrocnemius mRNA levels of different transcription factors ($n = 7$ CON and $n = 5$ NCoR1 MKO mice). RXR, retinoid X receptor; PPAR, peroxisome proliferator activated receptor; ERR, estrogen-related receptor; TFAM, transcription factor A, mitochondrial. (B and C) Luciferase activity of PPRE-luc and ERRE-luc reporter plasmids in COS-7 cells cotransfected with PPARβ/δ, ERRα, NCoR1, and PGC-1α as indicated in each figure ($n = 5$ independent experiments each performed in triplicate). (D and E) Adenoviral knockdown (KD) of LacZ (CON) or NCoR1 alone or in combination with 10 μM XCT790 or 1 μM GSK0660 for 48 h in C_2C_{12} myotubes ($n = 3$ independent experiments each performed in triplicate). Values represent means ± SEM. $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***) for CON versus NCoR1 MKO mice or as indicated.

next measured relative luciferase activity in the absence and presence of NCoR1 and PGC-1α. As predicted, NCoR1 decreased PPRE-luc and ERRE-luc luciferase activity by 43 and 36%, respectively (Fig. 6B and C). Inversely, PGC-1α induced a significant increase in PPRE-luc and ERRE-luc luciferase activity, 2,037 and 161%, respectively (Fig. 6B and C). Importantly, we have found that the activation of both PPRE-luc and ERRE-luc by PGC-1α was significantly decreased by NCoR1 (Fig. 6B and C). These data demonstrate that NCoR1 represses the transcriptional activity of both PPARβ/δ and ERRα, while PGC-1α competes with NCoR1 to exert a positive effect.

Finally, to study the relative contribution of PPARβ/δ and ERRα to the regulation of the oxidative phenotype exhibited in response to NCoR1 deletion in muscle, we used the PPARβ/δ selective antagonist GSK0660 (44) and the ERRα inverse agonist XCT790 (33, 49). As expected, GSK0660 induced a strong decrease in the mRNA level of the PPARβ/δ target gene uncoupling protein 3 (*UCP3*) (see Fig. S6A in the supplemental material), demonstrating the efficiency of the antagonist and the presence of functional PPARβ/δ in C_2C_{12} myotubes. Neither NCoR1 knockdown nor GSK0660 treatment changed *PPARβ/δ* mRNA levels (see Fig. S6A). Importantly, however, the induction of *SDHa*, *NDUFA5*, *NDUFB5*, *FH1*, *COX1*, and *ATP6* mRNA were not inhibited by GSK0660 in cells with a knockdown of NCoR1 (Fig. 6D). Consistent with this, the mRNA levels of different PPARβ/δ target genes, like the carnitine palmitoyltransferase 1b gene (*CPT1b*), the lipoprotein lipase gene (*LPL*), and *UCP3*, were not significantly increased in NCoR1 MKO mice (see Fig. S3C in the supplemental material). In contrast, ERRα inhibition with XCT790 completely blocked the effects of NCoR1 knockdown on nuclear and mitochondrial genes (Fig. 6E). However, unlike the NCoR1 MKO animals, NCoR1 knockdown induced an upregulation of *ERRα* in C_2C_{12} myotubes (see Fig. S6B). This upregulation of *ERRα* mRNA was only partially prevented by XCT790 (see Fig. S6B), indicating that ERRα activation, rather than its upregulation, is responsible for the effects of NCoR1 knockdown of oxidative metabolism in C_2C_{12} myotubes. Interestingly, similar to NCoR1 and PGC-1α microarray analysis, GO enrichment analysis of previously published ERRα chromatin immunoprecipitation (ChIP)-on-chip data from mouse liver (12) also revealed overrepresentation of transcripts related to oxidative phosphorylation (see Fig. S4G in the supplemental material). Furthermore, we found a high overlap between the NCoR1 MKO microarray and the ERRα ChIP-on-chip data sets when comparing the genes found with the GO term oxidative phosphorylation (see Fig. S4H). Therefore, these data suggest that ERRα and PGC-1α are essential for the effects of NCoR1 deletion on oxidative metabolism in

skeletal muscle, while PPARβ/δ and PGC-1β seem to play minor roles in this experimental context.

DISCUSSION

In stark contrast to obese and type 2 diabetic subjects, endurance athletes exhibit an increased metabolic fitness and consequently lower risk for metabolic disorders (9, 35). Importantly, most of the adaptations to muscle use (e.g., endurance training) and disuse (e.g., physical inactivity) are under the coordinated control of different transcription factors and coregulators (7, 19). NCoR1 and its homolog NCoR2 have been recently suggested as important modulators of energy metabolism in several tissues, including skeletal muscle (28, 40, 46, 51). Consistent with this, we observed that NCoR1 MKO animals exhibit an increased VO_2 during high-intensity exercise. Surprisingly, while Yamamoto et al. (51) have reported improved exercise performance in NCoR1 MKO mice, we did not observe significant differences in distance, time, or work in the exercise trial in our experimental context. Considering the mild effect of NCoR1 deletion on muscle oxidative metabolism, it is possible that small differences in the exercise test protocol significantly affect exercise performance. In addition, as previously shown (51), we observed a higher proportion of oxidative fibers in NCoR1 MKO skeletal muscle. Interestingly, our data also indicate a higher activation of AMPK in NCoR1 MKO mice, which has been associated with enhanced oxidative metabolism (20, 21). Importantly, we have now demonstrated that NCoR1 controls skeletal muscle oxidative metabolism primarily through the regulation of ERRα and PGC-1α.

NCoR1 deletion in striated muscle led to an increase in the mRNA content of a broad range of mitochondrial enzymes, thus supporting its potential role as a negative regulator of oxidative metabolism. In agreement with this idea, NCoR1 MKO mice recapitulate many aspects of the phenotype exhibited by PGC-1α mTg mice, such as the increased expression of mitochondrial enzymes, higher levels of oxidative fibers, and enhanced VO_{2peak} during maximal exercise (11, 30, 51). Moreover, as found in NCoR1 MKO animals, PGC-1α overexpression in skeletal muscle also results in a reduced maximal force and increased muscle fatigue resistance (30, 45). Curiously, only EDL exhibited a higher muscle fatigue resistance during the short-interval protocol in NCoR1 MKO mice. Since EDL is a highly glycolytic muscle, it might be more susceptible to benefitting from the mild improvement in oxidative metabolism as a consequence of NCoR1 deletion compared to the predominantly oxidative soleus. Analysis of NCoR1 MKO- and PGC-1α mTg-based microarrays also showed a high level of similarity between these mouse models, since all of the upregulated genes related to oxidative metabolism found in NCoR1 MKO mice were also increased by PGC-1α overexpression. Hence, our findings indicate that NCoR1 deletion facilitates PGC-1α action on its target genes. In fact, PGC-1α mRNA was not increased in NCoR1 MKO mice, suggesting that the higher transcription of its target genes is not associated with its upregulation. In contrast, NCoR1 knockdown in cultured cells induced a 133-fold increase in PGC-1α mRNA. Hence, it is possible that the bigger effects of NCoR1 deletion in C_2C_{12} myotubes compared to those on the mice are a consequence of both upregulation of PGC-1α and lower competition between these coregulators. Importantly, however, we found that knockdown of PGC-1α, but not PGC-1β, in C_2C_{12} myotubes completely blocked the effects of NCoR1 knockdown on oxidative metabolism, underlining the essential role of PGC-1α in this process. Similarly, prediction of the relative contribution of transcription factors to the gene expression pattern exhibited by NCoR1 MKO and PGC-1α mTg animals revealed a significant overlap between these mouse models. Interestingly, RXRs (heterodimerization partners for the PPARs) and ERRα stood out as top candidates, showing the strongest link to the control of energy metabolism (13, 16). In fact, Yamamoto et al. (51) have recently showed that in C_2C_{12} myotubes, NCoR1 is recruited to the PPRE and nuclear receptor half-sites (ERR binding site) of *UCP3* and *PDK4* promoter, respectively, though whether the effects of NCoR1 deletion on gene expression actually depend on PPARβ/δ or ERRα activation was not studied. Here, through reporter gene assays, we have demonstrated that NCoR1 is a direct corepressor of both PPARβ/δ and ERRα. Importantly, given that NCoR1 and PGC-1α compete for the transcriptional regulation of PPARβ/δ and ERRα, our data indicate that these transcription factors are common targets of both coregulators. Interestingly, we observed a striking effect of the ERRα inverse agonist XCT790, since it completely inhibited the increase in the mRNA level of several mitochondrial enzymes triggered by NCoR1 knockdown in cultured myotubes. These data are consistent with MARA results and suggest that ERRα is the main target of NCoR1 in the control of muscle oxidative metabolism (Fig. 7).

Unexpectedly, we found that the PPARβ/δ selective antagonist GSK0660 did not affect the effects of NCoR1 deletion on oxidative phosphorylation and gene regulation. PPARβ/δ plays an impor-

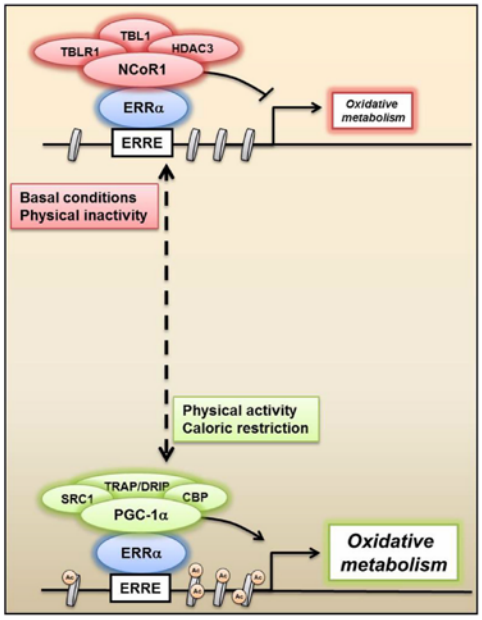


FIG 7 Antagonistic regulation of oxidative metabolism by NCoR1 and PGC-1α. Proposed mechanism by which NCoR1 and PGC-1α complexes compete for the transcriptional regulation of ERRα and, as a consequence, oxidative metabolism. Under basal and possibly pathological conditions, NCoR1 represses ERRα and increases histone deacetylation, thereby decreasing metabolic gene transcription. In contrast, PGC-1α is activated by exercise and caloric restriction in skeletal muscle, coactivates ERRα, and consequently enhances mitochondrial function. ERRE, ERR response elements; Ac, histone acetylation.

tant role in the transcriptional control of lipid metabolism (13). Intriguingly, our microarray analysis of NCoR1 MKO skeletal muscle shows a preferential enhancement of oxidative phosphorylation and the TCA cycle compared to the modulation of fatty acid β-oxidation. Thus, it is conceivable that in our experimental context of animals fed with normal chow, PPARβ/δ played less of a role than it did in studies using high-fat diets (51) or under conditions like obesity and physical inactivity. NCoR1 might control fatty acid metabolism through the transcriptional regulation of PPARβ/δ in an environment of elevated intramyocellular lipids that could act as PPARβ/δ ligands, but this hypothesis needs to be substantiated in future studies. Overall, it seems that a fully functional ERRα-PGC-1α complex is a prerequisite for the improved oxidative metabolism induced by muscle-specific NCoR1 deletion, at least in chow-fed mice.

Interestingly, NCoR1 MKO mice have a milder oxidative phenotype than PGC-1α transgenic mice. The weaker phenotype is also reflected in the quantitative differences in gene expression. Thus, while the muscle knockout of NCoR1 resulted in the upregulation of 21 genes involved in oxidative phosphorylation, 83 genes were increased in response to muscle PGC-1α overexpression. Consistent with this, PGC-1α mTg mice exhibit a higher increase in VO_{2peak} (24%) (11) than NCoR1 MKO animals (7.2%). However, it is important to note that NCoR1 is a basal corepressor, thus it represses transcription factors under basal conditions, and it is exchanged by coactivators upon a positive stimulus (17, 37). Therefore, our data suggest that under basal conditions, NCoR1 deletion would not result in a full activation of PPARβ/δ and ERRα, which subsequently could be amplified by a positive stimulus, such as exercise, that is well known to increase PGC-1α expression (4) and thereby unleashes the full activity of these transcription factors.

In conclusion, our data indicate competition between NCoR1 and PGC-1α in the regulation of PPARβ/δ and ERRα transcriptional activity in skeletal muscle, of which the regulation of ERRα represents the predominant regulatory mechanism of oxidative metabolism during basal conditions (Fig. 7). The elucidation of different pharmacological or nonpharmacological (e.g., exercise training) strategies to modulate NCoR1 activity and thus facilitate PGC-1α action represents an attractive strategy for the treatment of metabolic diseases.

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SUPPLEMENTAL MATERIAL

Fig. S1

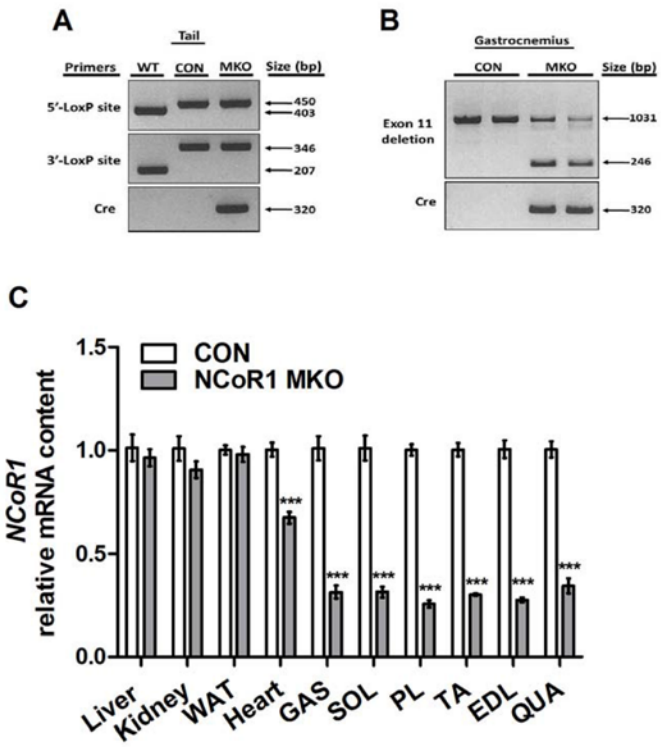

FIG S1 Specific deletion of NCoR1 in striated muscle. (*A* and *B*) PCR genotyping of wild type (WT), CON and NCoR1 MKO mice. (*A*) LoxP sites flanking exon 11 of *Ncor1* gene and the presence of *Cre* were detected from genomic DNA from the tail. (*B*) Recombination was assessed from genomic DNA from gastrocnemius. (*C*) NCoR1 mRNA levels in liver, kidney, white adipose tissue (WAT), heart, gastrocnemius (GAS), soleus (SOL), plantaris (PL), tibialis anterior (TA), extensor digitorum longus (EDL) and quadriceps (QUA) of CON (n = 7) and

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NCoR1 MKO (n = 5) animals. Values represent mean ± SEM. ***$p < 0.001$ CON vs. NCoR1 MKO.

2

Fig. S2

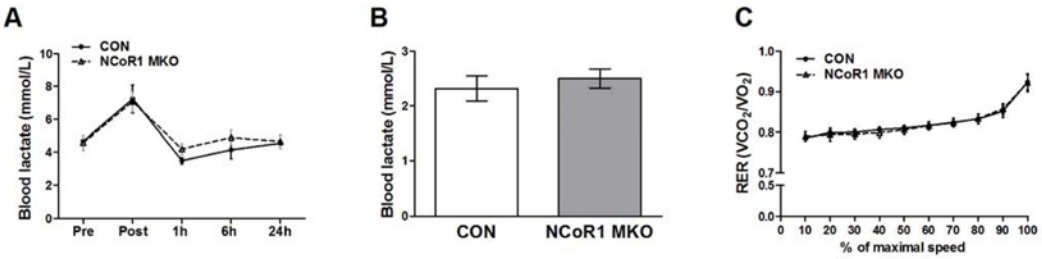

FIG S2 Indirect calorimetry during maximal exercise and blood lactate measurement. (A and B) Blood lactate determination before and after exercise (A), or in fasted (B) animals (n = 6 CON and n = 7 NCoR1 MKO). (C) Respiratory exchange ratio (RER) during the maximal exercise test (n = 8 CON and n = 7 NCoR1 MKO).. Values represent mean ± SEM.

Fig. S3

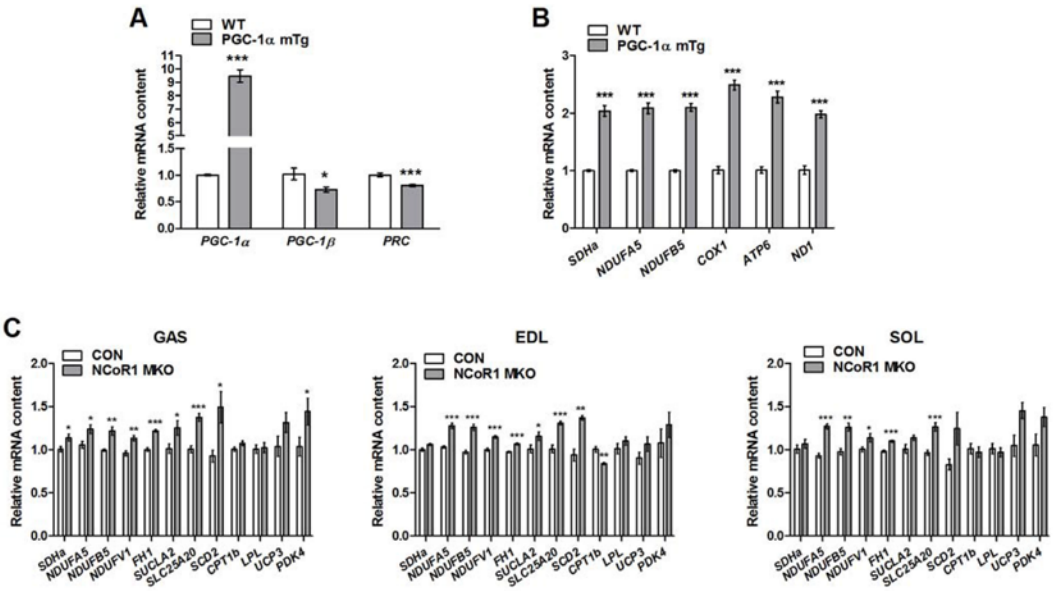

FIG S 3 PGC-1 levels in PGC-1α mTg mice and validation of microarray analysis. (*A*) mRNA level of PGC-1α, PGC-1β and PRC in gastrocnemius from wild type (WT) and PGC-1α mTg animals (n = 5 WT and n = 5 PGC-1α mTg). (*B*) Real-Time PCR analysis of several up-regulated genes from the microarray and mitochondrial encoded genes in WT and PGC-1α mTg gastrocnemius (n = 5 WT and n = 5 PGC-1α mTg). (*C*) Real-Time PCR analysis of several up-regulated genes from the microarray and different PPARβ/δ target genes in gastrocnemius (GAS), extensor digitorum longus (EDL) and soleus (SOL) (n = 7 CON and n = 5 NCoR1 MKO). Values represent mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ CON vs. NCoR1 MKO or WT vs. PGC-1α mTg.

Fig. S4

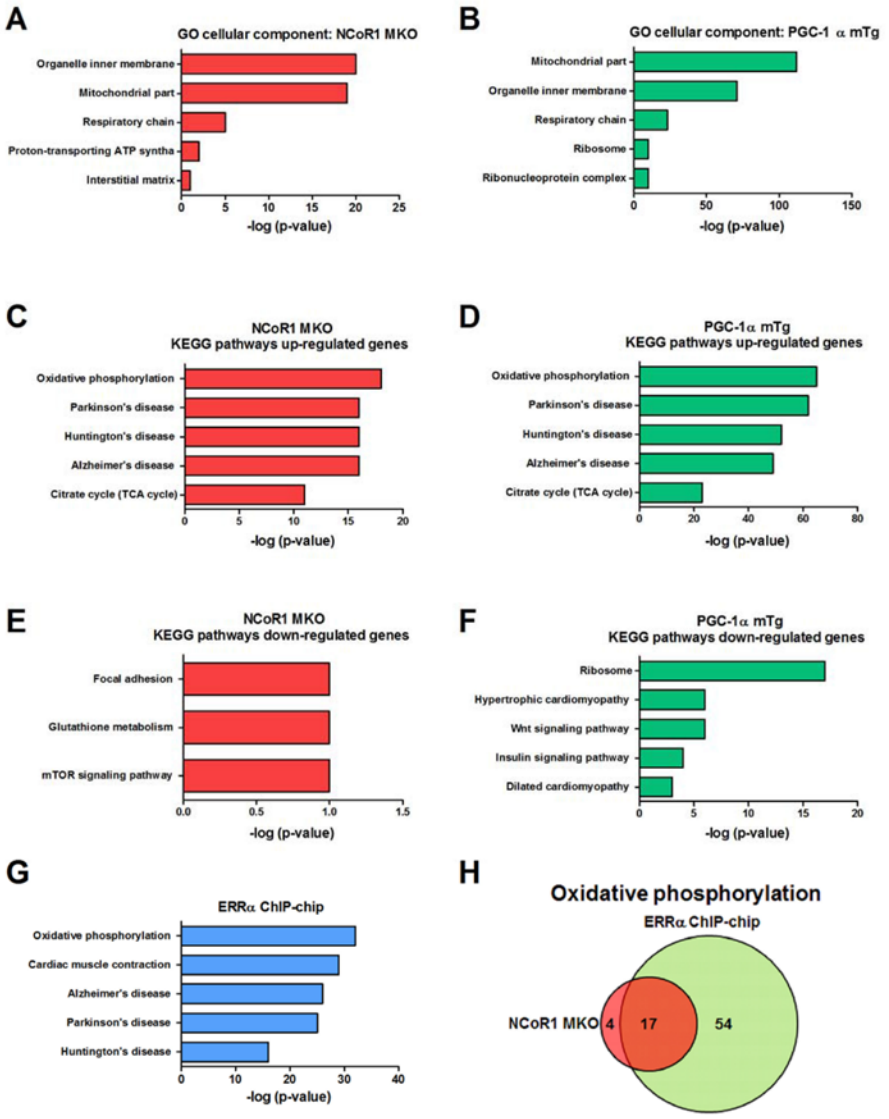

FIG S4 NCoR1 MKO mice exhibit enhanced oxidative metabolism. (*A* and *B*) Top 5 cellular component from GO analysis of the up- and down-regulated genes from the NCoR1 MKO and PGC-1α mTg microarray data sets (n = 5 per group). (*C-F*) Independent GO analyses of

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the up- and down-regulated genes from the NCoR1 MKO and PGC-1α mTg microarray data sets (n = 5 per group). (G) Top 5 KEGG pathways from GO analysis of the ERRα ChIP-chip data set (see reference #12 in the main text). (H) Venn diagrams showing the overlap between NCoR1 MKO microarray and ERRα ChIP-chip data set for the genes present in the GO terms "oxidative phosphorylation".

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Fig. S5

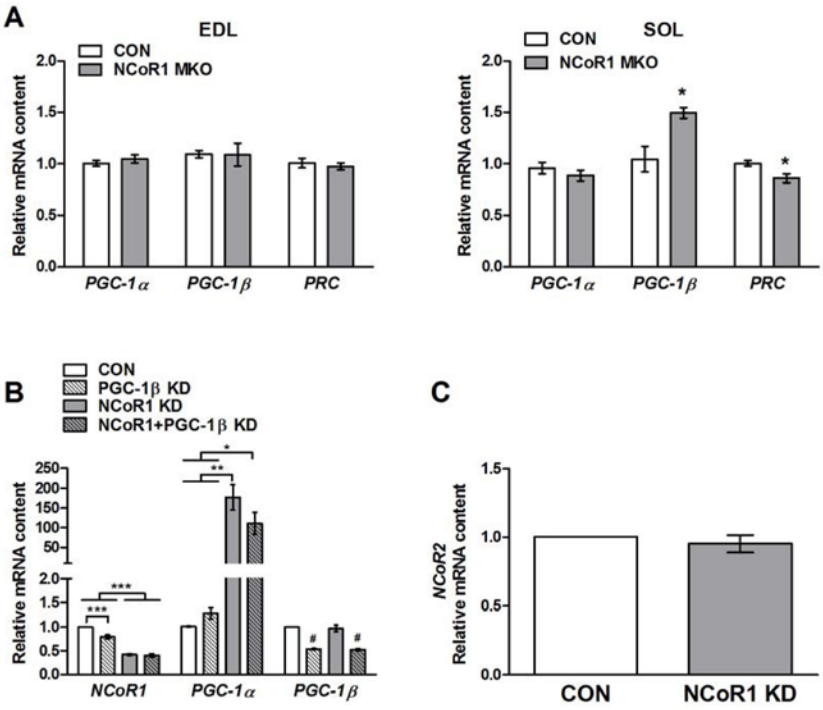

FIG S5 Role of PGC-1α and PGC-1β in NCoR1 modulation of oxidative metabolism. (A) Extensor digitorum longus (EDL) and soleus (SOL) mRNA levels of PGC-1 family of coactivators (n = 7 CON and n = 5 NCoR1 MKO). (B) NCoR1 and LacZ (CON) knockdown (KD) alone or in combination with PGC-1β KD for 48 h in C_2C_{12} myotubes (n = 3 independent experiments performed in triplicate each). (C) NCoR2 mRNA level in C_2C_{12} myotubes transfected with LacZ (CON) or NCoR1 shRNA adenovirus (NCoR1 KD; n = 3 independent experiments performed in triplicate each). Values represent mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 CON vs. NCoR1 MKO or as indicated; $^{\#}p < 0.001$ vs. CON and NCoR1 KD.

Fig. S6

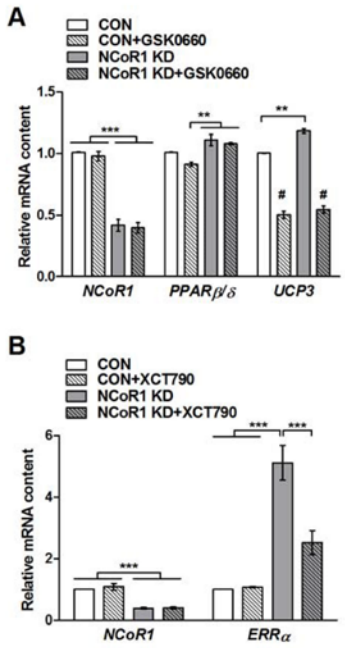

FIG S6 Role of ERRα and PPARδ in NCoR1 modulation of oxidative metabolism. (A and B) NCoR1 knockdown (KD) alone or in combination with 1 μM GSK0660 or 10 μM XCT790 for 48 h in C_2C_{12} myotubes (n = 3 independent experiments performed in triplicate each). Values represent mean ± SEM. $^{**}p < 0.01$, $^{***}p < 0.001$.

Table S1 In vivo and ex vivo assessment of muscle function

In vivo: exercise performance	Maximal test		Endurance test	
	CON	NCoR1 MKO	CON	NCoR1 MKO
Speed (m/min)	17.8 ± 1.2	19 ± 0.4	13.5 ± 7	14.4 ± 0.5
Distance (m)	219 ± 28	248 ± 15	556 ± 160	561 ± 45
Time (sec)	1013 ± 96	1120 ± 48	2778 ± 674	2789 ± 190
Work (W)	449 ± 67	494 ± 26	979 ± 294	972 ± 100
Power (J)	0.42 ± 0.03	0.44 ± 0.01	0.32 ± 0.02	0.35 ± 0.01
Ex vivo: contractile kinetics	**EDL**		**Soleus**	
	CON	NCoR1 MKO	CON	NCoR1 MKO
Twitch				
Time to peak (ms)	12.6 ± 0.9	11.3 ± 0.3	21.4 ± 0.8	23.0 ± 1.2
Half time to peak (ms)	3.5 ± 0.2	3.2 ± 0.0	5.6 ± 0.3	5.9 ± 0.3
Half relaxation time (ms)	18.5 ± 1.9	16.4 ± 0.5	38.5 ± 2.1	32.1 ± 2.3
Tetanus				
Half contraction time (ms)	22.1 ± 1.4	20.8 ± 1.3	35.9 ± 2.4	34.3 ± 2.6
Half relaxation time (ms)	24.7 ± 1.6	21.8 ± 1.4	58.6 ± 2.0	53.3 ± 2.8

In vivo: n = 8 CON and n = 7 NCoR1 MKO mice; Ex vivo: n = 10 CON and n = 14 NCoR1 MKO muscles. Values represent mean ± SEM.

Table S2 MARA prediction of motifs with increased and decreased activity in NCoR1 MKO and PGC-1α mTg skeletal muscle (n = 5 per group)

NCoR1 MKO	
Motif with increased activity	**z-value**
ESR1.p2	2.40
RXR{A,B,G}.p2	2.29
RXRA_VDR{dimer}.p2	2.26
ESRRA.p2	1.85
FOXO1,3,4.p2	1.79
RXRG_dimer.p3	1.68
EVI1.p2	1.62
ELF1,2,4.p2	1.57
NFE2.p2	1.51
Motif with decreased activity	**z-value**
NKX6-1,2.p2	-3.41
ZFP161.p2	-3.10
FOXA2.p3	-2.82
ZBTB6.p2	-2.26
MAFB.p2	-2.10
DBP.p2	-1.74
ZBTB16.p2	-1.57
PGC-1α mTg	
Motif with increased activity	**z-value**
RXR{A,B,G}.p2	10.12
ESRRA.p2	9.14
ESR1.p2	7.52
NR5A1,2.p2	7.35
RXRA_VDR{dimer}.p2	4.61
IKZF1.p2	3.08
FEV.p2	2.83
ZNF148.p2	2.40
HNF4A_NR2F1,2.p2	2.36
SPZ1.p2	2.28
NFE2L1.p2	2.17
CTCF.p2	2.01
POU3F1..4.p2	1.95
SPIB.p2	1.94
NANOG.p2	1.75
HMX1.p2	1.73
ONECUT1,2.p2	1.73
POU6F1.p2	1.67
ZEB1.p2	1.65
ZNF238.p2	1.61
FOXO1,3,4.p2	1.60
bHLH_family.p2	1.59
FOXP3.p2	1.57
NHLH1,2.p2	1.57
Motif with decreased activity	**z-value**
TFDP1.p2	-5.83
MZF1.p2	-4.66
ARNT_ARNT2_BHLHB2_MAX_MYC_USF1.p2	-4.35
EGR1..3.p2	-3.91
EOMES.p2	-3.88
MYBL2.p2	-3.87
POU5F1.p2	-3.64
MAZ.p2	-3.43
PAX2.p2	-3.23

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ZBTB6.p2	-3.22
MYB.p2	-3.19
MEF2{A,B,C,D}.p2	-2.89
YY1.p2	-2.72
GLI1..3.p2	-2.72
EHF.p2	-2.70
RFX1..5_RFXANK_RFXAP.p2	-2.44
TLX2.p2	-2.31
XBP1.p3	-2.31
NFATC1..3.p2	-2.20
ZIC1..3.p2	-2.18
NKX3-2.p2	-2.18
GATA1..3.p2	-2.11
ATF5_CREB3.p2	-2.02
TFAP2B.p2	-2.01
HOX{A4,D4}.p2	-1.95
HOX{A6,A7,B6,B7}.p2	-1.86
STAT5{A,B}.p2	-1.82
BPTF.p2	-1.80
HBP1_HMGB_SSRP1_UBTF.p2	-1.79
HSF1,2.p2	-1.77
GFI1B.p2	-1.76
ELK1,4_GABP{A,B1}.p3	-1.71
FOXN1.p2	-1.69
MAFB.p2	-1.69
CEBPA,B_DDIT3.p2	-1.67
RBPJ.p2	-1.63
FOS_FOS{B,L1}_JUN{B,D}.p2	-1.59
ATF6.p2	-1.58
PITX1..3.p2	-1.55
NFE2L2.p2	-1.52
NRF1.p2	-1.50

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Table S3 Real-Time PCR primer list.

Target gene	Forward primer	Reverse primer
Ncor1	GACCCGAGGGAAGACTACCATT	ATCCTTGTCCGAGGCAATTTG
Ncor2	CCTTCCGTGAGAAGTTTATGCA	CACACTCAGCGACCGTCTTTC
Ppargc1a	TGATGTGAATGACTTGGATACAGACA	GCTCATTGTTGTACTGGTTGGATATG
Ppargc1b	CCATGCTGTTGATGTTCCAC	GACGACTGACAGCACTTGGA
Pparc1	CACCCTGCCGGAGTGAAAT	CGCATTGACTGCTGCTTGTC
Rxra	AACCCCAGCTCACCAAATGACC	AACAGGACAATGGCTCGCAGG
Rxrb	GCCAAGCTGCTGTTACGTCTT	ACAGGTGCTCCAGACACTTGAG
Rxrg	CCGCTGCCAGTACTGTCG	ACCTGGTCCTCCAAGGTGAG
Ppara	GCGTACGGCAATGGCTTTAT	ACAGAACGGCTTCCTCAGGTT
Ppard	GCAAGCCCTTCAGTGACATCA	CCAGCGCATTGAACTTGACA
Pparg	CCCACCAACTTCGGAATCAG	AATGCGAGTGGTCTTCCATCA
Esrra	CGGTGTGGCATCCTGTGA	CTCCCCTGGATGGTCCTCTT
Esrrb	CCCTGACCACTCTCTGTGAATTG	TGGCCCAGTTGATGAGGAA
Esrrg	GCCCAGCCACGAATGAAT	GCAGGCCTGGCAGGATTT
Tfam	GGTCGCATCCCCTCGTCTA	GGATAGCTACCCATGCTGGAAA
Sdha	GCTGGTGTGGATGTCACTAAGG	CCCACCCATGTTGTAATGCA
Ndufa5	ACATGCAGCCTATAGAAAATACACAGA	TCCGCCTTGACCATATCCA
Ndufb5	TTTTCTCACGCGGAGCTTTC	TGCCATGGTCCCCACTGT
Ndufv1	CTTCCCCACTGGCCTCAA	ATACTTGGGCCTGCCATCTG
Fh1	TGCTCTCAGTGCAAAATCCAA	CGTGTGAGTTCGCCCAATT
Sucla2	CGCCACAGTCCAGCAAGTAA	AGCCTGCACCTTTTTATCTGAAGT
Slc25a20	CTGCGCCCATCATTGGA	CAGACCAAACCCAAAGAAGCA
Ucp3	TTTTGCGGACCTCCTCACTT	TGGATCTGCAGACGGACCTT
Pdk4	AAAATTTCCAGGCCAACCAA	CGAAGAGCATGTGGTGAAGGT
Lpl	GGGAGTTTGGCTCCAGAGTTT	TGTGTCTTCAGGGGTCCTTAG
Cpt1b	ATCATGTATCGCCGCAAACT	CCATCTGGTAGGAGCACATGG
Scd2	CGCCGTGGCTTCTTTTTC	CGGGTGTTTGCGCACAA
COX1	TGCTAGCCGCAGGCATTACT	GCGGGATCAAAGAAAGTTGTG
ATP6	ACTATGAGCTGGAGCCGTAATTACA	TGGAAGGAAGTGGGCAAGTG
ND1	TCTGCCAGCCTGACCCATA	GGGCCCGGTTTGTTTCTG
Tbp	TGCTGTTGGTGATTGTTGGT	CTGGCTTGTGTGGGAAAGAT

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CHAPTER 4: PGC-1α regulates skeletal muscle oxidative metabolism independently of PPARβ/δ

PGC-1α Regulates Skeletal Muscle Oxidative Metabolism Independently of PPARβ/δ

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Running head: PGC-1α-PPARβ/δ axis and skeletal muscle metabolism

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Abstract

Physical activity improves oxidative capacity and exerts therapeutic beneficial effects, particularly in the context of metabolic diseases. The peroxisome proliferator-activated receptor γ (PPARγ) coactivator-1α (PGC-1α) and the nuclear receptor PPARβ/δ have both been independently discovered to play a pivotal role in the regulation of oxidative metabolism in skeletal muscle. Surprisingly, despite the obvious overlap in phenotypic changes, the interaction of these two factors in skeletal muscle in vivo remains unclear. In this study, we have found that the disruption of the PGC-1α-PPARβ/δ axis does not affect whole body metabolism under basal conditions. Importantly, PPARβ/δ seems dispensable for most of the effects of PGC-1α on VO_2 and lactate metabolism during maximal exercise as well as the PGC-1α-mediated enhancement of an oxidative phenotype in skeletal muscle. Collectively, these results indicate that PPARβ/δ is not an essential partner of PGC-1α in the control of skeletal muscle remodeling.

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Introduction

The regulation of energy metabolism in skeletal muscle is highly controlled by the peroxisome proliferator-activated receptor γ (PPARγ) coactivator-1α (PGC-1α) [1]. PGC-1α drives the expression of a number of genes involved in catabolic processes leading to aerobic ATP synthesis [1], while concomitantly promoting anabolic processes, including *de novo* lipogenesis [2]. As a transcriptional coactivator, once activated, PGC-1α binds to and hence boosts the activity of different transcription factors to control various gene programs resembling an endurance-trained phenotype in skeletal muscle [1,3]. These metabolic adaptations are associated with an enhanced oxidative capacity, which consequently contributes to an increased skeletal muscle fatigue resistance ex vivo and exercise performance in vivo [4,5,6]. Importantly, exercise is in fact one of the most efficient stimuli to induce PGC-1α in skeletal muscle [3].

Among the transcription factors regulated by PGC-1α, the nuclear receptor PPARβ/δ has been proposed to be a key partner of PGC-1α in the regulation of skeletal muscle metabolism and function, though to a big extent based on cell culture and pharmacological based studies [7]. In this context, PGC-1α acts as a coactivator of PPARβ/δ [8,9,10], while PPARβ/δ can directly regulate PGC-1α expression [11,12], indicating that this nuclear receptor acts both upstream as well as downstream of PGC-1α. Furthermore, transgenic mouse models for PPARβ/δ exhibit a very similar phenotype compared to their counterparts for PGC-1α [4,5,13,14]. Nevertheless, even though the PGC-1α-PPARβ/δ axis appears to play a key role in the regulation of energy metabolism, the epistatic interaction between these two proteins is currently unclear. In this study, we therefore aimed at directly assessing the functional interplay between PGC-1α and PPARβ/δ in the regulation of skeletal muscle oxidative metabolism in vivo.

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Results

PGC-1α Overexpression and PPARβ/δ Deletion in Mouse Skeletal Muscle

To elucidate the functional requirement for PPARβ/δ in the metabolic adaptations induced by PGC-1α, we crossed PPARβ/δ muscle-specific knockout (mKO) mice with PGC-1α muscle-specific transgenic (mTg) mice, referred as mKO/mTg animals. As expected, both mKO and mKO/mTg mice showed a significant reduction of PPARβ/δ mRNA specifically in skeletal muscle, while PGC-1α mRNA was strongly up-regulated by ~12 fold in skeletal muscle of mTg and mKO/mTg mice compared to control (CON) animals (Figure 1A and B). To validate the functional consequence of PPARβ/δ deletion in skeletal muscle, we next assessed the effects of the PPARβ/δ agonist GW0742 on the expression levels of PPARβ/δ target genes [7,15]. Acute treatment with GW0742 did not affect PPARβ/δ mRNA in gastrocnemius and plantaris muscles whereas uncoupling protein 3 (UCP3) mRNA levels were induced in CON, but not in mKO mice (Figure 1C and D). Moreover, as previously reported [15], angiopoietin-like 4 (ANGPTL4) was strongly up-regulated by GW0742 in a way that was partially dependent on PPARβ/δ (Figure 1C and D). Importantly, PPARβ/δ deletion did not affect the transcript levels of PPARα and PPARγ (Figure 1E). We subsequently measured the expression levels of other transcription factors and coactivators that regulate energy metabolism, including the estrogen related receptors (ERRs), mitochondrial transcription factor A (TFAM), PGC-1β and PGC-1-related coactivator (PRC). The expression of these genes was altered in skeletal muscle of mTg and mKO/mTg mice, thus independent of PPARβ/δ gene ablation (Figure 1E).

Effects of Skeletal Muscle Disruption of the PGC-1α-PPARβ/δ Axis on Whole Body Metabolism

Body composition assessment revealed equal body weight, fat mass and lean mass in mKO, mTg, mKO/mTg and CON mice (Figure 2A). Analysis of plasma triglycerides (TG), cholesterol (CHOL), low density lipoproteins (LDL) and high density lipoproteins (HDL) during fed and fasted state exhibited no differences except for a significant decrease in fed CHOL in the mKO/mTg mice (Figure 2B and C). Whole body metabolism was further assessed by indirect calorimetry during 48 h. No differences in oxygen consumption (VO_2) or respiratory exchange ratio (RER) were observed between any of the genotypes (Figure 2D and E, Figure S1A and B).

Pharmacological activation of PPARβ/δ attenuates the detrimental effects of obesity and type 2 diabetes on systemic glucose homeostasis [13,16,17]. In our animals, neither glucose nor insulin tolerance tests were significantly affected by PGC-1α overexpression and/or PPARβ/δ deletion in skeletal muscle in mice fed a regular chow diet (Figure 2F and G). Moreover, we did not find any significant differences in blood glucose levels in fed mice of the four different genotypes (Figure 2H). These findings were corroborated by unchanged mRNA expression of gene involved in glucose transport and catabolism such as the glucose transporter 4 (GLUT4), TBC1 domain family member 1 (TBC1D1), phosphofructokinase (PFK) and hexokinase 2 (HK2) in skeletal muscle of mKO, mTg and mKO/mTg mice (Figure 2I). In contrast, TBC1D4 (also known as Akt substrate of 160 kDa, AS160) was significantly up-regulated exclusively in mTg animals (Figure 2I). Finally, we observed a small increase in total and phosphorylated Akt levels in mTg mice, with no substantial effect of PPARβ/δ deletion (Figure 2J). These data hence suggest that the PGC-1α-PPARβ/δ axis is not essential for the modulation of whole body metabolism and glucose homeostasis under basal conditions in chow fed animals.

Modulation of Skeletal Muscle Metabolism by the PGC-1α-PPARβ/δ Axis

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Skeletal muscle PGC-1α and PPARβ/δ have both been proposed to be key regulators of exercise performance and lactate metabolism [18,19]. Consequently, we next assessed exercise performance in treadmill-based tests, which revealed a higher exercise performance in mTg mice as expected (Figure 3A and B). Interestingly, genetic ablation of PPARβ/δ in muscle did not reduce this difference when mTg/mKO mice were compared to CON animals (Figure 3A and B). Moreover, VO_2 was significantly enhanced in mTg and mKO/mTg mice during maximal exercise (Figure 3C), thus altered by PGC-1α independent of PPARβ/δ. In contrast however, the decrease in the RER in mTg animals was attenuated by concomitant knockout of PPARβ/δ (Figure 3D). Blood lactate concentration significantly increased following maximal exercise in CON animals (Figure 3E). This effect was attenuated in mKO mice and virtually abolished in both mTg and mKO/mTg mice (Figure 3E). Similarly, pre-exercise blood lactate levels were reduced only in the mouse models with elevated skeletal muscle PGC-1α (Figure 3E). Consistently, lactate dehydrogenase A (LDHA) and monocarboxylic acid transporters 4 (MCT4) mRNA levels were reduced only by PGC-1α overexpression in skeletal muscle, while in the same mice, LDHB and MCT1 were up-regulated (Figure 3F) reflecting an attenuated lactate production as well as higher catabolism. These data show that, in response to maximal exercise, skeletal muscle PGC-1α is a pivotal regulator of whole body metabolism, mainly in a PPARβ/δ independent manner.

Next, we determined the functional relevance of PGC-1α and PPARβ/δ interaction in the regulation of skeletal muscle metabolism. We therefore determined the mRNA levels of different genes regulating skeletal muscle oxidative metabolism, several of which have been suggested to be both PGC-1α and PPARβ/δ targets. Interestingly, we observed that PPARβ/δ deletion in skeletal muscle did not change the transcript abundance of key genes involved in the TCA cycle, fatty acid β-oxidation and the mitochondrial electron transport chain (ETC) (Figure 4A and B). In contrast, most of these genes were strongly up-regulated by PGC-1α overexpression in a

PPARβ/δ independent manner (Figure 4A and B). In line with mRNA data, the assessment of protein content of different components of mitochondrial complexes revealed an up-regulation of these proteins both in mTg and mKO/mTg animals, whereas mKO and CON mice were indistinguishable (Figure 4C). We then assessed the metabolic muscle phenotype by determining the proportion of oxidative fiber using NADH staining, which revealed a higher oxidative activity and accordingly increased proportion of oxidative fibers in mTg and mKO/mTg mice independent on presence or absence of a functional PPARβ/δ gene (Figure 4D). Finally, the total protein content and phosphorylation levels of the key metabolic regulator AMP-activated protein kinase (AMPK) were not different in mKO, mTg or mKO/mTg mice, suggesting no altered energy status in any of these models (Figure 4E).

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Discussion

The oxidative phenotype of skeletal muscle is strongly linked to physical activity levels and it has been associated with health beneficial effects in metabolic diseases and other pathologies. Even though the molecular mechanisms that control exercise-induced adaptation in skeletal muscle have not been fully elucidated, the transcriptional coactivator PGC-1α is thought to promote mitochondrial function, myofibrillar gene expression, vascularization and other gene programs that are characteristic of oxidative muscle fibers [1]. Interestingly, the nuclear receptor PPARβ/δ is able to recapitulate several of these effects [7]. However, despite the phenotypic overlap, the functional interaction between PGC-1α and PPARβ/δ has not been elucidated in this tissue so far. We now provide strong evidence indicating almost complete PPARβ/δ independence of PGC-1α overexpression on the metabolic phenotype of skeletal muscle.

Importantly, supporting our hypothesis, contrary to the robust effects observed in PGC-1α muscle-specific transgenic mice, the enhancement of skeletal muscle oxidative metabolism is significantly weaker in a bona fide muscle-specific PPARβ/δ gain-of-function mouse model [14]. Moreover, ligand-based activation of PPARβ/δ only increases exercise performance in trained mice, but not in sedentary animals [10]. Interestingly, oxidative metabolism and exercise performance can be boosted by fusing the PPARβ/δ protein to the heterologous VP16 activation domain that strongly increases its transcriptional activity in the absence of ligand or coactivator recruitment [13]. These data demonstrate that the reported functions of PPARβ/δ upstream and downstream of PGC-1α thereby are dispensable for PGC-1α function in an overexpression context (Figure 5A). These observations are consistent with cell culture-based experiments showing that PGC-1α strongly increased oxidative metabolism in the absence of PPARβ/δ in skeletal muscle cells [20]. It appears hence that PGC-1α regulates skeletal muscle oxidative metabolism by increasing the transcriptional activity of alternative transcription factors, some of which might even compensate for the loss of PPARβ/δ. In fact, PPARα, ERRα and ERRγ were

significantly up-regulated in both mTg and mKO/mTg skeletal muscle, suggesting that these transcription factors might play a more relevant function in the regulation of skeletal muscle metabolism by PGC-1α.

The contribution of PPARβ/δ to the regulation of skeletal muscle metabolism seems to be more relevant in the context of ligand-induced activation. Accordingly, PPARβ/δ activation with synthetic ligands have been shown to be an efficient treatment for metabolic disorders [13,16,17,21], though it remains unclear whether this effect is mediated by skeletal muscle PPARβ/δ. Conversely, overexpression of PGC-1α in skeletal muscle is insufficient to evoke similar therapeutic benefits in young mice and even accelerates the development of insulin resistance when such animals are fed a high-fat diet [22], unless the mice are concomitantly exercised [23]. In old animals however, overexpression of PGC-1α in muscle prevents age-induced insulin resistance [24]. These findings indicate that in some pathological settings, PPARβ/δ activation might be more relevant than PGC-1α, in particular in the absence of physical activity (Figure 5B).

Surprisingly, in our study, mKO animals had a similar phenotype compared to CON mice, with minimal or no changes in body composition, blood parameters and gene expression. In contrast, Shuler et al. have reported higher body weight and fat, in addition to increased serum levels of glucose, insulin and TG in the same mouse model [11]. Intriguingly, similar discrepancies have been reported in global PPARβ/δ KO mouse models in regard to whole body metabolism assessed under basal conditions [17,25,26,27,28]. These differences in the phenotype of PPARβ/δ KO mouse models in chow fed sedentary condition might stem from different environmental factors (e.g. diet and temperature) that could lead to a partial PPARβ/δ activation in CON animals and thus lead to more pronounced phenotypic differences in metabolic parameters when compared to KO mice.

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During exercise, skeletal muscle exerts a bigger impact on whole body metabolism compared to rest. Accordingly, mTg mice exhibit a higher VO_2 and lower RER during treadmill running, reflecting an enhanced oxidative capacity and increased fatty acid oxidation [4]. Interestingly, while the PGC-1α-mediated improvement in VO_2 during exercise is maintained in the absence of a functional PPARβ/δ gene, knockout of PPARβ/δ attenuated the decrease in the RER in mKO/mTg mice. In line with our observations, it has been shown that PPARβ/δ overexpression in skeletal muscle does not affect VO_2 and RER during treadmill running [19]. Moreover, PPARβ/δ has been proposed to specifically regulate fatty acid metabolism and only to a smaller extent other oxidative metabolic genes in cultured muscle cells [20]. Together, these data suggest that the positive effects of skeletal muscle PGC-1α on VO_2 are not dependent of PPARβ/δ, even though this nuclear receptor appears to be at least partially involved in the PGC-1α-mediated increase in fatty acid β-oxidation during exercise. In addition, by using these mouse models, our findings further support previous data suggesting that PGC-1α-controlled lactate metabolism is predominantly regulated by ERRα and not PPARβ/δ [18].

In summary, our results reveal important insights into the regulatory networks that control skeletal muscle plasticity. Here, we show that in normal/physiological conditions, PPARβ/δ is dispensable for the potent effect of PGC-1α on skeletal muscle remodeling. Importantly, the different therapeutic effects of PPARβ/δ and PGC-1α in the context of metabolic diseases during sedentary vs. exercise/aging state, strongly suggest that the relative importance of these molecules in controlling the metabolic phenotype of skeletal muscle varies significantly depending on the physiological and pathological context. Therefore, we hope that these findings will allow a more targeted dissection and modulation of skeletal muscle plasticity in health and disease in the future.

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Materials and methods

Animals

Mice were housed in a conventional facility with a 12 h night and day cycle, with free access to food and water. All experiments were performed on adult male mice with approval of the Swiss authorities. PGC-1α muscle-specific transgenic (mTg) mice have been described previously [5]. PPARβ/δ muscle-specific knockout (mKO) mice were generated by crossing PPARβ/$\delta^{loxP/loxP}$ mice with HSA-Cre transgenic mice [11]. Finally, PGC-1α mTg and PPARβ/$\delta^{loxP/loxP}$ mKO mice were crossed to generate mKO/mTg mice. PPARβ/$\delta^{loxP/loxP}$ animals without *Cre* and PGC-1α transgene expression were used as control (CON) mice. All mice had mixed sv129 and C57BL/6 background. Genotypes were confirmed through standard PCR procedures (data not shown), in addition to qPCR analysis in kidney, gastrocnemius and tibialis anterior (Supplemental Figure 1, A and B).

PPARβ/δ Agonist Administration

CON and mKO mice were subjected to an intra-peritoneal injection of either 0.9% NaCl (control) or 1 mg/kg of body weight of the PPARβ/δ agonist GW0742 (Tocris #2229), as previously described [29]. Gastrocnemius and plantaris were collected 8 h following drug administration and immediately frozen in liquid nitrogen.

Body Composition Analysis

Lean and fat mass were measured via magnetic resonance (EchoMRI^{TM}).

Blood and Plasma Analysis

Blood samples were collected from fed and overnight fasted (16 h) mice. Blood glucose and lactate were measured with a glucose (Roche) and lactate (Nova Biomedical) meter under basal condition, while blood lactate was also measured following maximal exercise (at exhaustion; see maximal exercise test). For plasma analysis, blood was collected in lithium/heparin coated tubes (Microvette®) and plasma concentration of different lipids was measured using a cobas c 111 instrument (Roche).

Glucose and Insulin Tolerance Test

Intra-peritoneal glucose tolerance tests (GTT) were carried out by injecting 2 g/kg of body weight of glucose after 16 h of fasting (overnight). Insulin tolerance tests (ITT) were performed by injecting 0.8 U/kg of body weight of insulin (Novo Nordisk) after 6 h of fasting. In both tests, blood samples were obtained from the tail vein before and 30, 60, 90 and 120 min after glucose or insulin injection. Blood glucose was measured as described above.

Indirect Calorimetry

Animals were individually housed in a Comprehensive Lab Animal Monitoring System (Columbus Instruments) during an acclimatization period of 48 h with free access to food and water. Subsequently, indirect calorimetry was performed during 48 h and data analyzed with the Oxymax software (Columbus Instruments).

Maximal Exercise Test

Animals were acclimatized to treadmill running for two days. On the first day, mice ran in a closed treadmill (Columbus Instruments) for 5 min at 8 m/min, followed by 5 min at 10 m/min (5° slope was used during acclimatization and test). On the second day, animals ran for 5 min at 10 m/min, followed by 5 min at 12 m/min. To

determine maximal exercise performance, VO_{2peak} and RER indirect calorimetry was performed during the test. Thus, two days after the acclimatization, mice were placed in a closed treadmill for 5 min at 0 m/min, the test subsequently started at 8 m/min for 3 min and the speed increased 2 m/min every 3 min until exhaustion.

Histology

NADH staining was performed on 12 µm cross sections from plantaris by exposing them to 0.8 mg/mL NADH (Sigma #N8129) in the presence of 1 mg/mL nitro blue tetrazolium (Sigma #N5514).

RNA Isolation and Quantitative PCR (qPCR)

Total RNA was isolated by using lysing matrix tubes (MP Biomedicals) and TRI Reagent® (Sigma-Aldrich) according to the manufacturer's instructions. RNA concentration was measured with a NanoDrop 1000 spectrophotometer (Thermo Scientific). One microgram of RNA was treated with DNase I (Invitrogen) and then reversed transcribed using hexanucleotide mix (Roche) and SuperScript™ II reverse transcriptase (Invitrogen). Relative mRNA was quantified by qPCR on a StepOnePlus system (Applied Biosystems) using Power SYBR® Green PCR Master Mix (Applied Biosystems). The sequences of the qPCR primers are depicted in Supplemental Table 1. Analysis was performed by the $\Delta\Delta C_T$ method using TATA binding protein (TBP) as endogenous control. TBP transcript levels were not different between genotypes or different experimental conditions.

Protein Isolation and Western Blot

Samples were powdered on dry ice and homogenized in 300 µL of ice-cold sucrose lysis buffer (50 mM Tris-HCl pH 7.5, 250 mM Sucrose, 1 mM EDTA, 1 mM EGTA, 0.25% Nonidet P 40 substitute, 50 mM NaF, 5 mM $Na_4P_2O_7$, 0.1% DTT, fresh protease

and phosphatase inhibitor cocktail). Then, samples were shaken at 1300 rpm for 30 min at 4°C. Samples were subsequently centrifuged at 13000 g for 10 min at 4°C and the protein concentration of the supernatant determined by the Bradford assay (Bio-Rad). Equal aliquots of protein were boiled for 5 min in Laemmli sample buffer (250 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 0.01% bromophenol blue and 5% β-mercaptoetanol).

Samples were separated on SDS-polyacrylamide gels and then transferred to nitrocellulose membranes for 1 h. Membranes were blocked for 1 h in 3% milk in Tris-buffered saline and 0.1% tween-20 (TBST) before overnight incubation at 4°C with appropriate primary antibody in TBST (1:1000 dilution). Proteins were detected with a primary antibody to Akt (Cell Signaling #9272), p-Akt^{T308} (Cell Signaling #4056), AMPKα (Cell Signaling #2603), p-AMPKα^{T172} (Cell Signaling #2535) and total OXPHOS (Abcam #ab110413). As loading control, eEF2 (Cell Signaling: 2332) was used. Following incubation, membranes were washed 3 times with TBST before addition of an appropriate peroxidase-conjugated secondary antibody (Dako) in TBST (1:10000 dilution). Antibody binding was detected using enhanced chemiluminescence HRP substrate detection kit (Pierce: 32106).

Statistical Analysis

Values are expressed as mean ± standard error of the mean (SEM). Statistical significance was determined with unpaired two-tailed t-tests or one-way ANOVA with Tukey's post-hoc test. Significance was considered with a $p < 0.05$.

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Competing Interest: The authors declare no conflict of interest.

Author Contributions

The author(s) have made the following declarations about their contributions: Conceived and designed the experiments: JPS WW CH. Performed the experiments: JPS EVF GS KS. Analyzed the data: JPS EVF KS CH. Contributed reagents/materials/analysis tools: WW. Wrote the paper: JPS CH.

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Figures

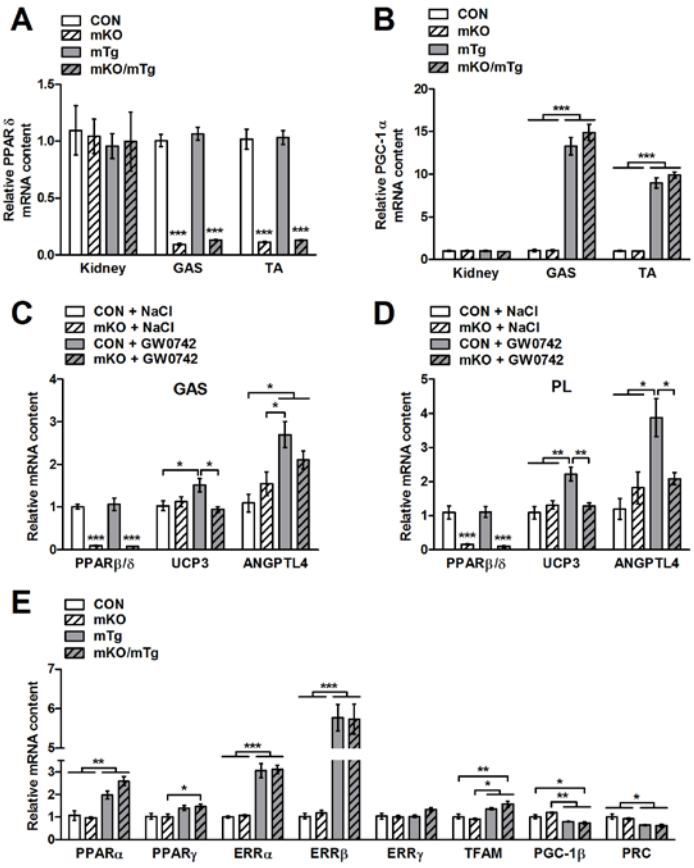

Figure 1. PGC-1α and PPARβ/δ mouse models. (A and B) PGC-1α and PPARβ/δ mRNA levels in kidney, gastrocnemius (GAS) and tibialis anterior (TA) (n = 6 per group). (C and D) PPARβ/δ, UCP3 and ANGPTL4 mRNA levels in GAS and plantaris (PL) 8 h after the injection of 0.9% NaCl (as control) or 1 mg/kg of body weight of the PPARβ/δ agonist GW0742 (n = 6 per group). (E) mRNA level of different transcription factors and coregulators modulating energy metabolism in GAS (n = 6 per group). Values are mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001.

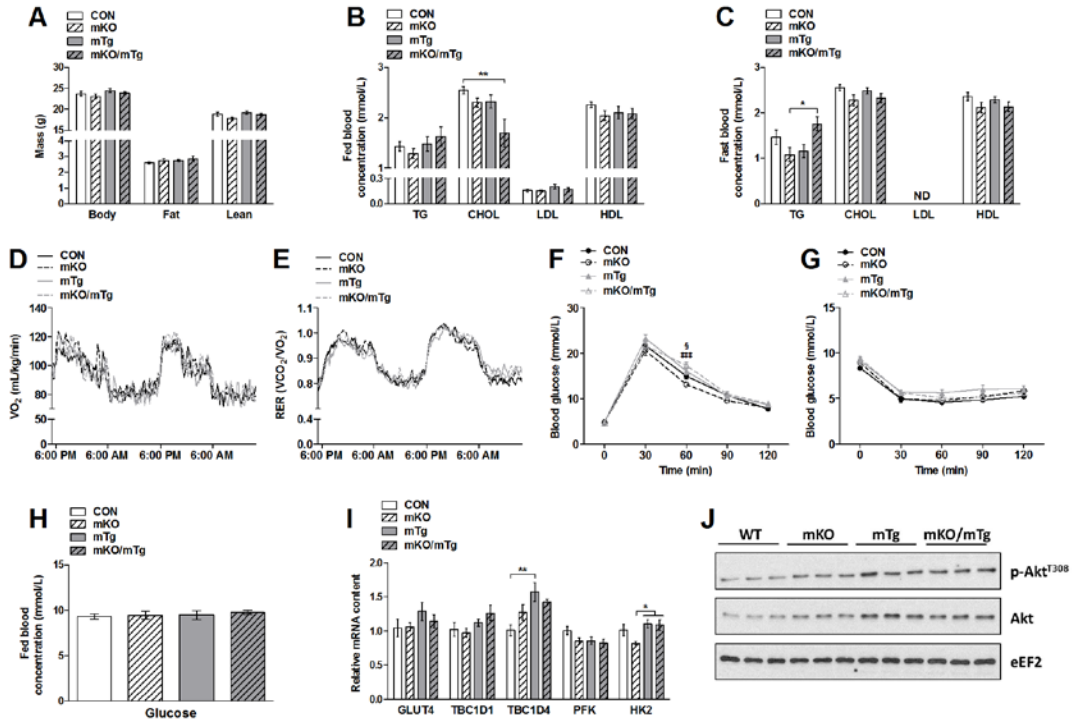

Figure 2. Body composition, systemic parameters and glucose handling in PGC-1α and PPARβ/δ mouse models. (A) Assessment of body composition (n = 10-12 per group). (B and C) Plasma concentration of triacylglycerol (TAG), cholesterol (CHOL), low density lipoprotein (LDL) and high density lipoprotein (HDL) under fed and fasted conditions (n = 10-12 per group). (D and E) Measurement of oxygen consumption (VO_2) (D) and respiratory exchange ratio (RER) (E) over a period of 48 h (n = 10-14 per group). (F-H) Blood glucose levels during glucose (F) and insulin tolerance (G) tests. (H) Blood glucose levels in fed animals (n = 10-12 per group). (I) Gastrocnemius mRNA levels of genes involved in glucose metabolism (n = 6 per group). (J) Western blot assessment of Akt phosphorylation status in gastrocnemius (n = 6 per group). Values are mean ± SEM. *p < 0.05, **p < 0.01. In F §p < 0.05 mKO vs. mTg; ‡‡‡p < 0.001 mKO vs. mKO/mTg.

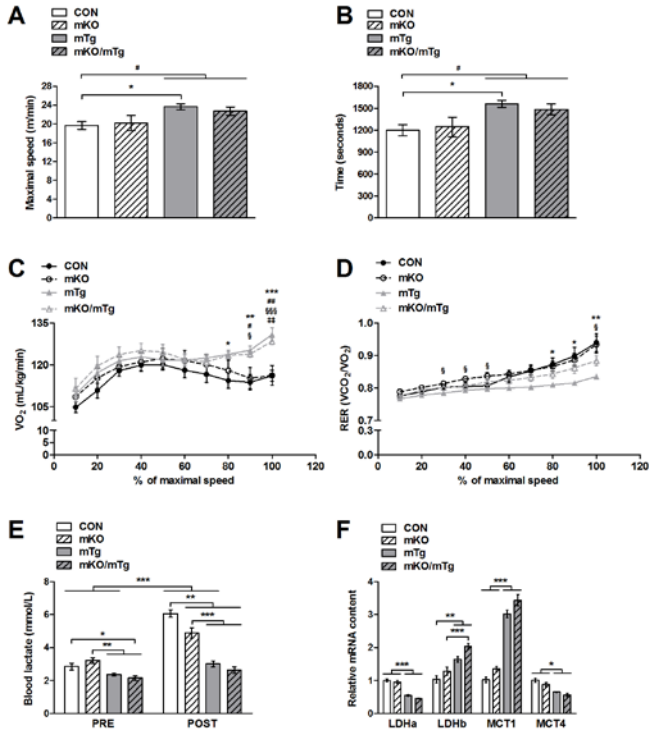

Figure 3. Skeletal muscle PGC-1α modulates whole body metabolism during maximal exercise. (A and B) Maximal speed and time achieved during exercise tests to exhaustion (n = 10-12 per group). (C and D) Measurement of oxygen consumption (VO_2) and respiratory exchange ratio (RER) during the maximal exercise test (n = 10-12 per group). (E) Blood lactate levels before (PRE) and after (POST) maximal exercise (n = 10-12 per group). (F) mRNA levels of key genes of lactate metabolism in gastrocnemius (n = 6 per group). Values are mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001. In C and D *p < 0.05, **p < 0.01, ***p < 0.001 CON vs. mTg; #p < 0.05, ##p < 0.01 CON vs. mKO/mTg; §p < 0.05, §§§p < 0.001 mKO vs. mTg; ‡‡p < 0.01 mKO vs. mKO/mTg.

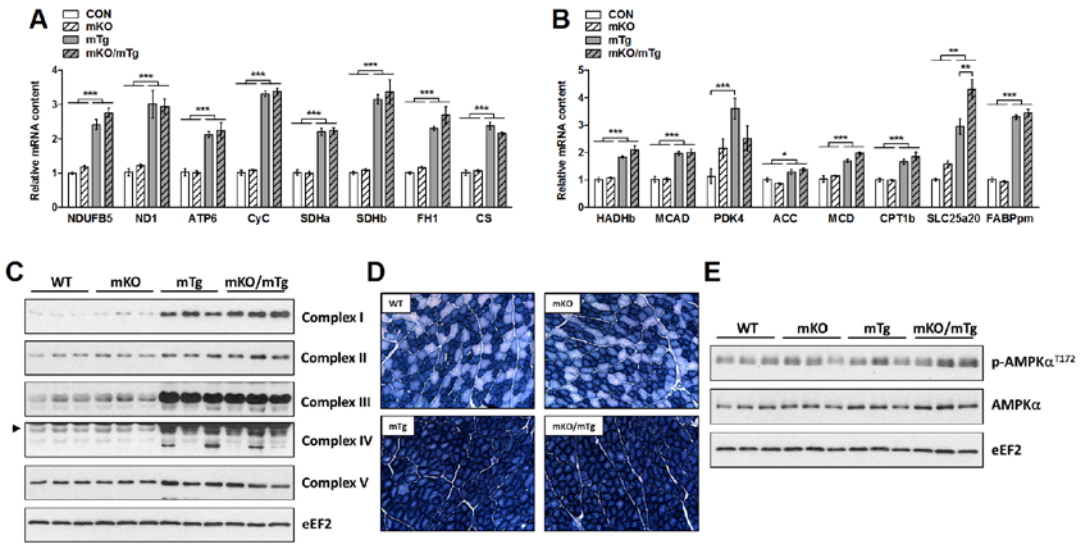

Figure 4. Oxidative metabolism of gastrocnemius is enhanced by PGC-1α even in the absence of PPARβ/δ. (A and B) mRNA levels of genes regulating oxidative and fatty acid metabolism (n = 6 per group). (C) Western blot analysis of key proteins regulating the ETC (n = 6 per group). (D) Assessment of oxidative muscle fibers (dark blue) via NADH staining (n = 3 per group). (E) Western blot analysis of AMPK phosphorylation status (n = 6 per group). Values are mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001.

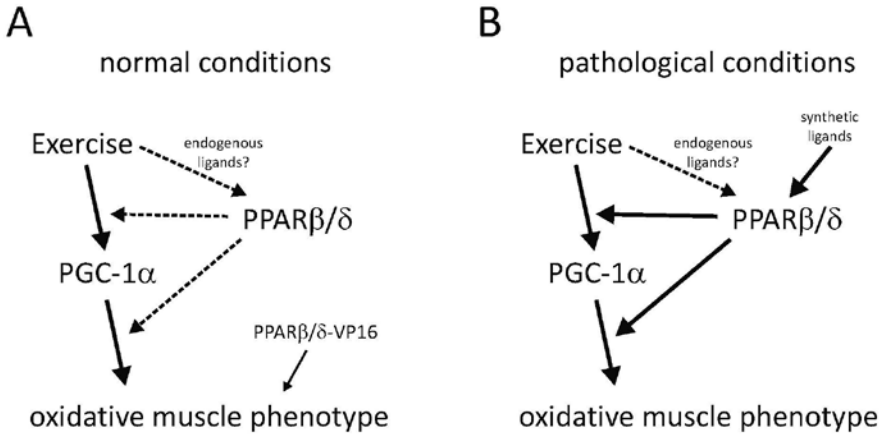

Figure 5. Regulation of the oxidative phenotype of skeletal muscle cells in health and disease. (A) In normal, physiological conditions, PPARβ/δ function is dispensable for PGC-1α-mediated remodeling of muscle metabolism. (B) In metabolic diseases, activation of PPARβ/δ with synthetic agonists relieves many disease symptoms whereas overexpression of muscle PGC-1α is insufficient to ameliorate insulin sensitivity in the absence of concomitant bona fide exercise.

CHAPTER 4: PGC-1α regulates skeletal muscle oxidative metabolism independently of PPARβ/δ

Supplemental information

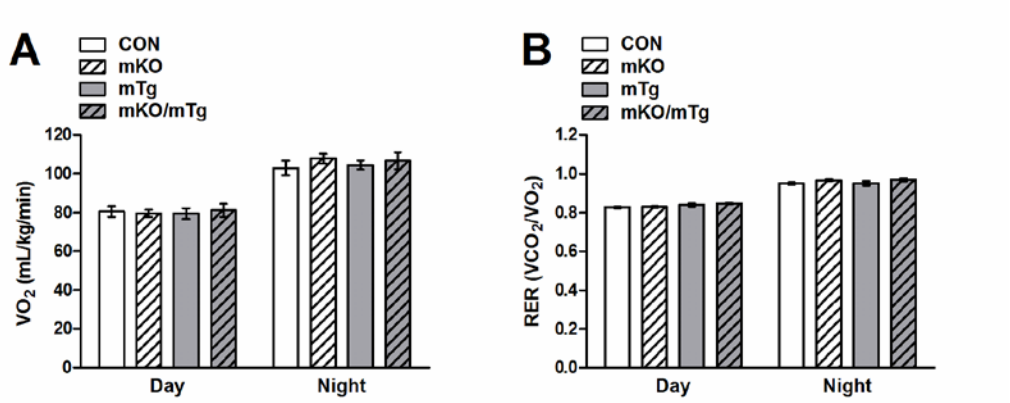

Figure S1. Influence of the PGC-1α-PPARβ/δ axis on whole body metabolism. (A and B) Analysis of oxygen consumption (VO_2) (A) and respiratory exchange ratio (RER) (B) over a period of 48 h (n = 10-14 per group). Values are mean ± SEM.

CHAPTER 4: PGC-1α regulates skeletal muscle oxidative metabolism independently of PPARβ/δ

Table S1 qPCR Primer sequences

Target gene	Forward primer	Reverse primer
NDUFB5	TTTTCTCACGCGGAGCTTTC	TGCCATGGTCCCCACTGT
SDHa	GCTGGTGTGGATGTCACTAAGG	CCCACCCATGTTGTAATGCA
CS	CCCAGGATACGGTCATGCA	GCAAACTCTCGCTGACAGGAA
FH1	TGCTCTCAGTGCAAAATCCAA	CGTGTGAGTTCGCCCAATT
MCAD	AACACTTACTATGCCTCGATTGCA	CCATAGCCTCCGAAAATCTGAA
HADHb	TGCTGTCAGGCACTTCGTATAAA	AAACCCGAAAGTGCAGCTCTAG
PFK	TGTGGTCCGAGTTGGTATCTT	GCACTTCCAATCACTGTGCC
HK2	CCCTGCCACCAGACGAAA	GACTTGAACCCCTTAGTCCATGA
LDHa	CATTGTCAAGTACAGTCCACACT	TTCCAATTACTCGGTTTTTGGGA
LDHb	CATTGCGTCCGTTGCAGATG	GGAGGAACAAGCTCCCGTG
CPT1b	ATCATGTATCGCCGCAAACT	CCATCTGGTAGGAGCACATGG
ACC	GGGCTCGGGCATGATTG	CAGGTAAGCCCCGATTCCA
MCD	ACTCCATCAGCCTGACCCAG	ACCCCTTGAGGCTCTCGTGA
TBC1D1	CATAAAGAACACACTCCCCAACCT	TGCTTGGCGATGTCCATCT
TBC1D4	GTACCGACCGGATATGATGTCA	CGGTGGTAGTCATGAAGGAGTCT
GLUT4	CATGGCTGTCGCTGGTTTC	AAACCCATGCCGACAATGA
MCT1	GTGACCATTGTGGAATGCTG	CTCCGCTTTCTGTTCTTTGG
MCT4	TCACGGGTTTCTCCTACGC	GCCAAAGCGGTTCACACAC
PGC-1α	AGCCGTGACCACTGACAACGAG	GCTGCATGGTTCTGAGTGCTAAG
PGC-1β	CCATGCTGTTGATGTTCCAC	GACGACTGACAGCACTTGGA
PRC	CACCCTGCCGGAGTGAAAT	CGCATTGACTGCTGCTTGTC
ND1	TCTGCCAGCCTGACCCATA	GGGCCCGGTTTGTTTCTG
ATP6	ACTATGAGCTGGAGCCGTAATTACA	TGGAAGGAAGTGGGCAAGTG
CyC	AAATCTCCACGGTCTGTTCG	TATCCTCTCCCCAGGTGATG
SDHb	TGACGTCAGGAGCCAAAATGG	CCTCGACAGGCCTGAAACTG
PDK4	AAAATTTCCAGGCCAACCAA	CGAAGAGCATGTGGTGAAGGT
UCP3	TTTTGCGGACCTCCTCACTT	TGGATCTGCAGACGGACCTT
ANGPTL4	GCGTAAAAAGGGTATCTTCTGGAA	GGTGGTAGCCTGCAGAGGATAG
SLC25a20	CTGCGCCCATCATTGGA	CAGACCAAACCCAAAGAAGCA
FABPpm	AGCGGCTGACCAAGGAGTT	GACCCCTGCCACGGAGAT
PPARβ/δ	GCAGACCTCTCCCAGAATTCC	ACACCCGACATTCCATGTTGA
PPARα	GCGTACGGCAATGGCTTTAT	ACAGAACGGCTTCCTCAGGTT
PPARγ	CCCACCAACTTCGGAATCAG	AATGCGAGTGGTCTTCCATCA
ERRα	CGGTGTGGCATCCTGTGA	CTCCCCTGGATGGTCCTCTT
ERRβ	CCCTGACCACTCTCTGTGAATTG	TGGCCCAGTTGATGAGGAA
ERRγ	GCCCAGCCACGAATGAAT	GCAGGCCTGGCAGGATTT
TFAM	GGTCGCATCCCCTCGTCTA	GGATAGCTACCCATGCTGGAAA
TBP	TGCTGTTGGTGATTGTTGGT	CTGGCTTGTGTGGGAAAGAT

The transcriptional coactivator PGC-1α is dispensable for chronic overload-induced skeletal muscle hypertrophy and metabolic remodeling

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Skeletal muscle mass loss and dysfunction have been linked to many diseases. Conversely, resistance exercise, mainly by activating mammalian target of rapamycin complex 1 (mTORC1), promotes skeletal muscle hypertrophy and exerts several therapeutic effects. Moreover, mTORC1, along with peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α), regulates skeletal muscle metabolism. However, it is unclear whether PGC-1α is required for skeletal muscle adaptations after overload. Here we show that although chronic overload of skeletal muscle via synergist ablation (SA) strongly induces hypertrophy and a switch toward a slow-contractile phenotype, these effects were independent of PGC-1α. In fact, SA down-regulated PGC-1α expression and led to a repression of energy metabolism. Interestingly, however, PGC-1α deletion preserved peak force after SA. Taken together, our data suggest that PGC-1α is not involved in skeletal muscle remodeling induced by SA.

muscle overload | transcriptional regulation | resistance training

Skeletal muscle size exhibits drastic changes throughout life, mainly dependent on mechanical load and nutritional supply (1). For example, loss of muscle mass is observed during immobilization and starvation, but also under pathological conditions like heart failure and cancer (2). To date, resistance exercise is considered as one of the most efficient ways to induce skeletal muscle hypertrophy and to revert the adverse effects of muscle wasting (2, 3). However, because pharmacological interventions are scarce, identification of the molecular regulation of muscle remodeling via resistance exercise is of great therapeutic interest.

Activation of mammalian target of rapamycin complex 1 (mTORC1) is the main regulatory step by which resistance exercise enhances protein synthesis and skeletal muscle size (4). In fact, inhibition of mTORC1 drastically abrogates plantaris hypertrophy via chronic overload (5), even though muscle size is not affected uniformly by muscle knockout of the mTORC1 inhibitor tuberous sclerosis complex 1 (6). Interestingly, the activity of the mTORC1 protein kinase complex positively correlates with oxidative capacity (7, 8). Moreover, mTORC1 is recruited to the promoter of a wide range of metabolism-related genes to regulate their expression (9–11). In skeletal muscle, mTORC1 regulates oxidative metabolism by facilitating the activation of Yin Yang 1 by the peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α) (10, 12). The physiological context in which the mTORC1–PGC-1α axis regulates energy metabolism, however, is unknown.

Protein degradation is an important limiting factor of skeletal muscle hypertrophy. This process is fine-tuned by the transcription factor forkhead box O3 (FOXO3), which regulates the expression of the E3 ubiquitin ligases muscle ring finger protein 1 (MuRF1) and F-box protein 32 (Fbxo32/atrogin-1) (13). Interestingly, PGC-1α represses FOXO3 activity and consequently ameliorates skeletal muscle mass loss during denervation and aging (14–16). Finally, a recently discovered transcript variant of PGC-1α called PGC-1α4 also protects against skeletal muscle wasting and even promotes skeletal muscle hypertrophy (17). Inversely however, the functional requirement for PGC-1α in muscle hypertrophy and its associated adaptations has not been studied so far. Here, we therefore examined the role of PGC-1α in the molecular and functional adaptations to chronic overload of skeletal muscle.

Results

PGC-1α Is Not Required for Chronic Overload-Induced Plantaris Hypertrophy. To induce skeletal muscle hypertrophy via mechanical overload, we have taken advantage of the well-established method of synergist ablation (SA) (18, 19). The role of PGC-1α in skeletal muscle was studied using WT, PGC-1α muscle-specific transgenic (mTg), and PGC-1α muscle-specific knockout (mKO) mice (Fig. S1*A* and *B*), thereby avoiding confounding factors of whole-body KO and Tg models (20). Under basal conditions, neither body weight nor wet weight of gastrocnemius, plantaris, tibialis anterior, and quadriceps showed significant differences between genotypes (Table S1). However, soleus was slightly heavier in mTg mice, whereas extensor digitorum longus was slightly lighter in both mTg and mKO animals (Table S1).

After SA, we observed a ~87% increase in the relative mass of WT and mTg overloaded plantaris (OVL) (Fig. 1*A*). Interestingly, this response was slightly attenuated in mKO mice (Fig.

Significance

Skeletal muscle hypertrophy is mainly induced by growth hormones and mechanical overload and exerts health beneficial effects. The mammalian target of rapamycin complex 1 (mTORC1) and the peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α) are key regulators of skeletal muscle mass and energy metabolism, respectively. Thus, acting in concert, mTORC1 and PGC-1α interplay is thought to regulate skeletal muscle function. Our results indicate that PGC-1α is not required for skeletal muscle hypertrophy and a slow-contractile phenotype induced by chronic overload of the plantaris muscle. In fact, PGC-1α gene expression and global energy metabolism were repressed in this experimental context of muscle hypertrophy. Hence, these results exclude PGC-1α as the main regulator of skeletal muscle remodeling after chronic overload.

Author contributions: J.P.-S. and C.H. designed research; J.P.-S., S.S., G.S., and F.Z. performed research; G.S. and F.Z. contributed new reagents/analytic tools; J.P.-S., S.S., F.Z., and C.H. analyzed data; and J.P.-S. and C.H. wrote the paper.

The authors declare no conflict of interest.

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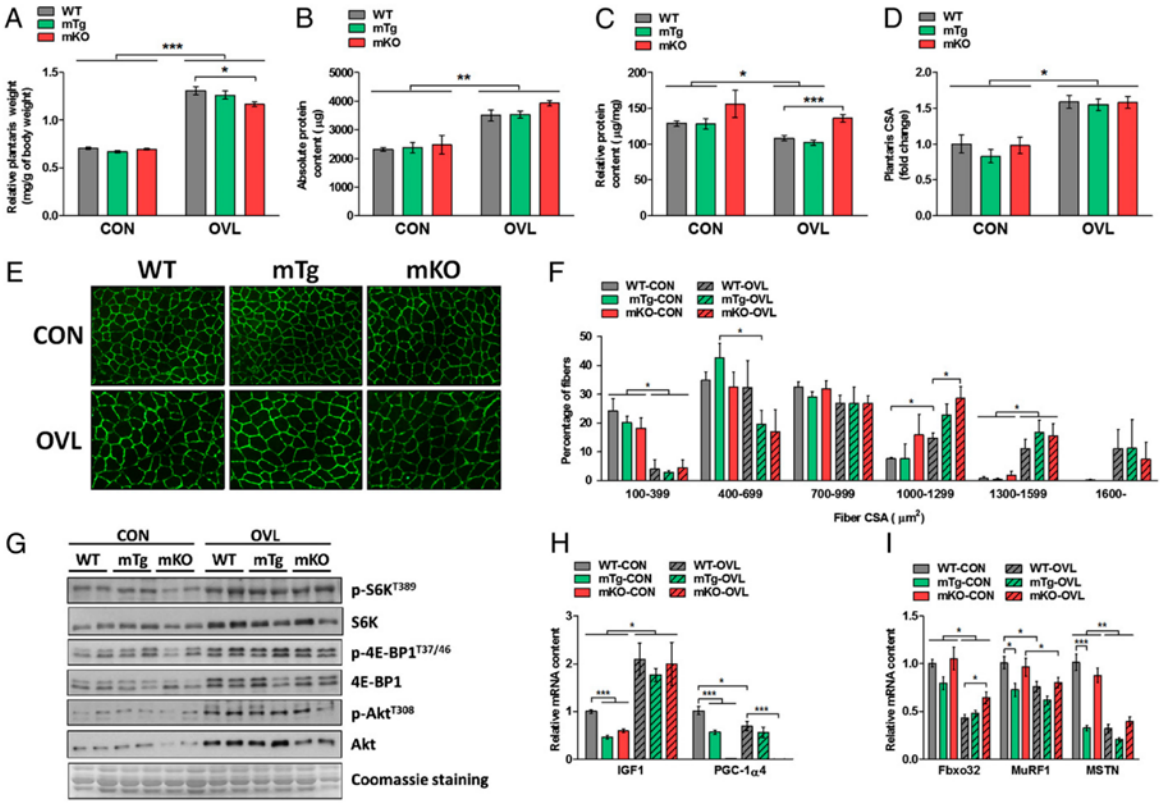

Fig. 1. Effects of SA on plantaris hypertrophy. (*A*) Relative plantaris weight (n = 15–18 per group). (*B* and *C*) Absolute and relative plantaris protein levels (n = 6 per group). (*D*) Whole plantaris CSA (n = 3 per group). (*E* and *F*) Representative pictures of laminin staining and quantification of fiber CSA (n = 3 per group). (*G*) Western blot analysis of mTORC1 and PI3K target proteins (n = 6 per group). (*H* and *I*) Quantitative PCR (qPCR) analysis of prohypertrophic and proatrophic genes (n = 6 per group). Values are mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001.

1*A*). Moreover, absolute protein and RNA content were elevated by ~53% and ~63%, respectively, in OVL of all genotypes (Fig. 1*B* and Fig. S1*C*). Whereas relative RNA content remained higher after SA (Fig. S1*D*), relative protein content was decreased in WT and mTg OVL (Fig. 1*C*). All genotypes showed an increase in whole plantaris cross-sectional area (CSA) in response to SA (Fig. 1*D*). The analysis of single muscle fiber CSA also revealed an overall shift toward a higher proportion of large fibers in WT, mTg, and mKO OVL (Fig. 1 *E* and *F*).

Next, plantaris hypertrophy was further studied at the molecular level. Interestingly, relative phosphorylation levels of the mTORC1 target protein ribosomal protein S6 kinase (S6K) were increased and decreased in mTg and mKO control plantaris (CON), respectively (Fig. 1*G* and Fig. S1*E*). After SA, relative p-S6K^{T389} levels remained elevated in mTg OVL, whereas they significantly increased in WT and mKO mice compared with CON (Fig. 1*G* and Fig. S1*E*). Surprisingly, this positive effect on S6K phosphorylation was particularly pronounced in mKO mice (Fig. 1*G* and Fig. S1*E*). In contrast, relative phosphorylation levels of the mTORC1 and PI3K target proteins eukaryotic translation initiation factor 4E binding protein 1 (4E-BP1) and Akt, respectively, were not increased by SA (Fig. 1*G* and Fig. S1*E*). The total protein content of these proteins was elevated by SA in all genotypes (Fig. 1*G* and Fig. S1*F*).

Next, transcriptional analysis revealed lower levels of insulin-like growth factor 1 (IGF1) mRNA in mTg and mKO CON, whereas IGF1 expression was increased by SA in all genotypes (Fig. 1*H*). Importantly, PGC-1α4 mRNA levels were significantly decreased in both mTg and mKO CON (Fig. 1*H*). Unexpectedly, we found that SA decreased PGC-1α4 mRNA levels by 32% in WT mice (Fig. 1*H*). The analysis of genes involved in muscle atrophy showed lower mRNA content of MuRF1 and myostatin (MSTN) in mTg CON (Fig. 1*I*). SA down-regulated Fbxo32 and MSTN in all genotypes, but the effect on Fbxo32 was slightly reduced in mKO mice (Fig. 1*I*). MuRF1 mRNA was also lower in WT and mKO OVL, whereas expression remained low in mTg OVL (Fig. 1*I*). Thus, these data suggest that PGC-1α does not modulate the overall effects of SA on plantaris hypertrophy.

Metabolic Remodeling Induced by Chronic Overload. Considering the proposed metabolic role of the mTORC1–PGC-1α axis, we also explored the metabolic adaptations to SA. Most of the genes involved in the electron transport chain (ETC), tricarboxylic acid cycle, β-oxidation, and fatty acid transport showed higher mRNA levels in mTg CON, whereas the opposite effects were observed in mKO CON (Fig. 2*A* and Fig. S2*A*). Genes involved in glucose and lactate metabolism revealed a different pattern, mainly reflecting an enhanced lactate catabolism in mTg CON (Fig. 2*A* and Fig. S2*A*). Surprisingly, SA down-regulated most of these genes, regardless of the metabolic function (Fig. 2*A* and Fig. S2*A*). Protein content of different components of the ETC also showed a similar expression pattern, both under basal conditions and after SA (Fig. 2*B* and Fig. S2*B*). To further explore the

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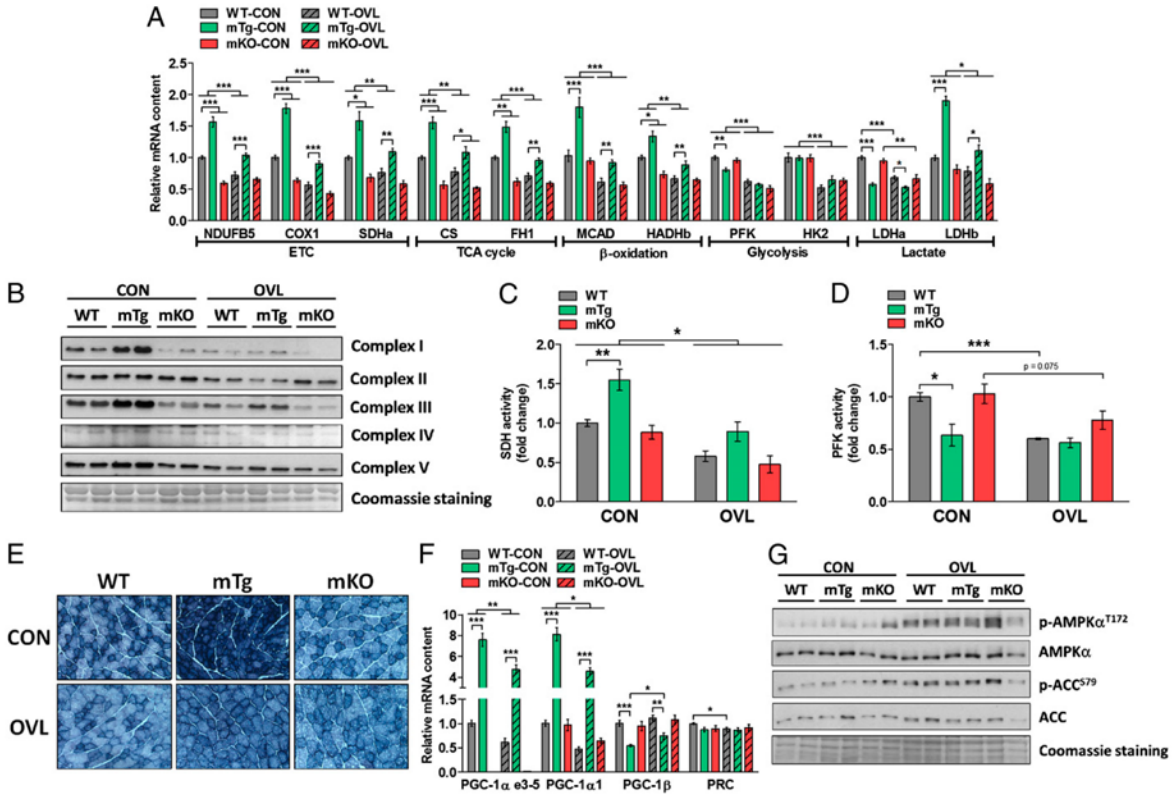

Fig. 2. Plantaris metabolism is repressed by SA. (*A*) qPCR analysis of metabolic-related genes ($n = 6$ per group). (*B*) Western blot analysis of different components of the ETC ($n = 6$ per group). (*C* and *D*) SDH and PFK activity assessment ($n = 6$ per group). (*E*) Representative pictures of NADH staining ($n = 3$ per group). (*F*) qPCR analysis of PGC-1 family of coactivators ($n = 6$ per group). (*G*) Western blot analysis of AMPK activation ($n = 6$ per group). Values are mean ± SEM. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

functional effect of SA on oxidative and glycolytic metabolism, we assessed the enzymatic activities of succinate dehydrogenase (SDH) and phosphofructokinase (PFK), respectively. As expected, SDH and PFK activities were higher and lower in mTg CON, respectively (Fig. 2*C* and *D*). After SA, SDH activity was significantly decreased in all genotypes (Fig. 2*C*), whereas PFK activity decreased in WT OVL and showed a trend ($P = 0.075$) toward a reduction in mKO OVL (Fig. 2*D*). Consistently, NADH staining implied an increase and slight decrease in the proportion of oxidative fibers in mTg and mKO CON, respectively, which was reduced in WT and mTg upon SA (Fig. 2*E*). Additionally, on the basis of NADH staining, either positive fibers (oxidative) and negative fibers (glycolytic) separately or all muscle fibers collectively showed a similar increase in CSA after SA (Fig. S2*C*). We also found that SA significantly decreased PGC-1α1 mRNA content by 53% and 44% in WT and mTg OVL, respectively (Fig. 2*F*), similar to the reduction in PGC-1α4 content (Fig. 1*H*). Importantly, it should be noted that the genetic ablation of PGC-1α in mKO mice was assessed with primers targeting the floxed region of the gene (exon 3–5; PGC-1α e3-5) that is shared in all different PGC-1α isoforms (17). In contrast, because specific detection of PGC-1α1, -2, -3, and -4 relies on amplification of transcript regions outside this deleted area, mRNA is detected in mKO mice with primers that are specific for PGC-1α1 (Fig. 2*F*). Nevertheless, however, because of the knockout strategy aimed at introducing a frame shift in the gene, no protein will be made from these nonfunctional transcripts in PGC-1α knockout models (21, 22). In contrast to PGC-1α, no major changes in PGC-1β- and PGC-1-related coactivator were induced by SA (Fig. 2*F*). The AMP-activated protein kinase (AMPK) is another key regulator of cellular energy metabolism in various tissues (23). Interestingly, relative phosphorylation levels of AMPK and its target protein acetyl-CoA carboxylase (ACC) were highly increased by SA, whereas absolute levels of ACC were only increased in WT OVL (Fig. 2*G* and Fig. S2 *D* and *E*). Our results imply a negative effect of SA on energy metabolism, which seems to be related to the down-regulation of PGC-1α and was consequently attenuated by PGC-1α overexpression.

Functional Adaptations to Chronic Overload. Finally, we investigated the effects of SA on plantaris contractility and fatigue resistance. Peak force in response to a single twitch and tetanic stimulation was lower in mTg CON (Fig. 3 *A*–*C*). In WT, SA significantly decreased force generation independently of the stimulation pattern, whereas in mTg mice only twitch force was further decreased (Fig. 3 *A*–*C*). Surprisingly, force generation did not change in mKO mice after SA (Fig. 3 *A*–*C*). SA also delayed the contractile kinetics observed in response to tetanic stimulation (Table S2). Furthermore, fatigue resistance was higher in mTg CON, and SA significantly improved this parameter in all genotypes (Fig. 3 *D* and *E*). Thus, the area under the curve of the fatigue resistance protocol was increased by SA in all genotypes (Fig. 3*F*).

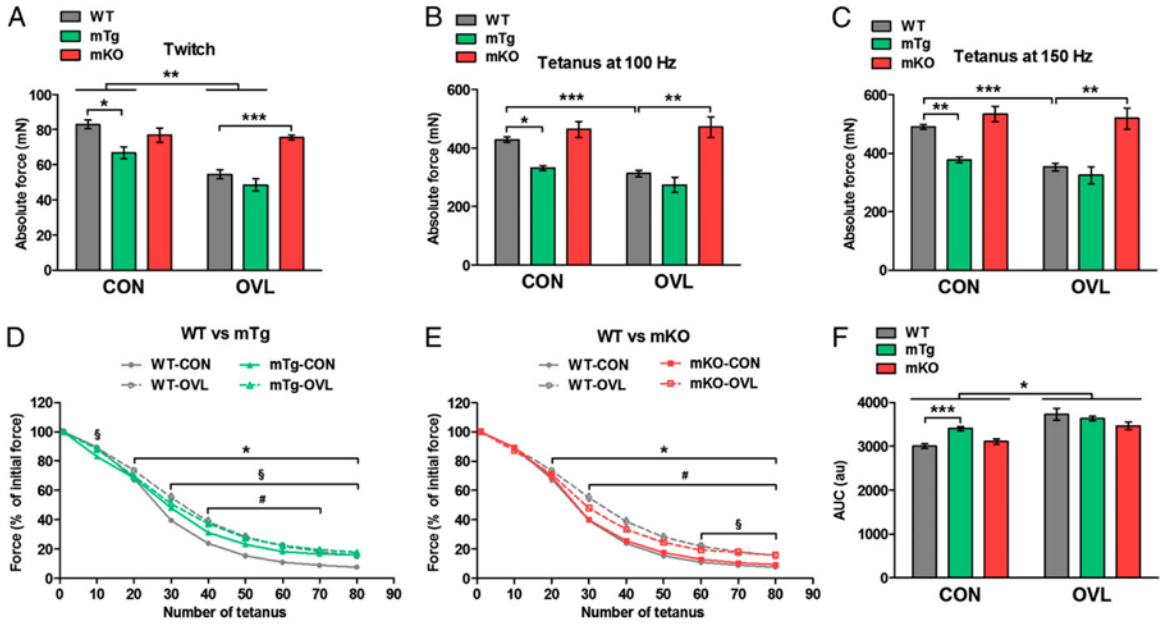

Fig. 3. Effects of SA on plantaris contractility. (*A–C*) Peak force in response to a single twitch or tetanic stimulation ($n = 5–6$ per group). (*D–F*) Assessment of fatigue resistance and the corresponding area under the curve ($n = 5–6$ per group). Values are mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. In *D* and *E*: $^{*}P < 0.05$ CON vs. OVL WT, $^{\#}P < 0.05$ CON vs. OVL mTg or mKO, $^{\S}P < 0.05$ CON WT vs. mTg or mKO.

The contractile properties of muscle cells are in part determined by the specific expression of genes involved in calcium handling and excitation–contraction coupling (24, 25). Thus, mTg CON showed a shift toward a slow phenotype, mainly reflected by a lower mRNA level of calsequestrin 1 (CSQ1) and myosin heavy chain 2 B (MyHC-2B), in contrast to higher levels of the respective slow isoform of these genes, CSQ2 and MyHC-1 (Fig. 4 *A* and *B*). On the other hand, mKO CON showed a higher mRNA level of CSQ1, sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase 1 (SERCA1), and actin α1 (ACTC1) (Fig. 4 *A* and *C*). In line with the functional analysis, SA induced CSQ2, SERCA2, MyHC-2A, ACTC1, and troponin C1 (TNNC1) gene expression in WT OVL (Fig. 4 *A–C*). Moreover, mRNA level of SERCA1, MyHC-2B, ACTN3, and TNNC2 were lower in WT OVL (Fig. 4 *A–C*). These effects of SA were not substantially altered in mTg or mKO mice (Fig. 4 *A–C*). Importantly, the genes up- and down-regulated by SA are characteristic of slow- and fast-twitch skeletal muscle, respectively (24, 25). At the

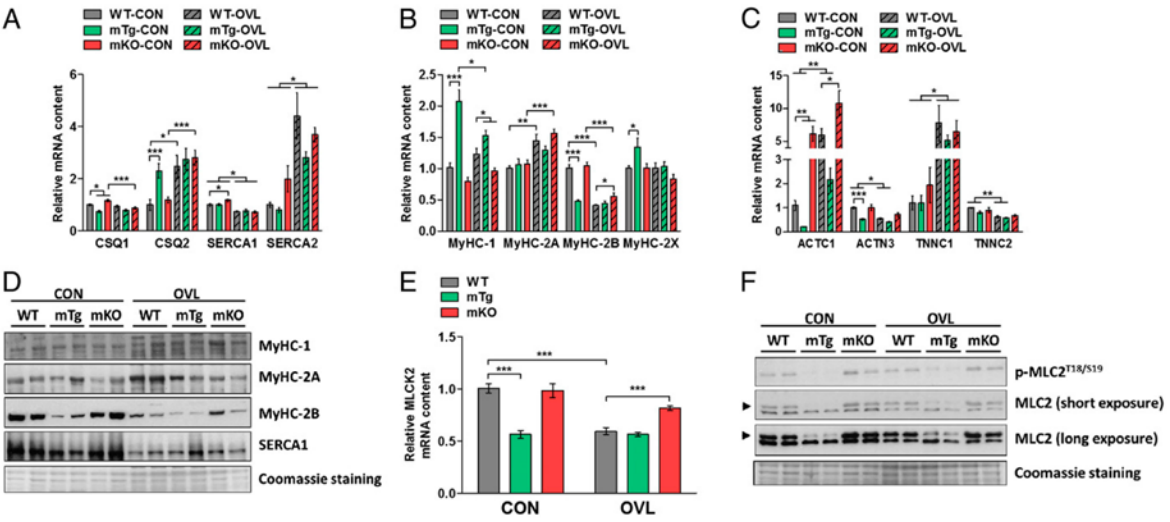

Fig. 4. SA modulates the expression of excitation–contraction coupling-related genes. (*A–F*) qPCR and Western blot analysis of key regulators of excitation–contraction coupling in plantaris (arrowhead indicates MLC2; $n = 6$ per group). Values are mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

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protein level, we observed a reduction of MyHC-2B and SERCA1 in mTg CON, whereas SA strongly decreased MyHC-2B in all genotypes and SERCA1 in WT and mKO OVL, further supporting the functional analysis (Fig. 4*D* and Fig. S3*A*). We finally analyzed myosin regulatory light chain (MLC) phosphorylation by MLC kinase 2 (MLCK2), a key process for the potentiation of force generation (26). MLCK2 mRNA content decreased by ~42% in mTg CON and WT OVL (Fig. 4*E*). In contrast, the effect of SA on MLCK2 mRNA was attenuated in mKO OVL (Fig. 4*E*), strongly supporting the ex vivo assessment of peak force. Interestingly, although relative levels of p-MLC2$^{T18/S19}$ were lower in mTg mice, the opposite effect was found in mKO CON and OVL (Fig. 4*F* and Fig. S3*B*). In addition, we observed that MLC2 protein content was strongly reduced in mTg CON, and it was increased by SA in mTg and mKO OVL (Fig. 4*F* and Fig. S3*C*). Thus, taken together, the functional and molecular analysis strongly suggests that SA induces a switch toward a slow-contractile phenotype.

Discussion

Different exercise-based and pharmacological strategies have been proposed to prevent skeletal muscle atrophy under pathological conditions, with mTORC1 activation playing a key role in this process (2, 3). In fact, mTORC1 is a well-known mediator of protein synthesis and cell growth and has recently emerged also as a metabolic regulator (27). The link between mTORC1 and skeletal muscle metabolism is thought to be PGC-1α (10, 12). PGC-1α and PGC-1α4 positively modulate skeletal muscle mass under catabolic conditions (14, 16, 17). However, we have now found that regardless of mTORC1 activity, PGC-1α is not required for skeletal muscle remodeling via chronic overload.

The role of PGC-1α in anabolic pathways is poorly understood. In aged mice, PGC-1α preserves skeletal muscle mass along with Akt and mTOR phosphorylation (15). We have found that mTg CON showed higher p-S6K^{T389} relative levels, whereas the opposite effect was observed in mKO CON. Interestingly, enhanced oxidative metabolism is associated with increased mTORC1 activity (7, 8), implying that PGC-1α might modulate mTORC1 through its effects on mitochondrial function. Moreover, mTg CON exhibited lower levels of MuRF1 and MSTN mRNA, but neither mTg nor mKO CON showed signs of skeletal muscle hypertrophy, similar to previous reports (16, 28–31). Consistently, we have previously shown that a cluster of genes related to ribosomes and anabolic pathways is down-regulated in mTg skeletal muscle (32). Finally, in vitro, PGC-1α does not regulate the rate of protein synthesis in C_2C_{12} myotubes (14). As a consequence, the assessment of a wide range of parameters related to skeletal muscle hypertrophy revealed that the overall effect of SA was not different in mTg or mKO mice. In fact, endogenous PGC-1α1 and PGC-1α4 were down-regulated by SA in WT mice. Importantly, the fact that both of these coactivators were fully deleted in mKO mice strongly indicates that they are not required for chronic overload-induced skeletal muscle hypertrophy. In line with these data, PGC-1α overexpression does not overcome skeletal muscle atrophy induced by mTORC1 inhibition via raptor knockout (28). It is important to note that PGC-1α4 was proposed to promote muscle growth via IGF1 and MSTN expression (17), which seem to play a minor role in the context of SA. In fact, even though IGF1 was up-regulated by SA, p-Akt^{T308} relative levels were not increased. Therefore, these data support the concept that mechanical overload-induced skeletal muscle hypertrophy is PI3K-independent (4) and thus might not involve PGC-1α4. Our results do not preclude PGC-1α4 to be required for muscle hypertrophy in other contexts. Accordingly, some studies show no changes in PGC-1α expression in human skeletal muscle after resistance exercise (33–35), whereas others have reported opposite results (17, 36).

PGC-1α is a central mediator of mitochondrial biogenesis (37). Consistently, concomitantly with a reduction in PGC-1α mRNA, SA strongly repressed the expression and activity of several metabolic-related proteins in both WT and mTg mice. Microarray analysis of human skeletal muscle has also revealed that resistance exercise does not increase the expression of genes involved in oxidative metabolism (38, 39). In fact, resistance-trained athletes exhibit the same (38) or lower relative peak oxygen consumption compared with untrained healthy people (40). These data disagree with the metabolic role of mTORC1, even though it should be noted that this aspect of mTORC1 function has been mainly studied using genetic and pharmacological approaches. In stark contrast, mechanical overload of skeletal muscle modulates a wide spectrum of signal pathways (1), suggesting that the regulation of additional molecules could blunt the metabolic effects of mTORC1, at least in part via PGC-1α down-regulation.

Interestingly, SA has previously been demonstrated to lower peak force (41, 42), which might be interpreted as a negative effect. However, our data suggest that it actually reflects an extreme switch to a slow-contractile phenotype. In fact, SA increased the expression of the slow-twitch specific isoform of several genes regulating excitation–contraction coupling in a PGC-1α–independent way, whereas the fast-twitch specific isoforms of these genes were concomitantly down-regulated. Importantly, our findings imply that the reduction in peak force induced by SA is mainly related to MyHC-2B, SERCA1, MLCK2, and MLC2 expression. These proteins are highly expressed in fast-twitch muscles, and they play a pivotal role in muscle force potentiation (25, 26). In accordance, we found that peak force, MyHC-2B mRNA/protein and MLCK2 mRNA levels were preserved in mKO OVL, whereas MLC2 protein levels were even increased. Moreover, p-MLC2$^{T18/S19}$ relative levels were higher in mKO mice, and the opposite results were observed in mTg CON, supporting thus the effects of PGC-1α on skeletal muscle contractility (29). Interestingly, SA improved fatigue resistance primarily in a PGC-1α–independent way. The metabolic remodeling induced by SA seems to disagree with the functional adaptations. However, our data as well as previous reports (5, 43) show that SA strongly increases AMPK activity, and considering its metabolic role (23), it seems likely that AMPK activation compensates for the negative effects of SA on energy metabolism.

In summary, we have now shown that besides inducing skeletal muscle hypertrophy, SA strongly promotes a switch toward a slow-contractile phenotype that seems to be dissociated from the metabolic phenotype. Moreover, we have identified MyHC-2B, MLCK2, and MCL2 as PGC-1α targets involved in skeletal muscle contractility. However, our data demonstrate that PGC-1α and PGC-1α4 are not involved in skeletal muscle remodeling induced by chronic overload. In fact, SA seems to disrupt the mTORC1–PGC-1α axis by down-regulation of this coactivator. It should be noted that SA does not fully resemble the effects of resistance exercise in human skeletal muscle in several aspects, and therefore the relevance of PGC-1α as a therapeutic target aiming at promoting skeletal muscle growth remains to be further explored under different conditions.

Materials and Methods

Animals. Mice were housed in a conventional facility with a 12-h night and day cycle, with free access to food and water. All experiments were performed on adult male mice with approval of the Swiss authorities. Description of mouse generation and genotyping is provided in *SI Materials and Methods*.

Synergist Ablation. Mechanical overload of plantaris was performed as previously described (43). Briefly, unilateral SA was performed under anesthesia (2.5% isoflurane) and sterile conditions, then soleus and gastrocnemius were surgically removed, and animals were allowed to recover for a period of 2 wk. The contralateral nonoperated leg was used as control.

Ex Vivo Assessment of Muscle Function, Histology and Immunohistochemistry, RNA Isolation and Quantitative PCR, Protein Isolation and Western blot, and SDH and PFK Activity Assays. These analyses were performed by standard procedures as described in *SI Materials and Methods*. See Table S3 for details on primer sequences.

Statistical Analysis. Values are expressed as mean ± SEM. Statistical significance was determined with unpaired two-tailed *t* tests or one-way ANOVA with Dunnett post hoc test. Significance was considered with a *P* value of <0.05.

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Supporting Information

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SI Materials and Methods

Animals. Peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α) muscle-specific transgenic (mTg) mice were generated by expressing PGC-1α transgene under the control of the muscle creatine kinase promoter as previously described (1). On the other hand, PGC-1α muscle-specific knockout (mKO) mice were generated by crossing PGC-1α$^{loxP/loxP}$ mice with HSA-Cre transgenic mice. PGC-1α$^{loxP/loxP}$ mice were generated as previously described (2). The genotype of mTg and mKO animals was assessed by PCR using specific primer pairs to detect the presence of the PGC-1α transgene, 5′ loxP site and recombination of the PGC-1α floxed allele in heart, quadriceps, and tibialis anterior (Fig. S1*A*). To further confirm the genotypes, quantitative PCR (qPCR) analysis of these tissues was performed, revealing a ∼10-fold increase and full deletion of PGC-1α mRNA in skeletal muscle of mTg and mKO mice, respectively (Fig. S1*B*).

Ex Vivo Assessment of Muscle Function. Proximal tendon of plantaris was left inserted into the distal femoral stub. The femur stub and the distal tendon were then ligated to silk and mounted onto a Muscle Tester chamber (SI-Heidelberg). Muscles were stimulated by supramaximal electrical field pulses of 0.5-ms duration via platinum electrodes. After determining the optimal length, isometric twitch and tetanic force were recorded at 4 kHz with a Power Lab AD Instruments converter. Tetanic contraction was assessed by stimulating the muscles for 800 ms with a train of pulses of 0.5-ms duration delivered at 100 Hz or 150 Hz. After tetanic stimulation, the muscle rested for 10 min. Fatigue resistance was assessed in response to a train of 100 Hz tetani of 400-ms duration delivered at 0.27 Hz for 5 min. Changes in force generation during the fatigue protocol are expressed as percentage of initial force.

Histology and Immunohistochemistry. NADH staining was performed on 12-μm cross-sections from plantaris by exposing them to 0.8 mg/mL NADH in the presence of 1 mg/mL nitro blue tetrazolium.

For immunohistochemistry, 10-μm cross-sections from plantaris were fixed in 100% methanol for 6 min, rinsed two times with 0.1 M glycine in PBS (pH 6.8), and subsequently microwave antigen retrieval was performed using 0.01 M citric acid (pH 6.0). After rinsing with PBS, sections were blocked in 3% (wt/vol) BSA in PBS for 1 h at room temperature, rinsed with PBS, and then incubated overnight with laminin antibody (Abcam #ab11575) in 3% BSA in PBS (1:500 dilution). The following day, sections were rinsed three times with PBS and incubated with appropriated secondary antibody (Invitrogen #A-11008) in 3% BSA in PBS (1:500 dilution) for 1.5 h at room temperature. Finally, sections were rinsed three times with PBS and mounted using Vectashield medium (Vector #H-1500). Images were acquired with a fluorescent microscope (Leica) and analyzed using analySIS software (Soft Imaging System). Muscle fiber cross-sectional area (CSA) was quantified according to standard procedures as previously described (3).

RNA Isolation and qPCR. Total RNA was isolated from heart, plantaris, tibialis anterior, and quadriceps from WT, mTg, and mKO mice using lysing matrix tubes (MP Biomedicals) and TRI Reagent (Sigma-Aldrich) according to the manufacturer's instructions. RNA concentration was measured with a NanoDrop 1000 spectrophotometer (Thermo Scientific). One microgram of RNA was treated with DNase I (Invitrogen) and then reversed transcribed using hexanucleotide mix (Roche) and SuperScript II reverse transcriptase (Invitrogen). Relative mRNA was quantified by qPCR on a StepOnePlus system (Applied Biosystems) using Power SYBR Green PCR Master Mix (Applied Biosystems). The sequences of the qPCR primers are depicted in Table S3. Specific primers for PGC-1α1 and PGC-1α4 have been previously reported (4). Analysis was performed by the $\Delta\Delta C_T$ method using TATA binding protein (TBP) as endogenous control. TBP transcript levels were not different between genotypes or different experimental conditions.

Protein Isolation and Western Blot. Plantaris was powdered on dry ice and homogenized in 300 μL of ice-cold lysis buffer [50 mM Tris·HCl (pH 7.5), 250 mM sucrose, 1 mM EDTA, 1 mM EGTA, 0.25% Nonidet P 40 substitute, 50 mM NaF, 5 mM $Na_4P_2O_7$, 0.1% DTT, fresh protease, and phosphatase inhibitor mixture]. Then, samples were shaken at 1,300 rpm for 30 min at 4 °C. Samples were subsequently centrifuged at 13,000 × *g* for 10 min at 4 °C and the protein concentration of the supernatant determined by the Bradford assay (Bio-Rad). Equal aliquots of protein were boiled for 5 min in Laemmli sample buffer [250 mM Tris·HCl (pH 6.8), 2% (vol/vol) SDS, 10% (vol/vol) glycerol, 0.01% bromophenol blue, and 5% (vol/vol) β-mercaptoetanol].

Samples were separated on SDS-polyacrylamide gels and then transferred to nitrocellulose membranes for 1 h. Membranes were blocked for 1 h in 3% milk in Tris-buffered saline and 0.1% Tween-20 (TBST) before overnight incubation at 4 °C with appropriate primary antibody in TBST (1:1,000 dilution). Proteins were detected with a primary antibody to S6K (Cell Signaling #2708), p-S6K^{T389} (Cell Signaling #9206), 4E-BP1 (Cell Signaling #9644), p-4E-BP1$^{T37/46}$ (Cell Signaling #2855), Akt (Cell Signaling #9272), p-Akt^{T308} (Cell Signaling #4056), AMPKα (Cell Signaling #2603), p-AMPKα^{T172} (Cell Signaling #2535), ACC (Cell Signaling #3662), p-ACC^{S79} (Cell Signaling #3661), MLC2 (Cell Signaling #3672), p-MLC2$^{T18/S19}$ (Cell Signaling #3674), SERCA1 (Cell Signaling #4219), and total OXPHOS (Abcam #ab110413). The MyHC-1 (BA-F8), MyHC-2A (SC-71), and MyHC-2B (BF-F3) antibodies developed by Dr. Stefano Schiaffino (Venetian Institute of Molecular Medicine, Padova, Italy) were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the Eunice Kennedy Shriver National Institute of Child Health and Human Development and maintained by the University of Iowa Department of Biology (Iowa City, IA). Coomassie staining was used as loading control by using SimplyBlue SafeStain (Invitrogen: lc6060). After incubation, membranes were washed three times with TBST before incubation with an appropriate peroxidase-conjugated secondary (Dako) antibody in TBST (1:10,000 dilution). Antibody binding was detected using enhanced chemiluminescence HRP substrate detection kit (Pierce: 32106) and quantified using ImageJ.

Succinate Dehydrogenase and Phosphofructokinase Activity Assays. Succinate dehydrogenase (SDH) and phosphofructokinase (PFK) enzymatic activities were determined colorimetrically by incubating 50 μL of protein extracts (50 μg of protein) with 50 μL of SDH assay buffer (50 mM sodium succinate, 0.5 mg/mL nitro blue tetrazolium, 0.03 mg/mL phenazine methosulfate, and 50 mM sodium phosphate buffer, pH 7.6) or PFK assay buffer (2 mM D-fructose 6-phosphate, 1 mM β-NAD, 1 mM ATP, 1 mM magnesium sulfate, 0.4 mg/mL nitro blue tetrazolium, 0.03 mg/mL phenazine methosulfate, and 10 mM sodium arsenate, pH 7.4) at 37 °C for 10–70 min. A second set of samples was incubated in background

control solution without substrate (sodium succinate or D-fructose 6-phosphate) to measure endogenous NADH oxidation. Background values were subtracted for further analysis. Absorbance at 450 nm was measured at two different time points, and the difference was used to calculate relative enzymatic activity.

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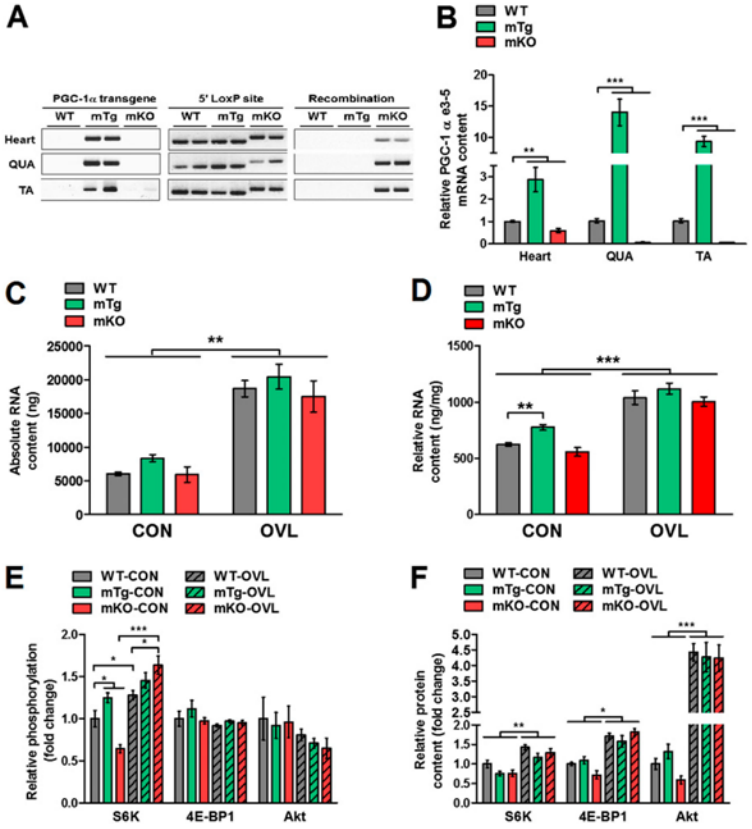

Fig. S1. Determination of genotypes and hypertrophic effects of synergist ablation (SA). (*A*) Detection of PGC-1α transgene, 5′ loxP site, and recombination of the PGC-1α floxed allele from genomic DNA via PCR genotyping in heart, quadriceps (QUA), and tibialis anterior (TA) of WT, mTg, and mKO mice. (*B*) qPCR analysis of total PGC-1α mRNA levels (exons 3–5) in heart, quadriceps, and tibialis anterior of WT, mTg, and mKO mice ($n = 6$ per group). (*C* and *D*) Absolute and relative plantaris RNA levels ($n = 6$ per group). (*E* and *F*) Western blot quantification of phosphorylation and total protein content of mammalian target of rapamycin complex 1 (mTORC1) and PI3K target proteins ($n = 6$ per group). Values are mean ± SEM. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

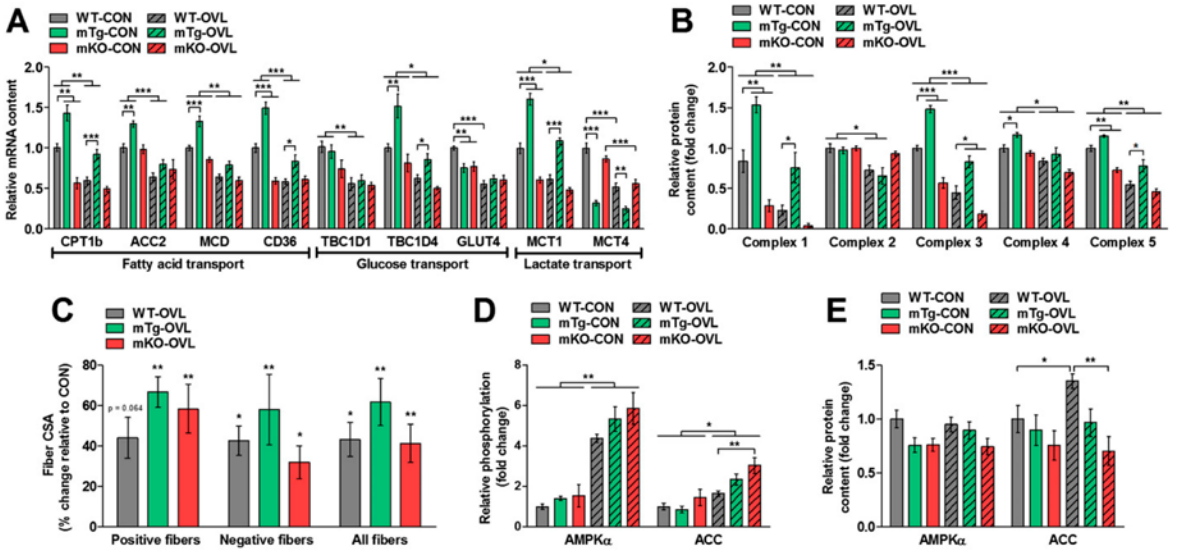

Fig. S2. Metabolic effects of SA. (*A*) qPCR analysis of metabolic-related genes ($n = 6$ per group). (*B*) Western blot quantification of different components of the electron transport chain ($n = 6$ per group). (*C*) Changes in average CSA of positive, negative, and all fibers assessed by NADH staining ($n = 3$ per group). (*D* and *E*) Western blot quantification of AMPK activity ($n = 6$ per group). Values are mean ± SEM. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. In *D*: $^*P < 0.05$, $^{**}P < 0.01$ vs. CON.

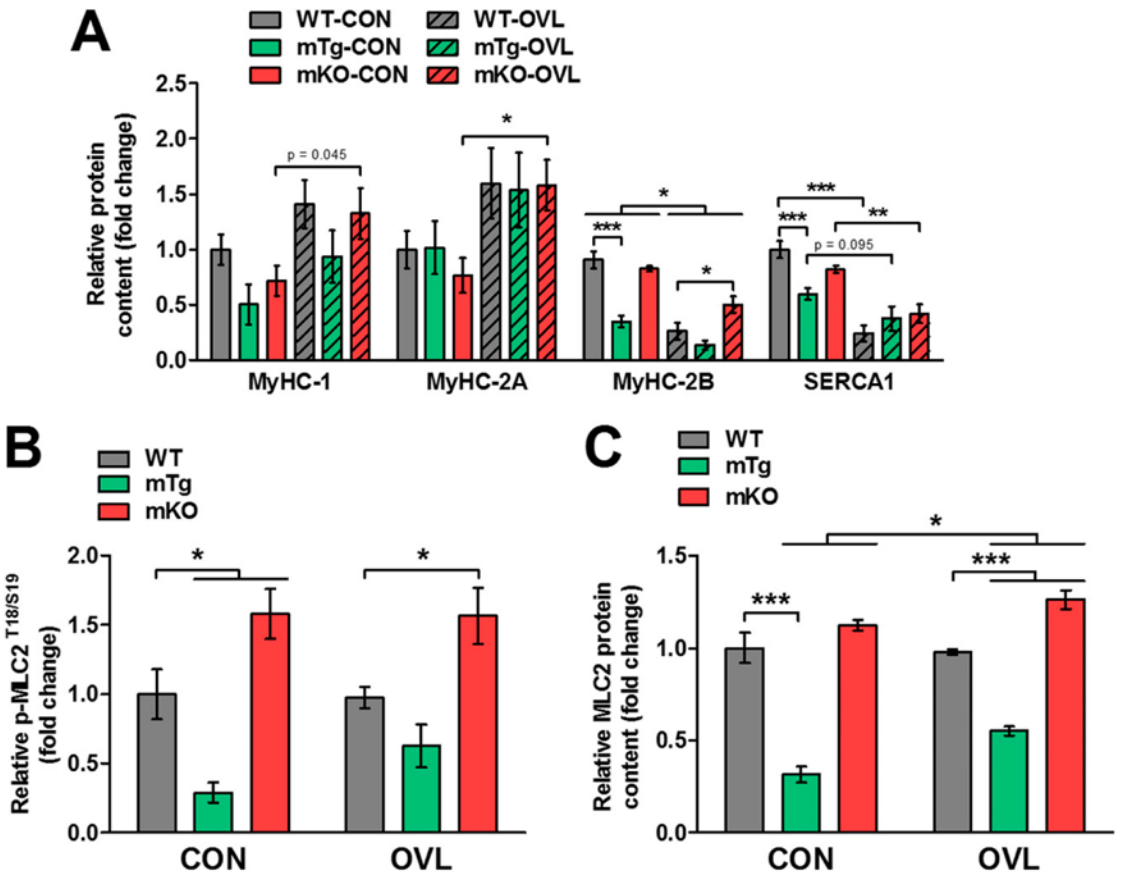

Fig. S3. SA decreases plantaris contractility. (*A–C*) Western blot quantification of total protein content of MyHC-1, MyHC-2A, MyHC-2B, SERCA1, p-MLC2$^{T18/S19}$, and MLC2 (n = 6 per group). Values are mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001.

Table S1. Body and muscle weight of WT, mTg, and mKO mice

Weight	WT	mTg	mKO
Body weight (g)	25.5 ± 0.21	26.8 ± 0.58	25.7 ± 0.27
Gastrocnemius (mg)	141.3 ± 2.24	139.0 ± 4.17	139.3 ± 2.51
Soleus (mg)	9.4 ± 0.19	10.5 ± 0.36*	9.7 ± 0.29
Plantaris (mg)	18.9 ± 0.42	18.3 ± 0.62	18.0 ± 0.29
Tibialis anterior (mg)	50.2 ± 0.70	47.1 ± 1.29	47.3 ± 0.77
Extensor digitorum longus (mg)	11.4 ± 0.14	10.2 ± 0.38**	9.8 ± 0.18***
Quadriceps (mg)	232.8 ± 3.08	222.7 ± 7.29	215.8 ± 3.84

Values are mean ± SEM (n = 10–25 per group). *P < 0.05, **P < 0.01, ***P < 0.001 vs. WT.

Table S2. Contractile kinetics of plantaris from WT, mTg, and mKO mice

	CON			OVL		
Parameter	WT	mTg	mKO	WT	mTg	mKO
Twitch						
TTP (ms)	14.8 ± 0.7	13.9 ± 0.3	13.9 ± 0.7	12.7 ± 0.3*	12.6 ± 0.4*	12.8 ± 0.4
1/2TTP (ms)	4.3 ± 0.2	4.2 ± 0.2	4.2 ± 0.2	4.1 ± 0.1	3.8 ± 0.1	4.1 ± 0.2
1/2RT (ms)	16.6 ± 1.1	16.4 ± 2.6	13.8 ± 1.9	17.0 ± 1.2	13.3 ± 1.5	12.1 ± 1.0$^{\#}$
Tetanus 100 Hz						
1/2CT (ms)	29.4 ± 2.0	30.4 ± 0.8	27.6 ± 1.4	36.6 ± 2.2*	36.3 ± 1.1**	36.8 ± 2.4*
1/2RT (ms)	22.9 ± 0.3	20.8 ± 0.7	25.8 ± 1.1	29.8 ± 0.9***	23.6 ± 0.5*,$^{\#\#\#}$	34.6 ± 0.8***,$^{\#\#}$
Tetanus 150 Hz						
1/2CT (ms)	23.3 ± 0.7	26.1 ± 0.2	25.4 ± 1.2	27.9 ± 1.7*	28.1 ± 1.3	28.9 ± 1.4
1/2RT (ms)	27.6 ± 0.6	23.0 ± 0.3$^{\#\#\#}$	29.0 ± 0.8	36.6 ± 1.1***	28.2 ± 0.7***,$^{\#\#\#}$	39.8 ± 0.8***

TTP, time to peak; 1/2TTP, half time to peak; 1/2RT, half relaxation time; 1/2CT, half contraction time. Values are mean ± SEM (n = 5–6 per group). *P < 0.05, **P < 0.01, ***P < 0.001 CON vs. OVL; $^{\#}P$ < 0.05, $^{\#\#}P$ < 0.01, $^{\#\#\#}P$ < 0.001 vs. WT of the same group.

Table S3. qPCR primer sequences

Target gene	Forward primer	Reverse primer
IGF1	CTGGACCAGAGACCCTTTGC	CCTCGGTCCACACACGAACT
Fbxo32	AAAGCCCTCTCTTGGTTCTGACT	GAGAAGAGGTGCAGGGACTGA
MuRF1	AGGCAGCCACCCGATGT	TCACACGTGAGACAGTAGATGTTGA
MSTN	GCTGGCCCAGTGGATCTAAA	GCCCCTCTTTTTCCACATTTT
NDUFB5	TTTTCTCACGCGGAGCTTTC	TGCCATGGTCCCCACTGT
COX1	TGCTAGCCGCAGGCATTACT	GCGGGATCAAAGAAAGTTGTG
SDHa	GCTGGTGTGGATGTCACTAAGG	CCCACCCATGTTGTAATGCA
CS	CCCAGGATACGGTCATGCA	GCAAACTCTCGCTGACAGGAA
FH1	TGCTCTCAGTGCAAAATCCAA	CGTGTGAGTTCGCCCAATT
MCAD	AACACTTACTATGCCTCGATTGCA	CCATAGCCTCCGAAAATCTGAA
HADHb	TGCTGTCAGGCACTTCGTATAAA	AAACCCGAAAGTGCAGCTCTAG
PFK	TGTGGTCCGAGTTGGTATCTT	GCACTTCCAATCACTGTGCC
HK2	CCCTGCCACCAGACGAAA	GACTTGAACCCCTTAGTCCATGA
LDHa	CATTGTCAAGTACAGTCCACACT	TTCCAATTACTCGGTTTTTGGGA
LDHb	CATTGCGTCCGTTGCAGATG	GGAGGAACAAGCTCCCGTG
CPT1b	ATCATGTATCGCCGCAAACT	CCATCTGGTAGGAGCACATGG
ACC2	GGGCTCGGGCATGATTG	CAGGTAAGCCCCGATTCCA
MCD	ACTCCATCAGCCTGACCCAG	ACCCCTTGAGGCTCTCGTGA
CD36	GGCAAAGAACAGCAGCAAAAT	TGGCTAGATAACGAACTCTGTATGTGT
TBC1D1	CATAAAGAACACACTCCCCAACCT	TGCTTGGCGATGTCCATCT
TBC1D4	GTACCGACCGGATATGATGTCA	CGGTGGTAGTCATGAAGGAGTCT
GLUT4	CATGGCTGTCGCTGGTTTC	AAACCCATGCCGACAATGA
MCT1	GTGACCATTGTGGAATGCTG	CTCCGCTTTCTGTTCTTTGG
MCT4	TCACGGGTTTCTCCTACGC	GCCAAAGCGGTTCACACAC
PGC-1α e3-5	AGCCGTGACCACTGACAACGAG	GCTGCATGGTTCTGAGTGCTAAG
PGC-1β	CCATGCTGTTGATGTTCCAC	GACGACTGACAGCACTTGGA
PRC	CACCCTGCCGGAGTGAAAT	CGCATTGACTGCTGCTTGTC
CSQ1	ACTCAGAGAAGGATGCAGCT	CTCTACAGGGTCTTCTAGGA
CSQ2	AGCTTGTGGAGTTTGTGAAG	GGATTGTCAGTGTTGTCCC
SERCA1	AGCCAGTGATGGAGAACTCG	CACCACCAACCAGATGTCAG
SERCA2	GAGAACGCTCACACAAAGACC	CAATTCGTTGGAGCCCCAT
MyHC-1	CCTCCTCACATCTTCTCCATCTCT	TGGACTGATTCTCCCGATCTG
MyHC-2A	CCCCGCCCCACATCTT	TGACTGATTCTCCCTGTCTGTTAGC
MyHC-2B	CAACCCATATGACTTTGCTTACGT	TCCCAGGATATCAACAGCAGTGT
MyHC-2X	GGCCCCACCCCACATC	CTCCCGATCTGTCAGCATGA
ACTC1	CACCACTGCTGAACGTGAAATT	TCCAGGGCGACGTAACACA
ACTN3	AGGAGCAGCTCAACGAATTCC	TCGTCGGGCTCCATCATC
TNNC1	GGCACAGTGGACTTCGATGA	TTCCCTTTGCTGTCGTCCTT
TNNC2	AAAGAGTTGGGCACCGTGAT	GGCATCCAATTCCTCTTTGG
MLCK2	AGCCAAGGTCATCAAGAAACAGA	GACCTCGATCTCCAGCAACAC

CHAPTER 6: Discussion and outlook

Discussion

Skeletal muscle tissue can exhibit drastic changes in its phenotype and thus directly affect whole body homeostasis. The effect of skeletal muscle in the modulation of whole body homeostasis and health status has been well reported in trained subjects, which in contrast to sedentary subjects, show lower risk of metabolic diseases and premature death (Booth et al., 2011; Booth and Roberts, 2008). Skeletal muscle remodelling mediated by physical activity and inactivity is determined by the expression and repression of a highly specific subset of genes. While a number of transcription factors have been implicated in the control of these gene expression signatures, transcriptional coactivators and corepressors have emerged as key players in skeletal muscle remodelling (Egan and Zierath, 2013; Gundersen, 2011). Coregulators are able to modulate the activity of a subset of transcription factors regulating common biological processes, with significant physiological relevance and potential as therapeutic targets for diseases related to transcriptional deregulation (Lonard and O'Malley, 2012). Different coregulators have been studied in skeletal muscle tissue, including SRC-1, TIF2 and RIP140 (Duteil et al., 2010; Seth et al., 2007), but the coactivator PGC-1α and the corepressor NCoR1 show a high potential as regulators of skeletal muscle remodelling (Handschin, 2010; Mottis et al., 2013). However, the mechanism by which these coregulators modulate skeletal muscle plasticity is not fully understood.

Interestingly, several transcription factors targeted by NCoR1 and some components of this corepressor complex are well-known regulators of skeletal muscle function (Bailey et al., 1999; Busson et al., 2005; Ishizuka and Lazar, 2005; Kitamura et al., 2007; McKenna and O'Malley, 2010), strongly suggesting a role of NCoR1 in skeletal muscle remodelling. Accordingly, during the first study of this thesis we used NCoR1 skeletal muscle-specific knockout (mKO) mice to define the function of skeletal muscle NCoR1. During the course of this study, an article describing the phenotype of this mouse model under basal conditions and following high-fat diet feeding reported that genetic ablation of NCoR1 in skeletal muscle improves exercise performance, enhance oxidative capacity and lower respiratory

exchange ratio (RER), primarily under high-fat diet feeding (Yamamoto et al., 2011). In line with these data, our study revealed new evidence supporting the metabolic function of NCoR1, including an enhanced VO_{2peak} during treadmill running, higher ex vivo fatigue resistance and increased proportion of oxidative fibres. These data demonstrate that NCoR1 is a negative regulator of skeletal muscle oxidative metabolism. Importantly, the fact that whole body oxidative capacity and skeletal muscle function are altered in NCoR1 mKO mice strongly highlight the physiological relevance of this corepressor. Interestingly, the gene expression signature and functional adaptations induced by skeletal muscle NCoR1 ablation resemble some aspects of endurance training (Egan and Zierath, 2013; Holloszy and Coyle, 1984). Hence, NCoR1 emerges as a potential modulator of skeletal muscle plasticity in contexts like physical activity and inactivity in which this corepressor would exhibit decreased and increased activity, respectively.

We further studied the possible mechanisms by which NCoR1 modulates skeletal muscle remodelling. The phenotype of NCoR1 mKO mice suggest that this corepressor exerts opposite effects than PGC-1α on oxidative metabolism and skeletal muscle function (Calvo et al., 2008; Lin et al., 2002; Summermatter et al., 2012), which has led to the idea of a functional interplay between these coregulators (Yamamoto et al., 2011). This interaction between NCoR1 and PGC-1α has been previously proposed to control PPARγ activity in adipocytes (Guan et al., 2005). In this study, by using bioinformatics and cell culture approaches, we directly explored this hypothesis. Microarray studies showed that, as speculated, NCoR1 and PGC-1α target a common subset of genes related to skeletal muscle oxidative metabolism, while cell culture experiments confirmed their functional interplay. PPARβ/δ and ERRα have been previously proposed to be directly repressed by NCoR1 (Yamamoto et al., 2011), while PGC-1α is well-known to activate both of these nuclear receptors (Ehrenborg and Krook, 2009; Giguere, 2008; Handschin, 2010). Consistently, further analysis of our microarray data predicted ERRα and the PPARs heterodimer partners RXRs as common targets of NCoR1 and PGC-1α. Importantly, both of these transcription factors have been demonstrated to promote the expression of key genes regulating energy metabolism (Ehrenborg and Krook, 2009; Giguere, 2008). As

predicted, we demonstrated that both of these transcription factors are directly regulated by NCoR1 and PGC-1α. To our surprise however, we found that only ERRα was required for NCoR1 to regulate skeletal muscle oxidative metabolism. This study therefore has revealed the key role of NCoR1 in skeletal muscle remodelling and function, providing new insights in the molecular mechanism by which the interplay between NCoR1 and PGC-1α regulate gene transcription. These results also suggest NCoR1 as a therapeutic target for the treatment of metabolic diseases, where NCoR1 target genes seems to be down-regulated. In fact, NCoR1 mKO animals appear to be slightly protected against high-fat diet induced insulin resistance and whole body metabolic deregulation (Yamamoto et al., 2011). Inhibition of NCoR1 via exercise or pharmacological compounds would be expected then to improve skeletal muscle metabolic fitness and thus prevent the development of metabolic diseases. However, future studies are required to determine the therapeutic potential of skeletal muscle NCoR1 in the context of metabolic diseases.

The molecular mechanism by which the coactivator PGC-1α regulates skeletal muscle metabolism was further examined during the second study of this thesis, where the functional interaction between PGC-1α and PPARβ/δ was directly assessed in vivo. As mentioned above, the transcriptional activity of the nuclear receptor PPARβ/δ is directly enhanced by PGC-1α (Dressel et al., 2003; Narkar et al., 2008; Perez-Schindler et al., 2012) and it has been proposed to play an important role in skeletal muscle plasticity (Ehrenborg and Krook, 2009). Actually, exercise has been shown to up-regulate PPARβ/δ levels in skeletal muscle (Ehrenborg and Krook, 2009). Moreover, genetic activation of PPARβ/δ strongly increases skeletal muscle oxidative metabolism and exercise performance (Wang et al., 2004). However, the phenotype exhibited by the mouse model mentioned above seems to exert supra-physiological effects, since pharmacological activation of PPARβ/δ or the overexpression of a normal non-hyperactive form this nuclear receptor result in milder effects on skeletal muscle and whole body metabolism (Gan et al., 2011; Luquet et al., 2003; Narkar et al., 2008). Our results validate the well-known effects of PGC-1α overexpression on skeletal muscle metabolism, whereas we surprisingly found that PPARβ/δ was dispensable for these effects. In fact, skeletal muscle PGC-

1α overexpression enhanced both VO_{2peak} and the proportion of oxidative fibres in the absence and presence of functional PPARβ/δ. However, we found that the higher fatty acid oxidation observed in PGC-1α mTg mice during exercise was attenuated by PPARβ/δ ablation, consistent with similar studies performed in cultured muscle cells (Kleiner et al., 2009). Unexpectedly, we also found that PPARβ/δ mKO mice showed a similar phenotype than wild type mice, contrasting previously published results using the same mouse model (Schuler et al., 2006). These results however, might be related to different environmental factors modulating PPARβ/δ activity, such as temperature, food and activity. In fact, similar discrepancies have been reported in PPARβ/δ global knockout mice (Akiyama et al., 2004; Feng et al., 2011; He et al., 2010; Lee et al., 2006; Peters et al., 2000). Thus, studying the interplay between PGC-1α and PPARβ/δ in the context of ligand-mediated activation of this nuclear receptor might reveal some levels of interdependence.

Interestingly, these results are in line with the data from the first study of this thesis. In fact, while PGC-1α was completely required for NCoR1 knockdown to up-regulate metabolic-related genes in C_2C_{12} cells, pharmacological inhibition of PPARβ/δ had no effect. Similarly, PPARβ/δ knockout did not impair the increase of mRNA and protein content of metabolic-related genes observed in skeletal muscle from PGC-1α mTg animals. Further supporting these results, it has been demonstrated that, irrespectively of PPARβ/δ expression levels, PGC-1α efficiently enhances oxidative metabolism in C_2C_{12} muscle cells (Kleiner et al., 2009). Furthermore, our data show that the regulation of common target genes of NCoR1 and PGC-1α is fully dependent of ERRα activation. Interestingly, we observed that PGC-1α overexpression significantly enhanced the expression levels of ERRα, ERRβ and PPARα in a PPARβ/δ independent manner. It therefore seems that similar to the cell culture experiments performed during the first study, the regulation of skeletal muscle oxidative metabolism in vivo might require the activation of ERRα or other of the up-regulated transcription factors. However, the fact that PPARβ/δ was found to be directly regulated by NCoR1 and PGC-1α implies that this nuclear receptor might play a relevant function in skeletal muscle plasticity in a context dependent manner.

In fact, PPARβ/δ mKO animals have been reported to be more susceptible to the metabolic dysregulation associated with aging and high-fat diet feeding (Schuler et al., 2006). In addition, administration of PPARβ/δ specific agonists are highly efficient in preventing the pathological effects of obesity and type 2 diabetes (Lee et al., 2006; Tanaka et al., 2003; Wang et al., 2004), while when combined with exercise training, it enhances skeletal muscle adaptations (Narkar et al., 2008). Impairments in skeletal muscle PGC-1α activity have been suggested to contribute to the development of metabolic diseases (Mootha et al., 2003; Patti et al., 2003), while our results suggest a possible pathogenic role of NCoR1. Therefore, regulation of PPARβ/δ by NCoR1 and PGC-1α might be relevant in conditions like metabolic diseases and exercise training.

Finally, we investigated the potential function of PGC-1α in mechanical overload-induced skeletal muscle remodelling, which represents a context in which this coactivator has not been previously studied. PGC-1α and its transcript variant PGC-1α4 have been demonstrated to efficiently prevent skeletal muscle loss under catabolic contexts such as denervation (Brault et al., 2010; Ruas et al., 2012; Sandri et al., 2006). In addition, the key regulator of skeletal muscle protein synthesis mTORC1 has been shown to regulate skeletal muscle metabolism through the modulation of PGC-1α-YY1 complex (Blattler et al., 2012; Cunningham et al., 2007). Hence, the mTORC1-PGC-1α axis seems to be a potential mechanism by which resistance exercise mediates skeletal muscle remodelling. Interestingly, our data show that while chronic skeletal muscle overload via synergist ablation (SA) strongly activates mTORC1 and promotes skeletal muscle hypertrophy, it actually down-regulates PGC-1α and PGC-1α4 levels. Consistently, PGC-1α and PGC-1α4 were found to be dispensable for SA-induced skeletal muscle hypertrophy. Actually, microarray data generated in the first study of this thesis revealed that PGC-1α overexpression in skeletal muscle down-regulates the expression of genes related to ribosomes and anabolic process. These data suggest that PGC-1α does not promote skeletal muscle growth, which is consistent with cell culture experiments showing no effects of PGC-1α overexpression on protein synthesis (Brault et al., 2010). Moreover, resistance exercise in humans seems to induce different results, with

some studies showing no effect and others showing increased levels of skeletal muscle PGC-1α following resistance exercise (Apro et al., 2013; Coffey et al., 2006; Donges et al., 2012; Item et al., 2013; Ruas et al., 2012). The mechanism regulating mechanical overload-induced skeletal muscle hypertrophy is not fully understood, but it seems that PI3K-insulin-like growth factor 1 (IGF-1) and myostatin pathways play a minor role (Philp et al., 2011). Therefore, it remains to be determined whether the mTORC1-PGC-1α axis might play a role in mediating skeletal muscle remodelling in a context in which skeletal muscle hypertrophy is induced by growth factors.

An important aim of this study was to investigate metabolic and functional adaptations induced by mechanical overload of skeletal muscle. Interestingly, we observed that the experimental model of SA does not fully resemble the effects induced by resistance exercise. While resistance exercise exerts mild or no effects on energy metabolism (Egan and Zierath, 2013), we found that SA induced a global repression of both glycolytic and oxidative metabolism in skeletal muscle, consistent thus with the negative effects of SA on PGC-1α levels. Actually, PGC-1α overexpression attenuated this metabolic repression. Further supporting these results, microarray studies have recently revealed that SA indeed represses a subset of genes related with mitochondrial function and oxidative metabolism (Chaillou et al., 2013). At the functional level, the results were also discordant, since resistance exercise increases maximal force (Egan and Zierath, 2013), whereas SA was found to decrease peak force and increase fatigue resistance. In fact, further analysis of genes regulating excitation contraction coupling revealed an increased expression of genes characteristic of slow-twitch muscle, while the respective fast isoforms of these genes were down-regulated. Interestingly however, while this fibre type switch toward a slow-contractile phenotype was not affected by PGC-1α overexpression, genetic ablation of this coactivator preserved muscle contractility after SA. These functional results are strongly supported by data demonstrating that expression of the fast-twitch related proteins MyHC-2B and myosin regulatory light chain (MLC) kinase 2 (MLCK2) was higher in skeletal muscle from PGC-1α mKO mice following SA, while MLC2 phosphorylation levels were also enhanced. Importantly, these data revealed a novel mechanism by which PGC-1α modulates skeletal muscle

contractility, which significantly complements the mechanisms previously reported (Lin et al., 2002; Summermatter et al., 2012). Although SA did not fully resemble skeletal muscle remodelling induced by resistance exercise, the data obtained in this study strongly demonstrate that PGC-1α and PGC-1α4 do not regulate skeletal muscle hypertrophy and the metabolic remodelling induced by chronic overload of skeletal muscle. The relevance of these coactivators in different mouse models of resistance exercise remains to be elucidated. It is possible that different stimulation patterns (e.g. chronic vs. intermittent) better resembling resistance exercise would differentially regulate PGC-1α and PGC-1α4 expression, exerting then different effects on their target genes and thus skeletal muscle phenotype. However, there are clear limitations in terms of experimental models to achieve these aims, though chronic high frequency electrical stimulation of the sciatic nerve might represent a suitable approach.

Collectively, these data demonstrate that the coregulators NCoR1 and PGC-1α compete for the regulation of common transcription factors and target genes. Here, we have demonstrated that among these transcription factors, ERRα plays a major function in the control of skeletal muscle oxidative metabolism under basal conditions. Although we have demonstrated that these coregulators directly target PPARβ/δ, the relevance of this nuclear receptor remains to be studied under different contexts since it seems to be dispensable for the control of oxidative metabolism under basal conditions. Finally, we explored the potential role of the mTORC1-PGC-1α axis in skeletal muscle remodelling following mechanical overload. Our data strongly showed that neither PGC-1α nor PGC-1α4 is required for SA mediated hypertrophy, despite of strong mTORC1 activation. SA actually decreased PGC-1α and PGC-1α4 expression, repressing both glycolytic and oxidative metabolism. Importantly, this study revealed new PGC-1α targets involved in the regulation of skeletal muscle contractility under basal conditions and after SA. Overall, these results suggest that promoting NCoR1 exchange by PGC-1α at target genes represents a key step in the control of skeletal muscle remodelling, thus modulating this process represents an attractive strategy to improve skeletal muscle metabolic fitness and function.

CHAPTER 6: Discussion and outlook

Outlook

The relevance of gene expression as a regulatory mechanism of skeletal muscle phenotype has been extensively reported under both physiological and pathological contexts. In the present study, we have identified novel mechanisms by which the interplay between coactivators and corepressors modulate skeletal muscle remodelling. The identification of NCoR1 as repressor of skeletal muscle metabolism through competition with PGC-1α opens new opportunities in the treatment of metabolic diseases. However, the physiological mechanism regulating NCoR1 and PGC-1α interaction needs to be determined. Considering the effects of endurance exercise on PGC-1α and skeletal muscle metabolism, it seems likely that NCoR1 might be inhibited by skeletal muscle contraction. This remains as an important open question, which might be addressed by assessing the effects of exercise on NCoR1 cellular localization, corepressor complex formation and promoter recruitment. Moreover, NCoR1 muscle-specific transgenic mice might be generated to directly determine the negative effects of NCoR1 on skeletal muscle remodelling following exercise training or pharmacological treatment of metabolic diseases. Moreover, assessing skeletal muscle NCoR1 activity in the context of metabolic diseases would reveal its potential pathogenic role and relevance as therapeutic target.

Moreover, we have found that, under basal conditions, NCoR1 and PGC-1α regulate skeletal muscle remodelling in a PPARβ/δ independent manner. PPARβ/δ however has been demonstrated to be an efficient target for the treatment of metabolic diseases (Salvado et al., 2012). Hence, it is possible that these coregulators are required in the context of PPARβ/δ activation. It might be relevant thus to determine the functional interplay between NCoR1/PGC-1α and PPARβ/δ in either exercise training- or PPARβ/δ agonist-mediated skeletal muscle remodelling. In fact, it would be possible that under these contexts, PPARβ/δ activation by natural or pharmacological ligands might require the recruitment of PGC-1α to modulate biological functions different from those regulated by the PGC-1α-ERRα axis.

Finally, results from the last study indicate that PGC-1α does not mediate chronic overload-mediated skeletal muscle hypertrophy. However, the fact that this coactivator can inhibit protein degradation and prevent skeletal muscle atrophy strongly supports its therapeutic potential in the context of skeletal muscle wasting. Importantly, it remains to be determined if PGC-1α plays a regulatory role in an experimental model that better resembles the effects of resistance exercise in human skeletal muscle. Alternatively, PGC-1α might be more relevant in the context of skeletal muscle atrophy and re-growth following treatment with anabolic agents, such as IGF-1. The fact that PGC-1α4 was suggested to mediate skeletal muscle hypertrophy through IGF-1 and myostatin expression (Ruas et al., 2012) further support the idea that these coactivators might be relevant in the context of growth factor-induced hypertrophy. Therefore, even though these studies have revealed valuable information regarding coregulator-mediated skeletal muscle remodelling, future studies are required to fully determine the therapeutic potential of the new regulatory mechanisms that we have found.

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APPENDIX 1: New insights in the regulation of skeletal muscle PGC-1α by exercise and metabolic diseases

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New insights in the regulation of skeletal muscle PGC-1α by exercise and metabolic diseases

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Skeletal muscle energy metabolism is severely impaired in insulin resistant and type 2 diabetic patients. In particular, deregulated transcription of oxidative metabolism genes has been linked to the development of non-communicable metabolic diseases. The peroxisome proliferator-activated receptor γ (PPARγ) coactivator-1α (PGC-1α) is a key molecule in the regulation of oxidative metabolism in different tissues, including skeletal muscle. In this tissue, physical exercise is one of the most dominant physiological stimuli to induce PGC-1α. In addition, exercise training efficiently prevents the development of metabolic diseases. Hence, better knowledge about the regulation of PGC-1α by exercise would significantly help to design effective treatments for these diseases.

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Introduction

Physical inactivity is nowadays considered as a pandemic, representing an important etiological factor in the development of non-communicable diseases and a major cause of premature death worldwide [1,2]. The detrimental effects of a sedentary life style are largely associated with the impairment of skeletal muscle metabolic fitness, which is mainly characterized by a lower oxidative metabolism [3,4]. Although causality of mitochondrial dysfunction in the development of insulin resistance remains controversial, insulin-resistant and type 2 diabetic patients often present an impairment of skeletal muscle oxidative metabolism [5]. It is important to note that a pathological decrease in skeletal muscle insulin sensitivity is sufficient to promote the development of the metabolic syndrome [6,7]. Therefore, the metabolic remodeling of skeletal muscle cells plays a central role in the pathogenesis of metabolic diseases like type 2 diabetes.

The development of obesity and type 2 diabetes largely depends on different environmental factors, including the balance between physical activity and inactivity [8–10]. At the transcriptional level, the peroxisome proliferator-activated receptor γ (PPARγ) coactivator-1α (PGC-1α) plays a key role in exercise-linked mitochondrial remodeling [11,12]. This coactivator regulates a specific transcriptional network, in which the estrogen related receptors (ERRs), nuclear respiratory factors (NRFs) and PPARδ are the principal transcription factors involved in the enhancement of oxidative metabolism [11]. Consistent with its metabolic function, impairment of PGC-1α expression and activity has been identified as potential pathogenic factors in the development of insulin resistance and type 2 diabetes [5,13,14]. The expression of PGC-1α is sensitive to muscle contraction with high levels in trained subjects and a strong down-regulation in muscle from people with spinal cord injury [15]. Here, we review the specific mechanism by which different types of physical exercise regulate skeletal muscle PGC-1α and how

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this process is affected by non-communicable metabolic diseases.

Regulation of PGC-1α expression and activity by skeletal muscle contraction

Muscle contraction modulates different signal pathways that trigger the activation or repression of a very specific subset of genes, such as the slow-oxidative or fast-glycolytic gene program observed in type 1 and 2 skeletal muscle fibers, respectively [16]. PGC-1α specifically controls the expression of the slow-oxidative gene program, while the expression and activity of this coactivator are regulated by several protein kinases/phosphatases, transcription factors and coregulators in response to muscle contraction [12].

Transcriptional regulation of PGC-1α in skeletal muscle

The human and mouse PGC-1α promoters contain several binding sites for different transcription factors, including PPARs, myocyte enhancer factor 2 (MEF2), activating transcription factor 2 (ATF2), forkhead box O1 (FOXO1), p53 and others [17–19]. Primarily however, muscle contraction mediates the activation of this promoter via the MEF2 binding sites and the cAMP response element (CRE) [20]. The transcriptional activity of MEF2 can be increased by PGC-1α or repressed by the histone deacetylase 5 (HDAC5) [18,21]. Muscle contraction can decrease the inhibitory effect of HDAC5 through its phosphorylation by protein kinase D (PKD), 5′AMP-activated protein kinase (AMPK) and Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) [22–24], representing an essential process in the activation of the PGC-1α promoter [22]. By contrast, PGC-1α is strongly induced by exercise (see below) and its positive effect on MEF2 has been suggested to activate a positive feedback loop, thus increasing its own transcription [18]. CRE binding protein (CREB) and ATF2 are the main transcription factors recruited to the CRE site in the PGC-1α promoter in response to muscle contraction [22]. Interestingly, both MEF2 and ATF2 are activated by p38 mitogen-activated protein kinase (MAPK) [25,26], indicating that this protein kinase is intimately involved in the regulation of PGC-1α transcription. Consistently, Pogozelski *et al.* [27] demonstrated that muscle-specific deletion of p38γ MAPK completely blocked the up-regulation of PGC-1α induced by exercise. Accordingly, activation of p38 MAPK by reactive oxygen species (ROS) in response to exercise training is indeed an important mechanism regulating PGC-1α expression in skeletal muscle [28,29]. Similarly, PKD activation is required for the activation of the PGC-1α promoter because the expression of a catalytic inactive form of PKD can fully prevent the effects of muscle contraction on PGC-1α expression [22]. By contrast to p38 MAPK and PKD, the relevance of AMPK and CaMKII in the regulation of PGC-1α expression by exercise has been more difficult to elucidate. For instance, muscle-specific deletion of the β1 and β2 subunits of AMPK significantly decreases the expression and activity of different mitochondrial enzymes during basal conditions, although it paradoxically results in higher levels of PGC-1α mRNA [30] while, to our knowledge, no studies exist that investigated the requirement for CaMKII for exercise-mediated regulation of PGC-1α expression. Interestingly, endurance exercise induces the activation p53 [31], suggesting that it could promote the activation of the PGC-1α promoter. Moreover, a single bout of low-intensity endurance exercise results in higher mRNA levels of the transcription factors PPARδ and FOXO1 [32], both of which have been described to bind to the PGC-1α promoter [17,19]. In particular, PPARδ in complex with RXRα can bind the PPAR response elements (PPRE) of the PGC-1α promoter and increase its transcriptional activity [19]. Considering that PGC-1α co-activates PPARδ [33], this could represent another positive feedback loop in the transcriptional regulation of this coactivator, but it is currently unknown whether PPARδ plays a significant role during endurance exercise.

More recently, novel mechanisms involving epigenetic modifications of the DNA and post-transcriptional regulation by microRNAs have been proposed to modulate PGC-1α transcription in response to exercise and pathological conditions. Interestingly, Barrès *et al.* [34] demonstrated that the DNA methyltransferase 3B (DNMT3B) could repress PGC-1α expression through non-CpG dinucleotide methylation of its promoter in human skeletal muscle. Importantly, the PGC-1α promoter was found to be hyper-methylated in skeletal muscle from impaired glucose-tolerant and type 2 diabetic patients, showing a negative correlation with PGC-1α mRNA levels and a decreased expression of several mitochondrial enzymes [34]. Inversely, acute exercise in human skeletal muscle and *ex vivo* electrical stimulation of mouse skeletal muscle induce a significant decrease in the methylation levels of the PGC-1α promoter, which precede the increase in its transcript levels [35]. Finally, physical activity and inactivity induce the down- and upregulation of the microRNA miR-696, respectively, which negatively regulate PGC-1α expression in skeletal muscle [36]. Thus, overall, the PGC-1α promoter contains a high number of different transcription factor binding sites that control PGC-1α expression while the activation of additional signal pathways represent a potential mechanism by which PGC-1α transcript levels are further fine-tuned (Fig. 1a).

Post-translational regulation of PGC-1α in skeletal muscle

In skeletal muscle, PGC-1α is a direct target of both AMPK and p38 MAPK, which both phosphorylate it at distinct sites and hereby induce its activation [37,38]. Interestingly, it has been suggested that p38 MAPK-mediated phosphorylation and thereby stabilization of the PGC-1α protein precede transcriptional induction of PGC-1α gene expression in contracting muscle [39]. The balance between the acetylation and

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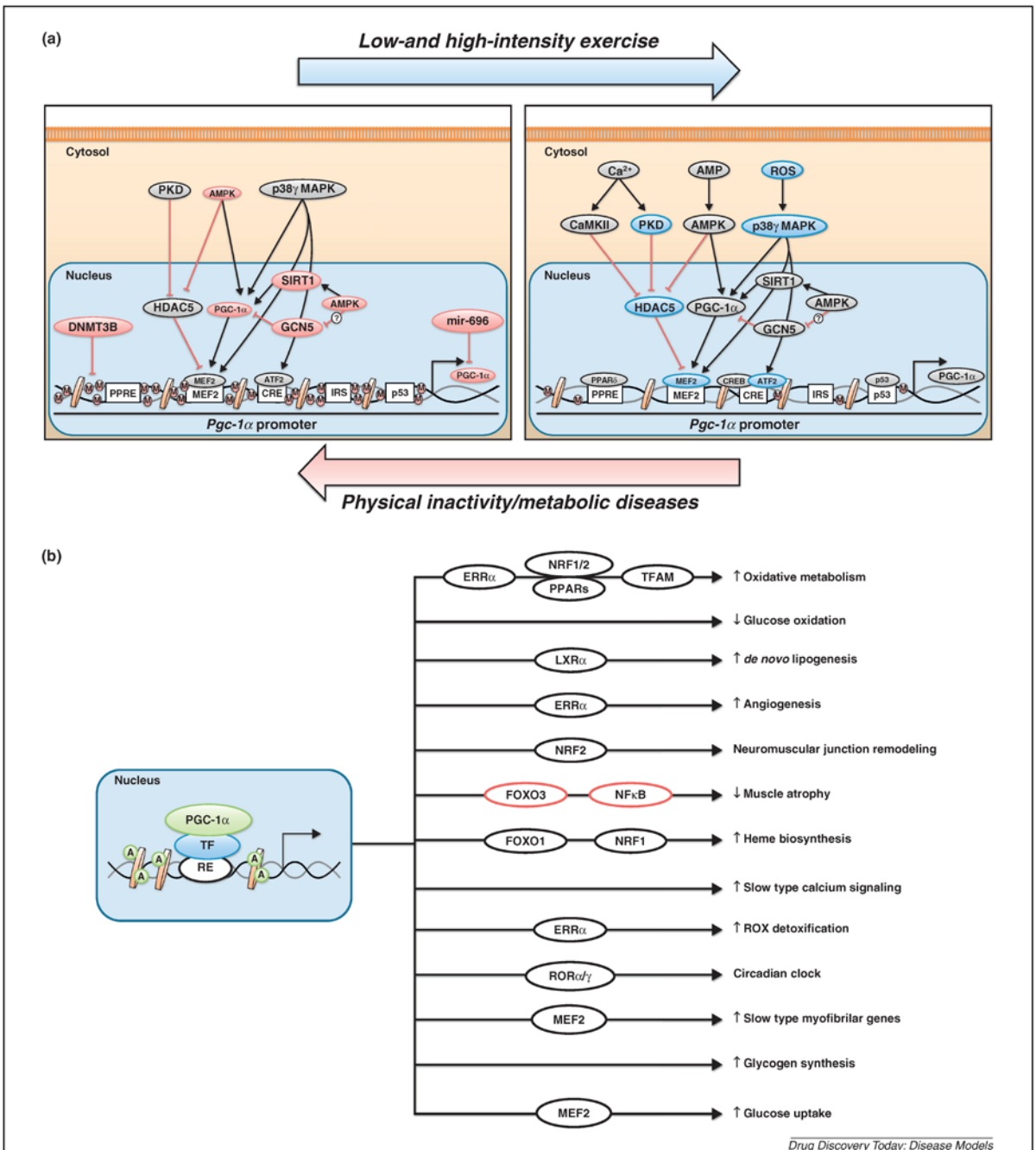

Figure 1. Schematic representation of the signal pathways regulating PGC-1α expression and activity in skeletal muscle cells. **(a)** Under pathological conditions such as a sedentary life style and obesity, PGC-1α expression is impaired due to aberrant activity and expression of negative and positive regulators (red circles) of this coactivator. By contrast, skeletal muscle contraction during low- and high-intensity exercise lead to the activation and inhibition of a subset of essential (blue circles) and accessory (grey circles) molecules involved in the control of the PGC-1α promoter activity, in addition to its post-translational modifications and thus protein activity. Consequently, physical activity results in the activation and up-regulation of this coactivator, thereby promoting an improvement of skeletal muscle metabolic fitness. Black and red arrows show activation and inhibition, respectively. **(b)** PGC-1α drives the activation of different putative transcription factors (TF), thus regulating different aspects of muscle physiology. In this figure, some of the most relevant effects of PGC-1α on skeletal muscle are shown (for more details, see Ref. [85]). *Abbreviations*: ERRα, estrogen-related receptor α; NRF1/2, nuclear respiratory factor 1 and 2; FoxO1/3, forkhead box O1/3; TFAM, mitochondrial transcription factor A; MEF2, myocyte enhancer factor 2; PPAR, peroxisome proliferator-activated receptor; RORα/γ, retinoic acid receptor-related orphan receptor α/γ; LXRα, liver X receptor α; NFκB, nuclear factor-kappaB; RE, TF response elements.

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deacetylation of PGC-1α is controlled by the deacetylase sirtuin 1 (SIRT1) and the acetyl transferase general control of amino-acid synthesis 5 (GCN5), respectively [40,41]. SIRT1-mediated deacetylation activates PGC-1α whereas GCN5 activity reverses this effect [40,41]. Interestingly, activation of AMPK is a prerequisite for SIRT1-controlled deacetylation of PGC-1α in response to exercise [42]. Recently however, the relevance of SIRT1 for the modulation of muscle PGC-1α acetylation by exercise has been questioned because the skeletal muscle-specific genetic inactivation of SIRT1 did not affect deacetylation of PGC-1α and subsequent induction of its target genes in mouse skeletal muscle after acute and chronic exercise [43]. In this study, reduced interaction with GCN5 has been proposed as the main mechanism controlling the acetylation status of PGC-1α [43]. Interestingly, AMPKγ3 knockout mice exhibit impaired decrease of PGC-1α acetylation levels induced by exercise [42], suggesting a potential role of AMPK in the regulation of PGC-1α acetylation by GCN5 rather than its deacetylation by SIRT1.

Effects of low- versus high-intensity exercise on PGC-1α

Endurance exercise

Physical activity represents one of the strongest physiological stimuli to promote skeletal muscle oxidative metabolism and stimulate PGC-1α expression. Initially, studies performed in rat skeletal muscle showed that three to seven days of low-intensity swimming training could efficiently induce a ~2-fold increase in PGC-1α mRNA and protein content [44,45]. Acutely, the completion of a total of six hours of low-intensity swimming or running resulted in a significant two to seven fold increase in PGC-1α transcript and protein levels [45–47]. Similar to rodents, a single bout of endurance exercise also results in a 4–12-fold increase of PGC-1α mRNA in human skeletal muscle, an effect that is observed immediately after exercise up to three to eight hours. Later, PGC-1α expression returns to basal levels within 4–24 hours post-exercise [31,32,48–51]. The magnitude by which acute endurance exercise modulates human skeletal muscle PGC-1α protein content is not well known, with some studies showing no increase three hours post-exercise [31,49] and others reporting a slight increase after endurance exercise to exhaustion [51], indicating that the intensity at which exercise is performed could be an important factor. However, in humans, endurance training over a period of six weeks induces a significant ~2-fold increase of both mRNA and protein levels of muscle PGC-1α [52]. Interestingly, the activation of an alternative promoter of PGC-1α leads to the expression of different splice variant of this coactivator (PGC-1α-a, PGC-1α-b and PGC-1α-c) in skeletal muscle [53,54]. Of these three transcript variants, PGC-1α-b exhibits a higher sensitivity to exercise and was indeed strongly upregulated by endurance exercise in mouse skeletal muscle [53,54].

Furthermore, there is some evidence implying cytoplasmic-nuclear translocation to contribute to the regulation of PGC-1α, however this concept remains controversial. Interestingly, a splice variant called novel truncated PGC-1α (NT-PGC-1α), which lacks key domains in nuclear localization, is thought to shuttle between cytoplasm and the nucleus [55]. Other studies imply that in rat skeletal muscle, low-intensity swimming significantly increased the nuclear abundance of 'normal' PGC-1α immediately after exercise [39]. In human skeletal muscle, a similar effect was reported, in which a ~54% increase in nuclear PGC-1α has been detected immediately after a single bout of endurance exercise [56]. In these studies, PGC-1α nuclear translocation after endurance exercise correlates with an increase in the phosphorylation levels p38 MAPK and its substrate ATF2 [39,56]. Interestingly, whereas McGee and Hargreaves [57] did not detect PGC-1α nuclear translocation after a single bout of endurance exercise, they found a decrease and increase in the interaction between HDAC5 and PGC-1α with MEF2, respectively. Moreover, the exchange between HDAC5 and PGC-1α was also associated with an increase in p38 MAPK and MEF2 phosphorylation [57]. Furthermore, endurance exercise has been recently described to also increase PGC-1α levels in the mitochondrial matrix immediately after and three hours post-exercise [58]. Specifically, PGC-1α was detected in complex with the mitochondrial transcription factor A (TFAM) at the D-loop region of the mitochondrial DNA in the mitochondrial matrix and this interaction was further enhanced by endurance exercise [58]. Therefore, endurance exercise activates a broad spectrum of proteins involved in the expression, activation and maybe also the cellular localization of PGC-1α.

Low-volume high-intensity interval training

During the past years, low-volume, high-intensity interval training (HIT) has been proposed as a time-efficient alternative to traditional endurance training to improve whole body and skeletal muscle oxidative metabolism [59]. For example, six weeks of low-volume HIT result in a higher VO_{2peak}, lower respiratory exchange ratio during exercise and increased activity of different mitochondrial enzymes (e.g. citrate synthase) in skeletal muscle [60]. Interestingly, the coactivator PGC-1α has also been considered as a potential mediator of the adaptations of energy metabolism to this kind of exercise training [59]. Accordingly, similar to low-intensity swimming, a single bout of high-intensity swimming in rats also induces a robust increase in skeletal muscle PGC-1α protein levels [61]. Importantly, in humans, a single bout of low-volume HIT has been showed to promote a 2–10-fold increase in skeletal muscle PGC-1α mRNA three to four hours post-exercise [31,62–64]. It seems that the transient increase in PGC-1α mRNA after HIT precedes the subsequent increase of its protein levels 24 hours post-exercise [63,64]. In fact,

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three hours after acute HIT, no increase in PGC-1α protein is detected [31,62,64], further suggesting that mRNA levels need to be elevated before an increase at the protein level can be observed. In the long term, cycling low-volume HIT over a period of two to six weeks can induce a significant ~2-fold increase in total and nuclear muscle PGC-1α protein levels in recreationally active and sedentary subjects, thus to a similar extent as classical endurance training [60,65,66]. Although HIT and endurance exercise result in similar effects on PGC-1α expression [60], the fact that the training volume during HIT is substantially lower strongly suggests that PGC-1α is highly sensitive to exercise intensity. Recent published data from rodents and humans studies further support this idea showing that PGC-1α mRNA is increased to a higher extent by high-intensity compared to low-intensity exercise [53,67,68].

Both chronic and acute low-volume HIT have been claimed to stimulate the translocation of PGC-1α from the cytosol to the nucleus, but different to endurance exercise, the acute effect is only detected three hours post-exercise in HIT [64,66]. The translocation of PGC-1α induced by HIT is associated with higher p38 MAPK phosphorylation levels [64]. Interestingly, besides p38 MAPK, acute HIT also increases AMPK, CaMKII and p53 phosphorylation in skeletal muscle [31,62,64,69]. Therefore, acute and chronic HIT activate the same signal pathways as endurance exercise (Fig. 1a), but it has been proposed that exercise at high intensity results in stronger effects overall. In fact, Egan *et al.* [68] compared cycling exercise trials at 40 and 80% of the VO_{2peak} and showed that only high-intensity exercise induced a significant increase in AMPK, CaMKII, ATF2 and HDAC5 phosphorylation in skeletal muscle. Moreover, although both low- and high-intensity cycling increased PGC-1α mRNA, the effects of cycling at 80% of the VO_{2peak} were significantly higher than that at 40% of the VO_{2peak} [68]. Interestingly, HIT elevates SIRT1 expression in skeletal muscle [66], suggesting that this kind of training modulates PGC-1α acetylation. Also similar to endurance training, HIT upregulates PPARδ in skeletal muscle [63]. Finally, high-intensity exercise is more efficient in decreasing the methylation levels of the PGC-1α promoter [35]. These data suggest that the activation of additional signal pathways in response to high-intensity compared to low-intensity exercise would promote a higher increase of PGC-1α activity and expression, representing thus a more efficient strategy in terms of time and potency compared to traditional endurance training.

Impairment of exercise effects on PGC-1α in metabolic diseases

Low expression levels of skeletal muscle PGC-1α are characteristic of physically inactive people and type 2 diabetes patients [3,14,15,70]. Accordingly, modulation of the PGC-1α axis in skeletal muscle in metabolic diseases has been proposed to be impaired at different levels. De Filippis *et al.* [71] demonstrated that insulin resistant skeletal muscle is also 'exercise resistant', because insulin resistant people exhibit diminished and delayed increase of PGC-1α expression in skeletal muscle after acute high-intensity exercise. However, endurance exercise at 40% or 70% of the VO_{2max} results in a normal increase in PGC-1α mRNA in human skeletal muscle from obese and type 2 diabetes patients [72]. Interestingly, only seven days of bed rest completely blunt the induction of PGC-1α by acute exercise in human skeletal muscle [73]. In animals, an attenuated or abolished up-regulation of PGC-1α mRNA and its target genes after chronic exercise has been reported in skeletal muscle from both mouse and rat models suffering from the metabolic syndrome [74,75]. The mechanism behind this response has been linked to impaired activation of AMPK by exercise, for example in acutely exercised mice fed a high fat diet [76]. In humans however, diminished induction of PGC-1α by high-intensity exercise was associated with normal activation of AMPK in insulin resistant subjects [71], while normal induction of PGC-1α by low-intensity exercise was associated with impaired activation of AMPK in obese and type 2 diabetes patients [72], although interestingly, skeletal muscle from *ob/ob* mice shows no increase in AMPK phosphorylation, SIRT1 protein and PGC-1α mRNA levels after chronic exercise [75]. Furthermore, the decrease of PGC-1α acetylation induced by endurance training was impaired in *ob/ob* mice skeletal muscle [75]. In *ob/ob* mice skeletal muscle, *ex vivo* muscle contraction induces a slightly attenuated increase of p38 MAPK phosphorylation although the effect was not significant compared to that observed in wild type animals [77]. However, despite of the lower levels of PGC-1α expression in type 2 diabetes patients [14], p38 MAPK phosphorylation seems to be elevated [78]. Altogether it appears that the effects of physical exercise on PGC-1α are negatively affected by metabolic diseases, affecting the expression and activation of this coactivator (Fig. 1a).

Conclusions

Low- and high-intensity exercise positively modulates PGC-1α activity and expression through different signal pathways, in which p38 MAPK seems to play a central role (Fig. 1a). This response to exercise seems to be impaired by physical inactivity and metabolic diseases (Fig. 1a). Upon activation, PGC-1α not only enhances oxidative metabolism, but also prevents muscle atrophy and improves ROS detoxification among other effects (Fig. 1b). Hence, the modulation of this coactivator in muscle cells is an attractive approach for the treatment of metabolic diseases [79], but it should be noted that as part of exercise-induced muscle plasticity, PGC-1α also enhances anabolic process such as *de novo* lipogenesis [80] (Fig. 1b). Accordingly, elevation of muscle PGC-1α in sedentary, high fat-fed mice accelerates the development of

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insulin resistance [81]. Importantly however, when combined with bona fide exercise, PGC-1α over-expression seems to be an efficient strategy to improve metabolic fitness under pathological conditions [82,83]. Importantly, HIT appears to be a more efficient approach to induce PGC-1α in skeletal muscle and improve whole body energy metabolism [59]. In fact, low-volume HIT efficiently reduces blood glucose levels in type 2 diabetic patients [84], further supporting its therapeutic effect. Therefore, the design of new therapies aiming to potentiate the effects of exercise on PGC-1α represents a potential path to combat non-communicable metabolic diseases.

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APPENDIX 1: New insights in the regulation of skeletal muscle PGC-1α by exercise and metabolic diseases

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APPENDIX 2: Skeletal muscle PGC-1α controls whole-body lactate homeostasis through estrogen-related receptor α-dependent activation of LDH B and repression of LDH A

Skeletal muscle PGC-1α controls whole-body lactate homeostasis through estrogen-related receptor α-dependent activation of LDH B and repression of LDH A

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The peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α) controls metabolic adaptations. We now show that PGC-1α in skeletal muscle drives the expression of *lactate dehydrogenase (LDH) B* in an estrogen-related receptor-α–dependent manner. Concomitantly, PGC-1α reduces the expression of *LDH A* and one of its regulators, the transcription factor *myelocytomatosis oncogene*. PGC-1α thereby coordinately alters the composition of the LDH complex and prevents the increase in blood lactate during exercise. Our results show how PGC-1α actively coordinates lactate homeostasis and provide a unique molecular explanation for PGC-1α–mediated muscle adaptations to training that ultimately enhance exercise performance and improve metabolic health.

transcriptional regulation | mitochondria | oxidative metabolism | metabolic reprogramming | muscle plasticity

Skeletal muscle adaptations to endurance exercise are largely mediated by peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α; reviewed in refs. 1, 2). Elevated expression of PGC-1α in skeletal muscle is consequently sufficient to mimic an endurance-trained phenotype (3), which is partially achieved by a pronounced fiber-type switching from fast, glycolytic fibers toward slow, oxidative fibers, including slow fiber type-specific calcium handling (4, 5). Additionally, PGC-1α improves oxygen supply to muscle by promoting angiogenesis (6) and remodels the neuromuscular junction (7). Most importantly, however, PGC-1α alters skeletal muscle metabolism by inducing mitochondrial biogenesis (5, 8) and promoting lipid oxidation (9). However, PGC-1α also drives anabolic processes like lipid (10) and glucose refueling in skeletal muscle (11). Concomitantly, substrate flux through glycolysis is inhibited by elevated levels of PGC-1α (11) while pentose phosphate pathway activity is increased (10).

Exercise performance and lactate synthesis are strongly linked (12). For example, strenuous exercise leads to the production of lactate in working skeletal muscles and to a steady increase in blood lactate levels. Inversely, regular training counters the excessive increase in blood lactate levels as indicated by the reduced blood lactate levels in trained athletes during acute exercise bouts (13). Similarly, reduced basal and postexercise blood lactate levels have previously been reported in mice with elevated levels of PGC-1α in skeletal muscle (11). However, it is unclear whether this observation is caused by diminished lactate generation, enhanced lactate clearance, or both. Moreover, the molecular mechanisms that underlie the putative PGC-1α–mediated reduction in blood lactate levels are unknown. We hypothesized that PGC-1α actively drives a transcriptional program to enhance lactate metabolism and have now unraveled the direct molecular mechanism by which PGC-1α remodels lactate homeostasis.

Results

PGC-1α in Skeletal Muscle Controls Whole-Body Lactate Levels. As exhaustive exercise boosts blood lactate levels, we first investigated the impact of PGC-1α in skeletal muscle on whole-body lactate homeostasis. During a maximal endurance performance test, blood lactate levels continuously increased in WT mice and returned to basal levels within 40 min following fatigue-induced exercise cessation (Fig. 1 *A* and *B*). In stark contrast, blood lactate levels did not substantially increase in muscle-specific PGC-1α transgenic (MPGC-1α TG) animals during exercise at any point (Fig. 1 *A* and *B*). As skeletal muscle is the main site for lactate production, we next assessed the mRNA expression of *lactate dehydrogenase (LDH) A*, which encodes for the LDH muscle subunit (LDH M) that metabolizes pyruvate to lactate (14). LDH A mRNA expression in tibialis anterior was decreased by 53.5% ($P < 0.001$) in MPGC-1α TG animals compared with control littermates (Fig. 1*C*). Consistently, the enzymatic activity of LDH-mediated pyruvate-to-lactate conversion was diminished by 40.1% ($P < 0.001$) in skeletal muscle of transgenic animals (Fig. 1*D*). These differences in LDH A mRNA expression and activity between MPGC-1α TG and control animals persisted in response to an acute bout of exercise (Fig. S1 *A* and *B*).

To test whether lactate removal is also altered by PGC-1α, lactate tolerance tests were performed. The excursion (Fig. 1*E*) and total area under the curves (Fig. 1*F*) clearly show that MPGC-1α TG animals more efficiently cleared lactate from the circulation compared with control animals. To further characterize the enhanced capacity of muscle for lactate clearance, we determined the mRNA expression of *LDH B*, which encodes for the LDH heart subunit (LDH H) that drives the conversion of lactate to pyruvate (14). LDH B mRNA expression in tibialis anterior was significantly elevated in MPGC-1α TG animals by 110.2% ($P < 0.001$; Fig. 1*G*). Moreover, the enzymatic activity of LDH to convert lactate to pyruvate was enhanced by 60.7% ($P < 0.01$; Fig. 1*H*). In response to exercise, LDH B mRNA levels did not change, but LDH B activity further increased in MPGC-1α TG animals (Fig. S1 *C* and *D*).

PGC-1α Drives LDH Isoenzyme-Type Switching in Skeletal Muscle. The tetrameric LDH complex consists of LDH M and H subunits (14, 15). According to its subunit composition, the complex is referred to as LDH 1 (H_4), LDH 2 (H_3M), LDH 3 (H_2M_2), LDH 4 (HM_3), or LDH 5 (M_4) (14, 16). To gain insights into the individual isoenzyme composition of WT and MPGC-1α TG animals, we performed native gel electrophoresis of muscle homogenates (Fig. 1*I*). MPGC-1α TG animals displayed a pronounced shift toward an isoenzyme composition enriched in LDH H subunits, namely LDH 1, 2, and 3 (Fig. 1 *I* and *J*). In contrast, protein levels of LDH 5, which exclusively contains LDH M subunits, were reduced in MPGC-1α TG animals (Fig. 1 *I* and *J*).

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The authors declare no conflict of interest.

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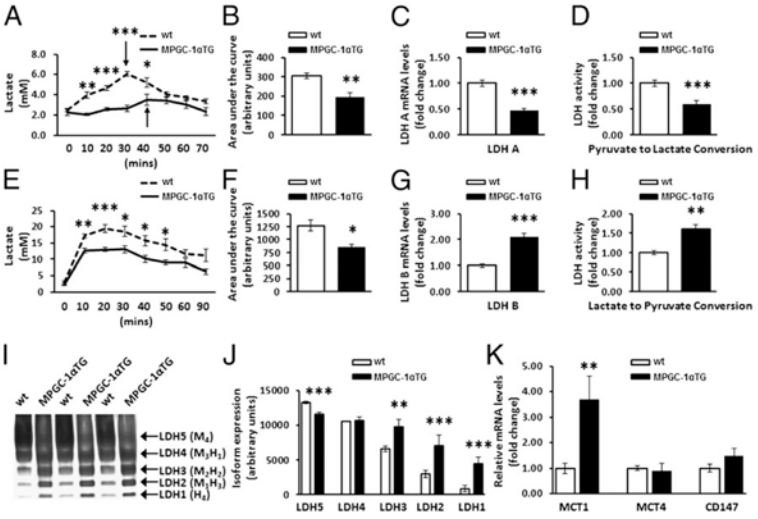

Fig. 1. Muscle PGC-1α controls blood lactate levels by shifting LDH composition. (*A* and *B*) Blood lactate excursion curves of WT (black dotted line) and MPGC-1α TG (black continuous line) animals during maximal endurance test (*A*) and corresponding area under the curve (*B*). Arrows indicate the time point of exhaustion. (*C* and *D*) LDH A mRNA levels (*C*) and activity (*D*) in tibialis anterior muscle. (*E* and *F*) Lactate tolerance test excursion curves of WT (dotted line) and MPGC-1α TG (continuous line) animals and (*E*) corresponding area under the curve (*F*). (*G* and *H*) LDH B mRNA levels (*G*) and activity (*H*) in tibialis anterior muscle. (*I*) LDH isoenzyme composition in tibialis anterior of MPGC-1α TG and control littermates. (*J*) Quantification of the LDH isoenzyme composition. (*K*) Relative mRNA levels of MCT1, MCT4, and CD147 in MPGC-1α TG and control littermates. All values are expressed as means ± SE ($n = 8$ per group; $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$).

Elevated Capacity for Lactate Uptake in MPGC-1α TG Animals. Given the enhanced potential of skeletal muscle of MPGC-1α TG animals to convert lactate to pyruvate, we also investigated the mRNA expression of key genes implicated in lactate import and export in this organ. Relative mRNA levels of monocarboxylate transporter 1 (MCT1), which mediates lactate import into muscle (17) and mitochondria (18), was significantly increased in MPGC-1α TG animals (Fig. 1*K*). In contrast, the mRNA levels of MCT4, which exports lactate (17), and CD147, an ancillary molecule of MCT1 and MCT4 (19), were unaltered (Fig. 1*K*). The elevated muscle MCT1 content in MPGC-1α TG animals is consistent with the enhanced whole-body lactate removal during lactate tolerance tests and with the previous demonstration that PGC-1α increases lactate uptake into skeletal muscle (20).

PGC-1α Promotes LDH B Transcription by Coactivating Estrogen-Related Receptor-α on LDH B Promoter. Biocomputational predictions were then applied to unravel the potential molecular mechanism by which PGC-1α promotes *LDH B* and *MCT1* transcription. To this end, microarray data from differentiated C2C12 myotubes adenovirally infected with GFP or bicistronic GFP-PGC-1α (21) were analyzed. We first used Motif Activity Response Analysis (MARA) to identify motifs that are most active following overexpression of PGC-1α (Fig. 2*A*). Then, we screened the *LDH B* and the *MCT1* promoters for putative binding sites for transcription factors. No transcription factors were found in the *MCT1* promoter that showed a high activity in MARA. In contrast, there were two transcription factors that displayed very high activities in MARA and concomitantly were predicted to bind to the *LDH B* promoter, namely the estrogen-related receptor-α (ERRα) and retinoid X receptors (RXRs; Fig. 2*A* and Fig. S2). Recruitment of ERRα to the *LDH B* promoter has previously been reported in global ChIP-on-ChIP assays in mouse liver cells; however, the functional consequences of this observation have not been investigated (22).

To experimentally verify our biocomputational predictions, pharmacological inhibitors and siRNA silencing technology for ERRα and RXR were used. In differentiated C2C12 myotubes, PGC-1α led to a very robust induction of LDH B, which was completely prevented by the ERRα inverse agonist XCT-790 (Fig. 2*B*). Inhibition of RXRs by HX-531 likewise resulted in a significant reduction in PGC-1α–mediated induction of LDH B, but to a smaller extent compared with ERRα inhibition (Fig. 2*B*). Similarly, RNA silencing of ERRα in myoblasts led to a significant reduction in LDH B expression, whereas silencing of RXRα or -β reduced LDH B expression to a smaller extent (Fig. S3).

Interestingly, analysis of the LDH B promoter (www.swissregulon.unibas.ch) (23) identified overlapping binding sites for ERRα and RXR in the LDH B promoter (Fig. S4*A*). By using chromatin immunoprecipitated DNA from muscle samples, we confirmed that PGC-1α is recruited to the ERRα/RXR binding site on the proximal *LDH B* promoter (Fig. 2*C*). Moreover, activation of a 684-bp fragment of the *LDH B* promoter by PGC-1α in reporter gene assays was dependent on functional integrity of the ERRα response element (Fig. S4 *B* and *C*). In contrast, association of PGC-1α with the recently identified distal myocyte enhancer factor 2 (MEF2) binding site in the *LDH* promoter region (24) was not elevated in MPGC-1α TG mice compared with WT controls (Fig. 2*C*). In line with this finding, pharmacological inhibition of the MEF2 upstream activator peroxisome proliferator-activated receptor-β/-δ did not prevent PGC-1α–mediated LDH B transcription (Fig. S5).

Decreased Levels of LDH A and Myelocytomatosis Oncogene in MPGC-1α TG Animals. *LDH A* transcription is regulated by hypoxia-inducible factor-1α (HIF-1α) and myelocytomatosis oncogene (Myc) (25). Although HIF-1α mRNA levels were similar in WT and transgenic animals, Myc transcript levels were reduced in skeletal muscle of MPGC-1α TG mice compared with controls (Fig. 2*D*). Consistently, protein levels of Myc were also significantly decreased in MPGC-1α TG mice compared with their control littermates (Fig. 2 *E* and *F*). Interestingly, the administration of AM6-36, a drug that binds to RXR responsive elements and mimics the effects of RXRs, has recently been reported to abrogate Myc expression (26, 27). Indeed, also in our experimental context, pharmacological inhibition of RXRs by HX-531 was sufficient to restore Myc (Fig. 2*G*) and consequently LDH A expression (Fig. 2*H*).

Increased Levels of MCT1 and Decreased Levels of LDH A Are Not Caused by Elevated LDH B. To investigate whether the increased MCT1 and the decreased LDH A levels constitute a secondary effect of LDH B induction, we next inhibited LDH B transcription in differentiated C2C12 myotubes. Although LDH B transcription was successfully reduced in this experiment (Fig. S6*A*), the effect of PGC-1α on MCT1 and LDH A persisted (Fig. S6 *B* and *C*).

Selective Regulation of Muscle Lactate Homeostasis by PGC-1β. Because PGC-1α and -β exert similar effects, we also examined the

PHYSIOLOGY

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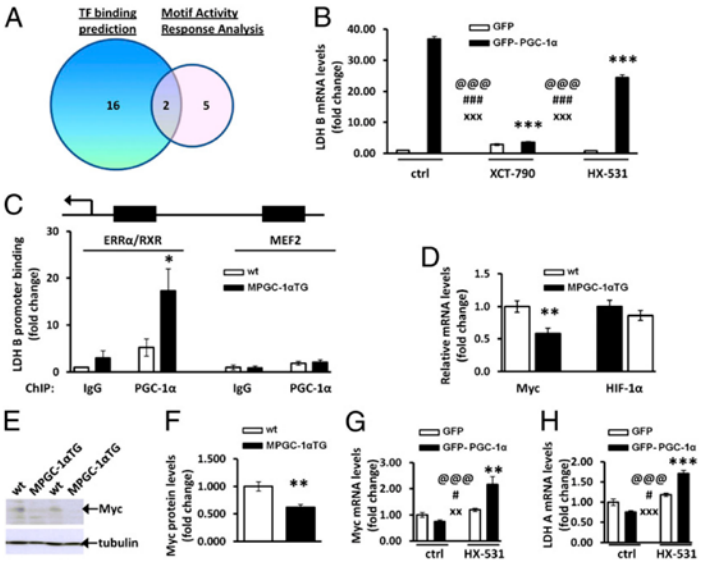

Fig. 2. PGC-1α interacts with ERRα on the LDH B promoter. (*A*) Venn diagram with red circle denoting the number of transcription factors from MARA of microarray data from C2C12 myotubes following adenoviral overexpression of GFP or bicistronic PGC-1α-GFP. Only transcription factors with *Z*-scores set above a cutoff of 2 were considered. The 18 transcription factors predicted to bind to LDH B are shown in the blue circle. Two predicted transcription factors were simultaneously found by MARA. (*B*) Relative mRNA levels of LDH B in C2C12 myotubes following adenoviral overexpression of GFP or bicistronic PGC-1α-GFP and in the absence or presence of XCT-790 or HX-531. All values are expressed as means ± SE ($n = 6$ per group). @, Effect of PGC-1α (GFP vs. PGC-1α–GFP); #, effect of treatment (DMSO vs. XCT-790 or HX-531); x, interaction. Triple symbols indicate $P < 0.001$. Symbols at left refer to the comparison of XCT-790–treated C2C12 myotubes vs. controls. Symbols at right refer to the comparison of HX-531–treated C2C12 myotubes vs. controls (*Results from post hoc analysis; ***$P < 0.001$ vs. GFP-PGC-α untreated). (*C*) ChIP assay on mouse skeletal muscle. Recruiting of PGC-lα to the ERRα and RXR or to the MEF2 binding site in the LDH B promoter of MPGC-1α TG mice and control animals. (*D*) Relative mRNA expression levels of Myc and Hif-1α. (*E* and *F*) Representative Western blot of Myc (*E*) and corresponding quantification (*F*). (*G* and *H*) Relative mRNA levels of Myc (*G*) and LDH A (*H*) in muscle cells following adenoviral overexpression of GFP or bicistronic PGC-1α–GFP and in the absence or presence of HX-531. All values are expressed as means ± SE ($n = 6$ per group). @, Effect of PGC-1α (GFP vs. PGC-1α-GFP); #, effect of treatment (DMSO vs. HX-531); x, interaction. Single symbols, $P < 0.05$; double symbols, $P < 0.01$; triple symbols, $P < 0.001$ (*Results from post hoc analysis; **$P < 0.01$ vs. GFP-PGC-α untreated).

potential impact of PGC-1β on LDH B, LDH A, and MCT1 mRNA levels. Overexpression of PGC-1β in differentiated C2C12 myotubes resulted in elevated levels of LDH B (Fig. S6*D*), but the effect was less pronounced than with PGC-1α (Fig. 2*B*). LDH A levels were reduced, but MCT1 levels were unaltered following overexpression of PGC-1β (Fig. 6 *E* and *F*). Importantly, however, PGC-1β is not induced in skeletal muscle by exercise (28), and might thus be less relevant in mediating altered lactate handling in the adaptation to exercise.

PGC-1α Is Important for Regulation of LDH B Transcription and Proper Lactate Handling. To corroborate the importance of PGC-1α in regulating lactate homeostasis, and especially LDH B expression, we then studied mice with a muscle-specific KO of *PGC-1α* (MPGC-1α KO). MPGC-1α KO animals fatigued rapidly and accumulated blood lactate to a higher extent than their control littermates in endurance exercise trials (Fig. 3 *A* and *B*). LDH A mRNA showed a nonsignificant trend toward higher expression in MPGC-1α KO animals (Fig. 3*C*). Myc mRNA did not differ between MPGC-1α KO and control animals (Fig. S7). In terms of lactate tolerance, MPGC-1α KO animals were less efficient in lactate clearance upon injection of lactate (Fig. 3 *D* and *E*). Consistently, MPGC-1α KO animals displayed a significant reduction of LDH B expression (Fig. 3*F*). Moreover, the analysis of LDH isoenzyme composition revealed lower levels of isoenzymes typically enriched in H subunits (Fig. 3 *G*– *I*). Together, these data clearly demonstrate that muscle PGC-1α is important for the transcription of LDH B and for maintaining whole-body lactate homeostasis.

Acute bouts of exercise had no effect on LDH A mRNA expression, but led to a significant increase in LDH A activity in control animals (Fig. S8 *A* and *B*). In MPGC-1α KO mice, LDH A activity was already elevated at the basal state and was not further inducible by exercise (Fig. S8*B*). LDH B mRNA levels and activity were lower in MPGC-1α KO animals, and these differences persisted after exercise (Fig. S8 *C* and *D*).

Low Blood Lactate Levels in MPGC-1α TG Are Not Caused by Altered Metabolism in Heart or Liver. Besides skeletal muscle, liver and heart play important roles in buffering blood lactate levels (29, 30). In the Cori cycle, the liver takes up lactate and converts it into pyruvate, which then serves as a substrate for gluconeogenesis. Glucose can subsequently be transported back to different organs including skeletal muscle, where it is again metabolized to lactate. In contrast, the heart directly uses lactate as energy source (29). Lactate is completely oxidized to water and carbon dioxide in the heart and is even preferred as energy source compared with glucose (29). We thus analyzed the mRNA levels of genes involved in lactate production, removal, and transport in these two tissues. In the heart, no differences in mRNA expression between WT and transgenic animals were observed (Fig. 4*A*). In the liver, there was a significant increase in CD147 mRNA levels, whereas the other genes remained unchanged (Fig. 4*B*). Analogous to our studies in skeletal muscle, we subsequently assessed the enzymatic activities of LDH in heart and liver. The conversion of pyruvate to lactate was significantly reduced in the heart (Fig. 4*C*), whereas the conversion of lactate to pyruvate was unaltered (Fig. 4*D*). In the liver, no changes in LDH activity were detectable (Fig. 4 *E* and *F*). Isoenzyme compositions between MPGC-1α TG and WT animals were undistinguishable for the heart (Fig. 4 *G* and *H*) and the liver (Fig. 4 *I* and *J*). An acute bout of exercise affected LDH in both tissues. In the heart, exercise increased LDH A activity in WT mice, but not in MPGC-1α TG mice (Fig. S9). Cardiac LDH B mRNA levels and activity were both induced by exercise to a similar extent in both genotypes (Fig. S9 *C* and *D*). The liver was less responsive to exercise, and only an elevation in LDH B mRNA levels was detected (Fig. S10).

Discussion

Disarranged metabolism in skeletal muscle is a common, early event in the etiology of obesity and type 2 diabetes. One trait of these diseases is a decreased oxidative capacity along with elevated lactate production (31, 32). Remodeling of the muscular metabolic profile might thus constitute a potential approach to treat or prevent such disorders (33). Exercise is one of the most important stimuli for improving the metabolic phenotype of skeletal muscle. Glucose and lipid metabolism are extensively altered in response to chronic exercise, largely mediated by PGC-1α (reviewed in refs.

APPENDIX 2: Skeletal muscle PGC-1α controls whole-body lactate homeostasis through estrogen-related receptor α-dependent activation of LDH B and repression of LDH A

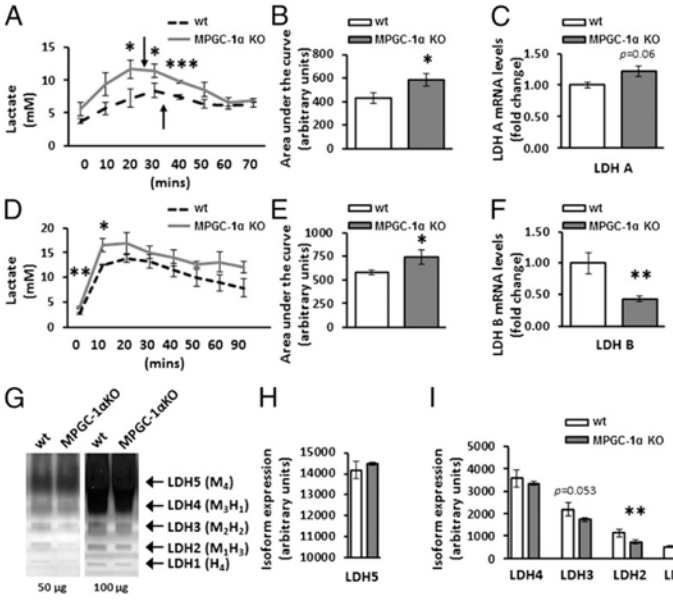

Fig. 3. PGC-1α is important for the regulation of LDH B transcription. (*A* and *B*) Blood lactate excursion curves of WT (dotted line) and MPGC-1α KO (continuous gray line) animals during maximal endurance test (*A*) and corresponding area under the curve (*B*). Arrows indicate the time point of exhaustion. (*C*) LDH A mRNA levels in tibialis anterior muscle of WT and KO mice. (*D* and *E*) Lactate tolerance test excursion curves of WT (dotted line) and MPGC-1α KO (continuous gray line) animals (*D*) and corresponding area under the curve (*E*). (*F*) LDH B mRNA levels in tibialis anterior muscle of WT and KO mice. (*G*) LDH isoenzyme composition in tibialis anterior from MPGC-1α KO and control littermates with 50 µg (*Left*) and 100 µg (*Right*) of protein extract. (*H* and *I*) Quantification of LDH isoenzyme composition. All values are expressed as means ± SE ($n = 6$ per group; $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$).

1, 2). We have now shown that PGC-1α also is a key regulator of tissue and systemic lactate homeostasis (Fig. 5).

Intriguingly, a shift toward a muscle LDH isoenzyme composition enriched in LDH H subunits has been observed in mice and humans in response to chronic electrical stimulation and regular endurance exercise, respectively (34, 35). The molecular mechanism that underlay the remodeling of the LDH complex remained, however, unresolved. Our study now demonstrates that this adaptation to exercise is directly mediated by the interaction of PGC-1α and ERRα and, moreover, that PGC-1α is required for the elevated LDH B transcription and proper lactate homeostasis during exercise. PGC-1α thus remodels LDH isoenzyme composition distinctly and independently of coactivation of peroxisome proliferator-activated receptor-β/-δ, which recently has been described to drive *LDH B* transcription via MEF2 activation in a distal enhancer region of LDH B (24). In addition, PGC-1α reduces *LDH A* gene expression, which could potentially counteract the enzymatic activity of LDH B of converting lactate into pyruvate. How PGC-1α, a transcriptional coactivator, can exert repressive effects remains elusive. A previous study revealed that stimulation of mitochondrial activity diminishes Myc expression (36). Conceivably, the PGC-1α–mediated boost in mitochondrial activity might repress Myc expression analogously. In addition, our results demonstrating restoration of Myc following RXR inhibition strongly suggest a role of RXRs in regulating Myc expression. This is further underlined by our previous data

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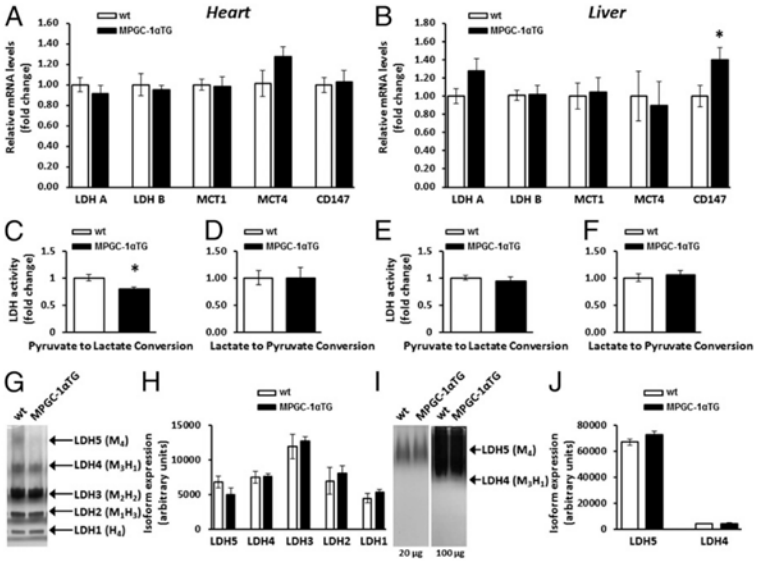

Fig. 4. Blood lactate levels are not controlled by heart or liver lactate metabolism following elevated PGC-1α expression. (*A*) Relative mRNA expression of LDH A, LDH B, MCT1, MCT4, and CD147 in the heart of MPGC-1α TG and control littermates. (*B*) Relative mRNA expression of LDH A, LDH B, MCT1, MCT4, and CD147 in the liver of MPGC-1α TG and control littermates. (*C* and *D*) Conversion of pyruvate to lactate (*C*) and reverse reaction (*D*) in the heart of MPGC-1α TG and control littermates. (*E* and *F*) Conversion of pyruvate to lactate (*E*) and reverse reaction (*F*) in the liver of MPGC-1α TG and control littermates. (*G* and *H*) LDH isoenzyme composition (*G*) and quantification (*H*) in the heart of MPGC-1α TG and control littermates. (*I* and *J*) LDH isoenzyme composition of 20 µg (*Left*) and 100 µg (*Right*) of protein extract (*I*) and quantification (*J*) in the liver of MPGC-1α TG and control littermates. All values are expressed as means ± SE ($n = 8$ per group; $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$).

APPENDIX 2: Skeletal muscle PGC-1α controls whole-body lactate homeostasis through estrogen-related receptor α-dependent activation of LDH B and repression of LDH A

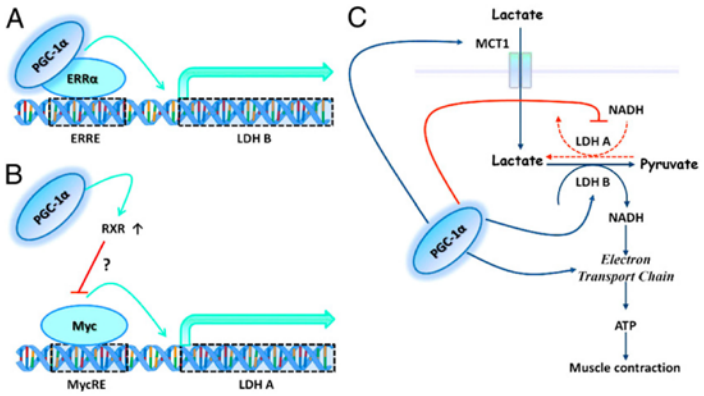

Fig. 5. PGC-1α promotes rapid energy provision by lactate oxidation. (*A*) Scheme illustrating the action of PGC-1α on the LDH B promoter. PGC-1α promotes the transcription of ERRα, which then binds to an ERRα-responsive element (ERRE) in the LDH B promoter. The subsequent activation of ERRα is enhanced by PGC-1α. (*B*) PGC-1α promotes the expression of RXRs, which, then, by unknown mechanisms, diminish Myc and thereby LDH A expression. (*C*) Scheme integrating the coordinate actions of PGC-1α on genes regulating lactate homeostasis. The enhanced transcription of MCT1 drives lactate import into skeletal muscle. Lactate is then converted to pyruvate through the action of LDH B. This process is further facilitated by the concomitant reduction in LDH A. NADH is then generated and serves as substrate for the electron transport chain. PGC-1α thereby promotes a lactate oxidizing phenotype, which is associated with improved endurance capacity and metabolic health.

showing induction of RXRs gene expression in skeletal muscle by PGC-1α (10). Furthermore, in the present study, we found RXR binding motifs to be predicted to be associated with PGC-1α–dependent gene expression in muscle cells by MARA. However, a detailed analysis of the mechanistic aspects of the repression of *LDH A* gene transcription by the transcriptional coactivator PGC-1α through Myc is hampered by the very low expression of Myc in skeletal muscle (37). Importantly, our findings that PGC-1α elevates LDH H and diminishes LDH M subunit expression are further corroborated by mouse and human studies on muscle beds with different fiber type compositions. In fact, fast-twitch muscles, which typically express low levels of PGC-1α, display high amounts of LDH M subunits, whereas the LDH complexes in slow-twitch muscles with higher expression of PGC-1α are enriched in LDH H subunits (38, 39).

Lactate production is important in working muscle to maintain glycolytic fluxes for ATP production (15, 40). Presumably, the conversion of pyruvate to lactate rapidly regenerates NAD^+ from NADH. This requirement is mainly a result of the limited pool of cytosolic NAD^+ in skeletal muscle (25). The prevention of NAD^+ regeneration by PGC-1α and the lower levels of the transcription factor Myc, a well-known activator of glycolysis (41), now suggest a molecular explanation for the reduced glycolytic rates occurring at high PGC-1α levels in muscle (11). Moreover, the potentially enhanced production of NADH by lactate oxidation triggered by PGC-1α ensures adequate levels of this cofactor for the electron transport chain and thus for ATP generation during muscle contractions. This concept of lactate as energy source during acute exercise is corroborated by previous studies showing that lactate oxidation was higher in exercising compared with resting muscle (42–44).

Importantly, our study also sheds light on the role of lactate in skeletal muscle fatigue. For many decades, lactate was considered as a side product of contracting muscle and viewed as a metabolite that causes muscle fatigue. MPGC-1α TG animals display reduced lactate levels and are more resistant to fatigue, which would further support the concept of lactate as fatiguing metabolite. Importantly, however, MPGC-1α TG animals reach exhaustion at a time point when blood lactate levels are still in a normal range, indicating that factors other than lactate contribute to muscle fatigue.

Recently, the perception of lactate as a harmful metabolite has drastically waned (12, 45). During exercise, lactate accumulation and mild lactic acidosis cause vasodilation and dissociation of oxygen from hemoglobin and thus oxygen transport to muscle. In this context, the reduction in blood lactate levels by PGC-1α might be viewed as a performance-limiting factor during strenuous exercise. However, PGC-1α likely overcomes this effect by increasing myoglobin expression (4) and enhancing angiogenesis (6).

In conclusion, we have demonstrated that PGC-1α and ERRα orchestrate the transcription of LDH B, that PGC-1α reduces the levels of the oncogenic LDH A activator Myc, and that the subsequent shift in LDH composition promotes lactate oxidation in skeletal muscle. Lactate produced during exercise by predominantly glycolytic muscles is thus used as fuel in oxidative muscle fibers that express elevated levels of PGC-1α. Importantly, our results suggest a direct regulatory role for PGC-1α in the metabolic remodeling of lactate metabolism. Thus, by driving lactate uptake and oxidation, PGC-1α promotes alternative metabolic pathways for energy generation during muscle contraction. Finally, overexpressed PGC-1α antagonizes the disarranged lactate metabolism observed under pathological conditions such as type 2 diabetes and obesity, in addition to restoring mitochondrial functions. Our results therefore provide insights into the molecular mechanisms by which PGC-1α metabolically enhances exercise performance and improves muscle function.

Materials and Methods

Animals. Male MPGC-1α TG (4), PGC-1α KO (46), and control littermates were maintained in a conventional facility with a fixed 12-h light/dark cycle on a commercial pellet chow diet and free access to tap water. Studies were performed with 8-wk-old animals according to criteria outlined for the care and use of laboratory animals and with approval of the veterinary office of the canton Basel.

Treadmill Running and Lactate Tolerance Tests. Detailed descriptions of treadmill running and lactate tolerance tests are provided in *SI Materials and Methods*.

RNA Extraction and RT-PCR. Frozen tissues were homogenized under liquid nitrogen, and total RNA was isolated by using TRIzol reagent (Invitrogen). RNA concentrations were adjusted, and reverse transcription was carried out by using random hexamer primers (Promega). Real-time PCR analysis (Power SYBR Green Master Mix; Applied Biosystems) was performed by using the StepONE Detector. Relative expression levels for each gene of interest were

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calculated with the $\Delta\Delta C_t$ method and normalized to the expression of the Tata box binding protein.

LDH Activity. Specific LDH activities (pyruvate-to-lactate and lactate-to-pyruvate conversions) were determined according to Howell et al. (47). The enzymatic reactions were carried out in the presence of 1 mmol sodium pyruvate and 150 μmol NADH or 7 mmol lactic acid and 5.5 mmol NAD^+. Changes in absorbance were assessed by a spectrophotometer at 340 nm and 30 °C.

Isoenzyme Shift. LDH isoenzyme patterns were determined colorimetrically. Protein isolation from tibialis anterior was performed as described previously (48). Twenty, 50, and/or 100 μg of protein were loaded onto a 6% (wt/vol) native polyacrylamide gel. Following electrophoresis, the gel was stained in 10 mL of staining solution containing 0.1 M sodium lactate, 1.5 mM NAD^+, 0.1 M Tris-HCl (pH 8.6), 10 mM NaCl, 5 mM $MgCl_2$, 0.03 mg/mL phenazine methosulfate, and 0.25 mg/mL nitro blue tetrazolium.

Cell Culture Experiments. C2C12 myoblasts were fused into myotubes and infected with adenovirus expressing GFP or bicistronic PGC-1α–GFP. The transfection efficiency was similar between GFP and PGC-1α–GFP, and PGC-1α–GFP efficiently increased PGC-1α protein levels (Fig. S11). In addition, cells were treated for 48 h with 0.1% DMSO (as control), 10 μM XCT-790 (Sigma-Aldrich) to inhibit ERRα, or 2 μM HX-531 (Tocris Bioscience) to inhibit RXRs, together with the corresponding adenovirus. Silencing of LDH B, ERRα, RXRα, and RXRβ was performed by using Dharmacon SMARTpool siRNAs according to the manufacturer's instructions.

ChIP, LDH B Promoter Cloning, Reporter Gene Assays, Bioinformatics, Data Analysis, and Statistics. Detailed descriptions of ChIP, LDH B promoter cloning, reporter gene assays, bioinformatics, data analysis, and statistics are provided in *SI Materials and Methods*.

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Supporting Information

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SI Materials and Methods

Treadmill Running. Treadmill running was performed on a motorized treadmill (Columbus Instruments) equipped with an electric shock grid. After acclimatization, the mice were forced to run until exhaustion. The detailed protocol was as follows: 10 m/min for 5 min and increase by 2 m/min every 5 min until 26 m/min, with 5° inclination. The speed of 26 m/min was then kept until exhaustion. The experiments were conducted according to established guidelines (1). Tissues were harvested 3 h after exercise.

Lactate Tolerance Test. Lactate tolerance test was performed following i.p. injection of 2 g/kg body weight of lactate. Blood was obtained at intervals of 10 min from the tail vein, and lactate levels were determined by using a standard lactometer (Nova Biomedical).

ChIP Assays. ChIP from muscle tissue was performed as previously described (1). In brief, muscle tissues were fixed in 1.5% formaldehyde at room temperature for 15 min. The reaction was stopped by adding glycine and tissues were homogenized on ice by using a Dounce homogenizer (20 strokes). Following cell lysis, chromatin was enzymatically sheared and subjected to immunoprecipitation by using anti– peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α) antibodies [PGC-1 (H-300); Santa Cruz Biotechnology] or unrelated IgG bound to protein G-coupled magnetic beads. Precipitated DNA was recovered and amplified by RT-PCR.

Bioinformatics. Previously published microarray data from differentiated C2C12 myotubes adenovirally infected with GFP or bicistronic PGC-1α–GFP under the CMV promoter (Gene Expression Omnibus dataset GSE4330) (2) were analyzed by using Motif Activity Response Analysis (www.mara.unibas.ch/cgi/mara). Prediction of potential transcription factors binding to the lactate dehydrogenase (LDH) B promoter were performed with SwissRegulon (www.swissregulon.unibas.ch) (3) and confirmed with Matinspector based on the TRANSFAC transcription factor binding elements database (4).

LDH B Promoter Cloning and Reporter Gene Assays. A 681-bp fragment of the LDH B promoter was amplified by using the primers LDHB prom forward (CGACGCGTGG AGGAACTGTG AAAGGCAGAA ATC) and LDHB prom reverse (GAAGATCTGC AAAGTCAGCA GGCTGCTGCT CTGC), which added an MluI and BglII restriction sites to the 5′ and 3′ ends of the PCR product, respectively. This promoter fragment was subcloned into the pGL3 basic luciferase reporter gene vector (Promega) and subsequently validated by sequencing. Site-directed mutagenesis of the estrogen-related receptor-α (ERRα) response element in the LDH B promoter was performed by using the following, overlapping mutagenesis primers: ERRa mut forward CAGCGGAAGG **GGTACC**CTAA CTTTAGAGAG C and ERRa mut reverse CTAAAGTTAG **GGTACC**CCTT CCGCTGAGGC AC. The nucleotides depicted in bold represent the mutagenesis of the core ERRα response element in the LDH B promoter (TGACCT) into a KpnI restriction site. The plasmid with the mutated promoter was verified by sequencing.

Reporter gene assays were performed on 12-well plates by using C2C12 myoblasts and COS-7 cells. Cells were transfected using Lipofectamine 2000 (Invitrogen) with 0.1 μg pRL-SV40 (E2231; Promega) and 0.7 μg pLDHB-luc or pLDHB-mut-luc. Total amount of plasmid DNA was kept constant at 1.6 μg per well by using the control plasmid pShuttle-CMV (ATCC). Twenty four hours after transfection cells were infected with GFP or PGC-1α adenovirus for another 24 h. Following a total period of 48 h, cells were lysed with 250 μL of 1× Passive Lysis 5× Buffer (Promega), and luciferase activity was measured in 75 μL of lysate in a 96-well plate by using the Dual-Glo Luciferase Assay System (Promega). Renilla (pRL-SV40) luciferase activity was used for normalization. Two independent experiments were performed in triplicate each.

Data Analysis and Statistics. All data are presented as means ± SE. Data were analyzed by Student two-tailed unpaired *t* test or Mann–Whitney test when the difference between the two SD values was significantly different. For the analysis of pharmacological inhibition, multifactorial ANOVA was performed followed by Tukey post hoc test or *t* test where indicated.

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APPENDIX 2: Skeletal muscle PGC-1α controls whole-body lactate homeostasis through estrogen-related receptor α-dependent activation of LDH B and repression of LDH A

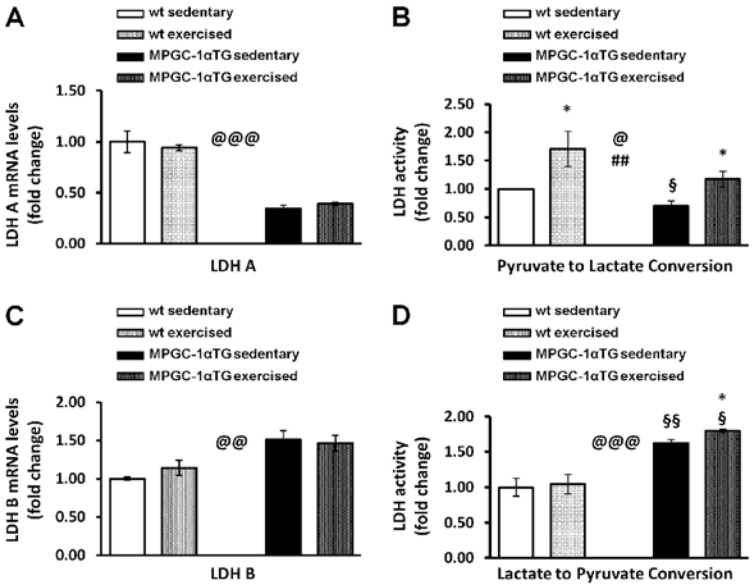


Fig. S1. Muscle LDH mRNA expression and activity in muscle-specific PGC-1α transgenic (MPGC-1α TG) mice after exercise. (*A* and *B*) Relative mRNA expression (*A*) and activity (*B*) of LDH A in gastrocnemius muscle of MPGC-1α TG and control littermates in response to exercise. (*C* and *D*) Relative mRNA expression (*C*) and activity (*D*) of LDH B in gastrocnemius muscle of MPGC-1α TG and control littermates in response to exercise. All values are expressed as means ± SE ($n = 4$–6 per group). @, Overall effect of genotype (WT vs. MPGC-1α TG); #, effect of training (sedentary vs. exercised); x, genotype-by-training interaction by ANOVA. Comparison between two individual groups: effects of training (sedentary vs. exercised, asterisk) and genotype (WT vs. MPGC-1α TG mice, §) were assessed by *t* test. Single symbols, $P < 0.05$; double symbols, $P < 0.01$; triple symbols, $P < 0.001$.

APPENDIX 2: Skeletal muscle PGC-1α controls whole-body lactate homeostasis through estrogen-related receptor α-dependent activation of LDH B and repression of LDH A

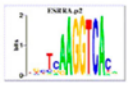
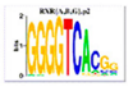
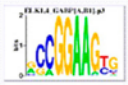
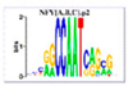
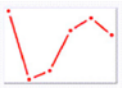
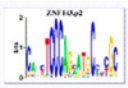
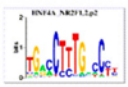
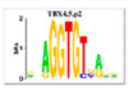
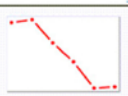
WM name	Z-value	Associated genes	Profile	Logo
ESRRA.p2	7.854	Esrra (ERRalpha, Estrra, Err1, Nr3b1)		
NR5A1,2.p2	6.836	Nr5a1 (Ad4BP, ELP, adrenal 4-binding protein, SF1, Ftz-F1, Ftzf1, SF-1, steroidogenic factor 1) Nr5a2 (D1Ertd308e, Ftf, LRH-1)		
RXR{A,B,G}.p2	3.004	Rxra (RXR alpha 1, RXRalpha1) Rxrb (Nr2b2, H-2RIIBP, RCoR-1, Rub) Rxrg (Nr2b3)		
ELK1,4_GABP{A,B1}.p3	2.858	Gabpa (GABPalpha) Gabpb1 (NRF2B2, E4TF1-47, BABPB2, E4TF1, NRF2B1, E4TF1B, GABPB1-1, GABPB1-2, E4TF1-53) Elk4 (Sap1) Elk1 (Elk-1)		
NFY{A,B,C}.p2	2.853	Nfyc Nfyb (Cbf-A) Nfya (Sez10, Cbf-b)		
ZNF143.p2	2.402	Zfp143 (KRAB14, Zfp80-rs1, Zfp79, Staf, D7Ertd805e, pHZ-1)		
HNF4A_NR2F1,2.p2	2.313	Nr2f1 (COUP-TF1, Tcfcoup1, Erbal3, COUP-TFI) Nr2f2 (Tcfcoup2, COUP-TFII, ARP-1, EAR3, COUP-TF2, Aporp1) Hnf4a (Tcf4, Hnf4, Tcf14, Nuclear receptor 2A1, Nr2a1, HNF4 alpha, HNF-4, MODY1)		
GATA1..3.p2	1.979	Gata1 (Gata-1, Gf-1) Gata2 (Gata-2) Gata3 (Gata-3)		
TBX4,5.p2	1.887	Tbx5 Tbx4		
TCF4_dimer.p2	1.887	Tcf4 (E2.2, bHLHb19, SEF-2, TFE, ME2, ITF-2b, ASP-I2, MITF-2A, SEF2-1, E2-2, ITF-2, MITF-2B)		

Fig. S2. Motif Activity Response Analysis of microarray data from muscle cells infected with GFP or bicistronic GFP–PGC-1α.

APPENDIX 2: Skeletal muscle PGC-1α controls whole-body lactate homeostasis through estrogen-related receptor α-dependent activation of LDH B and repression of LDH A

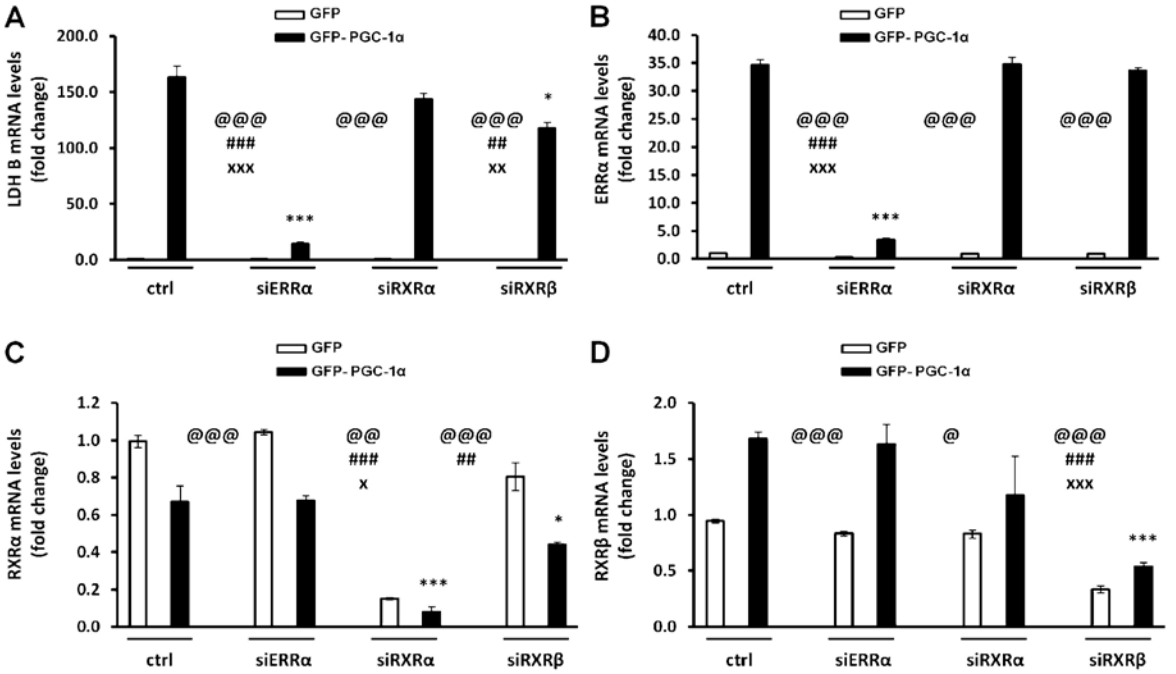


Fig. S3. LDH B mRNA levels in response to ERRα, retinoid X receptor (RXR)-α, or RXRβ silencing. (*A*) Relative mRNA levels of LDH B in confluent myoblasts transfected with siRNAs against ERRα, RXRα, or RXRβ followed by adenoviral transfection with GFP or PGC-1α–GFP. (*B–D*) Control experiments: silencing efficiency of ERRα (*B*), RXRα (*C*), and RXRβ (*D*) by their corresponding siRNA. Symbols at left, middle, and right refer to the comparison of siERRα-, siRXRα-, and siRXRβ-treated C2C12 myotubes vs. controls, respectively. @, Effect of PGC-1α (GFP vs. PGC-1α-GFP); #, effect of treatment (control vs. siRNA), x, interaction. Single symbols, $P < 0.05$; double symbols, $P < 0.01$; triple symbols, $P < 0.001$. Results from post hoc analysis: $*P < 0.05$ and $***P < 0.001$ vs. GFP–PGC-α untreated.

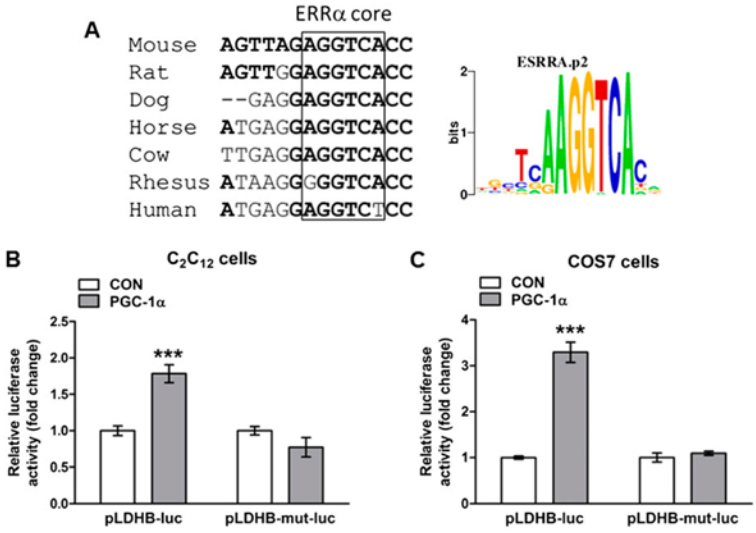


Fig. S4. Reporter gene activation of the LDH B promoter by PGC-1α depends on the integrity of the ERRα responsive element. (*A*) Evolutionary conservation of the ERRα response element in the LDH B promoter. This element was identified with Swissregulon (ESRRA.p2 motif logo, *Right*) and confirmed by using Matinspector scanning of the TRANSFAC database. Nucleotides that are conserved in comparison with the mouse sequence are indicated in bold. (*B* and *C*) Reporter gene assay by using LDHB promoter containing WT (pLDHB-luc) or mutated (pLDHB-mut-luc) ERRα response elements. (*B*) Autologous C2C12 and (*C*) heterologous COS-7 cells were transfected with pLDHB-luc or pLDHB-mut-luc for 24 h, followed by an additional 24 h in the presence of GFP [i.e., control (CON)] or PGC-1α adenovirus ($n = 2$ independent experiments performed in triplicate each). Values represent mean ± SEM ($***P < 0.001$, control vs. PGC-1α).

APPENDIX 2: Skeletal muscle PGC-1α controls whole-body lactate homeostasis through estrogen-related receptor α-dependent activation of LDH B and repression of LDH A

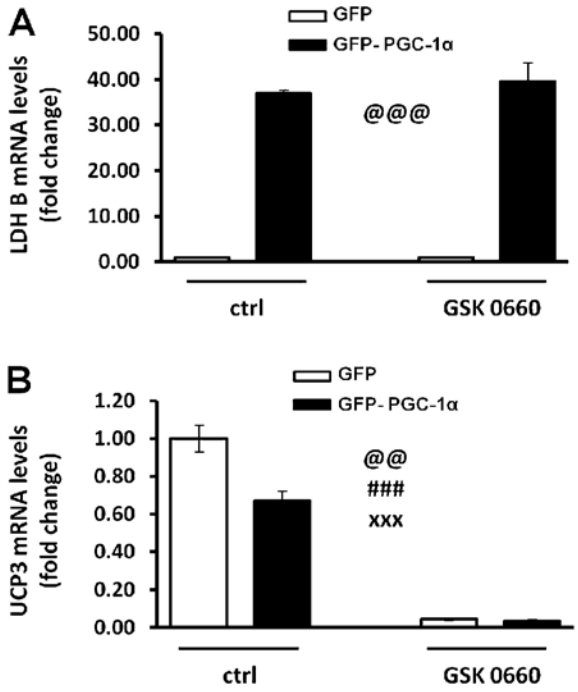


Fig. S5. LDH B mRNA levels in response to peroxisome proliferator-activated receptor-β/δ inhibition. (*A*) Relative mRNA levels of LDH B in differentiated C2C12 myotubes following adenoviral overexpression of GFP or bicistronic PGC-1α–GFP and in the absence or presence of GSK0660. (*B*) Control experiment: relative mRNA levels of the peroxisome proliferator-activated receptor-β/δ target gene uncoupling protein 3 (UCP3) in differentiated C2C12 myotubes following adenoviral overexpression of GFP or bicistronic PGC-1α-GFP and in the absence or presence of GSK0660. All values are expressed as means ± SE (n = 6 per group). @, Effect of PGC-1α (GFP vs. PGC-1α-GFP); #, effect of treatment (DMSO vs. GSK0660); x, interaction. Double symbols, $P < 0.01$; triple symbols, $P < 0.001$.

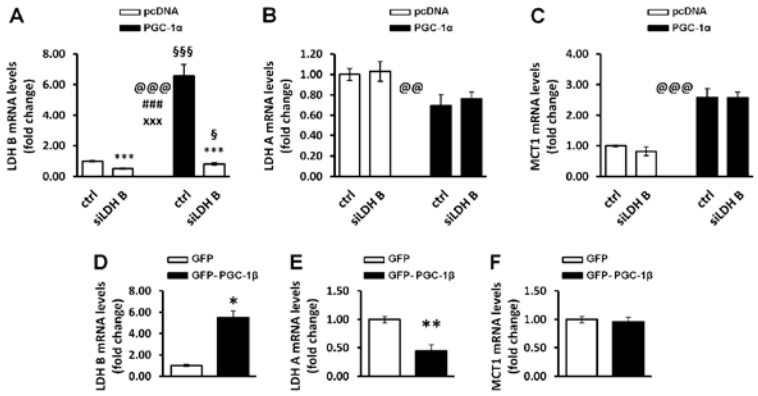


Fig. S6. Effect of LDH B silencing or PGC-1β overexpression on LDH B, LDH A, and monocarboxylate transporter 1 (MCT1). (*A–C*) Relative mRNA levels of LDH B (*A*), LDH A (*B*) and MCT1 (*C*) in confluent myoblasts transfected with pcDNA or PGC-1α expression plasmids in the absence or presence of siRNA against LDH B. All values are expressed as means ± SE (n = 4–6 per group). @, Overall effect of PGC-1α (pcDNA vs. PGC-1α); #, effect of treatment (control vs. siRNA); x, genotype-by-training interaction by ANOVA. Comparison between two individual groups: effects of treatment (control vs. siRNA, asterisk) and PGC-1α (pcDNA vs. PGC-1α, §) were assessed by t test. Single symbols, $P < 0.05$; double symbols, $P < 0.01$; triple symbols, $P < 0.001$. (*D–F*) Relative mRNA levels of LDH B (*D*), LDH A (*E*), and MCT1 (*F*) in differentiated C2C12 myotubes following adenoviral overexpression of GFP or bicistronic PGC-1β-GFP. Values are expressed as means ± SE (n = 6 per group).

APPENDIX 2: Skeletal muscle PGC-1α controls whole-body lactate homeostasis through estrogen-related receptor α-dependent activation of LDH B and repression of LDH A

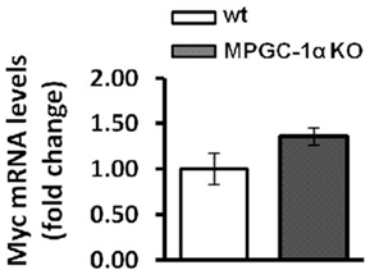


Fig. S7. Myelocytomatosis oncogene (Myc) mRNA levels in skeletal muscle of MPGC-1α KO mice. Relative mRNA levels of myelocytomatosis oncogene in MPGC-1α KO mice and control animals. Values are expressed as means ± SE (n = 8 per group).

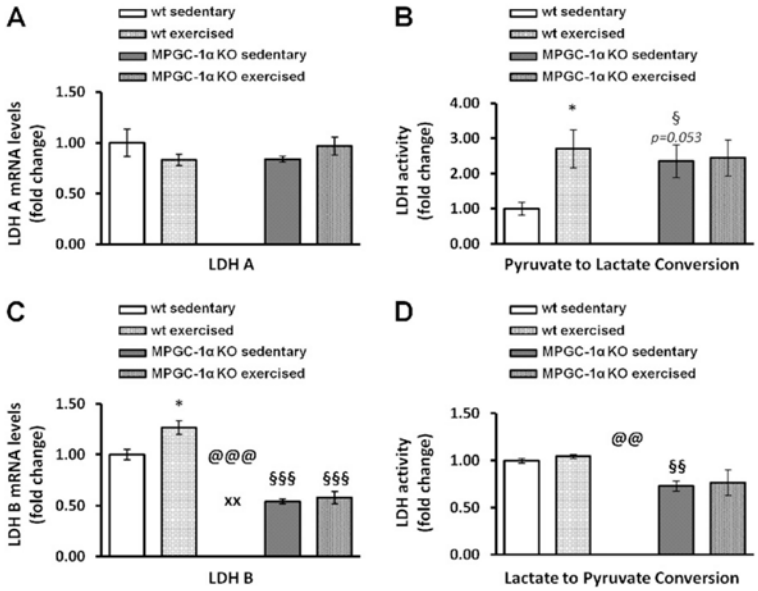


Fig. S8. Muscle LDH mRNA expression and activity in MPGC-1α KO mice after exercise. (*A* and *B*) Relative mRNA expression (*A*) and activity (*B*) of LDH A in gastrocnemius muscle of MPGC-1α KO and control littermates in response to exercise. (*C* and *D*) Relative mRNA expression (*C*) and activity (*D*) of LDH B in gastrocnemius muscle of MPGC-1α KO and control littermates in response to exercise. All values are expressed as means ± SE (n = 4–6 per group). @, Overall effect of genotype (WT vs. MPGC-1α KO); #, effect of training (sedentary vs. exercised); x, genotype-by-training interaction by ANOVA. Comparison between two individual groups: effects of training (sedentary vs. exercised, asterisk) and genotype (WT vs. MPGC-1α KO mice, §) were assessed by t test. Single symbols, $P < 0.05$; double symbols, $P < 0.01$; triple symbols, $P < 0.001$.

APPENDIX 2: Skeletal muscle PGC-1α controls whole-body lactate homeostasis through estrogen-related receptor α-dependent activation of LDH B and repression of LDH A

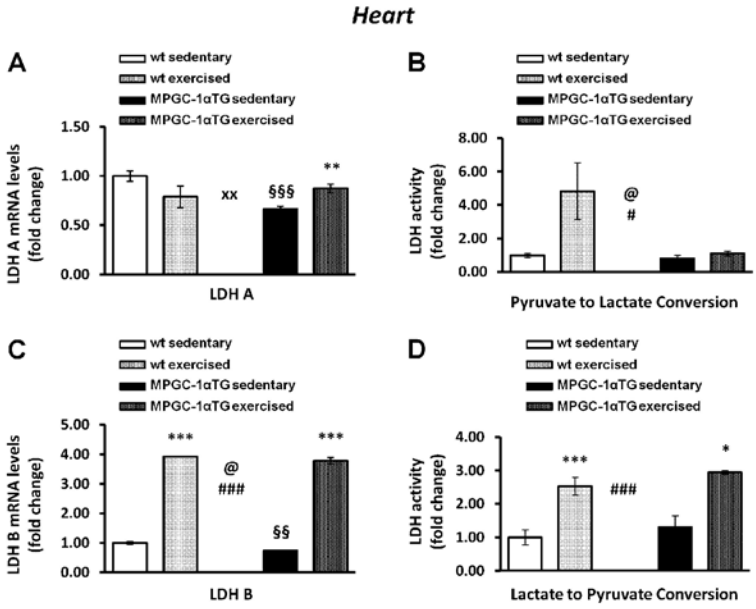


Fig. S9. LDH mRNA expression and activity in the heart of MPGC-1α TG mice after exercise. (*A* and *B*) Relative mRNA expression (*A*) and activity (*B*) of LDH A in the heart of MPGC-1α TG and control littermates in response to exercise. (*C* and *D*) Relative mRNA expression (*C*) and activity (*D*) of LDH B in the heart of MPGC-1α TG and control littermates in response to exercise. All values are expressed as means ± SE ($n = 4$–6 per group). @, Overall effect of genotype (WT vs. MPGC-1α TG); #, effect of training (sedentary vs. exercised); x, genotype-by-training interaction by ANOVA. Comparison between two individual groups: effects of training (sedentary vs. exercised, asterisk) and genotype (WT vs. MPGC-1α TG mice, §) were assessed by *t* test. Single symbols, $P < 0.05$; double symbols, $P < 0.01$; triple symbols, $P < 0.001$.

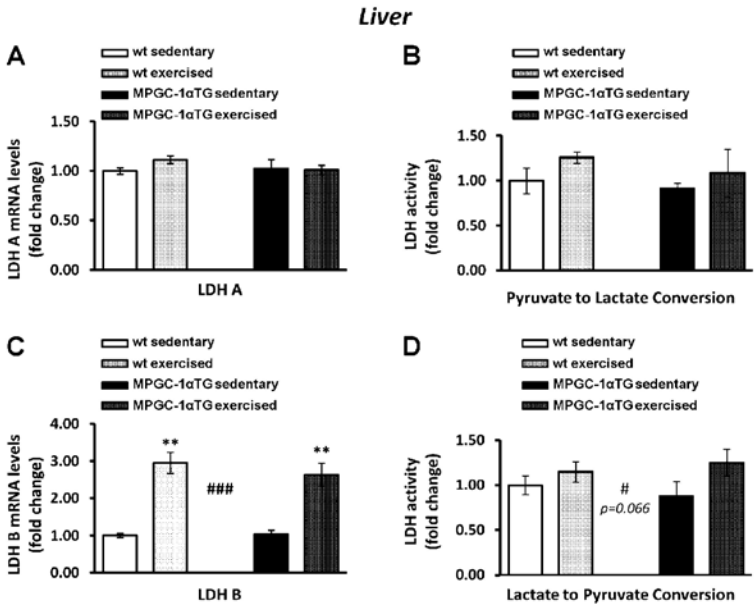


Fig. S10. LDH mRNA expression and activity in the liver of MPGC-1α TG mice after exercise. (*A* and *B*) Relative mRNA expression (*A*) and activity (*B*) of LDH A in the liver of MPGC-1α TG and control littermates in response to exercise. (*C* and *D*) Relative mRNA expression (*C*) and activity (*D*) of LDH B in the liver of MPGC-1α TG and control littermates in response to exercise. All values are expressed as means ± SE ($n = 4$–6 per group). @, Overall effect of genotype (WT vs. MPGC-1α TG); #, effect of training (sedentary vs. exercised); x, genotype by training interaction by ANOVA. Comparison between two individual groups: effects of training (sedentary vs. exercised, asterisk) and genotype (WT vs. MPGC-1α TG mice, §) were assessed by *t* test. Single symbols, $P < 0.05$; double symbols, $P < 0.01$; triple symbols, $P < 0.001$.

APPENDIX 2: Skeletal muscle PGC-1α controls whole-body lactate homeostasis through estrogen-related receptor α-dependent activation of LDH B and repression of LDH A

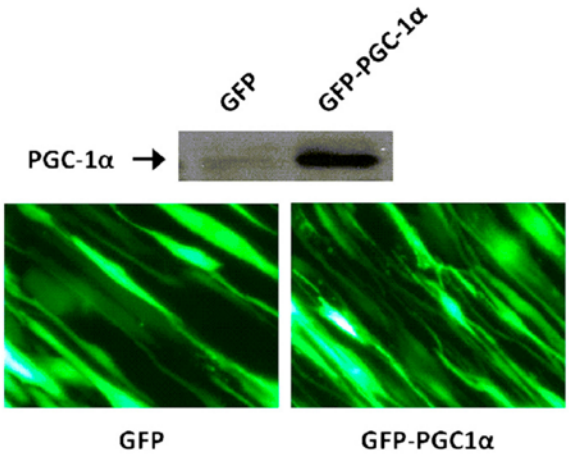


Fig. S11. Transfection efficiency and PGC-1α protein expression in differentiated C2C12 myotubes. Green fluorescence in differentiated C2C12 myotubes adenovirally infected with GFP or PGC-1α–GFP and resulting overexpression of PGC-1α protein.

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Languages	Spanish and English
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EDUCATION

10.2009-12.2013	Ph.D. in Cellular Biology, Biozentrum, University of Basel, Switzerland (Thesis supervisor: Dr. Christoph Handschin)
10.2008-07.2009	M.Sc. in Biomedical Sciences, Faculty of Medicine, University of Murcia, Spain (Thesis supervisor: Dr. Jesús Hernández-Cascales)
03.2002-04.2007	B.Ed. in Physical Education, Sports and Recreation, Faculty of Education, Mayor University, Chile (Thesis supervisor: Jorge Cajigal M.Sc.)

ADDITIONAL EDUCATION AND RESEARCH EXPERIENCE

08.2010-09.2010	LTK Module 1: Introductory Course in Laboratory Animal Science, Institute of Laboratory Animal Science, University of Zürich, Switzerland
03.2008-05.2008	Visiting student at the Division of Molecular Physiology, College of Life Sciences, University of Dundee, Scotland (Supervisor: Dr. Keith Baar)
04.2007-01.2008	Postgraduate course in Cellular and Molecular Biology, Faculty of Sciences, University of Chile, Chile
08.2007-11.2007	Postgraduate course (journal club) in Intracellular Signals of Calcium, Faculty of Medicine, University of Chile, Chile
07.2006-01.2007	Postgraduate course in Exercise, Nutrition and Health, Institute of Nutrition and Food Technology, University of Chile, Chile
03.2006-04.2007	Undergraduate student at the Laboratory of Exercise Physiology, Sport Center for High Performance, Chile (Supervisor: Jorge Cajigal M.Sc.)
04.2006-07.2006	Course of Continuum Education in Obesity, Chilean Society of Obesity, Chile

AWARDS & FELLOWSHIPS

2014-2018	Sir Henry Wellcome Postdoctoral Fellowship (Wellcome Trust, UK)
09.2013	Award for outstanding poster presentation (EMBO Workshop)

PROFESSIONAL MEMBERSHIPS

08.2013-present	The Physiological Society, UK

PUBLICATION LIST

Original articles

1. **Pérez-Schindler, J.,** Vargas-Fernández, E., Santos, G., Svensson, K., Wahli, W., and Handschin, C. PGC-1α regulates skeletal muscle oxidative metabolism independently of PPARβ/δ. (*manuscript submitted*).
2. Soler, F., Fernández-Belda, F., **Pérez-Schindler, J.,** Handschin, C., Fuente, T., and Hernandez-Cascales, J. (2013). PDE2 activity is different and distinctly regulates β2-adrenoceptors mediated inotropy in right and left rat ventricular myocardium. (*manuscript under review*).
3. **Perez-Schindler, J.,** Summermatter, S., Santos, G., Zorzato, F., and Handschin, C. (2013) The transcriptional coactivator PGC-1alpha is dispensable for chronic overload-induced skeletal muscle hypertrophy and metabolic remodeling. *Proc Natl Acad Sci U S A* 110, 20314-20319
4. Summermatter, S., Santos, G., **Perez-Schindler, J.,** and Handschin, C. (2013) Skeletal muscle PGC-1alpha controls whole-body lactate homeostasis through estrogen-related receptor alpha-dependent activation of LDH B and repression of LDH A. *Proc Natl Acad Sci U S A* 110, 8738-8743
5. **Perez-Schindler, J.,** Summermatter, S., Salatino, S., Zorzato, F., Beer, M., Balwierz, P. J., van Nimwegen, E., Feige, J. N., Auwerx, J., and Handschin, C. (2012) The corepressor NCoR1 antagonizes PGC-1alpha and estrogen-related receptor alpha in the regulation of skeletal muscle function and oxidative metabolism. *Mol Cell Biol* 32, 4913-4924
6. McPhee, J. S., Williams, A. G., **Perez-Schindler, J.,** Degens, H., Baar, K., and Jones, D. A. (2011) Variability in the magnitude of response of metabolic enzymes reveals patterns of co-ordinated expression following endurance training in women. *Exp Physiol* 96, 699-707
7. **Perez-Schindler, J.,** Philp, A., Baar, K., and Hernandez-Cascales, J. (2011) Regulation of contractility and metabolic signaling by the beta2-adrenergic receptor in rat ventricular muscle. *Life Sci* 88, 892-897
8. McPhee, J. S., **Perez-Schindler, J.,** Degens, H., Tomlinson, D., Hennis, P., Baar, K., and Williams, A. G. (2011) HIF1A P582S gene association with endurance training responses in young women. *Eur J Appl Physiol* 111, 2339-2347
9. Philp, A., **Perez-Schindler, J.,** Green, C., Hamilton, D. L., and Baar, K. (2010) Pyruvate suppresses PGC1alpha expression and substrate utilization despite increased respiratory chain content in C2C12 myotubes. *Am J Physiol Cell Physiol* 299, C240-250
10. McPhee, J. S., Williams, A. G., Stewart, C., Baar, K., **Schindler, J. P.,** Aldred, S., Maffulli, N., Sargeant, A. J., and Jones, D. A. (2009) The training stimulus experienced by the leg muscles during cycling in humans. *Exp Physiol* 94, 684-694

Review articles

1. **Pérez-Schindler, J.,** and Handschin, C. (2013) New insights in the regulation of skeletal muscle PGC-1α by exercise and metabolic diseases. *Drug Discovery Today: Disease Models* 10, e79-e85
2. **Perez-Schindler, J.,** Philp, A., and Hernandez-Cascales, J. (2013) Pathophysiological relevance of the cardiac beta2-adrenergic receptor and its potential as a therapeutic target to improve cardiac function. *Eur J Pharmacol* 698, 39-47

SCIENTIFIC MEETINGS

09.2013	EMBO Workshop: Molecular mechanisms of muscle growth and wasting in health and disease (**poster presentation**)
05.2013	5th Basel Symposium on Skeletal Muscle, Switzerland (**attendee**)
04.2013	Biozentrum Symposium, Switzerland (**poster presentation**)
05.2012-06.2012	American College of Sports Medicine 59th Annual Meeting & World Congress on Exercise is Medicine, USA (**oral presentation:** *The Interplay Between NCoR1 and PGC-1α Regulates Skeletal Muscle Oxidative Metabolism*)
09.2011	International Conference on Muscle Wasting: Molecular Mechanisms of Muscle Growth and Wasting in Health and Disease, Switzerland (**poster presentation**)
04.2011	4th Basel Symposium on Skeletal Muscle, Switzerland (**attendee**)
06.2009	14th International Biochemistry of Exercise Conference, Canada (**poster presentation**)
09.2008	American Physiological Society Intersociety Meeting: Integrative Biology of Exercise, USA (**poster presentation**)
09.2007	International Congress of Sport Nutrition and Exercise Physiology, Chile (**attendee**)
08.2007	International Symposium on Signal Transduction in Endocrinology Health and Disease, Chile (**attendee**)