Transcriptional Regulation of Kidney and Skeletal Muscle Metabolism by the Coactivator PGC-1α

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Summary

Transcriptional regulation plays an intricate role in metabolic adaptation. Cellular metabolism is thus regulated through a system of transcriptional activators and repressors. These coregulators are able to modulate transcription of various metabolic programs to maintain energy homeostasis in response to altered energy demands or environmental cues. Peroxisome proliferator-activated receptor (PPAR) coactivator 1 alpha (PGC-1α) is a transcriptional coactivator, which is import for cellular metabolic adaptation. PGC-1α is expressed in mitochondria-rich tissues and regulates distinct metabolic gene programs, such as fatty acid oxidation and mitochondrial oxidative phosphorylation through interaction with various transcription factors. The pervasive role of PGC-1α in metabolic regulation has made this transcriptional coregulator a promising therapeutic target in several disorders with a mitochondrial bioenergetic etiology. However, to comprehend the therapeutic potential of PGC-1α activation, it is essential to gain a full understanding of the transcriptional networks modulated by this coactivator and its impact on cellular- and organismal physiology. Moreover, it is important to validate whether PGC-1α activation is necessary to mediate the beneficial metabolic effects (i.e. increased energy expenditure or improved glucose tolerance) of various pharmacological compounds. Such experiments will confirm the potential use of PGC-1α as a therapeutic target, and will give a better insight into the molecular mechanisms how these compounds exert their effects. Ultimately, the characterization of PGC-1α as a therapeutic target will aid in the design of more efficient drug therapies.

The role of PGC-1α is well established in organs such as liver and skeletal muscle. However, despite the prevalence of mitochondrial dysfunction in the pathogenesis of renal disorders, the role of PGC-1α in kidney physiology and its potential therapeutic use in this organ is still unknown. To define the role of PGC-1α in renal physiology, we generated and characterized a nephron-specific PGC-1α knockout mouse model. We observed that deletion of PGC-1α in kidney led to a reduced transcription of gene programs involved in mitochondrial oxidative metabolism. PGC-1α was also required for the induction of PPARα target genes and renal fatty acid oxidation during high fat diet feeding. Renal deletion of PGC-1α resulted in mild hypertension and increased urinary sodium excretion. However, mice deficient for PGC-1α in the kidney could still adapt their salt and water homeostasis in response to salt stress. This indicates that PGC-1α is dispensable for the adaptive regulation of tubular reabsorption and secretion. However, due to the high basal energy demand of the kidney, there is a strong link between mitochondrial dysfunction and renal disorders. While the loss of PGC-1α did not affect basal renal physiology, it has a central role as a regulator of metabolic and mitochondrial transcriptional programs in the kidney. Hence,

PGC-1α could be a potential therapeutic target to ameliorate renal metabolic disorders associated with mitochondrial dysfunction and lipotoxicity.

In the second study, we investigated the role of skeletal muscle PGC-1α in mediating the therapeutic effects of the SIRT1-activaiting compounds resveratrol (RSV) and SRT1720. The beneficial systemic effects of these compounds, such as an enhanced metabolic rate and improved glucose tolerance, were independent of skeletal muscle PGC-1α. PGC-1α was however necessary for transcriptional activation of mitochondrial genes in skeletal muscle with RSV and SRT1720 treatment. Intriguingly, while postulated to act through the same signaling pathways, we could also demonstrate differential effects of RSV and SRT1720 treatment on mitochondrial and metabolic processes in liver and white adipose tissue (WAT). Importantly, both RSV and SRT1720 enhanced transcription of PGC-1α target genes in WAT and liver, respectively. Finally, in the third part of this thesis, we investigated the role of PGC-1α in the regulation of skeletal muscle ketone body oxidation. Ketone bodies are important metabolic fuels during prolonged starvation and dietary ketosis has been postulated to possess several therapeutic effects, such as improved epileptic seizure control, reduced cancer growth rates and enhanced mitochondrial biogenesis in brown adipose tissue and brain. However, relatively little is known how ketolytic capacity in skeletal muscle is regulated. We demonstrated that PGC-1α, together with the estrogen-related receptor alpha (ERRα), regulates transcription of ketolytic enzymes in skeletal muscle, both in a basal state and in response to exercise. Importantly, modulation of PGC-1α levels in skeletal muscle affected systemic ketone body homeostasis during exercise, fasting and feeding of a low-carbohydrate ketogenic diet. Moreover, elevation of PGC-1α levels in skeletal muscle was sufficient to ameliorate diabetic ketoacidosis in mice. Hence, we identified PGC-1α as a potential therapeutic target to reduce hyperketonemia in diabetic patients.

In summary, the work presented in this thesis describes several new aspects of PGC-1α biology. We have revealed novel insights into the role of PGC-1α in renal physiology and its potential role as a therapeutic target in kidney. Additionally, we have evaluated the role of skeletal muscle PGC-1α as a molecular effector of the beneficial effects of RSV and SRT1720 on whole body metabolism. Finally, we have described a novel role of PGC-1α as a transcriptional regulator of ketone body oxidation. Collectively, our findings demonstrates a crucial role of PGC-1α in many different biological processes which are all ultimately connected to mitochondrial metabolism. These data expand our knowledge on the transcriptional networks and cellular processes regulated by PGC-1α and will help to develop more efficient therapeutic strategies against metabolic disorders.

Abbreviations

βOHB	Beta hydroxybutyrate
AcAc	Acetoacetate
ACAT1	Acetyl-CoA acetyltransferase
AMPK	AMP-activated protein kinase
ANGII	Angiotensin II
AP1	Activating protein 1
ASDN	Aldosterone-sensitive distal nephron
ATF2	Activating transcription factor 2
ATP	Adenosine triphosphate
BAF60a	BRG-1/Brm associated factor complex, subunit 60a
BAP1	BRCA1 associated protein-1
BAT	Brown adipose tissue
BDH1	3-hydroxybutyrate dehydrogenase, type 1
cAMP	Cyclic AMP
CBP	CREB-binding protein
CDK4	Cyclin-dependent kinase 4
ChIP-Seq	Chromatin immunoprecipitation sequencing
CLK2	CDC-like kinase 2
CnA	Calcineurin A subunit
Cpt1b	Carnitine palmitoyltransferase 1B
CR	Calorie restriction
CREB	cAMP response element-binding protein
CRTC2	CREB regulated transcription coactivator 2
CTRL	Control
DOX	Doxycycline
ENaC	Epithelial sodium channel
eNOS	Endothelial nitric oxide synthase
Epac1	Exchange protein directly activated by cAMP 1
ERα	Estrogen receptor alpha
ERR	Estrogen-related receptor
FGF21	Fibroblast growth factor 21
FOXA2	Forkhead box protein A2
FOXO1	Forkhead box protein O1
G6pc	Glucose-6-phosphatase catalytic-subunit
GABPA	GA binding protein transcription factor
GCN5	General control of amino acid synthesis protein 5
gKO	Global PGC-1α knockout
GLUT4	Glucose transporter 4
GO	Gene ontology
GR	Glucocorticoid receptor
HAT	Histone-acetyltransferase
HFD	High fat diet
HIF2α	Hypoxia-inducible factor 2 alpha
HMGCL	3-hydroxymethyl-3-methylglutaryl-CoA lyase
HMGCS2	3-hydroxy-3-methylglutaryl-CoA synthase 2
HNF4A	Hepatocyte nuclear factor 4 alpha
HSD	High salt diet
HSF1	Heat-shock factor 1
IRF4	Interferon regulatory factor 4
KKS	Kallikrein-kinin system
LCKD	Low-carbohydrate ketogenic diet
LSD	Low salt diet
MCT1	Monocarboxylate transporter 1
MEF2C/D	Myocyte enhancer factor-2 C/D
mKO	Muscle-specific PGC-1α knockout
MR	Mineralocorticoid receptor
mTG	Muscle-specific PGC-1α transgenic
mTORC1	Mammalian target of rapamycin complex 1
NCoR1	Nuclear receptor co-repressor 1
NEFA	Non-esterified fatty acid
NR	Nuclear receptor
NRF1/2	Nuclear respiratory factor 1/2

Abbreviations

NSD	Normal salt diet
OXCT1	Succinyl-CoA:3-ketoacid-coenzyme A transferase 1
P160^{MBP}	p160 myb binding protein
P300	E1A-associated protein p300
P38MAPK	p38 MAP Kinase
Pck1	Phosphoenolpyruvate carboxykinase
PDE	Phosphodiesterase
PERM1	PGC-1 and ERR-induced Regulator in Muscle 1
PGC-1α/β	Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha/beta
PiNKO	Inducible PGC-1α nephron knockout
PKA	Protein kinase A
PoPKO	Podocyte-specific PGC-1α knockout
PPAR	Peroxisome proliferator-activated receptor
PRC	PGC-1 related coactivator
PRDM16	PR domain containing 16
PRMT	Protein arginine methyltransferase
PTM	Post-translational modification
RAAS	Renin-angiotensin-aldosterone system
RIP140	Receptor-interacting protein of 140 kDa
ROS	Reactive oxygen species
RRM	RNA recognition motif
RSV	Resveratrol
RT-PCR	Reverse transcription polymerase chain reaction
rtTA	Reverse tetracycline-controlled transactivator
RXR	Retinoid X receptor
S6K	S6 kinase
SCF^{CDC4}	SKP1-CUL1-F-box protein/ cell division control protein 4
SF1	Steroidogenic factor 1
SGK1	Serum and glucocorticoid-regulated kinase 1
SHP	Small heterodimer partner
SIRT1/3	Sirtuin 1/3
SLC	Solute carrier
SRC1/3	Steroid receptor coactivator-1/3
SREBP	Sterol regulatory element-binding proteins
SRT	SRT1720
STARS	Striated muscle activator of Rho signaling
STZ	Streptozotocin
SWI/SNF	SWItch/Sucrose NonFermentable
TCA cycle	Tricarboxylic acid cycle
tetO	tet-operator
TF	Transcription factor
TNF	Tumor necrosis factor
TRAP/DRIP	Thyroid hormone receptor-associated proteins/vitamin D receptor interacting protein complex
TRβ	Thyroid receptor beta
UCP1	Uncoupling-protein 1
VEGFA	Vascular endothelial growth factor
WAT	White adipose tissue

1. General introduction

1.1 - PGC-1α - a multifaceted transcriptional regulator

Peroxisome proliferator-activated receptor gamma (PPARγ) co-activator 1 alpha (PGC-1α) is a protein, which belongs to the PGC-1 family of transcriptional coactivators **[Liang and Ward 2006]**. The other members of this family are PGC-1β **[Kressler et al. 2002, Lin et al. 2002a, Meirhaeghe et al. 2003]** and PGC-1-related coactivator (PRC) **[Andersson and Scarpulla 2001]**. PGC-1α was discovered through its ability to interact with PPARγ (hence its name), and thereby induce uncoupling protein 1 (UCP1) expression and a thermogenic gene program in brown adipose tissue (BAT) **[Puigserver et al. 1998]**. PGC-1α also interacts with several other transcription factors (TF) belonging to the nuclear receptor (NR) family of TFs (i.e. thyroid receptor beta, TRβ; retinoid X receptor alpha, RXRα) **[Puigserver et al. 1998]**. PGC-1α does not contain a DNA-binding domain, and can therefore not bind directly to DNA. Hence, TF-binding is essential for the function of PGC-1α **[Puigserver and Spiegelman 2003]**. PGC-1α is expressed at high levels in organs with a high basal oxidative capacity, such as skeletal muscle, BAT, brain, heart and kidney **[Puigserver et al. 1998, Larrouy et al. 1999, Handschin and Spiegelman 2006]**. Other organs such as liver and white adipose tissue (WAT) display lower basal levels of PGC-1α **[Puigserver et al. 1998, Liang and Ward 2006]**. However, transcription of PGC-1α can be strongly induced in these organs in response to external stimuli such as fasting in liver **[Handschin and Spiegelman 2006]**, or cold exposure in WAT depots **[Barbatelli et al. 2010]**. Cold exposure also increases PGC-1α levels in both BAT and skeletal muscle **[Puigserver et al. 1998]**, and exercise has been shown to induce PGC-1α expression in skeletal muscle **[Baar et al. 2002, Pilegaard et al. 2003, Terada and Tabata 2004]**. PGC-1α thus acts as a sensor of several distinct external stimuli, and in response enhances transcription of tissue-specific gene programs involved in mitochondrial function and oxidative capacity (discussed in more detail in section 1.2 of the introduction) to match the enhanced energy demand during cold adaptation or exercise.

PGC-1β was the second PGC-1 family member to be cloned, and was identified due to its sequence similarities with PGC-1α **[Kressler et al. 2002, Lin et al. 2002a, Meirhaeghe et al. 2003]**. PGC-1β shows high sequence homology with PGC-1α at the N-terminal and C-terminal regions of the protein (Figure 1) **[Lin et al. 2002a]**. These are the regions where the major effector domains are found, and PGC-1β thus contains similar NR-interaction domains and RNA-recognition motifs as PGC-1α **[Lin et al. 2002a, Meirhaeghe et al. 2003]**. The expression pattern of PGC-1β is almost identical to PGC-1α, with higher expression in oxidative organs **[Meirhaeghe et al. 2003]**. PGC-1β can also interact with several TFs known

to be coactivated by PGC-1α, such as nuclear respiratory factor 1 (NRF1), peroxisome proliferator-activated receptor alpha (PPARα) and estrogen related receptor alpha (ERRα), and can thus induce identical transcriptional programs as PGC-1α **[Lin et al. 2005a]**. This has led to the idea that PGC-1α and PGC-1β are functionally redundant. In line with this, whole body knock-out models of either PGC-1α **[Lin et al. 2004, Leone et al. 2005]** or PGC-1β **[Lelliott et al. 2006, Vianna et al. 2006, Sonoda et al. 2007]** are viable, while PGC-1α/PGC-1β double-knockout mice die soon after birth due to cardiac failure **[Lai et al. 2008]**. Moreover, double-knockout of PGC-1α/PGC-1β in skeletal muscle leads to exacerbated mitochondrial dysfunction compared to single-knockout models of either PGC-1α or PGC-1β **[Zechner et al. 2010]**. Despite the apparent similarities between PGC-1α and PGC-1β, they differ in some important aspects. In contrast to PGC-1α, PGC-1β is not induced in BAT with cold exposure **[Lin et al. 2002a]**, while it can still be induced with fasting in liver **[Lin et al. 2002a]**. Moreover, PGC-1α and PGC-1β regulate distinct gene programs in liver. PGC-1β coactivates the sterol responsive element binding protein (SREBP)-family of lipogenic TFs in liver, and can thus induce a lipogenic gene program **[Lin et al. 2005b]**. On the other hand, it cannot interact with HNF4A and FOXO1, and is thus unable to induce a gluconeogenic gene program to the same extent as PGC-1α **[Lin et al. 2003]**.

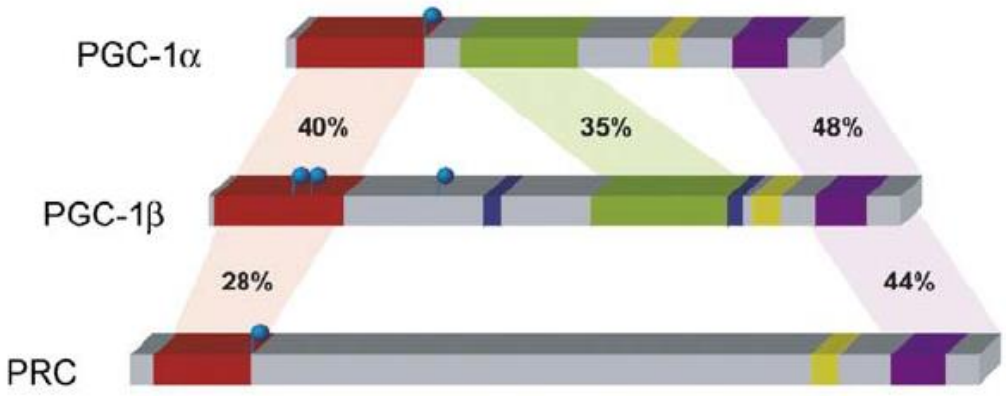

Figure 1 - The PGC-1 family of transcriptional coactivators

Members of the PGC-1 family display high sequence similarities, especially within the N-terminal activation domain (red) and the C-terminal RNA-binding domain (purple). Adapted from **[Lin et al. 2005a]**.

The third PGC-1 family member, PGC-1-related coactivator (PRC) shares the NR-interaction and RNA-recognition motifs with PGC-1α and PGC-1β **[Andersson and Scarpulla 2001]** (Figure 1). PRC can coactive several TFs involved in mitochondrial biogenesis, which are also coactivated by PGC-1α (i.e. NRF1 and ERRα) **[Andersson and Scarpulla 2001, Vercauteren et al. 2006, Mirebeau-Prunier et al. 2010]**. Consequently, overexpression of PRC is associated with increased mitochondrial biogenesis in skeletal muscle cells **[Philp et al. 2011a]**. PRC is ubiquitously expressed, and does not vary in expression levels between organs to the same extent as PGC-1α and PGC-1β. Moreover, PRC, in contrast to PGC-1α, is not induced in response to cold stimuli in either BAT or skeletal muscle **[Andersson and Scarpulla 2001]**. Conversely, PRC expression is increased by a variety of metabolic stressors **[Vercauteren et al. 2006, Gleyzer and Scarpulla 2011]**, and is also induced in fibroblasts upon cell-cycle entry. Hence, PRC is postulated to be important for maintaining mitochondrial transcription in proliferating cells **[Andersson and Scarpulla 2001, Vercauteren et al. 2006]**. Since this thesis is aimed at elucidating the specific role of PGC-1α in kidney and skeletal muscle physiology, the remainder of this introduction will accordingly be focused mainly on transcriptional regulation mediated by PGC-1α.

Transcriptional partners of PGC-1α

PGC-1α does not contain a DNA-binding domain, and it is therefore dependent on interactions with TFs to be able to associate with the DNA. PGC-1α binds TFs through several interaction domains, out of which, the best characterized is the NR-binding L2-motif (LXXLL) **[Knutti and Kralli 2001]** (Figure 2). Through the L2-motif, PGC-1α has been shown to interact in a ligand dependent manner with NRs such as PPARα **[Vega et al. 2000]**, estrogen receptor-α (ERα) **[Tcherepanova et al. 2000]** and RXRα **[Delerive et al. 2002]**, and in a ligand independent manner with hepatocyte nuclear factor 4 alpha (HNF4A) **[Yoon et al. 2001]**. Binding of ERRs (ERRα/β/γ) also occurs in a ligand independent manner, but is dependent on the closely related L3-motif (LLXYL) **[Huss et al. 2002]** (Figure 2). PGC-1α contains three NR-recognition motifs (LLXXL), but can also interact with TFs such as PPARγ **[Puigserver et al. 1998]**, forkhead box protein O1 (FOXO1) **[Puigserver et al. 2003]** and myocyte enhancer factor 2C (MEF2C) **[Michael et al. 2001]** through other regions along the protein (Figure 2). Once bound to the DNA together with its transcriptional partners, PGC-1α activates transcription through two main mechanisms; removal of corepressors and recruitment of transcriptional coactivators. For instance, PGC-1α has been shown to antagonize binding of the corepressor small heterodimer partner (SHP) to the glucocorticoid receptor (GR), thereby increasing transcription of gluconeogenic genes **[Borgius et al. 2002]**. Several transcriptional partners of PGC-1α (i.e. PPARs and ERRs) are also targeted by corepressors such as receptor-interacting protein 140 (RIP140) and

nuclear receptor co-repressor 1 (NCoR1) **[Qi and Ding 2012]**. Gene transcription is thus controlled through a balance between coactivator- and corepressor complexes binding to TFs. One example can be found in skeletal muscle, where NCoR1 acts as a repressor of ERRα-mediated transcription **[Yamamoto et al. 2011, Perez-Schindler et al. 2012]**. ERRα is furthermore coactivated by PGC-1α and this results in an opposite regulation of several mitochondrial and metabolic gene programs in muscle through binding of either NCoR1 or PGC-1α to ERRα **[Perez-Schindler et al. 2012]**.

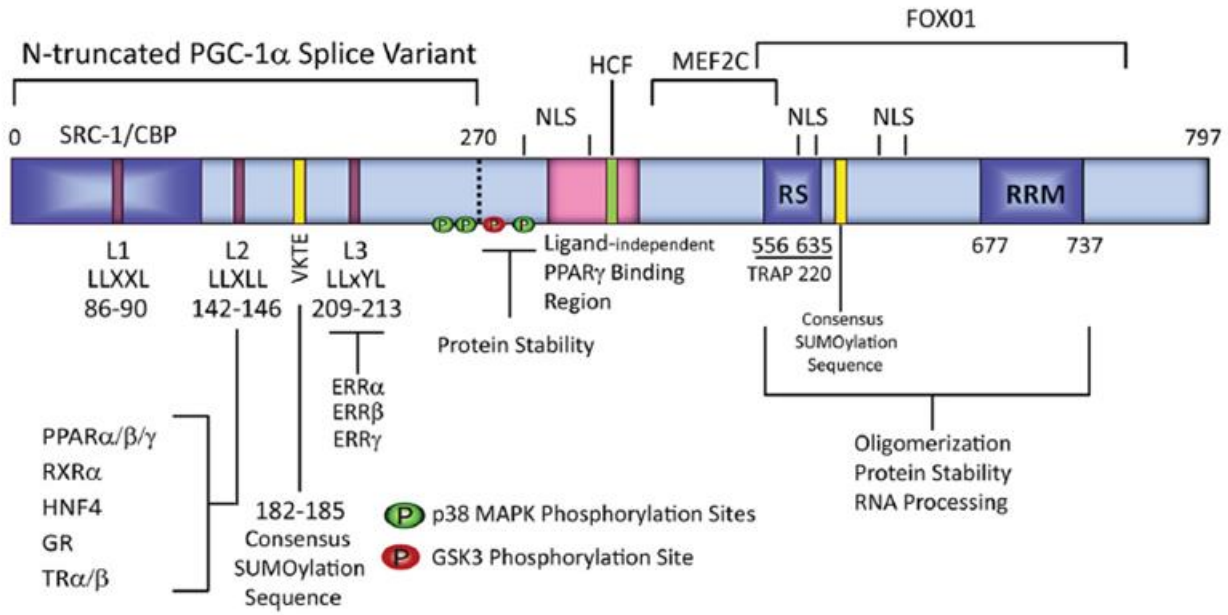

Figure 2 - Diagram of domain structure of PGC-1α and NT-PGC-1α

NLS, nuclear localization sequence; RS, arginine-serine-rich domain; ERR, estrogen-related receptor; RRM, RNA recognition motif. Adapted from **[Zhang et al. 2009]**.

To mediate full transcriptional activation, PGC-1α interacts with several other regulatory proteins to form a coactivator complex. Through its N-terminal domain, PGC-1α binds histone acetyltransferases (HAT) such as CREB-binding protein (CBP), steroid receptor coactivator-1 (SRC-1) and E1A binding protein p300 (p300) **[Puigserver et al. 1999, Wallberg et al. 2003]**. These proteins are known to increase local histone acetylation, which leads to a relaxation and enhanced accessibility of the chromatin structure for the transcriptional machinery. PGC-1α also increases DNA accessibility through recruitment of the nucleosome remodeling complex; SWItch/Sucrose NonFermentable (SWI/SNF) complex through the

interaction protein BRG1-associated factor 60a (BAF60a) **[Li et al. 2008]**. Moreover, PGC-1α also interacts with the multi-subunit; thyroid hormone receptor-associated protein/vitamin D receptor interacting protein (TRAP/DRIP) mediator complex through its C-terminal domain, which links PGC-1α to the pre-initiation RNA Polymerase II complex **[Wallberg et al. 2003]**, and thereby forms a link between PGC-1α and the transcriptional machinery. The C-terminal domain of PGC-1α also contains a serine/arginine-rich domain (RS) and a putative RNA recognition domain (RRM) (Figure 2), which are found in proteins involved in pre-mRNA processing **[Knutti and Kralli 2001]**. These domains have furthermore been shown to be necessary for correct transcriptional induction of several PGC-1α target genes **[Monsalve et al. 2000]**. However, the N-terminal truncated version of PGC-1α (NT-PGC-1α) (Figure 2) which consequently lacks the C-terminal domain can still induce transcription of several PGC-1α target genes despite absence of the RS and RRM domains **[Zhang et al. 2009]**. This indicates that the mRNA-processing abilities of PGC-1α are not necessary for correct transcription of all PGC-1α target genes.

PGC-1α isoforms and splice variants

PGC-1α can be transcribed from different promoters and subsequently spliced to yield several distinct PGC-1α protein isoforms **[Miura et al. 2008, Zhang et al. 2009, Ruas et al. 2012]**. Importantly, these different isoforms of PGC-1α can vary in their tissue distribution, response to external stimuli and target gene regulation. Two novel isoforms of PGC-1α were found to be transcribed from an alternative upstream promoter **[Miura et al. 2008]**. These were subsequently termed PGC-1α-b and PGC-1α-c, while PGC-1α-a in this case denoted the PGC-1α isoform transcribed from the classical promoter (Figure 3A). PGC-1α-b and PGC-1α-c were found to be similar in function to PGC-1α-a, but differed in their response to external stimuli. Exercise induces transcription of all three PGC-1α isoforms, however, transcriptional induction of PGC-1α-b and PGC-1α-c from the alternative promoter was dependent on β-adrenergic stimulation, while PGC-1α-a transcription was not **[Miura et al. 2008, Tadaishi et al. 2011]**. PGC-1α-a is the major isoform found in skeletal muscle and BAT in the basal state and furthermore the only PGC-1α isoform expressed in liver **[Miura et al. 2008]**. Also transcribed from the classical PGC-1α-promoter is the N-terminal truncated PGC-1α (NT-PGC-1α), which is produced through alternative 3' splicing **[Zhang et al. 2009]** (Figure 2). NT-PGC-1α contains all the NR-recognition domains of full-length PGC-1α, and can thus interact with and coactivate NRs to a similar extent. Similar to full-length PGC-1α, NT-PGC-1α is also induced by cold stimulus in BAT and in response to fasting in liver **[Zhang et al. 2009]**. This shorter splice variant of PGC-1α however lacks a nuclear localization signal, which drastically alters its subcellular localization and regulation. In its unstimulated form, NT-PGC-1α resides mainly in the cytoplasm **[Zhang**

et al. 2009], and is consequently transcriptionally inactive. This was shown to be dependent on nuclear exclusion of NT-PGC-1α through the nuclear export receptor chromosome region maintenance 1 (CRM1). However, protein kinase A (PKA)-mediated phosphorylation of NT-PGC-1α prevents interaction with CRM1, resulting in nuclear retention and increased transcriptional activity of NT-PGC-1α **[Chang et al. 2010]**. While NT-PGC-1α originates from the classical promoter, a similar 3' splice variant was also recently found to originate from the alternative promoter, yielding a truncated PGC-1α transcript variant termed PGC-1α4 **[Ruas et al. 2012]** (Figure 3B). In contrast to full-length PGC-1α (termed PGC-1α1 in the study by Ruas et al; Figure 3B) and NT-PGC-1α, PGC-1α4 does not increase transcription of classical PGC-1α target genes involved in mitochondrial and metabolic processes. PGC-1α4 instead regulates transcription of genes involved in hypertrophic response in skeletal muscle **[Ruas et al. 2012]**. Expression of PGC-1α4 matches the levels of full-length PGC-1α in skeletal muscle, BAT and heart, while this PGC-1α isoform is not expressed in liver and kidney. In the same study **[Ruas et al. 2012]**, two novel transcript variants, PGC-1α2 and PGC-1α3 were also discovered to be transcribed from the alternative promoter (Figure 3B), but not much is known about their functions. Taken together, these studies demonstrate that differential transcription and splicing of PGC-1α mRNA can regulate the responsiveness to external stimuli **[Miura et al. 2008, Tadaishi et al. 2011]**, subcellular localization **[Zhang et al. 2009, Chang et al. 2010]** and target specificity of the PGC-1α protein **[Ruas et al. 2012]**.

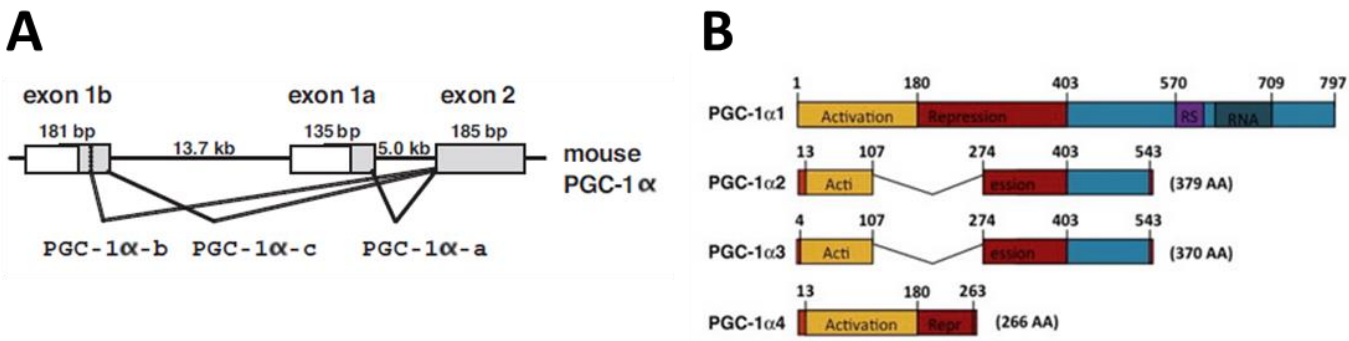

Figure 3 - PGC-1α isoforms and splice variants

(A) Alternative (exon 1b) and classical (exon 1a) exons of PGC-1α in mouse. PGC-1α-a (full-length PGC-1α). Adapted from **[Miura et al. 2008]**.

(B) Different splice variants of PGC-1α; PGC-1α1 (full-length PGC-1α), PGC-1α2, PGC-1α3 and PGC-1α4. Adapted from **[Ruas et al. 2012]**.

1.2 - Transcriptional networks regulated by PGC-1α

If we forego the upstream regulation of PGC-1α, then the transcriptional activity of PGC-1α is mainly dictated through interactions with its downstream transcriptional partners. As mentioned in the previous section, PGC-1α binds NRs in either a ligand bound (PPARα, RXRα) **[Vega et al. 2000, Delerive et al. 2002]** or ligand independent manner (ERRα) **[Huss et al. 2002]** through its NR recognition-motifs. Moreover, PGC-1α also interacts with several TFs outside the NR-family. One such TF is FOXO1, which PGC-1α binds through its C-terminal domain (Figure 2), thereby regulating transcription of the important gluconeogenic genes phosphoenolpyruvate carboxykinase 1 (*Pck1*) and glucose-6-phosphatase (*G6pc*) in liver **[Puigserver et al. 2003]**. These genes are however also regulated by PGC-1α through an LXXLL-domain dependent interaction with the hepatic NR HNF4a **[Yoon et al. 2001, Rhee et al. 2003]**. Another important interaction-partner of PGC-1α which is not dependent on the LXXLL NR-motif is MEF2C. PGC-1α was shown to interact with MEF2C through a region separate from the activation domain (Figure 2), and thereby increase glucose transporter 4 (*GLUT4*) expression in skeletal muscle **[Michael et al. 2001]**. In an effort to elucidate novel transcriptional partners of PGC-1α, Li et al. used a library of TF-Gal fusion proteins to screen for novel TFs coactivated by PGC-1α **[Li et al. 2008]**. Candidates from this screen were further evaluated for their ability to physically interact with PGC-1α, and yielded a list of 35 transcriptional partners of PGC-1α, a majority of these yet uncharacterized. This study highlighted a role for BAF60a in the recruitment of the SWI/SNF-complex (as mentioned in the section "Transcription factor and coregulator interactions"). Two recent studies successfully used a chromatin immunoprecipitation DNA-sequencing (ChIP-Seq) approach to predict PGC-1α partners based on the binding pattern of PGC-1α in the genome **[Charos et al. 2012, Baresic et al. 2014]**. Using either muscle- or liver cells, these studies could predict new transcriptional partners of PGC-1α such as; heat shock factor protein 1 (HSF1) **[Charos et al. 2012]** and the activator protein-1 complex (AP1) **[Baresic et al. 2014]**, along with known TF-partners such as ERRα **[Charos et al. 2012, Baresic et al. 2014]**. However, the majority of the predicted transcriptional partners does not overlap between the studies, and would likely represent inherent differences in the transcriptional programs targeted by PGC-1α in liver and muscle, respectively.

Another level of transcriptional control is mediated through coregulator proteins which guide the interactions of PGC-1α with other TFs, and thereby increase target specificity of PGC-1α-interactions. One such co-regulator protein is lipin 1, which is induced by PGC-1α in liver **[Finck et al. 2006]**. Lipin 1 increases transcription of PPARα and interacts with both PPARα and PGC-1α to boost activation of PGC-1α/PPARα regulated gene programs in liver in response to fasting **[Finck et al. 2006]**. Lipin 1 can furthermore be

induced through a PGC-1α-ERR pathway in heart **[Mitra et al. 2011]**. In skeletal muscle, three co-regulators of PGC-1α activity have been discovered; striated muscle activator of rho signaling (STARS) **[Wallace et al. 2011]**, hypoxia inducible factor 2 alpha (HIF2α) **[Rasbach et al. 2010]** and PGC-1- and ERR-induced regulator in muscle 1 (PERM1) **[Cho et al. 2013]**. Collectively, the transcription of these proteins is induced by exercise, and furthermore depends on PGC-1α/ERRs for their expression **[Rasbach et al. 2010, Wallace et al. 2011, Cho et al. 2013]**. Nonetheless, these co-regulators control separate processes in muscle. STARS was shown to be important for the PGC-1α-mediated transcription of carnitine palmitoyltransferase 1b (*Cpt1b*), while it did not affect the transcription of the shared PGC-1α/ERRα target vascular endothelial growth factor a (VEGFA) **[Wallace et al. 2011]**. HIF2α is instead important for the switch in fiber type induced by PGC-1α **[Rasbach et al. 2010]**. PERM1 is responsible for the PGC-1α-mediated induction of several genes (i.e. sirtuin 3, *SIRT3*), while other PGC-1α targets were not affected by modulation of PERM1 (i.e. mitochondrial transcription factor A, *TFAM*) **[Cho et al. 2013]**. Collectively, these PGC-1α-coregulatory proteins, together with the large number of TFs coactivated by PGC-1α, contribute to the target specificity of this coactivator. Since the first cloning of PGC-1α 16 years ago (1998 – 2014), its functions have been linked to a continuously expanding list of transcriptional partners. Thus, it is not surprising that PGC-1α is a versatile transcriptional regulator. As mentioned earlier, it is involved in the regulation of several tissue-specific gene programs (discussed in detail in section 1.3 as well as chapters 4 and 6) such as thermogenesis in BAT **[Puigserver et al. 1998]**, gluconeogenesis in liver **[Herzig et al. 2001, Yoon et al. 2001, Puigserver et al. 2003, Rhee et al. 2003]** and metabolic remodeling of skeletal muscle **[Handschin et al. 2007a, Handschin et al. 2007b, Handschin et al. 2007c]**. There are however certain processes which are globally regulated by PGC-1α. The most prominent of these are transcriptional regulation of mitochondrial function and oxidative phosphorylation **[Wu et al. 1999, Fernandez-Marcos and Auwerx 2011, Scarpulla 2011]**.

PGC-1α – a master regulator of mitochondrial function and biogenesis

PGC-1α is considered to be a transcriptional master regulator of mitochondrial genes **[Lin et al. 2005a, Scarpulla 2011]**, which is evident by the reduced mitochondrial transcription associated with deletion of PGC-1α in skeletal muscle **[Handschin et al. 2007b]**, brain **[Lin et al. 2004]**, heart **[Arany et al. 2005]** and intestine **[Bhalla et al. 2011]**, to name a few. In line with this, several studies have shown that overexpression of PGC-1α is sufficient to increase mitochondrial biogenesis and energy metabolism in organs such as skeletal muscle **[Lin et al. 2002b]**, adipose tissue **[Puigserver et al. 1998]** and heart **[Lehman et al. 2000, Russell et al. 2004]**. PGC-1α induces transcription of a range of mitochondrial

processes. Amongst these we find genes encoding proteins involved in the electron transport chain **[Puigserver et al. 1998]**, β-oxidation **[Vega et al. 2000]**, reactive oxygen species (ROS)-detoxification **[Valle et al. 2005, St-Pierre et al. 2006]** and mitochondrial fission/fusion **[Soriano et al. 2006]** amongst others. The versatility of PGC-1α in the regulation of mitochondrial transcription stems from its ability to coactivate several distinct TFs driving transcription of both nuclear- and mitochondrial-encoded genes. Two important TFs in this context are NRF1 and nuclear respiratory factor 2 (NRF2; also known as GA-binding protein alpha chain, GABPA). PGC-1α induces transcription of these TFs **[Wu et al. 1999, Mootha et al. 2004]**, and also acts as a transcriptional coactivator of NRF1 **[Wu et al. 1999]**. NRF1 and NRF2 increase transcription of nuclear-encoded mitochondrial genes involved in oxidative stress response and mitochondrial respiration. Importantly, PGC-1α coactivates NRF1 on the TFAM-promoter, and thereby indirectly influences transcription of mitochondrial-encoded genes **[Wu et al. 1999, Kelly and Scarpulla 2004]**. There is also evidence that PGC-1α directly interacts with TFAM on the mitochondrial DNA **[Aquilano et al. 2010, Safdar et al. 2011]**, implying a more direct role of PGC-1α in the transcription of mitochondrial-encoded genes. Apart from these central regulators of mitochondrial transcription, PGC-1α also coactivates other TFs to modulate transcription of mitochondrial genes. These TFs include ERRα **[Mootha et al. 2004, Schreiber et al. 2004]** and yin yang 1 (YY1) **[Cunningham et al. 2007]**. Especially ERRα has been shown to play an important role in the regulation of mitochondrial genes together with PGC-1α. This since ERRα constitutes a powerful feed-forward mechanisms to boost mitochondrial gene-transcription by acting as a transcriptional activator of NRF1, as well as its own promoter **[Mootha et al. 2004]**. ERRα also increases transcription of PPARα **[Huss et al. 2004]**, which is an important transcriptional regulator of mitochondrial fatty acid-import and β-oxidation, and which can be coactivated by PGC-1α **[Vega et al. 2000]**. These TF-interactions and feed-forward mechanisms constitute the basis of how PGC-1α induces transcription of several distinct but yet codependent mitochondrial processes such as β-oxidation, TCA-cycle and oxidative phosphorylation.

Upstream regulation of PGC-1α activity

PGC-1α transcription and activity is controlled by a range of upstream signaling pathways. Post-translational modifications (PTM) of PGC-1α regulates protein stability, subcellular localization as well as interaction of PGC-1α with its transcriptional partners. This ensures a precise regulation of this transcriptional activator, and allows PGC-1α to respond to various external stimuli **[Fernandez-Marcos and Auwerx 2011]**.

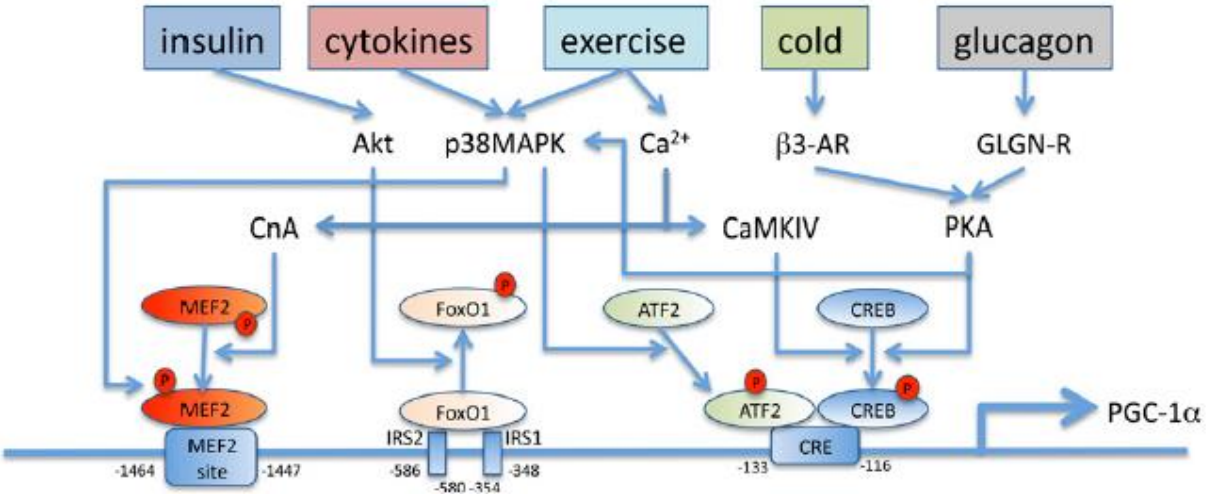

Figure 4 - Upstream signaling pathways regulating PGC-1a transcription.

Adapted from **[Fernandez-Marcos and Auwerx 2011]**.

PGC-1α induces a thermogenic gene program in BAT in response to cold stimulus, and this effect can be reproduced *in vitro* through the addition of cyclic AMP (cAMP) to primary adipocytes **[Puigserver et al. 1998]**. In liver, cAMP response element-binding protein (CREB) was shown to mediate the effects of cAMP signaling through binding and activation of the PGC-1α promoter. (Figure 4) **[Herzig et al. 2001]**. CREB is necessary for the induction of hepatic PGC-1α in response to fasting **[Herzig et al. 2001]**, and is an important transcriptional activator of PGC-1α in skeletal muscle **[Handschin et al. 2003, Akimoto et al. 2004]**. Induction of PKA-signaling also activates another important transcriptional regulator of PGC-1α; p38 mitogen-activated protein kinase (p38 MAPK) **[Cao et al. 2004]**. Importantly, p38 MAPK was shown to be necessary for CREB to induce PGC-1α transcription and hepatic gluconeogenesis in response to fasting **[Cao et al. 2005]**. In BAT, p38 MAPK is also required to induce PGC-1α transcription in response to β-adrenergic stimulation, however this is mediated primarily through activating transcription factor 2 (ATF2) (Figure 4), and not through CREB **[Cao et al. 2004]**. Two other important transcriptional regulators of PGC-1α are MEF2C and MEF2D **[Handschin et al. 2003, Akimoto et al. 2004]**. In skeletal muscle, MEF2C/D are necessary transcriptional induction of PGC-1α through calcineurin A (CnA) **[Handschin et al. 2003]** and in response to nerve stimulation **[Akimoto et al. 2004]**. Increased intracellular calcium during muscle contractions can modulate PGC-1α through CnA/MEF2, but also through activation of CREB

through calcium/calmodulin-dependent protein kinase IV (CaMKIV) **[Handschin et al. 2003]** (Figure 4). Importantly, PGC-1α increases the transcriptional activity of MEF2C/D on its own promoter, and thereby forms a positive autoregulatory loop **[Handschin et al. 2003]**, similar to what was mentioned earlier for ERRα **[Mootha et al. 2004]**.

Activation of PKA and p38 MAPK results in transcriptional activation of PGC-1α (Figure 4). However, these kinases have also been shown to directly modulate PGC-1α-activity through phosphorylation of PGC-1α. PKA phosphorylates NT-PGC-1α at three separate sites and thereby increases the nuclear retention and transcriptional activity of this PGC-1α isoform **[Chang et al. 2010]**. Full length PGC-1α furthermore contains 12 predicted PKA-phosphorylation sites, and PKA mediates stabilization of full length PGC-1α in response to cAMP signaling **[Shoag et al. 2013]**. Phosphorylation of PGC-1α by p38 MAPK **[Knutti et al. 2001, Puigserver et al. 2001]** (Figure 5) also increases protein stability **[Puigserver et al. 2001]**, and prevents the interaction of PGC-1α with its repressor p160 myb binding protein ($p160^{MBP}$) **[Fan et al. 2004]**, hence increasing its transcriptional activity. Other important kinases in the regulation of PGC-1α activity are Cdc2-like kinase 2 (CLK2), p70 ribosomal protein S6 kinase (S6K) and AKT **[Schmidt and Mandrup 2011]**. The activity of these kinases is influenced by insulin, and thus play an important role in the inhibition of hepatic PGC-1α activity during transition from a fasted to a fed state. In response to insulin signaling, AKT inhibits the gluconeogenic gene program through phosphorylation and nuclear exclusion of FOXO1, which is an important transcriptional partner of PGC-1α in the regulation of gluconeogenic genes **[Puigserver et al. 2003]**. Moreover, AKT can also directly phosphorylate PGC-1α (Figure 5) and inhibit its transcriptional activity **[Li et al. 2007]**. AKT also phosphorylates and activates CLK2, which subsequently inhibits PGC-1α activity through an inhibitory phosphorylation (Figure 5) **[Rodgers et al. 2010]**. Another AKT target is CREB-regulated transcription coactivator 2 (CRTC2, TORC2). In its phosphorylated state CRTC2 remains in the cytoplasm, while during fasting it becomes dephosphorylated and translocates to the nucleus, where it coactivates CREB-mediated transcription of PGC-1α and gluconeogenic genes **[Koo et al. 2005]**. CRTCs are also associated with increased PGC-1α transcription in skeletal muscle **[Wu et al. 2006]** and BAT **[Muraoka et al. 2009]**. S6K is another insulin-responsive kinase known to phosphorylate and inactivate transcription of gluconeogenic genes regulated by PGC-1α **[Lustig et al. 2011]**. However phosphorylation by S6K specifically inhibits the interaction of PGC-1α with HNF4a. PGC-1α can in this context still coactivate ERRα and PPARα, and thus still induce processes such as mitochondrial biogenesis and β-oxidation **[Lustig et al. 2011]**. S6K can thus in a similar

fashion as STARS, PERM1 and Lipin1 fine-tune the activity of PGC-1α towards certain promoters, and thus add another layer of complexity to the regulation of PGC-1α.

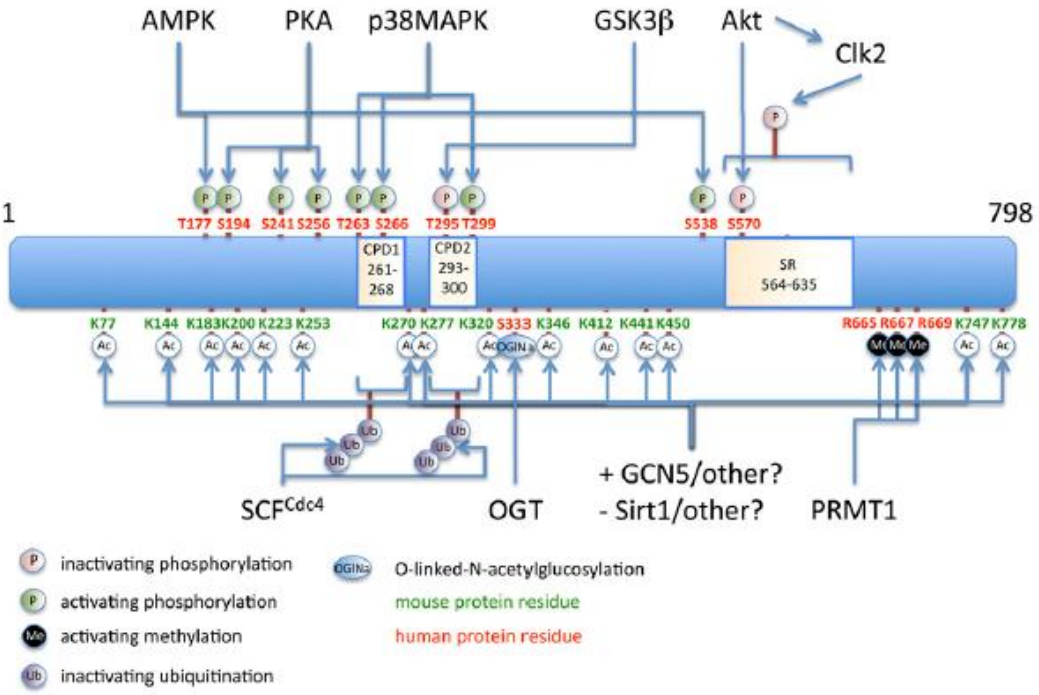

Figure 5 - Post-translational modifications of the PGC-1a protein

Adapted from **[Fernandez-Marcos and Auwerx 2011]**.

AMP-activated protein kinase (AMPK) is another kinase known to phosphorylate PGC-1α and thereby increase PGC-1α activity and transcriptional induction of mitochondrial genes in skeletal muscle cells **[Jager et al. 2007]**. Furthermore, AMPK signaling is also connected to the activation of another important energy sensor and regulator of PGC-1α activity; sirtuin 1 (SIRT1). SIRT1 is a nicotinamide adenine dinucleotide (NAD^+)-sensitive protein deacetylase, which can deacetylate and thereby activate PGC-1α **[Nemoto et al. 2005]**. PGC-1α activity is regulated through its acetylation status, since mutations of 13 of its acetylation residues (Figure 5) to arginine enhanced transcriptional activity of PGC-1α in liver, leading to increased gluconeogenic gene expression **[Rodgers et al. 2005]**. SIRT1 is thus an important regulator of hepatic gluconeogenesis through deacetylation and activation of PGC-1α in response to fasting **[Rodgers et al. 2005]**. Since PGC-1α is activated through deacetylation, it can consequently be inhibited by acetylation. One important acetyltransferase which acetylates PGC-1α in both muscle **[Philp**

et al. 2011b] and liver **[Lerin et al. 2006, Dominy et al. 2012]** is general control nonrepressed protein 5 (GCN5). Increased acetylation through GCN5 leads to an enhanced association of PGC-1α with inactive transcriptional domains within the nucleus, thereby decreasing PGC-1α target gene transcription **[Lerin et al. 2006]**. Additionally, insulin was shown to increase GCN5-activity through activation of its upstream kinase; cyclin D1- cyclin-dependent kinase 4 (CDK4), thereby reducing PGC-1α activation **[Lee et al. 2014]**. Other acetyltransferases such as p300, SRC-1 and SRC-3 have also been shown to interact with PGC-1α, however acetylation status and activation of PGC-1α was not affected through these interactions **[Jeninga et al. 2010]**. Apart from changes in phosphorylation- and acetylation status, PGC-1α activity is modulated by several other PTMs. Arginine methylation of PGC-1α through protein arginine methyltransferase 1 (PRMT1) is associated with increased transcriptional activity of PGC-1α **[Teyssier et al. 2005]**, and a similar activating effect has been observed for O-GlcNAcylation of PGC-1α. O-GlcNAcylation of PGC-1α leads to increased interaction of PGC-1α with the deubiquitinase BRCA1-Associated Protein 1 (BAP1), thereby relieving PGC-1α of its inhibitory ubiquitination **[Ruan et al. 2012]**. Moreover, Skp1/cullin/F-box-cell division control 4 (SCF^{Cdc4}) is an E3 ubiquitin ligase responsible for PGC-1α ubiquitination and proteosomal degradation **[Olson et al. 2008]**. Proteosomal degradation of PGC-1α was shown to mainly occur in the nucleus **[Trausch-Azar et al. 2010]**, which could explain the increased stability of the mainly cytoplasmic NT-PGC-1α isoform. In conclusion, PGC-1α activity is regulated through a variety of different PTMs, which are necessary for the responsiveness of PGC-1α to various external stimuli, and which ensures a controlled regulation of this transcriptional coactivator.

1.3 – Organ-specific effects of PGC-1α

In contrast to many other coactivator proteins, the expression of PGC-1α varies between organs and is highly regulated by environmental stimuli such as cold, fasting and exercise. The majority of the research on tissue-specific effects of PGC-1α has been focused on the major metabolic organs skeletal muscle, liver and BAT. In line with this, we will mainly focus on the role of PGC-1α in liver and adipose tissue in this section, and the role of PGC-1α in skeletal muscle will be discussed in more detail in chapters 6-9 of this thesis. However, several studies have also investigated the role of PGC-1α in other cell types, highlighting new tissue-specific transcriptional programs modulated by PGC-1α. In retina, PGC-1α protects against retinal deterioration **[Egger et al. 2012]**, and promotes angiogenesis **[Saint-Geniez et al. 2013]**. In the skin PGC-1α regulates melanin production and hence pigmentation **[Shoag et al. 2013]**. In the intestine

PGC-1α protects against formation of tumors by regulating mitochondrial biogenesis **[D'Errico et al. 2011]**. In adrenal glands, PGC-1α coactivates steroidogenic factor 1 (SF1) to regulate steroidogenesis **[Zhu et al. 2010]**, and in pancreatic β-cells, PGC-1α inhibits insulin release **[Yoon et al. 2003]**. Collectively, these studies give an indication of the multi-faceted role of PGC-1α based on the organ-specific context.

Liver

In a fed state, PGC-1α expression in liver is low compared to more oxidative organs such as heart, brain and skeletal muscle **[Finck and Kelly 2006]**. Upon fasting however, there is a strong induction of PGC-1α transcription and activity, resulting in an activation of genes involved in gluconeogenesis, β-oxidation, ketogenesis, heme biosynthesis and bile acid homeostasis **[Liu and Lin 2011]**. PGC-1α is an important regulator of hepatic gluconeogenesis, and this is evident in mouse models with a hepatic disruption of PGC-1α activity, which display hypoglycemia **[Koo et al. 2004]** and reduced transcription of gluconeogenic genes upon fasting **[Koo et al. 2004, Handschin et al. 2005]**. Surprisingly, global PGC-1α knockout mice display no impairment in the induction of gluconeogenic genes with fasting **[Lin et al. 2004, Handschin et al. 2005, Leone et al. 2005]**. These findings indicate that global deletion of PGC-1α leads to a complex systemic metabolic phenotype, which can preclude interpretation of the tissue-autonomous effects of PGC-1α on hepatic metabolism. PGC-1α regulates transcription of gluconeogenic genes mainly through coactivation of FOXO1 **[Puigserver et al. 2003]**, HNF4a **[Yoon et al. 2001, Puigserver et al. 2003, Rhee et al. 2003]** and GR **[Yoon et al. 2001]**. The increased hepatic PGC-1α transcription and activity in response to fasting, is induced through several distinct mechanisms (as discussed in the section "Upstream regulation of PGC-1α activity"). In short, binding of glucagon to its receptor leads to an increase in intracellular cAMP levels. This results in activation of PKA, and subsequent phosphorylation and induction of CREB and p38 MAPK, leading to transcriptional induction of PGC-1α during fasting **[Herzig et al. 2001, Cao et al. 2005, Handschin and Spiegelman 2006]**. Glucagon also leads to dephosphorylation and nuclear translocation of CRTC2, which increases the transcriptional activity of CREB on the PGC-1α promoter **[Koo et al. 2005]**. Another important regulator of hepatic gluconeogenesis is SIRT1, which increases the transcriptional activity of PGC-1α towards gluconeogenic genes through protein deacetylation **[Rodgers et al. 2005]**. GCN5 on the other hand increases PGC-1α acetylation in liver in response to insulin thereby leading to PGC-1α inactivation **[Lee et al. 2014]**. Insulin efficiently shuts down PGC-1α activity and the transcriptional activation of gluconeogenic genes in a fed state through activation of AKT. AKT directly phosphorylates and inactivates PGC-1α **[Li et al. 2007]**, as well as CRTC2 **[Koo et al. 2005]**. Insulin signaling also activates S6K, which prevents interaction of PGC-1α with HNF4a, thereby inhibiting gluconeogenesis

[Lustig et al. 2011]. In line with the repressive effect of insulin on PGC-1α transcriptional activity, PGC-1α activity is increased in livers of diabetic mice. Moreover, liver-specific deletion of PGC-1α reduces hyperglycemia **[Koo et al. 2004]**, which indicates that increased hepatic PGC-1α activity is involved in hepatic insulin resistance and development of diabetic hyperglycemia. PGC-1α is also an important regulator of hepatic β-oxidation and ketogenesis. This is evident in mice with reduced hepatic PGC-1α expression, which display a mild hypoketonemia in response to fasting. These mice also develop fasting induced hepatic steatosis as a result of reduced fatty acid oxidation **[Estall et al. 2009]**, a phenotype which is also observed in global PGC-1α knockout models **[Leone et al. 2005]**. PGC-1α regulates ketogenic and β-oxidation genes in liver through interaction with the TF PPARα, which is a known transcriptional partner of PGC-1α **[Vega et al. 2000]**. Consequently, knockout of PGC-1α in liver is associated with reduced transcription of β-oxidation genes **[Koo et al. 2004]**. PPARα is furthermore required for the induction of fibroblast growth factor 21 (FGF21) in liver during fasting. FGF21 has been demonstrated to be important for the induction of PGC-1α in liver with fasting **[Potthoff et al. 2009]**, thereby forming a feed-forward mechanism to enhance hepatic ketogenesis and β-oxidation upon fasting.

Adipose tissue

PGC-1α is expressed at a higher basal level in BAT compared to WAT, however thermogenic stimuli such as β-adrenergic stimulation, are known to increase PGC-1α levels in both depots. PGC-1α is important for the induction of the thermogenic gene *UCP1* through coactivation of PPARγ, TR **[Puigserver et al. 1998]** and interferon regulatory factor 4 (IRF4) **[Kong et al. 2014]** on the *UCP1*-promoter. This leads to enhanced mitochondrial uncoupling and subsequent energy dissipation in BAT. However, PGC-1α also induces mitochondrial metabolic processes to sustain the high metabolic activity in both WAT and BAT depots during cold adaptation. This was demonstrated through ectopic expression of PGC-1α in either mouse **[Puigserver et al. 1998]** or human **[Tiraby et al. 2003]** adipocytes, which led to a concomitant induction of *UCP1* expression and mitochondrial oxidative phosphorylation in these cells. Activation of PGC-1α in adipocytes in response to cold is attributed to the activation of CREB and p38 MAPK/ATF2 through cAMP/PKA signaling **[Cao et al. 2004]**. In line with this, knockdown of PGC-1α in cultured adipocytes blunted the thermogenic response induced by cAMP-stimulation **[Uldry et al. 2006]**. PGC-1α transcription in BAT is furthermore driven by PR domain containing 16 (PRDM16), an important co-regulator for BAT differentiation **[Seale et al. 2007]**, as well as the recently discovered thermogenic regulator IRF4 **[Kong et al. 2014]**. In line with the importance of PGC-1α in thermogenic adaptation in adipose tissue, whole-body PGC-1α knockout mice are cold sensitive **[Lin et al. 2004, Leone et al. 2005]**. This phenotype was evident

in both global PGC-1α knockout models **[Lin et al. 2004, Leone et al. 2005]**, however it was only associated with blunted induction of *UCP1* in response to cold in one of these models **[Lin et al. 2004]**. Surprisingly, adipose tissue specific PGC-1α knockout mice display no impairment in the basal expression of *UCP1* in either WAT or BAT **[Pardo et al. 2011]**. Moreover, these mice display no impairment in basal transcription of mitochondrial genes or the induction of mitochondrial genes in response to rosiglitazone-treatment in the absence of PGC-1α **[Pardo et al. 2011]**. Hence, further studies are needed to elucidate the complete role of PGC-1α in both WAT and BAT depots.

To fully understand the role that PGC-1α plays in the regulation of whole body metabolism, it is essential to study the effects of this coactivator in all major metabolic organs. Further studies on the tissue-specific transcriptional programs regulated by PGC-1α will give a greater insight into the global role of PGC-1α, and how modulation of PGC-1α in these organs can be used in a therapeutic context. In line with this, we will in chapter 4/manuscript 1 of this thesis present our data regarding the role of PGC-1α in the kidney. Moreover, in chapter 7/manuscript 2 and chapter 8/manuscript 3 of this thesis we will focus on our data regarding skeletal muscle and the role of pharmacological activation of PGC-1α, as well as the role of PGC-1α in the regulation of skeletal muscle ketone body oxidation.

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2. Aims of the thesis

Since the first cloning of PGC-1α over a decade-and-a-half ago, seminal studies have elucidated both the upstream- and downstream mechanisms responsible for regulating the effects of this coactivator. Importantly, these studies have also widened our knowledge of important metabolic processes such as thermogenesis, gluconeogenesis and exercise adaptation, which are all closely linked to PGC-1α activity. Consequently, our knowledge about the role of PGC-1α is best understood in metabolic and oxidative organs such as BAT, skeletal muscle and liver. However, the kidney is amongst the organs with the highest baseline PGC-1α expression. Despite its high basal expression of PGC-1α, relatively little is known regarding the role of this coactivator in the kidney. Intriguingly, several renal pathologies are associated with defects in mitochondrial and metabolic processes in tubule cells and are furthermore associated with reduced PGC-1α expression. This prompted us to generate a mouse model with an inducible nephron-specific knockout of PGC-1α. This model allowed us to study transcriptional networks regulated by PGC-1α in the kidneys, and how ablation of PGC-1α affects renal physiology. The data of this study are presented in chapter 4/manuscript 1 of this thesis. The first aim of the present thesis can thus be summarized:

1. **Elucidate the role of PGC-1α in transcriptional regulation of renal physiology, both in the basal state and in response to salt and dietary stress.**

The second part of the thesis is aimed at elucidating the role of PGC-1α as a therapeutic target for calorie restriction (CR)/exercise-mimetics in skeletal muscle. Resveratrol and SRT1720 are two CR/exercise mimetic compounds, which have been shown to activate PGC-1α in skeletal muscle, and to promote mitochondrial biogenesis and increase energy expenditure. This is furthermore associated with an improved metabolic phenotype in obese mice treated with these compounds. We were thus interested in whether skeletal muscle PGC-1α is the main mediator of the beneficial effects of resveratrol and SRT1720 in skeletal muscle, and in extension, how this affects whole body metabolism in obese mice. Moreover, we were interested in whether the structurally distinct compounds resveratrol and SRT1720 would elicit analogous or differential effects in major metabolic organs such as skeletal muscle, liver and white adipose tissue. These finding are presented in chapter 7/manuscript 2 of this thesis, and can be summarized with these two aims:

2. **Define the role of skeletal muscle PGC-1α as a potential mediator of the beneficial effects of resveratrol and SRT1720 in skeletal muscle, as well as on systemic metabolic parameters.**

3. **Compare the effects elicited by resveratrol and SRT1720 treatment in diet-induced obese mice; are the effects on organ-specific and systemic metabolic parameters analogous?**

Finally, we were interested in investigating the role of PGC-1α in the metabolic adaptation of skeletal muscle to a ketotic environment. PGC-1α is known to modulate several metabolic processes involved in glucose and fatty acid oxidation in muscle. Apart from glucose and fatty acids, ketone bodies are important metabolic fuels in skeletal muscle, especially during long-term fasting. However, despite the high basal ketolytic capacity of oxidative muscles, the role of PGC-1α in the regulation of ketone body oxidation has to our knowledge so far not been investigated. Our findings regarding the role of PGC-1α in the regulation of ketone body oxidation will be presented in chapter 8/manuscript 3 of this current thesis. The fourth aim of this thesis can thus be summarized:

4. **Investigate the role of PGC-1α in the regulation of skeletal muscle ketone body oxidation, and its impact on systemic ketone body metabolism.**

During the work on this thesis, several side-projects have branched of the main lines of investigation mentioned above. These are however not finished studies, and will thus be discussed in brief within the discussion-sections of the respective chapters (aim 5/chapter 5; aim 6/chapter 9). The aims of these side-projects can be summarized accordingly:

5. **Determine the role of PGC-1α in kidney glomeruli, and its role in the maintenance of the renal filtration barrier in physiological and pathophysiological states.**

6. **Define the mitochondrial and metabolic adaptations in skeletal muscle during long-term ketosis, and which role PGC-1α plays in this.**

THE ROLE OF PGC-1α IN KIDNEY

3. Introduction to renal physiology

3.1 - Kidney – structure and function

The major function of the renal system is to filter blood, and to ensure proper reabsorption of nutrients and electrolytes. At the same time, toxic compounds, metabolic waste products and excess acids are removed from the circulation. The importance for the kidneys in whole organism health is underscored by the prevalence of hypertension, acidosis, anemia, bone disorders, malnutrition, cognitive impairment and inflammation in patients suffering from chronic kidney disease **[Levey and Coresh 2012]**. The extent of these complications indicate that the kidney is responsible for more than just passive filtration of blood and urine formation. The kidney is an important endocrine organ involved in blood pressure regulation through secretion of renin, erythropoiesis through production of erythropoietin and mineral and bone homeostasis through activation of vitamin D3 within renal mitochondria **[Sahay et al. 2012]**. The kidneys are also important for systemic glucose homeostasis. It has been demonstrated that renal gluconeogenesis matches the capacity of hepatic gluconeogenesis in a post-prandial state **[Mitrakou 2011]**. However, the net glucose release from the kidney is lower due to the inability of renal cells to store and release glucose from glycogen stores **[Stumvoll et al. 1997]**. Renal gluconeogenesis has furthermore been shown to contribute significantly to hyperglycemia in diabetic patients **[Mitrakou 2011]**.

Apart from the important functions of the kidney in endocrine and metabolic processes, the main role of the kidney is to maintain systemic ion-, water- and nutrient-homeostasis through reabsorption and secretion of ions and nutrients across the renal tubular epithelium. Urine formation is a complex process which takes place within the functional subunit of the kidney, the nephron, consisting of several morphologically and functionally distinct cell types. The main function of the nephrons is to filter blood and excrete waste-products through urine formation. Approximately 180 liters of blood are filtered each day through the nephrons of an adult human **[Boron and Boulpaep 2003]**. Blood reaches the kidneys through the renal arteries, and is filtered in a complex capillary system within the renal glomeruli. Endothelial cells together with podocyte cells form a fenestrated filtration barrier around the glomerular capillaries, to ensure proper filtration of water and electrolytes while retaining plasma proteins and blood cells within the vascular compartment **[Miner 2011]**. The glomerulus constitute the initial filtration unit of the nephron, and empties into the proximal tubules (Figure 1).

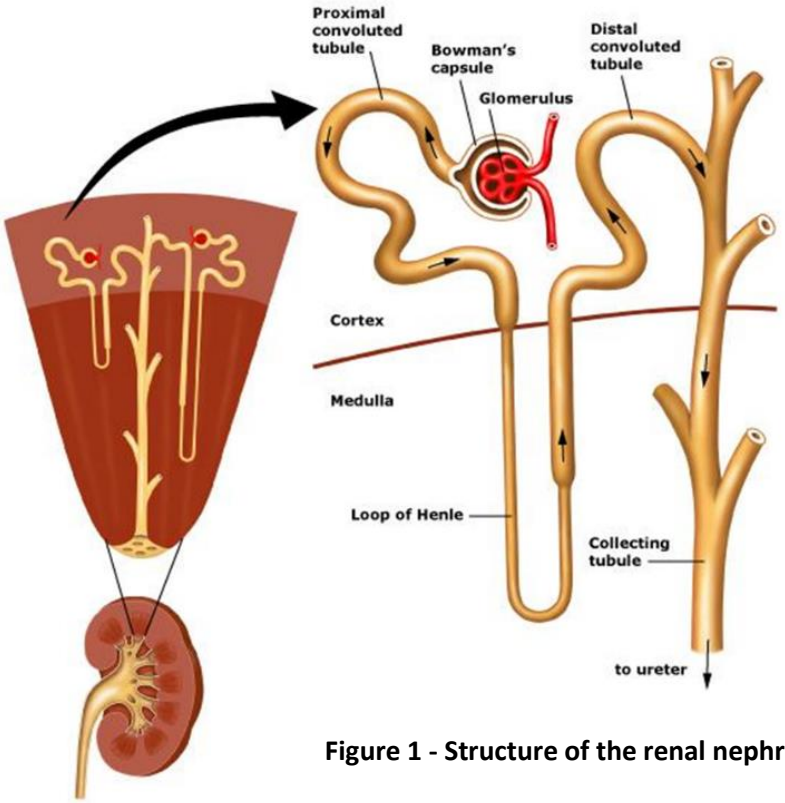

Figure 1 - Structure of the renal nephron.

The proximal tubules are responsible for the reabsorption of approximately 70% of the filtered water, the majority of electrolytes and all filtered nutrients such as glucose and amino acids. This efficient reabsorption is facilitated by the extensive brush border of the proximal tubule cells, coupled to a high basal transporter activity **[Guyton and Hall 2000, Rhoades and Bell 2013]**. Na^+-K^+-ATPases in the basolateral membrane of proximal tubule cells create a sodium gradient over the epithelial cells which drives reabsorption of water, but also glucose through sodium-dependent glucose transporters **[Wright 2001]** and amino acids through amino acid sodium symporters **[Verrey et al. 2009]** to name a few. The importance of the proximal tubules for the maintenance of systemic nutrient and ion homeostasis can be appreciated in patients suffering from renal Fanconi syndrome which is linked to a general dysfunction in sodium-linked transport in the proximal tubules. These patients display a urinary loss of glucose, amino acids and phosphates, together with renal tubular acidosis **[Sirac et al. 2011]**. The proximal tubules are connected to the thin loop of Henle, which stretches into the renal medulla and which is essential for urine concentration (Figure 1) **[Halperin et al. 2008]**. The thick ascending part of the loop of Henle is responsible for further ion reabsorption from the primary filtrate. The subsequent parts of the nephron are named the aldosterone-sensitive distal nephron (ASDN), which comprises parts

of the distal convoluted tubules, connecting tubules and collecting ducts **[Loffing and Korbmacher 2009]** and which are responsive to the steroid hormone aldosterone **[Rossier et al. 2013]**. While passive reabsorption in the initial parts of the nephron accounts for the majority (~90%) of salt and water re-uptake, the final balance of sodium- and potassium uptake/excretion occurs in the ASDN **[Rossier et al. 2013]**. Aldosterone binds to the mineralocorticoid receptor (MR) within ASDN cells, and induces transcription of several genes important for renal sodium uptake, such as sodium transporters (epithelial sodium channel; *ENaC*) and regulators of these channels (serum- and glucocorticoid-regulated kinase 1; *SGK1*) **[Loffing and Korbmacher 2009]**, thereby increasing renal sodium uptake. The primary filtrate, now considered urine, is then drained into the major calyx and subsequently the ureter. Closely linked to the regulation of salt- and water homeostasis, the kidneys also regulates systemic blood pressure. In response to a drop in blood pressure, the renal juxtaglomerular cells increase production and release of renin into the circulation. Renin is the initial component of the renin-angiotensin-aldosterone system (RAAS), which controls systemic blood pressure through regulation of extracellular fluid volume, cardiovascular activity and systemic vasoconstriction **[Brewster and Perazella 2004]**. Renin is the first step in a proteolytic pathway yielding angiotensin II (ANGII) from hepatic angiotensinogen (Figure 2). ANGII, which is a potent vasoconstrictor, also stimulates aldosterone-release from the adrenal cortex, which in turn acts within the ASDN to increase sodium and water retention from the nephron **[Brewster and Perazella 2004]**. In combination to the systemic effects of the RAAS, there is also an intrarenal RAAS, since all components of the RAAS are expressed within different segment of the nephron **[Moon 2013]**. This leads to generation of intrarenal ANGII, which can regulate sodium re-uptake independently of aldosterone **[Zaika et al. 2013]**. Another important system involved in regulation of blood pressure is the kallikrein-kinin system (KKS) **[Sharma et al. 1996]**. Similar to the RAAS, the KKS has both a systemic and an intrarenal component, but in contrast to the RAAS, activation of KKS instead leads to vasodilation **[Scicli and Carretero 1986]**, reduced sodium reabsorption **[Zaika et al. 2011]** and consequently reduced blood pressure.

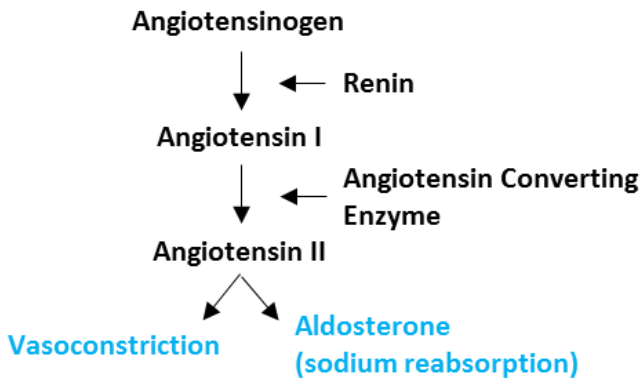

Figure 2 - The Renin-Angiotensin-Aldosterone system (RAAS).

3.2 - Renal energy metabolism and PGC-1α

The kidney is a highly metabolic organ which relies almost entirely on oxidative phosphorylation. The kidneys thus have a high oxygen consumption rate, only exceeded by that of the heart **[O'Connor 2006]**. Renal mitochondria are integral to the function of the kidneys, since the majority of the ATP produced in kidney is derived from oxidative metabolism **[Soltoff and Mandel 1984]**. Mitochondrial content and oxidative capacity is highest within cells of the proximal tubules and thick ascending limb of henle, while other nephron segments such as glomeruli, thin loop of henle, distal- and collecting tubules have a lower mitochondrial content **[Soltoff 1986]**. Oxidative phosphorylation in the proximal tubules and thick loop of henle is necessary to maintain the high transport capacity of these segments, since the majority of transepithelial transport in these segments is driven through ATP-hydrolysis by basolateral Na^+-K^+-ATPase channels **[Soltoff 1986]**. The constant high rate of filtration and reabsorption within the nephron results in a dependence on mitochondrial ATP production for proper renal function. This is evident in patients suffering from hereditary mitochondrial disorders, which often display a reduced proximal tubule function, associated with a loss of nutrients and electrolytes in the urine **[Emma et al. 2011, Rahman and Hall 2013, Che et al. 2014]**. Reduced mitochondrial function is also found in several disease states associated with reduced renal function, such as sepsis **[Tran et al. 2011, Parikh 2013]**, diabetes **[Sharma et al. 2013]** and ischemia/reperfusion injury **[Brooks et al. 2009, Funk and Schnellmann 2013, Lempiainen et al. 2013]**. The close link between mitochondrial dysfunction and renal disease implies that a reduction in mitochondrial oxidative capacity, which is often coupled to increased oxidative stress and reduced energy levels, could play an important role in both the etiology and the pathology of renal disease states.

The kidney is one of the organs that expresses the highest baseline levels of PGC-1α **[Larrouy et al. 1999]**, which is in agreement with the high mitochondrial content and oxidative capacity of this organ. Interestingly, a common feature associated with reduced mitochondrial function in sepsis, diabetes and ischemia/reperfusion-injury is a concomitant reduction in renal PGC-1α levels **[Tran et al. 2011, Funk and Schnellmann 2013, Lempiainen et al. 2013]**. Reduced PGC-1α levels and mitochondrial dysfunction in the kidney during renal disorders could point towards a therapeutic potential of activating PGC-1α in the context of renal diseases. Several therapeutic strategies aimed at improving the impaired mitochondrial phenotype associated with renal disease have already been tested in rodent models. One study could show that targeted peptides, which act as ROS-scavengers and inhibitors of mitochondrial permeability transition could ameliorate renal damage during ischemia/reperfusion-injury

[Szeto et al. 2011]. Also treatment with the SIRT1-activator SRT1720 **[Funk and Schnellmann 2013]** or calorie restriction **[Lempiainen et al. 2013]** have been shown to improve the mitochondrial phenotype in kidney and reduce ischemia/reperfusion-injury, an occurrence closely linked to increased PGC-1α activation with these treatments **[Funk and Schnellmann 2013, Lempiainen et al. 2013]**. The results obtained in these studies point towards a beneficial effect of activating PGC-1α in the kidney. Especially since PGC-1α overexpression is associated with improved oxidative phosphorylation and reduced oxidative stress in several transgenic rodent models **[Lin et al. 2002, Liang et al. 2009, Svensson and Handschin 2014]**. Aldosterone-induced epithelial-to-mesenchymal transition of proximal tubule epithelial cells is a pathological state associated with reduced mitochondrial function, and this phenotype could be ameliorated through overexpression of PGC-1α **[Yuan et al. 2012]**. Moreover, proximal tubule-specific ablation of PGC-1α has been shown to exacerbate endotoxemic acute kidney injury, and overexpression of PGC-1α could protect against tumor necrosis factor (TNF)-induced mitochondrial dysfunction in human proximal tubule cells **[Tran et al. 2011]**. Reduced PGC-1α activity has also been linked to high fat diet (HFD)-induced oxidative stress and lipotoxicity in the kidney **[Chung et al. 2012, Kim et al. 2013, Hong et al. 2014]**. Treatment of genetically obese mice with the PPARα-agonist fenofibrate has been shown to normalize expression and activity of PGC-1α, and thus ameliorate HFD-induced lipotoxicity **[Chung et al. 2012, Hong et al. 2014]**. Activation of PGC-1α through resveratrol-treatment has also been associated with reduced glomerular damage in genetically obese mice **[Kim et al. 2013]**. Specifically in the glomerular podocytes, PGC-1α has been implicated to play a dual role. Overexpression of PGC-1α can protect podocytes against aldosterone-induced damage **[Yuan et al. 2012]** while another study demonstrated a role of PGC-1α in mediating podocyte apoptosis and growth arrest **[Kim and Park 2013]**.

Despite the strong connection between reduced PGC-1α activity and numerous renal disorders, relatively little is known regarding the actual role of PGC-1α in renal physiology and pathophysiology. The high basal oxidative capacity of the kidney implies a prominent role for PGC-1α in renal physiology, which is further supported by its abundant expression in this organ. The mitochondrial impairment associated with several etiologically distinct renal disorders also indicates that decreased PGC-1α activity and a concomitant reduction in oxidative phosphorylation could be a common denominator for these renal disease states. In light of this, we were interested in elucidating the role of PGC-1α in the kidney, and how ablation of PGC-1α would affect renal physiology.

3.3 - The role of PGC-1α in kidney – Aims

The kidney is one of the organs with the highest energy demand in the body. A high basal rate of mitochondrial oxidative phosphorylation is needed to drive the constant reabsorption of salts and nutrients over the tubular epithelium. Considering the established role of PGC-1α in maintaining basal mitochondrial function and biogenesis, it is not surprising that this transcriptional coactivator is highly expressed in the kidneys. In addition, several renal pathologies are closely linked to reduced PGC-1α activity and impaired renal oxidative capacity. However, relatively little is known regarding the role of PGC-1α in the kidney. Hence, in the first manuscript of this thesis, we have generated a mouse model with an inducible nephron-specific knockout of PGC-1α. This allowed us to study transcriptional regulation by PGC-1α in the kidneys, and how the ablation of PGC-1α affects renal physiology. The specific aims for the first manuscript of this thesis can be summarized as follows:

1. **Determine the transcriptional networks regulated by PGC-1α in the kidney, and identify the relevant transcriptional partners of PGC-1α in this context.**

2. **Investigate how ablation of PGC-1α specifically in the nephron affects renal functions such as blood pressure regulation and salt/water homeostasis.**

3. **Study the impact of PGC-1α-ablation on transcriptional regulation of renal mitochondrial and metabolic processes, in both lean and diet-induced obese mice.**

With the first study in this thesis we aim to improve the understanding of the role of PGC-1α in renal physiology. This will not only further our understanding of renal transcriptional regulation and function, but will help to elucidate the potential therapeutic applications of modulating PGC-1α activity in a renal setting.

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Manuscript 1:

Transcriptional Regulation of Renal Function and Energy Metabolism by PGC-1α.

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4. Manuscript 1: Transcriptional Regulation of Renal Function and Energy Metabolism by PGC-1α

Abstract

The kidney is a vital organ for maintenance of systemic salt and water homeostasis. Reabsorption of nutrients and ions across the renal tubular membrane is driven by ATP-hydrolysis, and the kidneys are highly reliant on mitochondrial oxidative phosphorylation to maintain a high basal ATP-production rate. Consequently, renal mitochondrial dysfunction is a common denominator for several distinct renal pathologies. Hence, it is important to understand how renal mitochondrial function is regulated. Peroxisome proliferator-activated receptor coactivator 1 alpha (PGC-1α) is an important transcriptional regulator of mitochondrial biogenesis and function. To elucidate the role of PGC-1α in the regulation of renal oxidative energy metabolism and function, we generated inducible nephron-specific PGC-1α knockout (PiNKO) mice. PGC-1α-ablation in the kidneys results in reduced transcription of several mitochondrial and metabolic processes. PiNKO mice furthermore display a mild hypertensive and salt-losing phenotype associated with altered transcription of several genes involved in transmembrane ion transport. Finally, we demonstrate that PGC-1α is necessary for the induction of genes involved in fatty acid oxidation in the kidney during high fat diet feeding. PGC-1α thus constitutes a promising therapeutic target to ameliorate renal disorders associated with metabolic and mitochondrial dysfunction, such as diabetic nephropathy, ischemia/reperfusion injury or sepsis.

Introduction

The kidney is a highly complex organ responsible for removal of toxins and metabolic waste products from the body, through filtration of blood and subsequent urine formation **[Tryggvason and Wartiovaara 2005]**. This passive filtration is coupled to a system of transporters along the nephron, responsible for maintaining systemic nutrient- and salt homeostasis **[Zhuo and Li 2013]**. Tubular reabsorption is an energy-demanding process mediated through ATP-hydrolysis by Na^+-K^+-ATPases in tubular epithelial cells. Mitochondrial density is thus found to be highest in tubule segments associated with high basal transcellular transport, such as the proximal tubules and the thick loop of henle **[Soltoff 1986]**. The integral role of mitochondria in renal function is also underscored by the prevalence of renal dysfunction in patients suffering from hereditary mitochondrial disorders **[Emma et al. 2011, Rahman and Hall 2013, Che et al. 2014]**. The transcriptional coactivator, peroxisome proliferator-activated receptor coactivator 1 alpha (PGC-1α) is an important regulator of mitochondrial homeostasis **[Puigserver and Spiegelman 2003]**. PGC-1α was originally discovered through its ability to induce a thermogenic gene program in brown adipose tissue **[Puigserver et al. 1998]**. Since then, it has been shown that PGC-1α is a critical modulator of fasting-induced gluconeogenesis and β-oxidation in liver **[Liu and Lin 2011]**, and that it is furthermore important for the induction of an oxidative slow-twitch phenotype in skeletal muscle upon exercise **[Svensson and Handschin 2014]**. Apart from these tissue-specific effects, PGC-1α is a global regulator of mitochondrial biogenesis and oxidative function through coactivation and transcriptional activation of transcription factors such as nuclear respiratory factor 1 (NRF1), mitochondrial transcription factor A (TFAM) **[Wu et al. 1999]** and estrogen-related receptor α (ERRα) **[Mootha et al. 2004, Schreiber et al. 2004]**. Despite the importance of mitochondrial energy metabolism for renal function, relatively little is known how PGC-1α influences mitochondrial homeostasis in the kidney. Mice deficient for endothelial nitric oxide synthase (eNOS) display a strong reduction in renal PGC-1α levels, and reduced transcription of several mitochondrial PGC-1α target genes **[Borniquel et al. 2006]**. These mice furthermore have an increased susceptibility to develop diabetic nephropathy **[Alpers and Hudkins 2011]**. In line with this, several etiologically distinct renal disorders, such as diabetic nephropathy, ischemia/reperfusion injury and sepsis are associated with reduced mitochondrial function **[Brooks et al. 2009, Tran et al. 2011, Funk and Schnellmann 2013, Lempiainen et al. 2013, Rahman and Hall 2013, Sharma et al. 2013]**. These pathological states furthermore display a concurrent reduction in renal PGC-1α levels and activity **[Funk et al. 2010, Tran et al. 2011, Yuan et al. 2012, Funk and Schnellmann 2013, Lempiainen et al. 2013]**, which could contribute to mitochondrial impairments and renal dysfunction in these diseases. Indeed,

therapeutic strategies aimed at improving mitochondrial function **[Szeto et al. 2011, Funk and Schnellmann 2013, Lempiainen et al. 2013]** and increasing PGC-1α levels **[Tran et al. 2011, Funk and Schnellmann 2013, Lempiainen et al. 2013]** have been successful in ameliorating renal cellular pathologies. Furthermore, several lines of evidence point towards a role of PGC-1α in the transcriptional regulation of blood pressure regulation and salt and water homeostasis. Deletion or pharmacological inhibition of ERRα, which is a key transcriptional partners of PGC-1α, leads to a renal phenotype characterized by impaired blood pressure regulation and deregulated ion homeostasis **[Tremblay et al. 2010]**. Moreover, also ERRβ and ERRγ have been implicated in the regulation of renal ion homeostasis **[Alaynick et al. 2010, Krid et al. 2012]**. Another PGC-1α-related transcription factor, PPARα, has been postulated to mediate protective effects in mice during salt-induced hypertension **[Obih and Oyekan 2008, Lee et al. 2011]**. Hence, coactivation of these transcription factors by PGC-1α could impact renal physiology beyond its role in mitochondrial energy metabolism. In the current study, we have generated nephron-specific PGC-1α knockout mice to elucidate the transcriptional networks influenced by PGC-1α in the kidney, and its role in renal physiology.

Material and methods

Animals and diets **-** Animals were housed in a conventional facility with a 12-h light/12-h dark cycle with free access to food and water. All experiments were performed in accordance with federal guidelines and were approved by the Kantonales Veterinäramt of Kanton Basel-Stadt. To generate a tubule-specific PGC-1α knockout model, mice harboring a transgenic expression of the reverse tetracycline-dependent transactivator (rtTA) under the Pax8-promoter (Pax8-rtTA) **[Traykova-Brauch et al. 2008]** were crossed with transgenic (*tet*O-cre)-LC1 mice **[Schonig et al. 2002]**. These double-transgenic mice were subsequently crossed with mice harboring floxed PGC-1α alleles (PGC-1α$^{fl/fl}$) **[Lin et al. 2004]** to generate inducible PGC-1α nephron knockout mice (PGC-1α-iNKO; PiNKO). Addition of doxycycline (DOX) to the drinking water of PiNKO-mice induces binding of rtTA to the *tet*-operator (*tet*O)-promoter, and drives a Cre-mediated recombination of exons 3 to 5 of PGC-1α, specifically in Pax8-expressing cells. To induce the knockout of PGC-1α, DOX (Sigma) (0.2 mg/mL) was administered *ad libitum* in the drinking water of twelve week old mice, with the addition of 2% sucrose (Sigma) to enhance palatability. After two weeks, mice were switched back to regular drinking water, and were allowed one week of rest before experiments ensued. Recombination PCR was performed on DNA extracted from relevant organs, using primers binding

to a region surrounding exons 3–5 of PGC-1α; FWD 5'-TCCAGTAGGCAGAGATTTATGAC-3', REV 5'-CCAACTGTCTATAATTCCAGTTC-3'. This primer pair yields a product when exons 3-5 of PGC-1α are excised **[Lin et al. 2004]**. The experimental diets used for this study were either obtained from Harlan Teklad; normal salt diet (NSD, 1% NaCL) (TD.90229), low salt diet (LSD, <0.02% NaCL) (TD.90228), high salt diet (HSD, 8% NaCL) (TD.92012), or from Research Diets Inc; high fat diet (HFD, 60 kcal% fat, D12492).

Physiological measurements **–** Blood pressure and heart rate was measured in restrained conscious mice using a non-invasive tail-cuff blood pressure analyzer (BP-2000, Visitech system, Bioseb). For collection of urine, mice were housed in single mouse metabolic cages (3600M021, Tecniplast) for 24 hours. Amount of food and water consumed was recorded, and urine collected for further analysis. Body composition was measured using an EchoMRI-100™ analyzer (EchoMRI Medical Systems).

Blood & urine analysis **-** Blood glucose was measured in a tail vein blood sample using a handheld glucose meter (Accu-Chek, Roche). Levels of sodium, chloride, potassium, calcium and creatinine levels in urine were determined using an automated biochemical analyzer (Cobas c 111 analyzer; Roche). Urinary ion excretion was normalized to the urinary excretion of creatinine.

RNA extraction and qRT-PCR **-** Frozen tissue was homogenized and total RNA was extracted using TRIzol reagent (Invitrogen). RNA concentration was adjusted and cDNA synthesis was performed using 1 µg of total RNA. Semi-quantitative Real-time PCR analysis was performed using Fast SYBR Green master mix (Applied Biosystems) on a StepOnePlus Real-Time PCR System (Applied Biosystems). Relative expression levels for each gene of interest were calculated with the $\Delta\Delta C_t$ method, using eukaryotic elongation factor 2 (eEF2) as normalization control.

Gene expression array and analysis **–** Whole gene expression of CTRL and PiNKO kidneys were measured using 4 samples per group, with the Affymetrix GeneChip® Mouse Gene 2.0 ST microarrays. For gene ontology (GO)-analysis, gene lists were entered into the functional annotation tool FatiGO **[Al-Shahrour et al. 2004]** to identify genes over-represented within distinct GO categories. GO terms were considered significantly enriched if having a p-values <0.05. To calculate the key transcription factors driving the changes observed in our gene expression array we used the Integrated System for Motif Activity Response Analysis (ISMARA) **[Balwierz et al. 2014]**.

Statistical analysis **-** All data are presented as means ± SEM. Unpaired student two-tailed t test was used to determine differences between groups.

Results

The heterogeneity of the renal cell population prevents efficient targeting of a transgene to all cell populations within the kidney. However, by crossing transgenic Pax8rtTA-(tetO-cre)-LC1 mice **[Schonig et al. 2002, Traykova-Brauch et al. 2008]** with mice harboring floxed PGC-1α alleles (PGC-1α$^{fl/fl}$) **[Lin et al. 2004]**, we could generate mice carrying a doxycycline (DOX)-inducible nephron-specific ablation of PGC-1α (PGC-1α-iNKO; PiNKO). The knockout of PGC-1α was induced through administration of DOX in the drinking water of the mice. Successful recombination of exon 3-5 of PGC-1α was confirmed in PiNKO kidneys after DOX-administration (FIG 1A). While we could not detect recombination in skeletal muscle, brain or heart, a recombination band was detect in the liver (FIG 1A). Partial Pax8 expression is known to occur in a subset of periportal hepatocytes **[Traykova-Brauch et al. 2008]**, and could explain the presence of a recombination band in liver. PiNKO mice displayed roughly 80% reduction in PGC-1α transcript levels in kidney (FIG 1B), confirming an efficient knockout of PGC-1α in our model (FIG 1B). We could detect no significant reduction in PGC-1α transcript levels in liver (FIG 1B), which is in agreement with only a partial recombination in this organ. All other tissues tested showed unchanged PGC-1α transcript levels between CTRL and PiNKO mice (FIG 1B). This indicates that knockout of PGC-1α in PiNKO mice is targeted specifically to the kidneys. The ablation of PGC-1α in kidney led to no compensatory increase in the related family members, PGC-1β and PGC1-related coactivator (PRC) (FIG 1C), but resulted in significantly reduced transcript levels of known PGC-1α target genes fumarate hydratase 1 (*Fh1*) and citrate synthase (*Cs*) (FIG 1C). Deletion of PGC-1α in kidney affected neither body weight (FIG 1D) nor relative kidney weight (FIG 1E) at either one, fifteen or twenty-four months after DOX-administration compared to CTRL mice.

PGC-1α regulates transcription of metabolic and mitochondrial processes in kidney

PGC-1α is a robust transcriptional regulator, and we were interested in how ablation of PGC-1α impacts the renal transcriptome. We thus performed a gene expression array on samples from CTRL and PiNKO kidneys. Using a cut-off of $p<0.05$ and a fold-change of >1.2, we could find 523 genes that were significantly down-regulated, and 516 genes that were significantly up-regulated in kidneys from PiNKO compared to CTRL mice (FIG S1A). We performed gene ontology (GO)-analysis on the down-regulated transcripts and could find an overrepresentation of terms associated with metabolic and mitochondrial processes, such as; *electron transport chain* (GO:0022900), *oxidative reduction* (GO:0055114) (FIG 2A), *oxidative phosphorylation* and *tricarboxylic acid cycle (TCA cycle)* (FIG 2B). Within the GO-categories *oxidative phosphorylation*, *TCA cycle* and *glycolysis,* we detected several genes that were significantly down-

regulated in PiNKO compared to CTRL mice (FIG 2C), and a selection of these gene where further validated through RT-PCR (FIG 2D). These data indicate an important role for PGC-1α in the transcriptional regulation of metabolic and mitochondrial processes in the kidney, which is in line with the known function of PGC-1α also in other organs **[Liu and Lin 2011]**. Amongst the up-regulated transcripts we could detect an overrepresentation of terms associated with binding of vitamin A and isoprenoids, metal ion binding as well as glycoprotein binding (FIG S1B). Interestingly, *cation channel activity* (GO:0005261) was one of the overrepresented terms amongst the up-regulated transcripts (FIG S1B). Within this GO-category we could find several transcripts belonging to the KCN-family of potassium transporters, which would indicate a transcriptional induction of this process in the kidney in the absence of PGC-1α. We next performed motif activity response analysis of our gene expression array data using *Integrated System for Motif Activity Response Analysis* (ISMARA) **[Balwierz et al. 2014]**, to predict transcription factors (TF) with a significantly altered activity in response to PGC-1α deletion. We were especially interested in TFs that display a reduced activity in PiNKO mice, and could therefore be direct regulatory partners of PGC-1α in the kidney. Amongst the TFs displaying a reduced activity (Z-value>1.5) in kidneys from PiNKO mice were the known transcriptional partners of PGC-1α steroidogenic factor 1 (*SF-1*, *NR5A1.2.p2*), estrogen-related receptor α (*ERRα*, *ESRRA.p2*) and the retinoid x receptor (RXR)-family of transcription factors (*RXR(A,B,G).p2*) (FIG 2E) **[Delerive et al. 2002, Huss et al. 2002, Yazawa et al. 2010]**. ERRα transcription is known to be regulated by PGC-1α **[Schreiber et al. 2004]**, and in line with this we detected reduced ERRα transcript levels in PiNKO kidneys (FIG 2F), together with a reduction in the known ERRα target genes vascular endothelial growth factor A (*Vegfa*) and isocitrate dehydrogenase [NAD] subunit alpha (*Idh3a*) **[Schreiber et al. 2004, Arany et al. 2008]** (FIG 2F). Also ERRβ transcription was reduced in PiNKO mice, while expression of ERRγ was unchanged.

PiNKO mice display a mild hypertensive and salt-losing phenotype

ERRα has been shown to regulate several genes involved in renal salt-and water homeostasis and blood pressure regulation in the kidney **[Tremblay et al. 2010]**. Considering the close link between ERRα and PGC-1α, we were interested in whether blood pressure regulation would be altered in PiNKO mice. We thus measured resting blood pressure in CTRL and PiNKO mice. PiNKO mice displayed a mild hypertensive phenotype, with significantly increased systolic (FIG 3A) and diastolic (FIG 3B) blood pressure compared to CTRL mice. This slight increase in blood pressure was however not linked to any changes in heart rate (FIG S1C). Transcriptional analysis by RT-PCR revealed several genes involved in renal blood pressure regulation

that were significantly altered in PiNKO compared to CTRL mice. These were genes belonging to the renin-angiotensin-aldosterone system (RAAS); angiotensinogen (*Agt*), renin (*Ren1*), the kallikrein-kinin system (KKS); kallikrein 1 (*Klk1*) and carboxypeptidase N (*Cpn1*) and genes involved in prostaglandin synthesis; prostaglandin D2 synthase (*Ptgds*) and prostaglandin E synthase 2 (*Ptges2*) (FIG 3C). The enhanced *Ren1* transcription furthermore provides a likely explanation for the mild hypertensive phenotype of PiNKO mice (FIG 3A-B). Importantly, enhanced transcription of both *Ren1* and *Cpn1* is also detected in global ERRα knockout mice **[Tremblay et al. 2010]**. However, expression of *Ptgds* and *Agt* were differentially regulated in ERRα knockout mice compared to PiNKO mice, indicating that these two genetic models differ slightly in their renal transcriptional profiles, which could potentially explain the different effect on blood pressure regulation between global ERRα knockout and PiNKO mice. Closely linked to renal blood pressure regulation is the ability of the kidney to adjust the concentration of salts and other constituents of the primary filtrate. Hence, we investigated whether PGC-1α deletion affects transcription of genes involved in transcellular transport. To use an unbiased approach, we encompassed both up- and down-regulated genes ($p<0.05$, fold change >1.2; 1039 genes) and performed functional annotation for differentially regulated genes in kidney from PiNKO mice. GO-analysis revealed that the most highly enriched term was associated with energy metabolism, *oxidoreductase activity* (GO:0016491) (FIG 3D), confirming our earlier findings using only down-regulated genes (FIG 2A-B). Interestingly, we could also find an enrichment of terms associated with ion transport, such as *transmembrane transporter activity* (GO:0022857), *cation transmembrane transporter activity* (GO:0008324) and *ion transmembrane transporter activity* (GO:0015075) in kidney from PiNKO mice (FIG 3D). Manual inspection of the genes associated within the term *transmembrane transporter activity* (GO:0022857) revealed two major clusters of genes associated with the solute carrier (SLC) family or the KCN-family of potassium transporters (FIG 3E). These two clusters contained both up- and down-regulated transcripts (FIG 3E), suggesting both a direct- and an indirect regulation of these genes by PGC-1α. A selection of genes contained within these clusters were validated by RT-PCR (FIG 3F). Amongst the down-regulated genes was the known PGC-1α-target glucose transporter 4 (*Slc2a4*, *GLUT4*) **[Michael et al. 2001]**, as well as the other members of the SLC-family; solute carrier family 43 (amino acid system L transporter), member 2 (*Slc43a2*, *LAT4*) and solute carrier family 12 (potassium/chloride transporters), member 7 (*Slc12a7*, *KCC4*). Conversely, transcription of solute carrier family 8 (sodium/calcium exchanger), member 1 (*Slc8a1*, *NCX1*), solute carrier family 17 (organic anion transporter), member 4 (*Slc17a4*; *NPT-homologue*) and solute carrier family 34 (type II sodium/phosphate co-transporter), member 2 (*Slc34a2*, *NaPi-2b*) was upregulated in the absences of PGC-1α (FIG 3F). Since

several genes involved in renal ion transport were influenced by the ablation of PGC-1α, we investigated whether salt- and water homeostasis would be affected in PiNKO mice. However, PiNKO mice displayed no alteration in either water intake or diuresis over a 24 hour period (FIG 3G-H), and there was no difference in food consumption between genotypes (FIG S1D). PiNKO mice however displayed a mild increase in sodium excretion when normalized to urinary creatinine excretion (FIG 3I). No significant difference could be detected for the urinary excretion of other ions, such as chloride, potassium and calcium (FIG 3I). These data indicate that PiNKO mice display a largely normal regulation of water intake and urine concentration, but display a mild sodium-losing phenotype in the basal state.

Young PiNKO mice can adapt their salt and water homeostasis to an altered dietary salt intake

The small increase in sodium excretion in PiNKO mice could indicate a role for PGC-1α in maintenance of salt homeostasis. To test this hypothesis, we exposed PiNKO mice to a dietary salt stress. CTRL and PiNKO mice were administered a standardized diet supplemented with either 1% NaCl (normal salt diet, NSD), <0.02% NaCl (low salt diet, LSD) or 8% NaCl (high salt diet, HSD). LSD-feeding did not alter water intake (FIG 4A) or diuresis (FIG 4B) in either genotype, however this diet resulted in a significantly reduced urine/water ratio only in CTRL mice (FIG 4C). Conversely, HSD feeding increased both water intake (FIG 4A), diuresis (FIG 4B) and urine/water ratio (FIG 4C), and did so to the same extent in both CTRL and PiNKO mice. The administered diets did not alter food intake in any of the groups (FIG S1E). While urinary sodium excretion was slightly increased in PiNKO mice on a NSD (FIG 4D), PiNKO mice could efficiently adapt their urinary sodium and chloride excretion rates to both a decreased and an increased dietary sodium load (FIG 4D-E). Conclusively, these data indicate that young PiNKO mice display a mild salt losing phenotype in the basal state, but can efficiently adapt their salt- and water homeostasis to an altered dietary NaCl -load. Aging is associated with a reduced ability to adjust for alteration in urinary salt excretion **[Schlanger et al. 2010, Karam and Tuazon 2013]**. Since young PiNKO mice displayed a mild salt losing phenotype in the basal state, we wanted to investigate whether this phenotype would be exacerbated as the mice aged. We therefore repeated the LSD-feeding in aged mice (15 months old). In aged PiNKO mice, we could detect an increased loss of sodium in the urine compared to CTRL mice when fed an NSD (FIG 4F), corroborating our finding in young PiNKO mice (FIG 4D). While there was no significant difference in urinary chloride excretion (FIG 4G) at this time-point, we could however detect a trend towards increased calcium excretion in the urine (FIG 4H). Interestingly, although aged PiNKO mice could partially adapt to a reduced dietary salt load, they displayed increased urinary excretion of both sodium (FIG 4F) and calcium (FIG 4H)

compared to LSD-fed CTRL mice. This ion losing phenotype also correlated with a significantly increased diuresis (FIG 4I) and urine/water ratio (FIG 4J) in PiNKO mice fed a LSD compared to CTRL mice. Collectively, these data indicate that young PiNKO mice can adapt to altered dietary salt intake, while this is progressively impaired as the mice age.

HFD-feeding does not impact transcription of PGC-1α-regulated mitochondrial genes in kidney

Analysis of the kidney transcriptome of PiNKO mice suggested an important role for PGC-1α in transcriptional regulation of several metabolic and mitochondrial processes (FIG 2A-B). We were thus interested in whether PGC-1α was involved in renal adaptation to metabolic stress and more specifically, how the renal transcriptome would be affected by the ablation of PGC-1α in mice kept on a high fat diet (HFD). To investigate this, we fed CTRL and PiNKO mice a HFD for five months. After this period, CTRL and PiNKO mice were significantly heavier than their chow fed counterparts (CHOW) (FIG 5A). However, there was no significant difference in relative fat mass (FIG 5B) between HFD-fed CTRL and PiNKO mice. Moreover, both HFD-fed CTRL and PiNKO mice displayed significant hyperglycemia, characterized by increased fasting glucose levels (FIG 5C) compared to CHOW fed mice. To investigate how the renal transcriptome is affected by the ablation of PGC-1α during HFD feeding, we performed a gene expression array on kidneys from HFD fed CTRL and PiNKO mice. Using a cut-off of $p<0.05$ and a fold-change >1.2, we could find 418 genes that were significantly down-regulated, and 425 genes that were significantly up-regulated in kidneys from HFD-fed PiNKO compared to HFD-fed CTRL mice (FIG S1F). For the up-regulated genes, 38 out of 425 genes were associated with the GO-category *response to external stimulus* (GO:0009605). However, none of the up-regulated genes in HFD-fed PiNKO compared to HFD-fed CTRL mice were associated with similar terms as those detected in chow fed PiNKO versus CTRL mice (FIG S1B). On the other hand, similar to the GO-analysis performed on kidneys from chow-fed mice (FIG 2A-B), metabolic and mitochondrial processes were overrepresented amongst the downregulated genes between HFD-fed CTRL and PiNKO mice (FIG 5E-F). The GO-term *oxidative phosphorylation* (mmu00190) was amongst the highest ranking KEGG categories within the downregulated genes when comparing PiNKO and CTRL mice in both chow-fed (FIG 2B) and HFD-fed (FIG 5F) states. Since diet-induced obesity has been associated with reduced mitochondrial transcription and reduced PGC-1α activity in several organs **[Patti et al. 2003, Boudina et al. 2005, Sparks et al. 2005, Sivitz and Yorek 2010]**, we were interested in whether HFD-feeding would impact transcription of mitochondrial genes also in the kidney.

However, functional annotation of differentially regulated genes (p>0.05, fold change >1.2 or <-1.2) in kidney from HFD-fed CTRL mice compared to chow-fed CTRL mice did not reveal any significant enrichment of terms associated with mitochondrial structure or functions (unpublished observation). To further investigate whether PGC-1α-regulated oxidative phosphorylation genes would be affected by HFD feeding, we selected genes that were down-regulated in PiNKO compared to CTRL mice and functionally annotated to the GO-category *oxidative phosphorylation* (mmu00190) (SI Table I). We then cross-referenced this list with genes differentially regulated in HFD-fed CTRL mice compared to CHOW-fed CTRL mice. Surprisingly, only 8 out of 42 PGC-1α-regulated genes associated with mitochondrial oxidative phosphorylation were significantly (p<0.05) altered with HFD feeding in CTRL mice in our microarray data. However, validation of these genes with RT-PCR revealed that only expression of two of these predicted transcripts; NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 4 (*Ndufa4*) and NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 1 (*Ndufa1*) were significantly affected by HFD-feeding (FIG 5G). The other positive hits from our microarray-data were accordingly unaltered in kidney of HFD-fed CTRL mice compared to chow-fed CTRL mice when analyzed by RT-PCR using a large sample size (FIG 5G). Considering that only a small number of these predicted genes changed their transcription with HFD feeding, we can conclude that HFD feeding did not alter expression of PGC-1α-regulated genes involved in renal mitochondrial oxidative phosphorylation. In line with this, we did not detect any significant differences in PGC-1α transcript levels in the kidney after five months HFD feeding (FIG 5H). Interestingly, transcript levels of PGC-1β and PRC displayed a slight but significant increase in kidney with HFD-feeding (FIG 5H), irrespective of genotype. Moreover, when we analyzed the expression of TFs involved in regulation of mitochondrial genes, we could detect a slight induction of *NRF1* transcription, while expression levels of *ERRα* and *TFAM* were unaltered (FIG 5H). Conclusively, five months of HFD feeding does not significantly alter transcription of mitochondrial genes in kidney.

PGC-1α mediates induction of fatty acid metabolic genes in the kidney during HFD feeding

Since we only detected minor differences in PGC-1α-regulated mitochondrial genes with HFD-feeding, we were next interested in whether loss of PGC-1α in kidney would affect the renal metabolic adaptations during HFD feeding. GO-analysis of genes significantly (p<0.05, fold-change>1.2) altered in either CTRL mice (FIG 6A) or PiNKO mice (FIG 6B) with HFD feeding revealed an enrichment of down-regulated genes associated with the GO-term *steroid biosynthesis* (mmu00100) in both genotypes (FIG 6A-B). This was associated with a significant down-regulation of genes involved in cholesterol biosynthesis, such as

squalene epoxidase (*Sqle*), farnesyl-diphosphate farnesyltransferase 1 (*Fdft1*) and 7-dehydrocholesterol reductase (*Dhcr7*) in kidneys from both HFD-fed CTRL and PiNKO mice (FIG 6C), indicating that the loss of PGC-1α does not affect transcriptional adaptation of renal cholesterol metabolism upon HFD feeding. Next we focused on genes induced by HFD feeding, and could find the two related GO-terms; *PPAR signaling pathway* (mmu03320) and *fatty acid degradation* (mmu00071) significantly enriched amongst up-regulated genes in CTRL mice (FIG 6A). Interestingly, these GO-terms were not enriched amongst the up-regulated processes in PiNKO mice (FIG 6B), which suggests that the induction of these processes could be dependent on PGC-1α. To investigate this further, we measured transcript levels of PPARs in the kidney and detected an induction of PPARα, but not PPARβ/δ or PPARγ, in HFD fed mice (FIG 6D). Importantly, the induction of PPARα with HFD feeding was blunted in PiNKO mice (FIG 6D), and a similar transcriptional pattern could be observed for several PPARα target genes involved in fatty acid metabolism (FIG 6E). HFD feeding led to an increased transcription of acyl-CoA thioesterase 2 (*Acot2*), *Acot3*, solute carrier family 25 (carnitine/acylcarnitine translocase) member 20 (*SLC25A20*), acyl-CoA dehydrogenase very long chain (*Acadvl*) and acyl-CoA dehydrogenase long chain (*Acadl*) in kidneys of CTRL mice, and this induction was diminished in the absence of PGC-1α (FIG 6E). PGC-1α is a known transcriptional coactivator of PPARα in liver **[Finck and Kelly 2006]**, and these data indicate an important role for PGC-1α in regulation of PPARα-activity and fatty acid metabolism also in the kidney. To confirm our findings, we selected genes, which were significantly induced in CTRL mice with HFD feeding ($p<0.05$, fold change>1.1) and which showed a blunted induction in HFD-fed PiNKO mice ($p<0.05$, fold change <1.1) compared to HFD-fed CTRL mice (SI Table II). Using these cut-offs, out of the total number of genes induced in kidney with HFD feeding (1467 genes), approximately one fifth of these (279 genes) were significantly reduced in PiNKO mice (FIG S1G). GO-analysis of this subset of genes revealed an enrichment of genes within the GO categories *fatty acid degradation* (mmu00071) (FIG S1H) and *fatty acid metabolic process* (GO:0006631) (FIG 6F), further supporting our finding that PGC-1α is important for the transcriptional induction of β-oxidation in kidneys from HFD-fed mice. Moreover, within the GO category *fatty acid metabolic process* (GO:0006631), we could find several genes which are known target genes of PPARα, such as *Acot3*, *Acaa2* and hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase (*Hadha*) (FIG 6G). Collectively, these data highlight an important role for PGC-1α in the induction of PPARα-target genes involved in fatty acid metabolism in kidney. Impaired β-oxidation in kidney has been associated with increased lipotoxicity and increased renal inflammation during HFD-feeding **[Tanaka et al. 2011]**. Interestingly, several of the GO terms enriched amongst up-regulated genes in PiNKO mice with HFD-feeding were associated with

immune response, such as *primary immunodeficiencies* (mmu05340) and *B cell receptor signaling pathway* (mmu04664), or cancer; *small cell lung cancer* and *pathways in cancer* (mmu05222) (FIG 6B), while no such terms could be found in HFD fed CTRL mice (FIG 6A). Importantly, motif activity response analysis of our gene expression array revealed that the activity of nuclear factor 'kappa-light-chain-enhancer' of activated B-cells (*NF-κB*)-activity (*NFKB1_REL_RELA.p2*) was predicted to be increased in HFD fed PiNKO mice compared to both chow-fed PiNKO mice and HFD-fed CTRL mice (FIG 6H). Considering the role of NF-κB activation in the pathogenesis of renal inflammation **[Sanz et al. 2010]**, this suggests a worsened renal phenotype in PiNKO mice upon HFD-feeding compared to CTRL mice. Conclusively, these data highlight an important role of PGC-1α in the metabolic adaptation to HFD-feeding, through induction of PPARα-target genes involved in renal fatty acid metabolism.

Discussion

The kidney has a high basal energy demand, and derives the majority of its energy from oxidative phosphorylation. Renal mitochondrial activity is thus integral to the function of the kidney and for the maintenance of a constant rate of filtration and reabsorption along the nephron. This is evident by the high mitochondrial density in nephron segments associated with a high basal transmembrane transport activity **[Soltoff 1986]** and the prevalence of renal dysfunction in patients suffering from hereditary mitochondrial disorders **[Emma et al. 2011, Rahman and Hall 2013, Che et al. 2014]**. PGC-1α is an important regulator of mitochondrial oxidative phosphorylation and oxidative metabolism in several organs **[Liu and Lin 2011]**. We were thus interested in elucidating the role of PGC-1α in the regulation of renal energy metabolism and function. To this end, we have generated and characterized a nephron-specific PGC-1α knockout mouse.

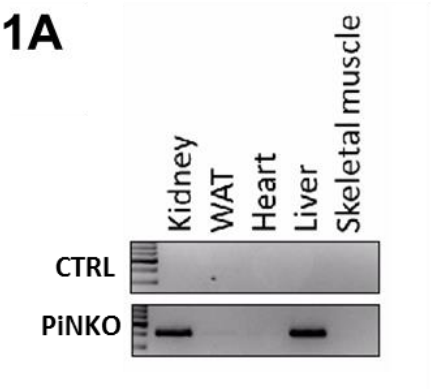
We could demonstrate that PGC-1α plays an important role in the transcriptional regulation of genes involved in mitochondrial oxidative phosphorylation, TCA cycle and glycolysis in kidney. These specific processes are known to be regulated by PGC-1α also in other metabolic organs, for instance in skeletal muscle **[Perez-Schindler et al. 2012]**. Our findings are thus in agreement with a global role of PGC-1α in the regulation of mitochondrial and metabolic processes, which have been demonstrated also in BAT, heart and skeletal muscle **[Liu and Lin 2011]**. The transcriptional congruency between these tissues could result not only from high basal expression of PGC-1α in these organs, but also from a uniform expression of transcription factors relevant for mitochondrial transcription, such as NRF1, TFAM and ERRα. Indeed, renal expression of ERRα has been shown to equal that of both BAT and heart, and to exceed that

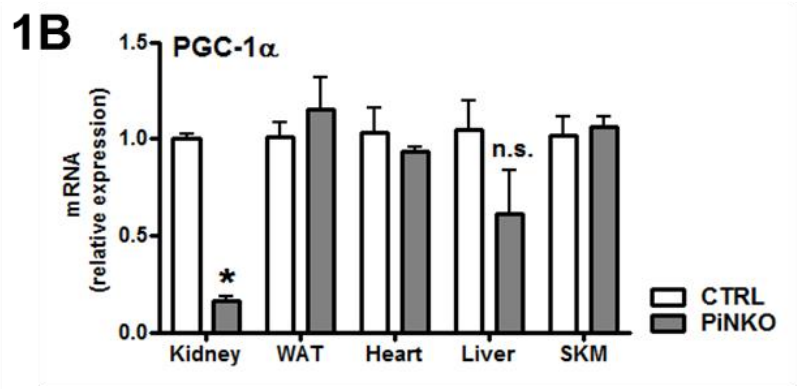
of skeletal muscle **[Sladek et al. 1997, Ranhotra 2009]**. Based on motif activity response analysis of our gene expression data, we predicted a significant blunting of ERRα transcriptional activity in our tubule-specific PGC-1α knockout mice. In line with this, we found that ERRα transcription was significantly reduced in PiNKO mice. ERRα is not only a transcriptional partner of PGC-1α in the regulation of mitochondrial genes, but has also been shown to be important for the transcriptional regulation of genes involved in salt and water homeostasis and blood pressure regulation in kidney. Global ERRα knockout mice are hypotensive and display a reduced sodium excretion in response to salt stress **[Tremblay et al. 2010]**. Interestingly, despite reduced ERRα transcription and a predicted reduction in ERRα transcriptional activity, PiNKO mice displayed the opposite phenotype, characterized by a mild hypertension and a salt-losing phenotype. This discrepancy could be partially explained by an opposite regulation of several genes (i.e. *Agt*, *Ptgds*) important for blood pressure regulation and salt homeostasis in PiNKO compared to global ERRα knockout mice **[Tremblay et al. 2010]**. Renin transcription was however increased in both knockout models. The effect on renin transcription is likely mediated through indirect effects, since PGC-1α has not been shown to function as a transcriptional corepressor. The opposite regulation of several genes in PiNKO mice compared to ERRα knockout mice could originate from compensatory mechanisms due the global ablation of ERRα in this particular model **[Tremblay et al. 2010]**. Knockout of ERRγ on the other hand has been shown to lead to a reduced renal transcription of *Klk1* in mice **[Alaynick et al. 2010]**, similar to what we observed in PiNKO mice. ERRγ transcription was however not altered in kidneys of PiNKO mice. Despite the effect of PGC-1α ablation on the transcription of genes involved in mitochondrial energy metabolism, and regulation of salt- and water homeostasis, PiNKO mice display a very mild renal phenotype, and can adjust for both increased and decreased NaCl-load in the diet. However, aged PiNKO mice displayed increasing difficulties to adjust their salt- and water balance, which was evident by the salt and water losing phenotype when fed a low NaCl-diet. Importantly, aging is associated with a reduced ability to retain sodium during salt depletion also in humans **[Schlanger et al. 2010, Karam and Tuazon 2013]** and is associated with reduced renal PGC-1α levels in mice **[Lim et al. 2012]**. Aging is also connected to increased renal oxidative damage, resulting in exacerbated albuminuria **[Kume et al. 2010]**, and has been linked to reduced renal mitochondrial number and function **[de Cavanagh et al. 2003, Benigni et al. 2009]**. Conclusively, this points towards an important role for PGC-1α in maintaining renal tubular homeostasis with aging.

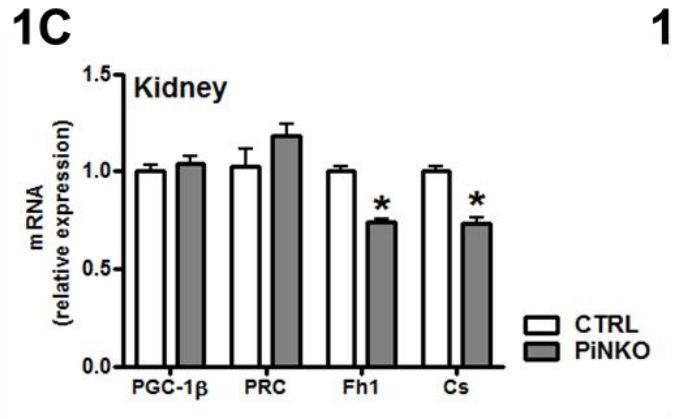
Obesity and diabetes have been associated with reduced PGC-1α levels and impaired mitochondrial transcription in several organs **[Patti et al. 2003, Boudina et al. 2005, Sparks et al. 2005,**

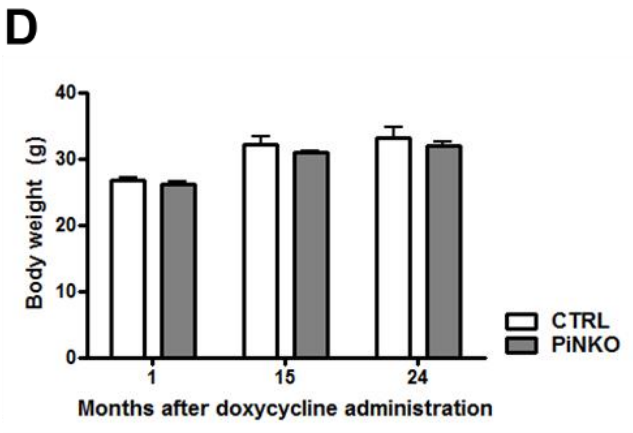
Sivitz and Yorek 2010]. We were thus interested in how diet-induced obesity affects transcription of mitochondrial genes in the kidney, and the role of PGC-1α in this regulation. After five months on a HFD, 1467 genes were found to be significantly altered in kidneys from CTRL mice. However, functional annotation of these genes did not reveal any association with GO-categories associated with mitochondrial structural components or oxidative function. Moreover, only a small part of PGC-1α target genes involved in oxidative phosphorylation were found to be altered by HFD-feeding in CTRL mice. Thus, HFD feeding does not lead to major alterations in mitochondrial transcription. Our findings are supported by a study from Ruggiero et al. **[Ruggiero et al. 2011]**, which demonstrates unchanged mitochondrial function in mouse kidney after 16 weeks HFD-feeding. While mitochondrial transcription was unaltered with HFD-feeding, we detected a significant induction of renal PPARα levels as well as several of its target genes involved in fatty acid metabolism. Interestingly, induction of PPARα and its target genes with HFD-feeding was significantly blunted in the absence of renal PGC-1α. It has been shown that ablation of PPARα in mice leads to an increased susceptibility to diabetic nephropathy **[Park et al. 2006]** and exacerbated free fatty acid-induced injury in the kidney **[Kamijo et al. 2007]**. Moreover, increased PPARα-activity in obese rodents has been shown to protect against renal lipotoxicity **[Shin et al. 2009, Tanaka et al. 2011]** and has been associated with increased levels of PGC-1α **[Chung et al. 2012]**. Our findings indicate that PGC-1α is necessary for the induction of renal PPARα expression in response to diet-induced obesity. Hence, PGC-1α might play an important role in protection against renal lipotoxicity.

In conclusion, our results show that ablation of PGC-1α in renal tubular cells does not lead to any impairments in basal renal function, indicating that PGC-1α is dispensable for maintenance of basal renal physiology. We can however demonstrate that PGC-1α is an important transcriptional regulator of mitochondrial and metabolic processes in the kidney, and would thus serve as a promising therapeutic target to ameliorate renal metabolic disorders. In line with this, two recent studies have shown that treatment with the SIRT1-activator SRT1720 **[Funk and Schnellmann 2013]** or calorie restriction **[Lempiainen et al. 2013]**, which both are linked to increased PGC-1α activation, could improve the renal mitochondrial phenotype and reduce ischemia/reperfusion injury in mice. Moreover, proximal tubule-specific ablation of PGC-1α has been shown to exacerbate endotoxemic acute kidney injury and overexpression of PGC-1α in cultured proximal tubule cells protected against both TNF- **[Tran et al. 2011]** and aldosterone-induced **[Yuan et al. 2012]** mitochondrial dysfunction. Hence, increased PGC-1α activity could be a valid therapeutic strategy to ameliorate renal mitochondrial dysfunction in a broad spectrum of renal disorders.


1A
Kidney
WAT
Heart
Liver
Skeletal muscle
CTRL
PiNKO


1B
PGC-1α
mRNA (relative expression)
1.5
1.0
0.5
0.0
n.s.
*
Kidney
WAT
Heart
Liver
SKM
CTRL
PiNKO


1C
Kidney
mRNA (relative expression)
1.5
1.0
0.5
0.0
*
*
PGC-1β
PRC
Fh1
Cs
CTRL
PiNKO


1D
Body weight (g)
40
30
20
10
0
1
15
24
Months after doxycycline administration
CTRL
PiNKO

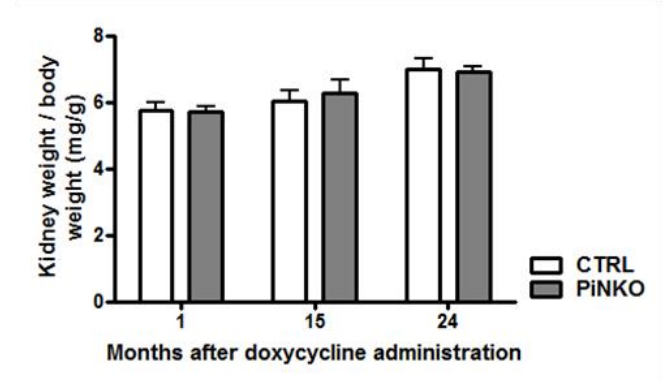

1E
Kidney weight / body weight (mg/g)
8
6
4
2
0
1
15
24
Months after doxycycline administration
CTRL
PiNKO

Figure 1 – PiNKO mice display a renal-specific ablation of PGC-1α

(A) Representative recombination PCR for PGC-1α on DNA extracted from kidney, white adipose tissue (WAT), heart, liver and skeletal muscle. (B) Transcript levels of PGC-1α in kidney, WAT, heart, liver and skeletal muscle (SKM) normalized to eukaryotic elongation factor 2 (eEF2) mRNA levels (n=5-7). (C) Transcript levels of indicated genes in whole kidney, normalized to eEF2 (n=7-8). (D) Absolute body weight of mice at 1, 15 and 24 months after doxycycline administration (n=5-11). (E) Average kidney weight normalized to body weight at 1, 15 and 24 months after doxycycline administration (n=5-11). Error bars represent mean ±SEM. Significant differences (p-value <0.05) between CTRL and PiNKO mice are indicated by an asterisk (*).

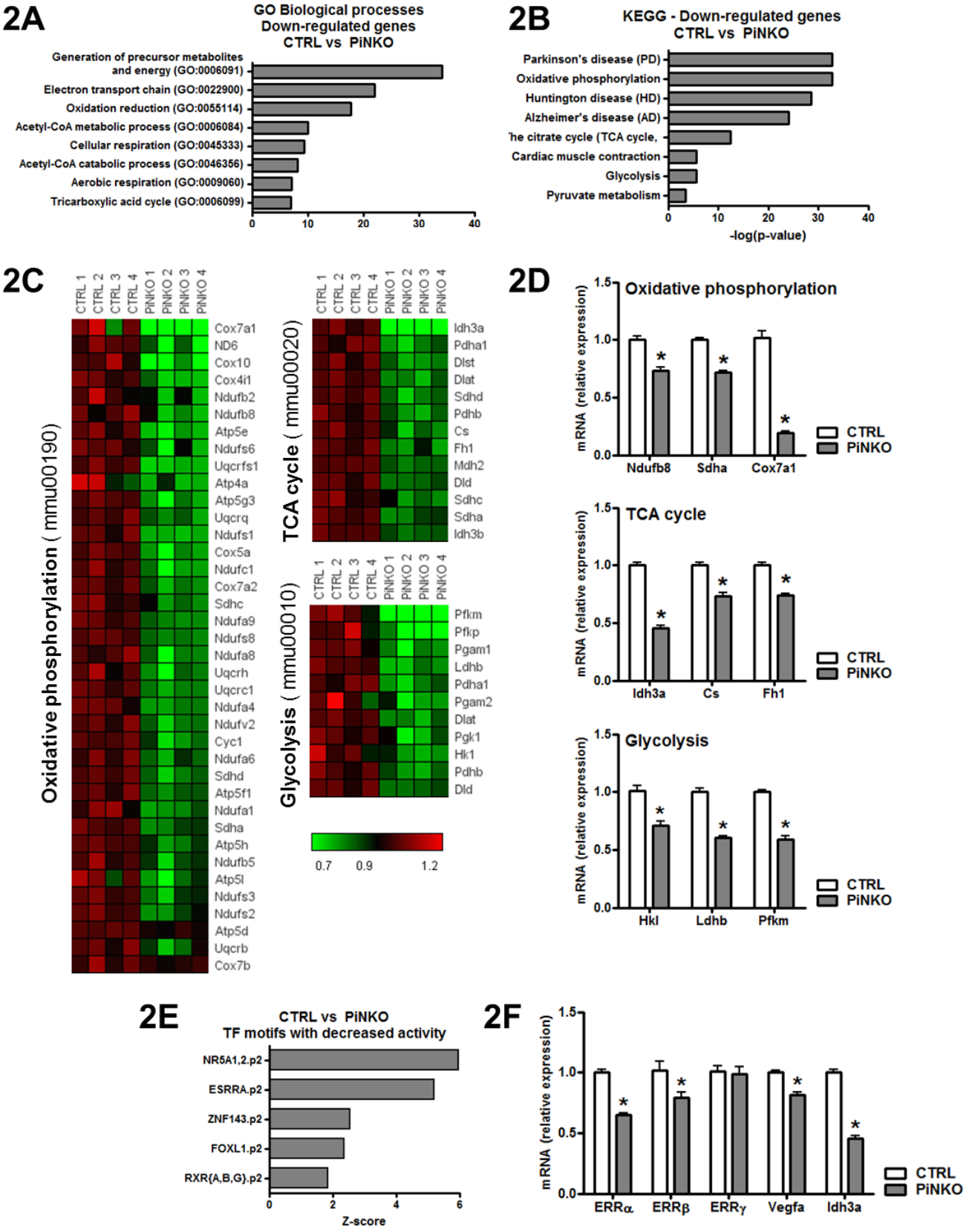

2A
GO Biological processes
Down-regulated genes
CTRL vs PiNKO
Generation of precursor metabolites and energy (GO:0006091)
Electron transport chain (GO:0022900)
Oxidation reduction (GO:0055114)
Acetyl-CoA metabolic process (GO:0006084)
Cellular respiration (GO:0045333)
Acetyl-CoA catabolic process (GO:0046356)
Aerobic respiration (GO:0009060)
Tricarboxylic acid cycle (GO:0006099)
0
10
20
30
40
2B
KEGG - Down-regulated genes
CTRL vs PiNKO
Parkinson's disease (PD)
Oxidative phosphorylation
Huntington disease (HD)
Alzheimer's disease (AD)
he citrate cycle (TCA cycle,
Cardiac muscle contraction
Glycolysis
Pyruvate metabolism
0
10
20
30
40
-log(p-value)
2C
CTRL 1
CTRL 2
CTRL 3
CTRL 4
PiNKO 1
PiNKO 2
PiNKO 3
PiNKO 4
Oxidative phosphorylation (mmu00190)
Cox7a1
ND6
Cox10
Cox4i1
Ndufb2
Ndufb8
Atp5e
Ndufs6
Uqcrfs1
Atp4a
Atp5g3
Uqcrq
Ndufs1
Cox5a
Ndufc1
Cox7a2
Sdhc
Ndufa9
Ndufs8
Ndufa8
Uqcrh
Uqcrc1
Ndufa4
Ndufv2
Cyc1
Ndufa6
Sdhd
Atp5f1
Ndufa1
Sdha
Atp5h
Ndufb5
Atp5l
Ndufs3
Ndufs2
Atp5d
Uqcrb
Cox7b
TCA cycle (mmu00020)
Idh3a
Pdha1
Dlst
Dlat
Sdhd
Pdhb
Cs
Fh1
Mdh2
Dld
Sdhc
Sdha
Idh3b
Glycolysis (mmu00010)
Pfkm
Pfkp
Pgam1
Ldhb
Pdha1
Pgam2
Dlat
Pgk1
Hk1
Pdhb
Dld
0.7
0.9
1.2
2D
Oxidative phosphorylation
mRNA (relative expression)
Ndufb8
Sdha
Cox7a1
CTRL
PiNKO
TCA cycle
Idh3a
Cs
Fh1
Glycolysis
Hkl
Ldhb
Pfkm
2E
CTRL vs PiNKO
TF motifs with decreased activity
NR5A1,2.p2
ESRRA.p2
ZNF143.p2
FOXL1.p2
RXR{A,B,G}.p2
Z-score
2F
mRNA (relative expression)
ERRα
ERRβ
ERRγ
Vegfa
Idh3a
CTRL
PiNKO

Figure 2 – PGC-1α regulates transcription of metabolic and mitochondrial processes in kidney

Top gene ontology (GO) terms enriched within the Biological processes (A) and KEGG (B) categories for down-regulated genes ($p<0.05$, fold change >1.2) in PiNKO compared to CTRL kidneys. (C) Heat maps generated using probe set intensities for the transcripts associated with the GO categories "Oxidative phosphorylation", "TCA cycle" and "Glycolysis". (D) Transcript levels of indicated genes in whole kidney, normalized to eEF2 (n=8). (E) Top transcription factor motifs exhibiting reduced activity in ISMARA analysis of microarray data for PiNKO compared to CTRL mice. (F) Transcript levels of indicated genes in whole kidney, normalized to eEF2 (n=8). Error bars represent mean ±SEM. Significant differences (p value < 0.05) between CTRL and PiNKO mice are indicated by an asterisk (*).

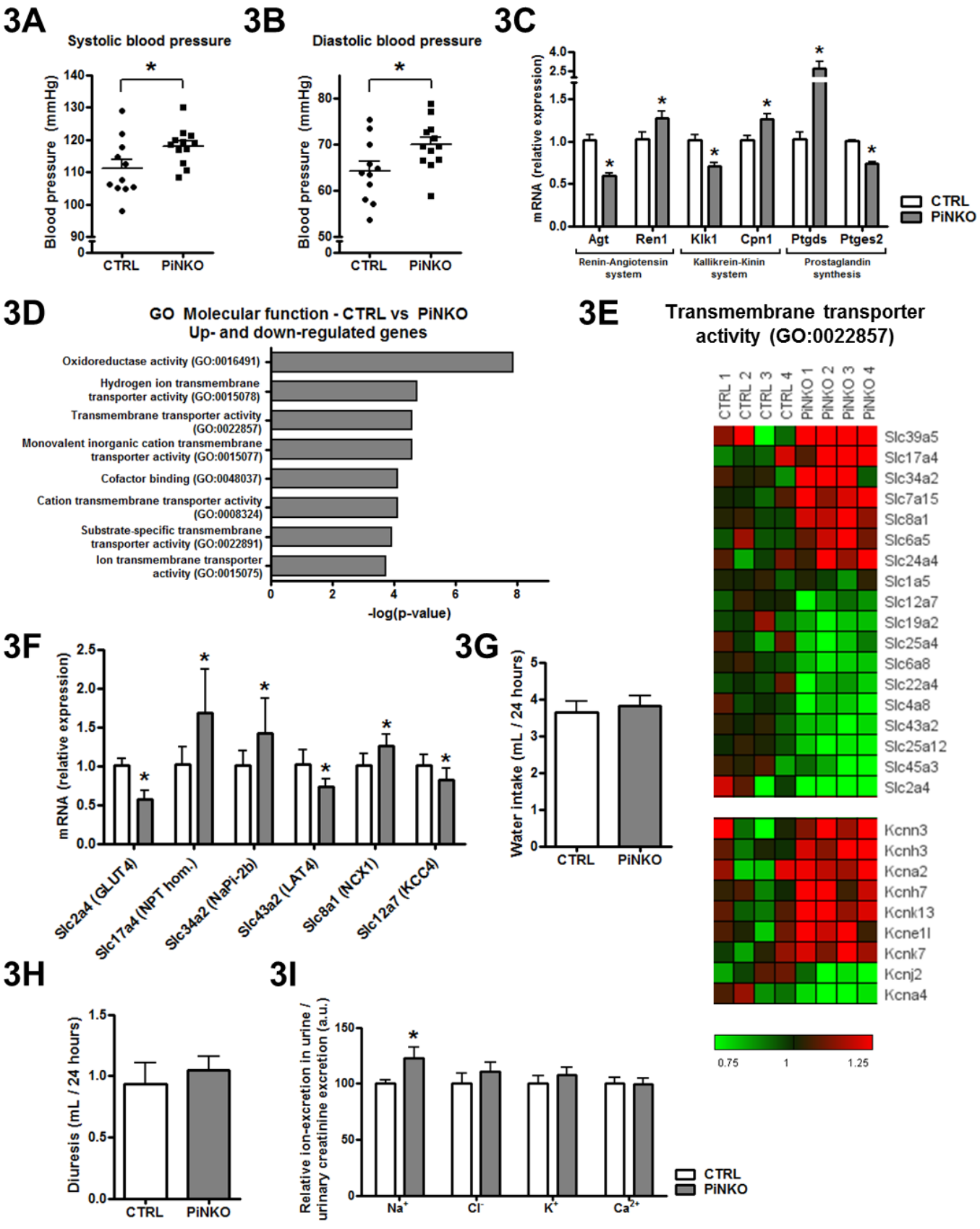

3A
Systolic blood pressure
Blood pressure (mmHg)
CTRL
PiNKO
3B
Diastolic blood pressure
Blood pressure (mmHg)
CTRL
PiNKO
3C
mRNA (relative expression)
Agt
Ren1
Klk1
Cpn1
Ptgds
Ptges2
Renin-Angiotensin system
Kallikrein-Kinin system
Prostaglandin synthesis
CTRL
PiNKO
3D
GO Molecular function - CTRL vs PiNKO
Up- and down-regulated genes
Oxidoreductase activity (GO:0016491)
Hydrogen ion transmembrane transporter activity (GO:0015078)
Transmembrane transporter activity (GO:0022857)
Monovalent inorganic cation transmembrane transporter activity (GO:0015077)
Cofactor binding (GO:0048037)
Cation transmembrane transporter activity (GO:0008324)
Substrate-specific transmembrane transporter activity (GO:0022891)
Ion transmembrane transporter activity (GO:0015075)
-log(p-value)
3E
Transmembrane transporter activity (GO:0022857)
CTRL 1
CTRL 2
CTRL 3
CTRL 4
PiNKO 1
PiNKO 2
PiNKO 3
PiNKO 4
Slc39a5
Slc17a4
Slc34a2
Slc7a15
Slc8a1
Slc6a5
Slc24a4
Slc1a5
Slc12a7
Slc19a2
Slc25a4
Slc6a8
Slc22a4
Slc4a8
Slc43a2
Slc25a12
Slc45a3
Slc2a4
Kcnn3
Kcnh3
Kcna2
Kcnh7
Kcnk13
Kcne1l
Kcnk7
Kcnj2
Kcna4
0.75
1
1.25
3F
mRNA (relative expression)
Slc2a4 (GLUT4)
Slc17a4 (NPT hom.)
Slc34a2 (NaPi-2b)
Slc43a2 (LAT4)
Slc8a1 (NCX1)
Slc12a7 (KCC4)
3G
Water intake (mL / 24 hours)
CTRL
PiNKO
3H
Diuresis (mL / 24 hours)
CTRL
PiNKO
3I
Relative ion-excretion in urine / urinary creatinine excretion (a.u.)
Na+
Cl-
K+
Ca2+
CTRL
PiNKO

Figure 3 – PiNKO mice display a mild hypertensive and salt-losing phenotype

Systolic (A) and diastolic (B) blood pressure measured via tail-cuff photoplethysmography. (C) Transcript levels of indicated genes in whole kidney, normalized to eEF2 (n=8). (D) Top gene ontology (GO) terms enriched within the molecular function category for differentially regulated genes ($p<0.05$, fold change >1.2 or <-1.2) in PiNKO compared to CTRL kidneys. (E) Heat maps generated using probe-set intensities for transcripts belonging to the solute carrier (SLC) family or KCN-family of potassium transporters and associated with the GO category "Transmembrane transporter activity". (F) Transcript levels of indicated genes in whole kidney, normalized to eEF2 (n=8). (G) Ad libitum water intake of mice measured over 24 hours (n=9-11). (H) Urine excretion over 24 hours (n=9-11). (I) Urinary levels of sodium (Na+), chloride (Cl-), potassium (K+) and calcium (Ca2+) over 24 hours. Values expressed relative to CTRL mice (n=8-9). Error bars represent mean ±SEM. Significant differences (p-value <0.05) between CTRL and PiNKO mice are indicated by an asterisk (*).

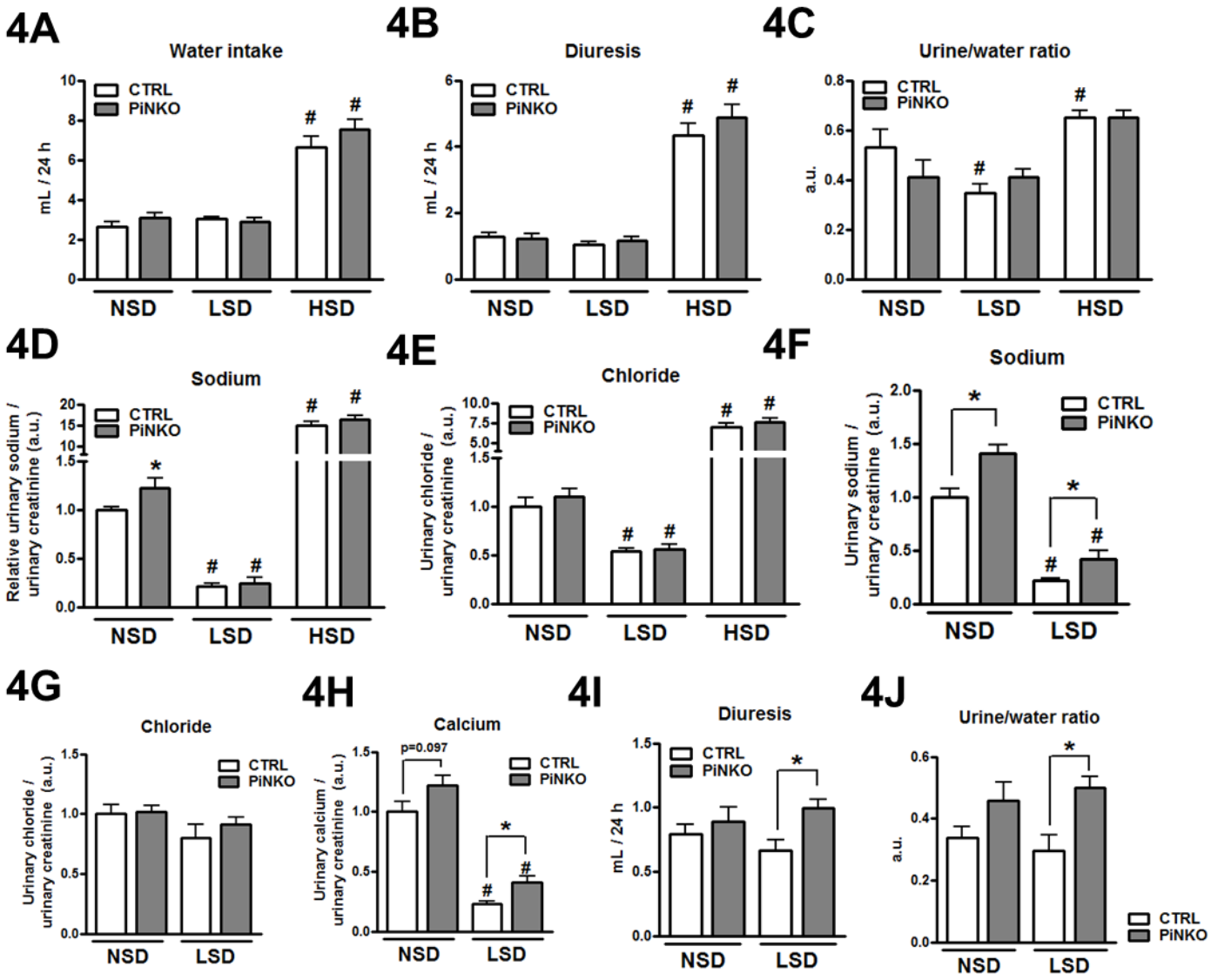

4A
Water intake
mL / 24 h
CTRL
PiNKO
NSD
LSD
HSD
4B
Diuresis
mL / 24 h
4C
Urine/water ratio
a.u.
4D
Sodium
Relative urinary sodium / urinary creatinine (a.u.)
4E
Chloride
Urinary chloride / urinary creatinine (a.u.)
4F
Sodium
Urinary sodium / urinary creatinine (a.u.)
4G
Chloride
Urinary chloride / urinary creatinine (a.u.)
4H
Calcium
p=0.097
Urinary calcium / urinary creatinine (a.u.)
4I
Diuresis
mL / 24 h
4J
Urine/water ratio
a.u.

Figure 4 – Young PiNKO mice can adapt their salt and water homeostasis to an altered dietary salt intake

(A) Ad libitum water intake over 24 hours (n=8-9). (B) Urine excretion over 24 hours (n=8-9). (C) Urine-to-water ratio over 24 hours (n=8-9). Urinary levels of (D) sodium and (E) chloride over 24 hours in mice fed a normal-salt diet (NSD), a low-salt diet (LSD) or a high-salt diet (HSD). All values relative to NSD-fed CTRL mice (n=8-9). Urinary levels of (F) sodium, (G) chloride and (H) calcium over 24 hours in aged mice fed a normal-salt diet (NSD) or a low-salt diet (LSD). All values relative to NSD-fed CTRL mice (n=6-13). (I) Urine excretion and (J) urine-to-water ratio over 24 hours in aged mice (n=6-13). Error bars represent mean ±SEM. Significant differences (p value < 0.05) between CTRL and PiNKO mice are indicated by an asterisk (*), and between LSD/HSD-fed and NSD-fed groups are indicated by a number-sign (#).

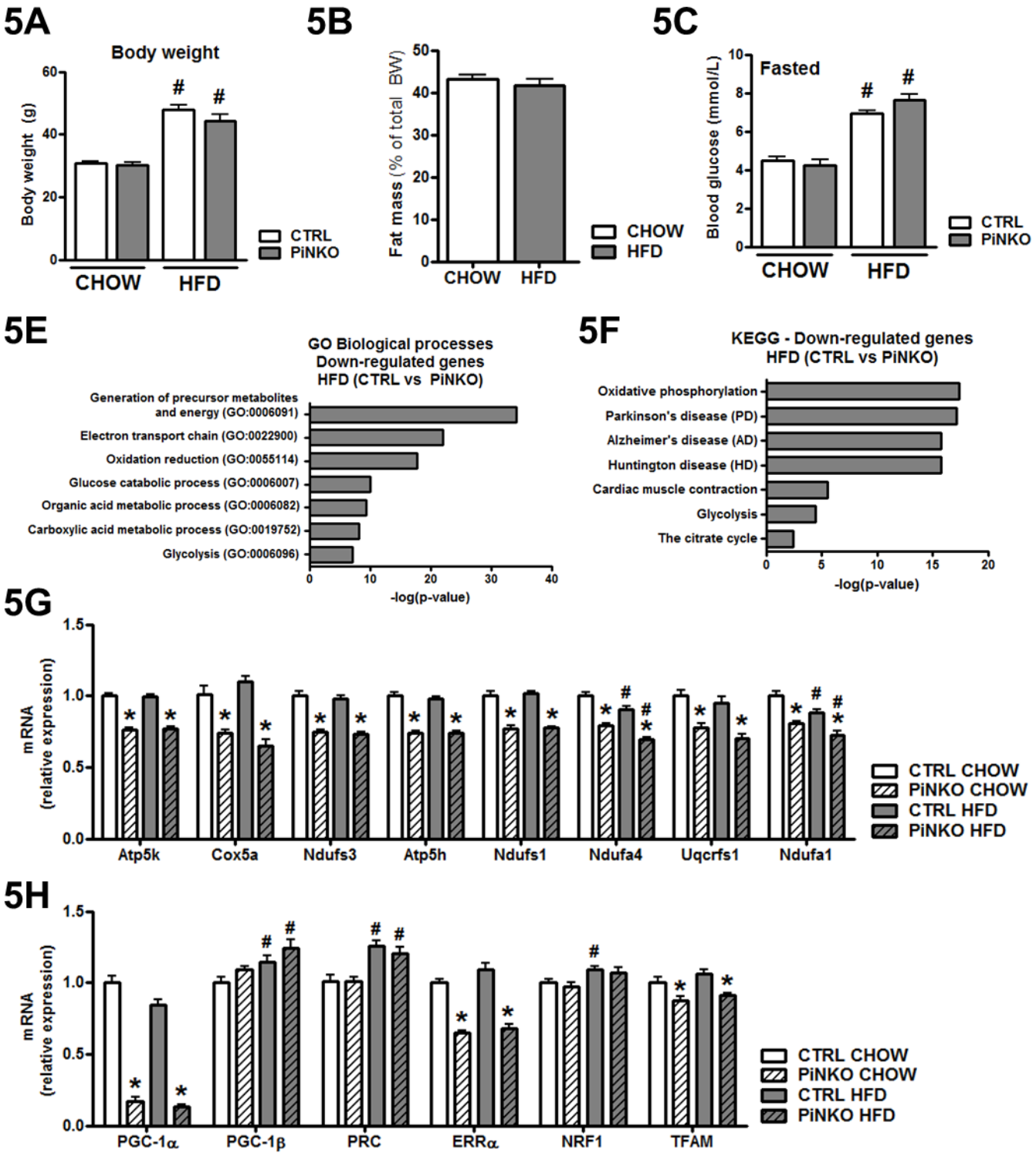
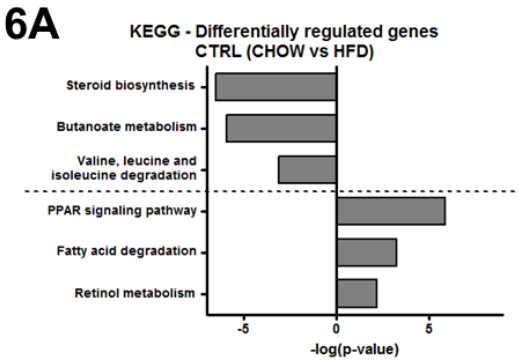
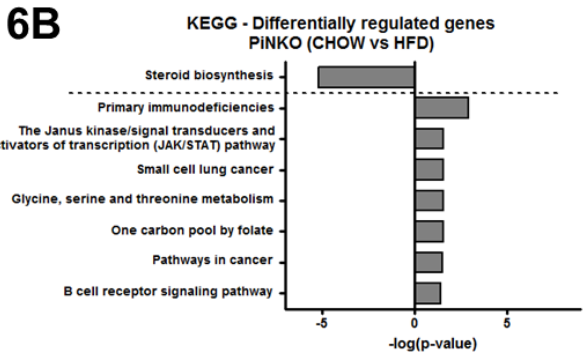

5A
Body weight
Body weight (g)
CTRL
PiNKO
CHOW
HFD
5B
Fat mass (% of total BW)
CHOW
HFD
5C
Fasted
Blood glucose (mmol/L)
CTRL
PiNKO
CHOW
HFD
5E
GO Biological processes
Down-regulated genes
HFD (CTRL vs PiNKO)
Generation of precursor metabolites and energy (GO:0006091)
Electron transport chain (GO:0022900)
Oxidation reduction (GO:0055114)
Glucose catabolic process (GO:0006007)
Organic acid metabolic process (GO:0006082)
Carboxylic acid metabolic process (GO:0019752)
Glycolysis (GO:0006096)
-log(p-value)
5F
KEGG - Down-regulated genes
HFD (CTRL vs PiNKO)
Oxidative phosphorylation
Parkinson's disease (PD)
Alzheimer's disease (AD)
Huntington disease (HD)
Cardiac muscle contraction
Glycolysis
The citrate cycle
-log(p-value)
5G
mRNA (relative expression)
Atp5k
Cox5a
Ndufs3
Atp5h
Ndufs1
Ndufa4
Uqcrfs1
Ndufa1
CTRL CHOW
PiNKO CHOW
CTRL HFD
PiNKO HFD
5H
mRNA (relative expression)
PGC-1α
PGC-1β
PRC
ERRα
NRF1
TFAM
CTRL CHOW
PiNKO CHOW
CTRL HFD
PiNKO HFD

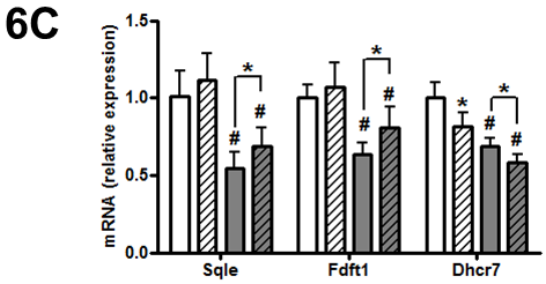
Figure 5 – High fat diet (HFD)-feeding does not impact transcription of PGC-1α regulated mitochondrial genes in kidney

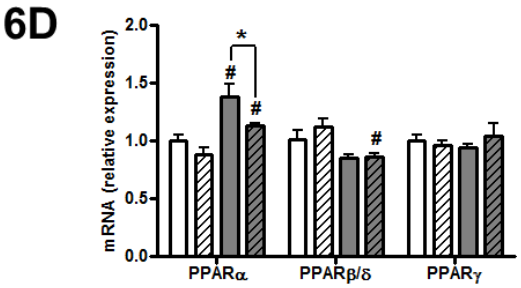
(A) Body weight of chow-fed (CHOW) and HFD-fed (HFD) mice after 20 weeks on their respective diets (n=10-15). (B) Relative fat mass expressed as % of total body weight (n=16). (C) Blood glucose measured in tail-vein blood after a 16 hour fast (n=8-15). Top gene ontology (GO) terms enriched within the Biological processes (E) and KEGG (F) categories for down-regulated genes ($p<0.05$, fold change >1.2) in HFD-fed PiNKO compared to HFD-fed CTRL kidneys. (G-H) Transcript levels of indicated genes in whole kidney, normalized to eEF2 (n=7-8). Error bars represent mean ±SEM. Significant differences (p-value <0.05) between CTRL and PiNKO mice are indicated by an asterisk (*), and between chow-fed and HFD-fed groups are indicated by a number-sign (#).

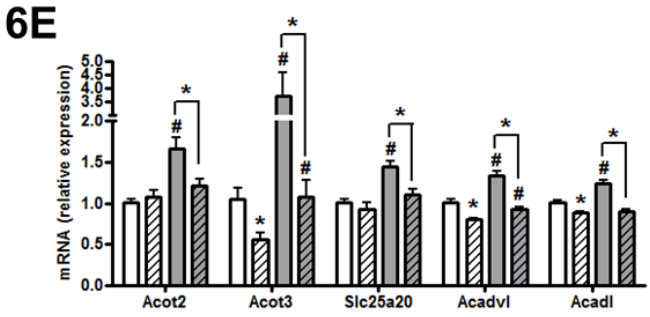
4. Manuscript 1: Transcriptional Regulation of Renal Function and Energy Metabolism by PGC-1α

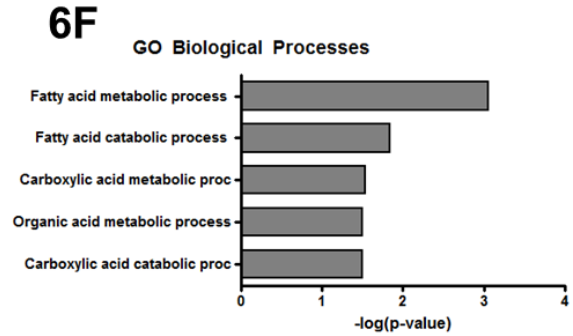


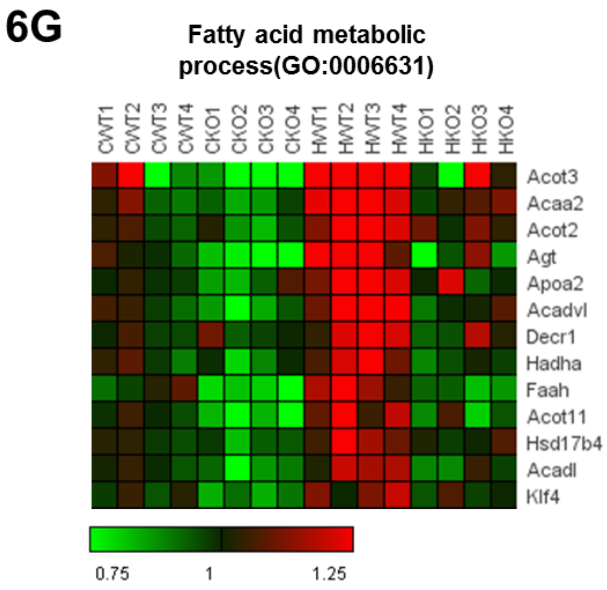












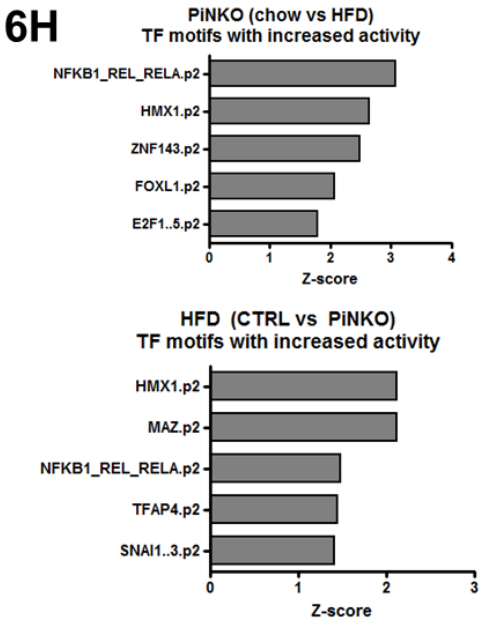
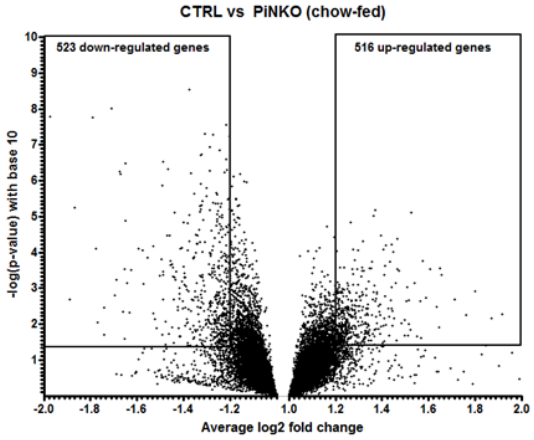


Figure 6 – PGC-1α mediates induction of fatty acid metabolic genes in the kidney during HFD feeding

Top gene ontology (GO) terms enriched within the KEGG category for up- and down-regulated genes ($p<0.05$, fold change >1.2) in kidneys from (A) HFD-fed CTRL mice compared to chow-fed CTRL mice or in (B) HFD-fed PiNKO mice compared to chow-fed PiNKO mice. (C-E) Transcript levels of indicated genes in whole kidney, normalized to eEF2 (n=7-8). (F) Top GO-terms enriched within the biological processes category for genes significantly up-regulated ($p<0.05$, fold change >1.1) in HFD-fed CTRL mice compared to chow-fed CTRL mice, and significantly blunted ($p<0.05$) in HFD-fed PiNKO mice compared to HFD-fed CTRL mice. (G) Heat map generated using probe set intensities for transcripts associated with the GO-category "Fatty acid metabolic process". Chow-fed CTRL mice (CWT), chow-fed PiNKO mice (CKO), HFD-fed CTRL mice (HWT) and HFD-fed PiNKO mice (HKO). (H) Top transcription factor motifs exhibiting increased activity in ISMARA analysis of microarray data for HFD-fed compared to chow-fed PiNKO mice, and HFD-fed PiNKO compared to HFD-fed CTRL mice. Error bars represent mean ±SEM. Significant differences (p value < 0.05) between CTRL and PiNKO mice are indicated by an asterisk (*), and between chow-fed and HFD-fed groups are indicated by a number-sign (#).

S1A



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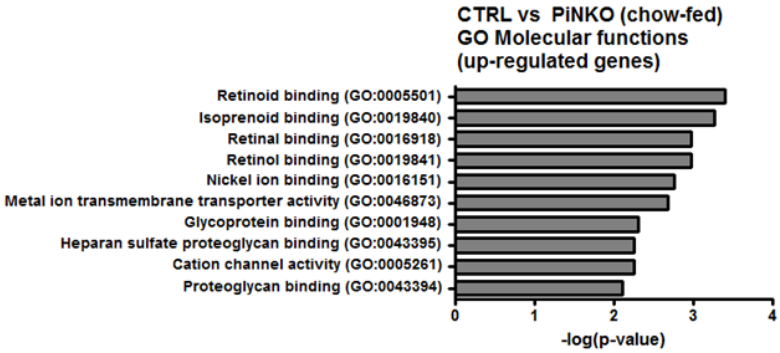
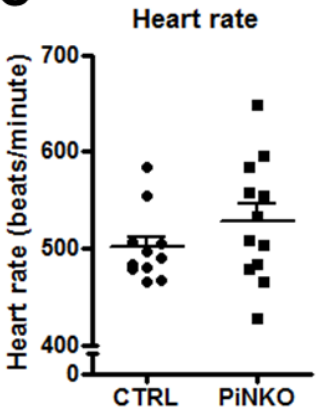
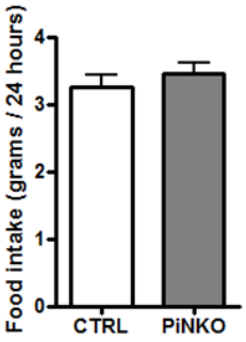


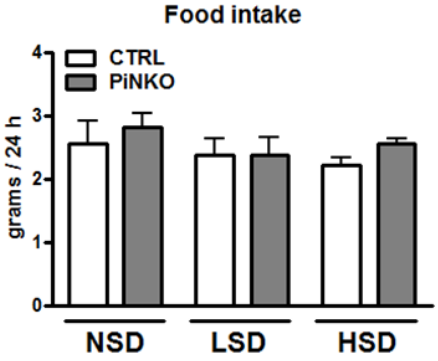
S1C



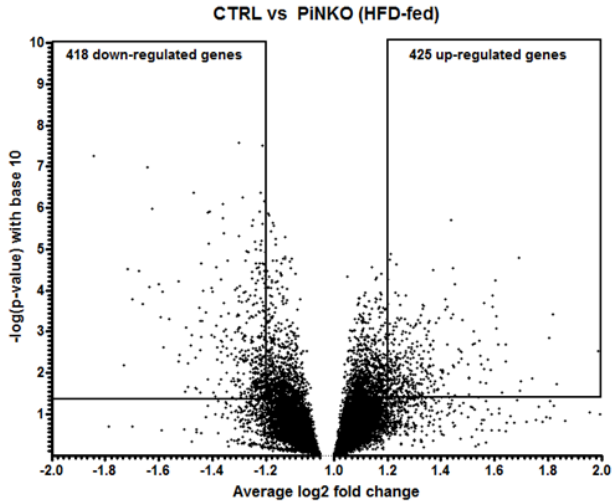
S1D



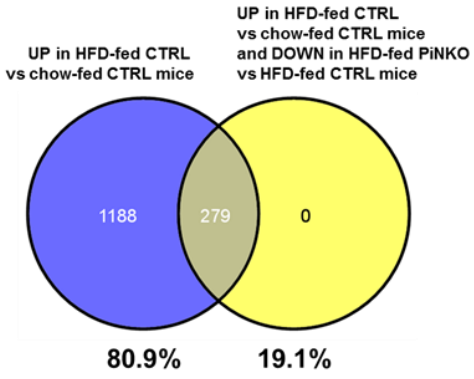
S1E



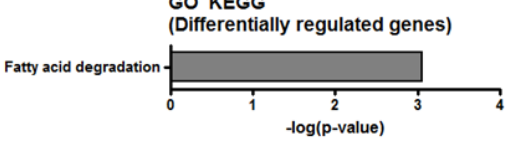
S1F



S1G



S1H



SI Figure 1

(A) Volcano-plot for probe intensities from gene expression array. P-values are plotted on the Y-axis and average log2 fold change is plotted on the X-axis. Boxes represents cut-offs ($p<0.05$, fold change >1.2 or <-1.2) for up- and down-regulated genes in kidneys from chow-fed CTRL and PiNKO mice. (B) Top gene ontology (GO)-terms enriched within the Molecular functions category for genes significantly up-regulated ($p<0.05$, fold change >1.2) in chow-fed PiNKO mice compared to chow-fed CTRL mice. (C) Heart rate measured via tail-cuff photoplethysmography. (D-E) Food intake over 24 hours (n=8-9). (F) Volcano-plot for probe intensities from gene expression array. P-values are plotted on the Y-axis and average log2 fold change is plotted on the X-axis. Boxes represents cut-offs ($p<0.05$, fold change >1.2 or <-1.2) for up- and down-regulated genes in kidneys from HFD-fed CTRL and PiNKO mice. (G) VENN diagram representing transcripts up-regulated in HFD-fed CTRL mice compared to chow-fed CTRL mice (blue) and the amount of these gene significantly blunted ($p<0.05$) in HFD-fed PiNKO compared to HFD-fed CTRL mice (yellow). (H) GO-term enriched within the KEGG category for genes significantly up-regulated ($p<0.05$, fold change >1.1) in HFD-fed CTRL mice compared to chow-fed CTRL mice, and significantly blunted ($p<0.05$) in HFD-fed PiNKO mice compared to HFD-fed CTRL mice. Significant differences (p value < 0.05) between CTRL and PiNKO mice are indicated by an asterisk (*).

SI Table I – Genes in this list are found to be significantly down-regulated ($p<0.05$, fold change < -1.2) in kidney between either chow-CTRL vs. chow-PiNKO or HFD-CTRL vs. HFD-PiNKO mice. All genes were furthermore associated with the KEGG GO category *oxidative phosphorylation* (mmu00190).

Gene	Fold change - Chow-fed PiNKO Vs. Chow-fed CTRL mice	p-value	Fold change - High fat diet-fed PiNKO Vs. High fat diet-fed CTRL mice	p-value
Atp4a	-1.25	0.030729	-1.21	0.059675
Atp5c1	-1.20	0.001949	-1.22	0.001114
Atp5d	-1.23	0.002976	-1.12	0.078392
Atp5e	-1.33	2.30E-05	-1.20	0.001115
Atp5f1	-1.22	0.000176	-1.21	0.00024
Atp5g1	-1.08	0.536452	-1.49	0.005845
Atp5g3	-1.32	3.69E-06	-1.23	5.84E-05
Atp5h	-1.21	3.19E-06	-1.21	2.45E-06
Atp5k	-1.18	0.00132	-1.24	0.000134
Atp5l	-1.26	0.002095	-1.15	0.03815
Cox10	-1.41	7.36E-05	-1.36	0.000207
Cox4i1	-1.30	0.000002	-1.19	0.000086
Cox5a	-1.31	0.000012	-1.36	0.000004
Cox5b	-1.24	0.088812	-1.37	0.019061
Cox7a1	-3.31	0.000001	-3.06	0.000002
Cox7a2	-1.25	0.000004	-1.24	0.000006
Cox7b	-1.24	0.027869	-1.03	0.727354
Cyc1	-1.26	0.000012	-1.20	0.000087
ND6	-1.40	0.002268	-1.16	0.123039
Ndufa1	-1.25	0.006665	-1.23	0.009967
Ndufa4	-1.26	0.000004	-1.24	0.000010
Ndufa6	-1.22	0.000299	-1.20	0.000604
Ndufa8	-1.28	0.000115	-1.30	0.000078
Ndufa9	-1.25	0.000000	-1.22	0.000000
Ndufb2	-1.21	0.004665	-1.09	0.131977
Ndufb5	-1.21	0.000370	-1.17	0.002029
Ndufb8	-1.25	0.002371	-1.18	0.014918
Ndufc1	-1.32	0.000094	-1.29	0.000195
Ndufs1	-1.31	0.000000	-1.14	0.000084
Ndufs2	-1.21	0.000017	-1.18	0.000062
Ndufs3	-1.22	0.000117	-1.25	0.000035
Ndufs6	-1.21	0.000488	-1.18	0.001373
Ndufs8	-1.24	0.000023	-1.23	0.000039
Ndufv2	-1.25	0.000008	-1.17	0.000220
Sdha	-1.21	0.000003	-1.17	0.000019
Sdhc	-1.21	0.000143	-1.13	0.004467
Sdhd	-1.25	0.000007	-1.20	0.000054
Uqcrb	-1.20	0.002694	-1.18	0.004626

Uqcrc1	**-1.26**	**0.000002**	**-1.25**	**0.000003**
Uqcrfs1	**-1.33**	**0.000005**	**-1.25**	**0.000051**
Uqcrh	**-1.29**	**0.000103**	**-1.28**	**0.000115**
Uqcrq	**-1.24**	**0.000238**	**-1.23**	**0.000323**

SI Table II – Genes within this table were significantly increased in kidney ($p<0.05$, fold change >1.1) from HFD-fed CTRL mice compared to chow-fed CTRL mice, and this induction was significantly blunted in HFD-fed PiNKO mice compared to HFD-fed CTRL mice ($p<0.05$).

0610038L08Rik	BC018473	Faah	Gm600	Mkl2	Slc22a5
1110007C09Rik	BC030499	Fadd	Gm6086	Mpl	Slc25a20
1200014J11Rik	C1s	Fam171a1	Gm8234	Msi1	Slc25a22
1700011F14Rik	C2cd4a	Fam18a	Gm9442	Myl1	Slc35e4
1700019E08Rik	C330011F03	Fam19a2	Gm9946	Myod1	Slc38a1
1700067G17Rik	C820005J03Rik	Fam5b	Grin2d	Ncapd2	Slc41a3
1810012K16Rik	Cadm4	Fam71f1	Gsto1	Ncr1	Slc6a5
2300002M23Rik	Ccdc146	Fancg	Gucy1a2	Ndufs4	Slc6a8
2310046A06Rik	Ccl4	Fchsd1	Gucy2e	Nfix	Smyd1
4632404H12Rik	Ccnyl1	Fgf14	Hadha	Nmur2	Snrpd2
4732471J01Rik	Cd80	Fgf16	Hbb-bh2	Olfr1100	Sowahb
4921509C19Rik	Cdh22	Fgfr4	Hck	Olfr391-ps	Spata21
4921509C19Rik	Cdkn1c	Frmd5	Hddc3	Olfr479	Speer4e
4921523A10Rik	Cdkn2d	G6pd2	Hepacam	Olfr574	Ssxa1
4921525B02Rik	Chchd2	Gabbr2	Hexdc	Olfr702	Ssxb1
4930440C22Rik	Chrm1	Gad2	Hlf	Olfr884	Stfa2l1
4930474M22Rik	Cisd3	Gbe1	Hras1	Olfr951	Stmn3
4930524N10Rik	Cisd3	Glra1	Hsd17b4	Osbpl5	Stoml2
4930583I09Rik	Clic6	Gm10696	Ifitm6	Pcdhb7	Syne1
4931403G20Rik	Cnr1	Gm10839	Ikbke	Pdk2	Taf7l
4933422A05Rik	Cox17	Gm10941	Il1rap	Pex5l	Tbx19
4933434M16Rik	Cox6b2	Gm11937	Il25	Pknox2	Tm6sf2
5430421F17Rik	Creb3l3	Gm12070	Inhbc	Pla2g16	Tmem236
5830473C10Rik	Csmd1	Gm12070	Itih4	Plekha2	Tmem40
A230077H06Rik	Cyp2d13	Gm12356	Kif15	Plekhg6	Tmem79
A430078I02Rik	Cyp46a1	Gm12503	Kifc2	Plvap	Tmem91
Abcd3	D0H4S114	Gm12830	Kiss1r	Ppcs	Tmie
Acaa2	D630042P16Rik	Gm13369	Klf4	Ppp1r32	Tspo
Acad11	Dbil5	Gm13430	Klhl34	Ppp2r5c	Tssk5
Acadl	Dcaf4	Gm13547	Klre1	Prickle1	Tyw1
Acadvl	Decr1	Gm13662	Krt42	Prl3a1	Ubac1
Accn5	Dgkh	Gm13941	Large	Pstk	Ube2d2b
Acot11	Dhrs2	Gm14317	Ldlrad2	Pttg1	Uqcc
Acot2	Dll1	Gm16701	LOC433944	Rab30	Vamp5

Acot3	Dmrt3	Gm17494	LOC434003	Rabep2	Vash1
Adam6a	Dnajb8	Gm17733	Lpin2	Rbpjl	Vmn1r2
Adcy2	Drd5	Gm17749	Lrrc39	Rcor3	Vmn1r212
Agt	Edil3	Gm19304	Lrrtm2	Rcvrn	Vmn2r20
Alyref2	Efcab4b	Gm19462	Lypd3	Rdh8	Vmn2r93
Apoa2	Eif4a3	Gm19747	Mettl21a	Rhox4f	Wnt8a
Aqp9	Eno1	Gm20095	Mir184	Rubie	Ypel4
Arc	Esp3	Gm20187	Mir1963	Sall4	Zbtb46
As3mt	Esrra	Gm20279	Mir3473c	Samsn1	Zeb2
Atp2a1	Etfb	Gm281	Mir466g	Scarb2	Zfp146
Atp5k	Expi	Gm4301	Mir5125	Siae	Zfp319
AW011738	F11	Gm5237	Mir744	Sla2	Zfp961
AY358078	F830002L21 Rik	Gm5938	Mir92b	Slc22a20	Zp4-ps

SI Table III – Sequences of primers used in this study

Target gene	Forward primer	Reverse primer
Acadl	CCAGCTAATGCCTTACTTGGAGA	GCAATTAAGAGCCTTTCCTGTGG
Acadvl	GTAGCCTCCATCCGAAGCTC	CAGGCCCCCATTACTGATCC
Acot2	AGTGCCTATGAAGGACTGAGG	TCTTACGGCACTGGGGAATG
Acot3	AGCTCTTGACCTTGTCTGTCTG	GAGAAAGTTCAAGCAAACTGGG
Agt	GCAGGAGAGGAGGAACAGCCGA	AGATGGCGAACAGGAAGGGGCT
Atp5h	GGGGGTCGGTGAAGTATCC	AATTGCCTTCTGGTTTTGGGG
Atp5k	GCCAAGCGCTACAGTTACCTA	GCCAGTTCTCTCTCAATCCGT
Cox5a	CCTGGGAATTGCGTAAAGGGA	TAACCGTCTACATGCTCGCAA
Cox7a1	TGTACAGACTGACCATGACGC	GTGTCACTTCTTGTGGGGGAA
Cpn1	AATTCCCCGACACCTGATGAC	GGTGATGCCGTCTGGAAAGTA
Cs	CCCAGGATACGGTCATGCA	GCAAACTCTCGCTGACAGGAA
Dhcr7	AGGCTGGATCTCAAGGACAATG	CAATGAATGGAGCGAAGAGCAG
eEF2	ATACCTGCCTGTCAATGAGTCCTT	CTGGCCGCCGGTGTT
ERRα	CGGTGTGGCATCCTGTGA	CTCCCCTGGATGGTCCTCTT
ERRβ	CAGATCGGGAGCTTGTGTTC	TGGTCCCCAAGTGTCAGACT
ERRγ	ACTTGGCTGACCGAGAGTTG	GCAAGGGACAGTGTGGAGAA
Fdft1	GTTCGTCAAGTGTCTAGGCCA	GTGAGTCCTGGTCCATCTTGG
Fh1	TGCTCTCAGTGCAAAATCCAA	CGTGTGAGTTCGCCCAATT
Hkl	ACAACGCCATCACTGGAATCT	CAAGGAAACACCACTCCGACT
Idh3a	GCTGGTGGTGTTCAGACAGTAA	CACTGAATAGGTGCTTTGGCAG
Klk1	CAAGCTGGGGAGCACATGCCTA	ACACTGGAGCTCATCTGGGTATTCA
Lcad	CCAGCTAATGCCTTACTTGGAGA	GCAATTAAGAGCCTTTCCTGTGG
Ldhb	TAAGATGGTGGTGGACAGTGC	GCATGGACTCGATGAGGTCAG
Ndufa1	TCCACTGCGTACATCCACAAA	CGTCTATCGCGTTCCATCAGA
Ndufa4	AACCCAGAGCCATGGAACAAA	AGTCTGGGCCTTCTTTCTTCAG
Ndufb8	CAAGAAGTATAACATGCGAGTGGAA	CCATACCCCATGCCATCATC
Ndufs1	GTTTTGAGGGGTCAGGGAGAC	TTCAACTTGCCCACTTCAACG
Ndufs3	AAAGACTTTCCCCTCACTGGC	AACTTGCGGAATTCTTGTGCC
Nrf1	CTCTGCATCTCACCCTCCAAAC	TCGCACCACATTCTCCAAAG
Pfkm	GGGGATCACCAATCTGTGTGT	ATCATTCAGCAAGTCGCTCCA
PGC-1α	AGCCGTGACCACTGACAACGAG	GCTGCATGGTTCTGAGTGCTAAG
PGC-1β	CCATGCTGTTGATGTTCCAC	GACGACTGACAGCACTTGGA
PPARα	ACAAGGCCTCAGGGTACCA	GCCGAAAGAAGCCCTTACAG
PPARβ/δ	GCAAGCCCTTCAGTGACATCA	CCAGCGCATTGAACTTGACA
PPARγ	CCCACCAACTTCGGAATCAG	AATGCGAGTGGTCTTCCATCA
PRC	CACCCTGCCGGAGTGAAAT	CGCATTGACTGCTGCTTGTC
Ptgds	GCTCCTTCTGCCCAGTTTTCC	AGGAGGACCAAACCCATCCAC
Ptges2	CCCAACGTGTACCGAACACCCG	CTCCACAGCCCCAAACTTGCCC
Ren1	AGGTAGCGACCCGCAGCATTAT	ACCCCACAGACACCCCCTTCAT

Sdha	GCTGGTGTGGATGTCACTAAGG	CCCACCCATGTTGTAATGCA
Slc12a7 (KCC4)	GTACCACCTCAGGATCAGTGC	ACCTCTGCTCCATCATTAGCG
Slc17a4 (NPT1 Hom.)	TGCAGAAAGCTGAGAAACCGA	CTTCTTCTAAGGTCCAGCATCT
Slc2a4 (GLUT4)	GATGAGAAACGGAAGTTGGAGAGA	GCACCACTGCGATGATCAGA
Slc25a20	CTGCGCCCATCATTGGA	CAGACCAAACCCAAAGAAGCA
Slc34a2 (NaPi-2b)	TCTACTTGTTCGTGTGCTCCC	TTGTTGCTGAAGAACTGTCCG
Slc43a2 (LAT4)	CTCCTACCTGTGTACGAAGCC	CATTCACCAGGCTCAACTCCT
Slc8a1 (NCX1)	GCATCTGCGTGTGTTCTTTGT	CTTCCCAGACCTCCACAACTC
Sqle	TCTCCGTTTCTTCCCACTTCG	ACTGGGGTTGACCAGAACAAG
Tfam	GGAATGTGGAGCGTGCTAAAA	TGCTGGAAAAACACTTCGGAATA
Uqcrfs1	CCTGAAGGGAAGAACATGGCT	TAACTGGGACACTTCGACTGC
Vegfa	ATCATGCGGATCAAACCTCAC	GGTCTGCATTCACATCTGCTG

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5. The role of PGC-1α in kidney – Discussion and future perspectives

5.1 - Discussion

The kidneys are vital organs for the maintenance of systemic salt and water homeostasis, acid-base balance, blood pressure regulation and excretion of toxic substances and metabolic by-products. This is achieved through an intricate system of plasma filtration and subsequent reabsorption of nutrients and ions along the renal nephron **[Guyton and Hall 2000, Boron and Boulpaep 2003]**. Trans-tubular transport is driven by a sodium gradient, generated by Na^+-K^+-ATPases in the basolateral membrane of the renal epithelium. Transcellular transport in the kidney relies on ATP production through oxidative phosphorylation. Hence, the kidney is one of the organs with the highest basal metabolic rate **[Soltoff 1986]**. Mitochondrial dysfunction is appreciated as a central part of the etiology of several disorders affecting metabolic organs like skeletal muscle, liver and kidney **[Pieczenik and Neustadt 2007]**. Mitochondrial cytopathies are closely associated with a reduced renal function, development of proximal tubulopathy and subsequent loss of both nutrients and ions in the urine **[Niaudet 1998]**. In recent years, several studies **[Brooks et al. 2009, Tran et al. 2011, Funk and Schnellmann 2013, Lempiainen et al. 2013, Parikh 2013, Sharma et al. 2013]** have demonstrated a strong association between mitochondrial dysfunction and renal disorders, such as diabetic nephropathy, sepsis and ischemia/reperfusion injury. Interestingly, treatments aimed at ameliorating disease progression in these disorders have been closely linked to a concurrent increase in renal mitochondrial function **[Funk and Schnellmann 2013, Lempiainen et al. 2013]**. Accordingly, these findings have sparked an interest in finding ways to ameliorate renal mitochondrial dysfunction associated with kidney pathologies. In this context, PGC-1α might be a promising therapeutic target, considering its role as a master regulator of mitochondrial biogenesis and function **[Liu and Lin 2011]**. Additionally, this coactivator is highly expressed in the kidney **[Puigserver et al. 1998, Larrouy et al. 1999]** and its expression is reduced in several renal pathologies **[Tran et al. 2011, Funk and Schnellmann 2013, Lempiainen et al. 2013]**. Hence, PGC-1α might constitute a potential therapeutic candidate to ameliorate renal mitochondrial and metabolic disorders. However, the role of PGC-1α in the kidney has not been conclusively investigated. Thus, in the first part of this thesis we addressed this open question and accordingly studied the role of PGC-1α in the kidney and its impact on renal physiology. Global- and tissue-specific knockout models for PGC-1α have been instrumental in elucidating the role of PGC-1α in various organs, such as heart **[Arany et al. 2005]**, liver **[Handschin et al. 2005]**, skeletal muscle **[Handschin et al. 2007a, Handschin et al. 2007b]**, brain **[Ma et al. 2010]**, and white adipose tissue **[Pardo et al. 2011]**. Despite the high basal expression of PGC-1α and the oxidative

phenotype of the kidney, a renal-specific knockout model of PGC-1α has to this date not been published. One reason for this is likely due to technical reasons, since the heterogeneity of the renal cell population makes it difficult to target a transgene to all cells within the renal nephron. However, in 2008, Traykova-Brauch et al. published a study which provided an elegant solution to this problem **[Traykova-Brauch et al. 2008]**. The authors generated a transgenic mouse model, which expressed the reverse tetracycline-dependent transactivator (rtTA) under the *Pax8* promoter, thereby efficiently targeting it to all renal tubular cells **[Traykova-Brauch et al. 2008]**. When crossed with the transgenic LC1-Cre mouse line originally published by Schönig et al. **[Schonig et al. 2002]**, this resulted in a mouse model with a doxycycline-inducible expression of Cre in all renal tubular cells. We utilized this specific double-transgenic mouse model to generate a nephron-specific, inducible PGC-1α knockout (PiNKO) mouse, with which we could study the role of PGC-1α in the kidney.

After doxycycline induction, PiNKO mice were healthy and displayed no obvious aberrant systemic or renal phenotypes. Transcriptome analysis of RNA extracted from whole kidney confirmed a significant reduction in PGC-1α-regulated metabolic gene programs, such as mitochondrial oxidative phosphorylation, TCA cycle and glycolysis. These findings implicate an important role for PGC-1α in transcriptional regulation of mitochondrial processes in kidney, which is in agreement with the role of PGC-1α as a global transcriptional regulator of mitochondrial processes in other organs **[Lin et al. 2004, Arany et al. 2005, Handschin et al. 2007b, Pardo et al. 2011]**. Next, we investigated whether the reduction in renal mitochondrial biogenesis affected transcellular ion reabsorption. This is a pertinent question, since inhibition of mitochondrial ATP-production has been shown to reduce transtubular transport activity in perfusion studies **[Gullans et al. 1982]**. Moreover, the loss of PGC-1α in PiNKO mice resulted in differential regulation of several mRNAs coding for transcellular transporters from both the solute carrier (SLC) and potassium transporter (KCN) families, which could further affect transtubular transport. Intriguingly, PiNKO mice showed no major alterations in either salt or water handling, and only displayed a mild sodium-losing phenotype. To challenge this aspect of renal physiology further, we decided to use two separate paradigms to stress the renal salt and water homeostasis in our mice; modulation of dietary salt intake and aging. Altered dietary NaCl-intake leads to a drastic adaptation of renal tubular homeostasis and in extension increased tubular energy demand. Indeed, low-NaCl diets have been suggested to boost renal energy metabolism to facilitate the increased transtubular transport activity needed to retain sufficient quantities of salts in the body **[Siragy and Linden 1996, Klawitter et al. 2012]**. Interestingly, young PiNKO mice efficiently adapted to both reduced and increased dietary NaCl

loads. Hence, despite a reduced mitochondrial transcription in PiNKO mice and an altered transcription of genes involved in transmembrane- and ion transport processes, PGC-1α is not necessary for maintaining renal ion and water homeostasis. A reduced ability to adjust for altered dietary sodium intake is associated with aging in humans **[Schlanger et al. 2010, Karam and Tuazon 2013]**. Interestingly, in line with this, we detected a mild urinary sodium-loss in aged PiNKO mice and a concomitant impairment in the adaptation to a low-NaCl diet. Hence, our data indicates that reduced expression of PGC-1α in the kidney during aging might result in a worsening of the renal phenotype as the mice age. Importantly, PGC-1α levels have been shown to be reduced in kidneys from aged mice **[Lim et al. 2012]**. Lim et al. demonstrated a significant reduction in renal PGC-1α levels at 24 month old mice compared to younger mice, and this was furthermore associated with a significant worsening of renal function and an increased renal damage **[Lim et al. 2012]**. Interestingly, it has been shown that calorie restriction or treatment with the PPARγ agonist Pioglitazone ameliorates renal injuries and improves mitochondrial function in aged mice **[Yang et al. 2009, Kume et al. 2010]**. Both calorie restriction and thiazolidinedione treatment are known to increase PGC-1α activity **[Nisoli et al. 2005, Hondares et al. 2006]** and elevated PGC-1α activity could therefore have a protective role in the kidney during aging. Despite extensive research on the connection between PGC-1α and aging-related injuries such as sarcopenia in muscle and neurodegenerative disorders in brain **[Wenz 2011, Dillon et al. 2012]**, little is known how PGC-1α influences aging-related dysfunctions in the kidney. Hence, based on our findings that PGC-1α is a regulator of metabolic and mitochondrial processes in the kidney, an important future direction would be to elucidate the role of PGC-1α in aging-related renal dysfunction. This could be achieved through a series of experiments designed to investigate the protective role of PGC-1α in aging-related renal dysfunction. The initial aim of such a study would be to map renal PGC-1α expression and oxidative function in aging mice and to correlate these parameters with aging-related alterations in blood pressure regulation, renal salt and water homeostasis as well as proteinuria and glomerular permselectivity. This would be complemented with post mortem histological and molecular analysis of the kidneys. A good age-span of the groups would be to use 3, 12 and 24 month old mice, which would represent young adult mice and two separate aging time points. Since we detected a progressive impairment in the adaptation to altered dietary salt intake in PiNKO mice in our study, this indicates that loss of PGC-1α and the concomitant reduction in mitochondrial gene transcription could sensitize the kidneys to aging-related dysfunction. Hence, it would be imperative to perform the above-mentioned experiments in control and PiNKO mice. Such a setup would allow a closer examination of the relationship between mitochondrial dysfunction and renal impairment during aging and whether these pathological alterations are accelerated upon loss of

renal PGC-1α expression. Ultimately, for a deeper mechanistic insight, it would be interesting to use the PiNKO model to examine whether PGC-1α is necessary to mediate the beneficial effects of calorie restriction and thiazolidinedione treatment on renal parameters in aged mice.

The orphan nuclear receptor ERRα is an established transcriptional partner of PGC-1α **[Mootha et al. 2004]**. Importantly, in our microarray data, ERRα was predicted to be one of the top transcription factors with a reduced activity in kidneys from PiNKO mice compared to CTRL mice. This indicates that ERRα is a transcriptional partner of PGC-1α in the kidney. Interestingly, in a study by Tremblay et al. global deletion of ERRα was associated with altered transcriptional regulation of several genes involved in ion homeostasis and blood pressure regulation in kidney **[Tremblay et al. 2010]**. In this study, global ablation of ERRα led to a hypotensive phenotype associated with reduced urinary sodium excretion in mice **[Tremblay et al. 2010]**. Surprisingly, even though ERRα levels were also reduced in the kidney of PiNKO mice, these mice displayed an opposite phenotype as compared to ERRα knockout mice. This dichotomy was also evident at the transcriptional level, since several differentially regulated genes in the global ERRα knockout mice (*Ptgds*, *Agt*, *Slc34a2*, *and Slc22a4*) **[Tremblay et al. 2010]** were regulated in an opposite direction in the kidney of PiNKO mice. These differences could originate from ERRα-independent effects in PiNKO mice, or simply through compensatory mechanisms due to the systemic ablation of ERRα in the study by Tremblay et al. An outstanding question would thus be to elucidate the transcriptional networks regulated by PGC-1α-ERRα interactions in renal tubular cells. These experiments would preferably be done in an *in vitro* setup to avoid compensatory effects originating from other cell types within the kidney or other organs. Considering the high expression of PGC-1α together with the high basal oxidative capacity of proximal tubule cells, this cell type would be the preferable model to use. Overexpression of PGC-1α in proximal tubule cells would identify a transcriptional signature of PGC-1α-regulated genes in this cell type. When combined with knockdown or pharmacological inhibition of ERRα, this would yield a subgroup of genes regulated by PGC-1α together with ERRα. Transcriptome analysis of these two gene signatures and subsequent GO-analysis would reveal the functional annotations of PGC-1α/ERRα-regulated genes in proximal tubule cells. This would allow the identification of cell autonomous transcriptional networks of PGC-1α and ERRα in proximal tubule cells associated with energy metabolism, renal ion homeostasis and blood pressure regulation. Importantly, such an approach can be extended to other renal cell types or transcriptional partners of PGC-1α. Considering the complex organization of the kidney, *in vitro* approaches are essential to map the molecular mechanisms through which PGC-1α regulates renal transcription in a specific cell type. Ultimately, these findings could be validated in an *in*

vivo setting, for example through the use of the PiNKO mouse model to specifically study the effect of PGC-1α deletion in a specific nephron segment. Microdissection techniques could be used to excise specific regions of the nephron for molecular analysis and immunohistological and enzymatic stainings could be applied to whole kidney sections to study alteration induced by PGC-1α deletion. There are also several nephron segment-specific drugs (i.e. loop diuretics, thiazides, amiloride) which modulate salt and water homeostasis in the kidney by acting only on certain parts of the nephron **[Brenner and Stevens 2013]**. Hence, these diuretic drugs could be useful tools to modulate salt and water homeostasis only in specific nephron segments in PiNKO mice and would allow us to study the role of PGC-1α specifically on these localized processes.

Obesity is associated with a reduced mitochondrial function in several organs **[Patti et al. 2003, Boudina et al. 2005, Sparks et al. 2005, Sivitz and Yorek 2010]**. However, this phenomenon has not been extensively studied in the kidney. Hence, in the second part of this study we focused on metabolic adaptation in the kidney occurring during diet-induced obesity and how this is influenced by the loss of PGC-1α. Five months of HFD-feeding did not lead to any major changes in renal mitochondrial transcription. Despite a differential regulation ($p<0.05$, fold-change >1.2) of 1467 genes in kidney with HFD-feeding, none of these genes were primarily associated with GO-categories representing mitochondrial function or oxidative phosphorylation. Furthermore, HFD-feeding led to a small decrease in PGC-1α levels in kidney and only a small number of PGC-1α-target genes associated with oxidative phosphorylation were affected by HFD-feeding in CTRL mice. Hence, our data demonstrates that diet-induced obesity does not affect transcription of mitochondrial genes in the kidney. This finding is corroborated by a study from Ruggiero et al. from 2011, which shows that HFD-feeding does not alter mitochondrial oxidative function or biogenesis in kidney **[Ruggiero et al. 2011]**. However, the authors of this study detected increased oxidative stress and signs of renal dysfunction, such as fibrosis and glomerular hypertrophy with HFD-feeding. Interestingly, in our study we detected a significant increase in glomerular hypertrophy in HFD-fed PiNKO mice compared to CTRL mice (Figure 1). While, these alterations did not lead to an exacerbated proteinuria in PiNKO mice (unpublished data), this finding suggests that the overall renal health with HFD-feeding is significantly worsened in the absence of PGC-1α. A likely explanation for the worsened renal phenotype in HFD-fed PiNKO mice could be an increased susceptibility to renal lipotoxicity. We demonstrated that renal PPARα-signaling and several known PPARα target genes involved in fatty acid oxidation were increased in kidneys of HFD-fed CTRL mice, which could represent an adaptive mechanism to cope with increased circulating levels of fatty acids.

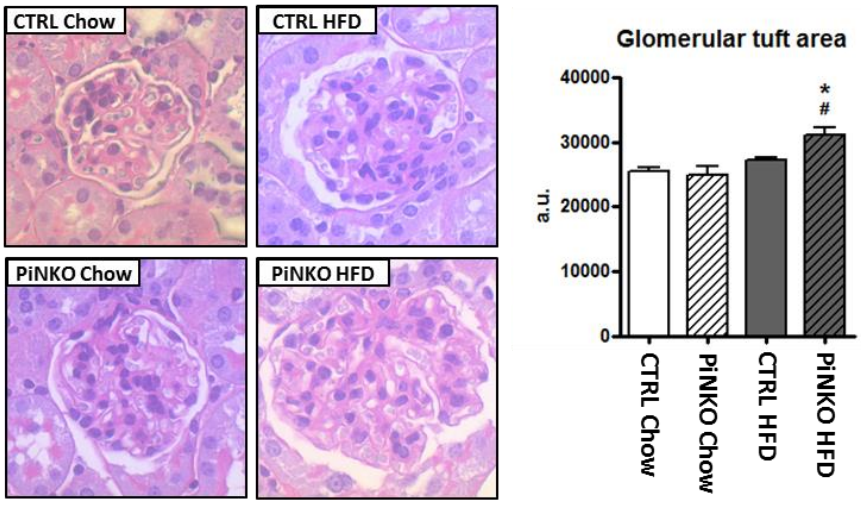

Figure 1 - HFD-fed PiNKO mice display exacerbated glomerular hypertrophy

Glomerular tuft area was quantified in periodic acid–schiff (PAS)-stained kidney cross-sections from chow- or HFD-fed CTRL and PiNKO mice. Error bars represent mean ±SEM. Significant differences (p value < 0.05) between CTRL and PiNKO mice are indicted by an asterisk (*), and between chow-fed and HFD-fed groups are indicated by a number-sign (#).

Importantly, this induction was blunted in the absence of PGC-1α, which demonstrates a role for PGC-1α in the transcriptional regulation of renal PPARα-signaling and fatty acid metabolism. This is in line with the known role of PGC-1α as a coactivator of PPARα-transcription **[Vega et al. 2000]**. Importantly, several recent studies using PPARα-knockout mice and/or pharmacological PPARα agonists **[Park et al. 2006, Kamijo et al. 2007, Shin et al. 2009, Tanaka et al. 2011]** have shown that PPARα signaling is important for the protection against renal lipotoxicity and inflammation. Hence, it will be of interest in future studies to elaborate on our current findings and further elucidate the importance of PGC-1α/PPARα signaling in protection against renal lipotoxicity. This could be achieved through an experimental model of fatty acid-induced renal damage such as albumin overload **[Kamijo et al. 2002]**. Increased circulating levels of albumin lead to impaired glomerular permselectivity and thereby an increased urinary filtration of albumin. Moreover, BSA-bound fatty acids have been demonstrated to elicit pathological alteration in proximal tubule cells, such as increased apoptosis and tubulointerstitial inflammation and fibrosis **[Kamijo et al. 2002, Tanaka et al. 2011]**. Importantly, pharmacological activation of PPARα has been shown to protect against both HFD- and BSA-induced renal lipotoxicity in mice **[Tanaka et al. 2011]**. Considering that several PPARα target genes displayed a blunted induction in HFD-fed PiNKO mice in our study, it would be interesting to perform a BSA-loading experiment in PiNKO mice. We could thereby evaluate whether the loss of PGC-1α and hence a blunted induction of PPARα target genes would lead to exacerbated lipotoxic injury in the kidney. This setup would provide a controlled and more potent lipotoxic stimuli than HFD feeding, especially since fatty acid-depleted BSA can be used as a control for these experiments. Hence, this setup would allow us to characterize the direct effect of PGC-1α deletion

on the sensitivity of the kidney to a lipotoxic stimuli. In this context, CTRL and PiNKO mice would also be treated with the PPARα agonist fenofibrate, which has been demonstrated to ameliorate renal lipotoxicity **[Tanaka et al. 2011]**. This would help to provide mechanistic insight into how PGC-1α/PPARα signaling influences the protection against renal lipotoxicity. These findings could eventually be applied to a more clinically relevant model of renal lipotoxicity, such as HFD-feeding and obesity, with the ultimate goal of developing therapeutic strategies to protect against fatty acid induced renal damage in obese and diabetic patients. In our current study, we have focused mainly on functions associated with the renal tubular system. However, another important component of basal renal function is the glomerulus, which largely consists of three distinct cell types, namely endothelial cells, mesangial cells and podocytes. Podocytes are highly specialized cells found within kidney glomeruli and their foot processes play an integral role in the establishment and maintenance of the glomerular filtration barrier **[Miner 2011]**. This filtration barrier prevents loss of proteins and larger blood constituents into the urine. Importantly, increased urinary protein excretion is a well-established prognostic risk factor for kidney disease progression in humans **[Abbate et al. 2006]**. Podocytes have a lower mitochondrial number compared to the surrounding proximal tubules and thus also express lower levels of PGC-1α (unpublished observation). However, despite its low basal expression, PGC-1α has been shown to play an important role in the response to podocyte injury. Podocyte injury induced through experimental obesity/diabetes **[Bao et al. 2014]** or through administration of adriamycin **[Zhu et al. 2014]** or aldosterone **[Yuan et al. 2012a]** has been associated with reduced PGC-1α levels and mitochondrial transcription in podocytes. Overexpression of PGC-1α in podocytes was furthermore sufficient to rescue mitochondrial function in response to both adriamycin- and aldosterone treatment in this cell-type **[Yuan et al. 2012b, Zhu et al. 2014]**. These findings point towards an important therapeutic potential of increased PGC-1α-activity in podocytes. However, PGC-1α has also been shown to mediate high-glucose induced apoptosis in cultured podocytes **[Kim and Park 2013]**. Therefore, in the context of using PGC-1α as a therapeutic target to ameliorate glomerular disorders, it is crucial to gain a full understanding of the role of PGC-1α in podocytes and glomerular function. Hence, we were also interested in elucidating the role of PGC-1α in podocyte function and structural integrity. To this end, we generated podocyte-specific PGC-1α knockout (PoPKO) mice by crossing mice expressing Cre under a podocyte-specific promoter (podocin; *NHPS2*-Cre) **[Moeller et al. 2003]** with mice harboring floxed PGC-1α alleles (PGC-1α$^{fl/fl}$) **[Lin et al. 2004]**.

At 10 weeks of age, PoPKO mice did not display any significant differences in either body- or kidney weight (FIG 2A-B), and did not show any alterations in food intake, water intake or urine output

over 24 hours (FIG 2C). To evaluate the function of the renal filtration barrier, we measured urinary protein excretion in PoPKO and CTRL mice. Proteinuria is an important read-out of defects in glomerular permselectivity and increased urinary protein excretion is closely associated with early glomerular dysfunction in both rodents and humans **[Miner 2011]**. Intriguingly, we detected a strong trend towards increased absolute protein content in the urine of PoPKO mice compared to CTRL mice (FIG 2D). This difference was still evident when protein levels were normalized to urinary creatinine levels (/crea) (FIG 2D), indicating that this phenotype was not due to a general increase in urinary flow rate. Next, we measured albumin and total protein levels in plasma and found no differences between PoPKO and CTRL mice (FIG 2E). Consequently, these data point towards a mild defect in permselectivity in young PoPKO mice. To stress podocyte function, we performed a BSA-loading experiment, where mice are injected with high levels of BSA which leads to acute glomerular and tubular damage **[Yoshida et al. 2008]**. Interestingly, one day after BSA injection, PoPKO mice displayed reduced levels of both creatinine (FIG 2F) and total protein (FIG 2G) in the urine compared to CTRL mice. These findings point towards a reduced glomerular filtration rate, which is a predictor of reduced glomerular function in PoPKO mice. Next we performed adriamycin-injections in a small pilot group of CTRL and PoPKO mice. Adriamycin-induced nephropathy is an established model of progressive glomerulosclerosis in mice **[Okuda et al. 1986, Lee and Harris 2011]**. Intriguingly, at 4 weeks post-injection, PoPKO mice displayed a strong trend towards reduced urinary creatinine and total protein levels (FIG 2H). Moreover, both urinary uric acid and urea/blood urea nitrogen (BUN) levels were reduced in PoPKO mice, while they remained unchanged when normalized to urinary excretion of creatinine (FIG 2I). Taken together, these data indicate that the ablation of PGC-1α in podocytes increases the susceptibility of podocytes to BSA-loading and adriamycin-induced podocyte damage. This is furthermore in line with the protective effect of PGC-1α overexpression in primary podocytes treated with adriamycin **[Zhu et al. 2014]** and could be linked to a protective effect of PGC-1α against the development of glomerulosclerosis. Although preliminary, our findings regarding the role of PGC-1α in glomerular function are promising and this project is currently being pursued by another member of our lab. Considering the reduced glomerular PGC-1α levels associated with several glomerular disease states **[Yuan et al. 2012b, Bao et al. 2014, Zhu et al. 2014]**, our mouse model will provide an important tool to assess the causal link between reduced podocyte PGC-1α levels and glomerular dysfunction. Further experiments are needed to expand upon the basal renal phenotype of the PoPKO mouse model. We did not find any major alterations in renal function in young PoPKO mice (10 weeks). However, glomerular function declines with age in both rodents and humans **[Wiggins 2009]** and it is thus imperative to study the function of the glomerular filtration barrier in old PoPKO mice.

Further experiments should preferably expand on our work on BSA- and adriamycin-induced nephropathy, especially considering the recent study by Zhu et al. were PGC-1α was shown to have a protective effect against adriamycin-induced damage in cultured podocytes **[Zhu et al. 2014]**. Hence, it would be interesting to induce glomerular damage in PoPKO mice through systemic administration of either adriamycin, puromycin aminonucleoside (PAN) or lipopolysaccharides (LPS). This would be followed by analysis of urinary protein content, glomerular morphology and ultrastructure as well as an assessment of general pathological renal markers such as fibrosis and inflammation. These experiments would indicate whether the loss of PGC-1α in podocytes sensitizes the glomerulus to these damaging stimuli and whether there is a causal link between reduced podocyte PGC-1α levels and glomerular dysfunction. Another important issue would be to study the role of PGC-1α in the protection against podocyte lipotoxicity during HFD-feeding or during acute lipotoxicity such as encountered with BSA-loading. For these specific experiments, it would be interesting to monitor the maintenance of the glomerular filtration unit by measuring urinary protein content. Subsequent molecular analysis would help to unravel whether loss of PGC-1α specifically in podocytes increases the susceptibility of the kidney to lipotoxic stimuli and thereby results in pathological alterations in glomerular and tubulointerstitial morphology and inflammation. Further characterization of the PoPKO mouse model generated during the scope of this thesis will hopefully help to form a more comprehensive picture of the role of PGC-1α in podocytes. These findings, together with the data generated from the PiNKO mouse model, will help to increase our understanding of the complex role of PGC-1α in renal physiology.

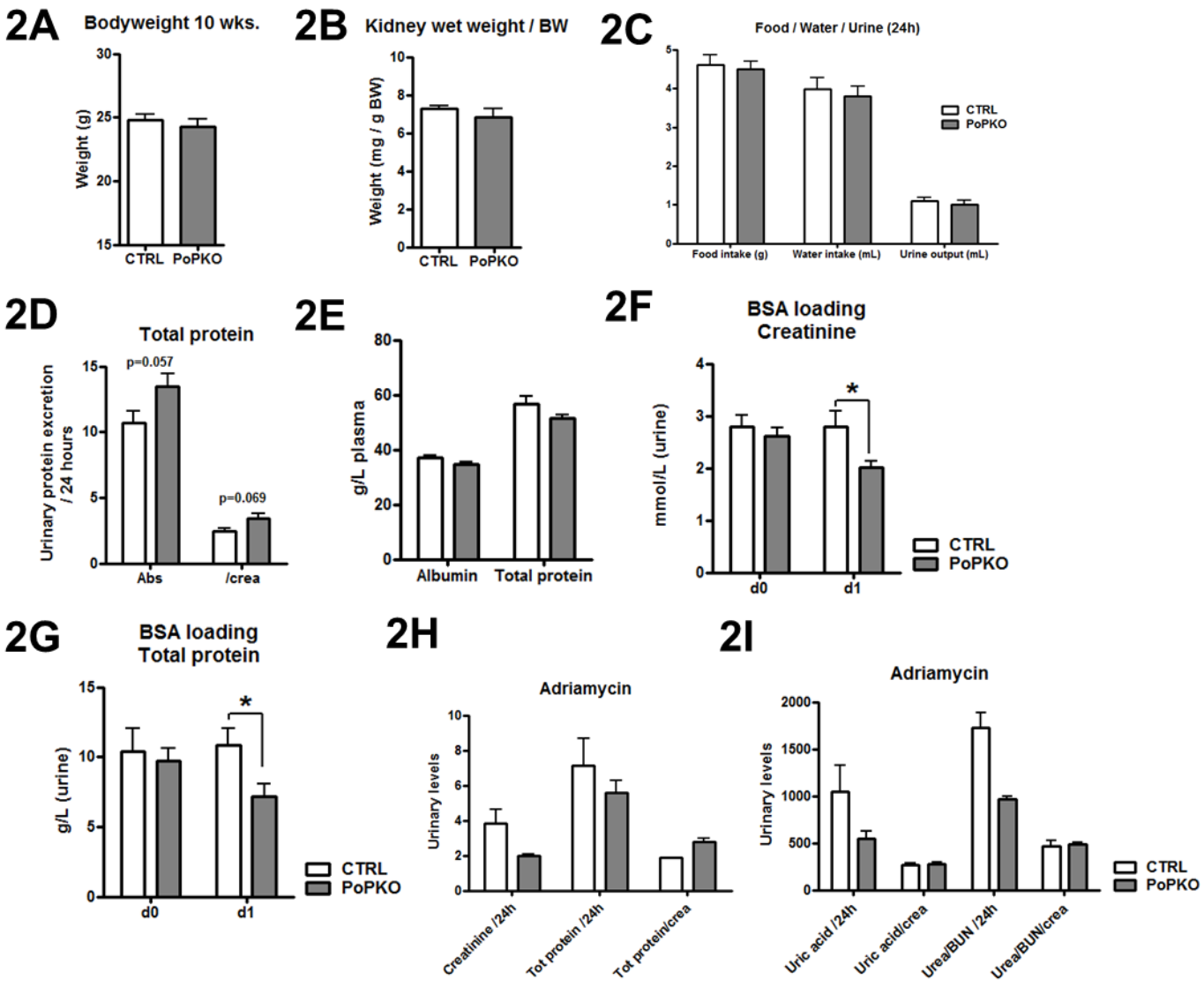

Figure 2 - Podocyte-specific PGC-1α knockout (PoPKO) mice display minor impairments in glomerular filtration

(A) Body weight of CTRL and PoPKO mice at 10 weeks (n=8-9). (B) Kidney weight normalized to body weight (n=3). (C) Food intake, water intake and diuresis over 24 hours (n=8). (D) Urinary excretion of protein over 24 hours, absolute levels and total protein levels normalized to creatinine excretion (/crea) (n=8). (E) Levels of albumin and total protein in plasma (n=8). Urinary levels of (F) creatinine and (G) and total protein in CTRL and PoPKO mice before BSA injection (d0) and after BSA injection (d1) (n=4). Urinary levels of (H) creatinine, total protein and (I) uric acid and urea/blood urea nitrogen (BUN) in CTRL and PoPKO mice 4 weeks after a single i.v. adriamycin injection (n=2). Error bars represent mean ±SEM. Significant differences (p-value <0.05) between CTRL and PoPKO mice are indicated by an asterisk (*).

5.2 – Future perspectives

In the present study, we have generated and characterized a novel knockout model of PGC-1α in the kidney. Transcriptome analysis confirmed that loss of PGC-1α in the kidney leads to a down-regulation of gene programs encoding mitochondrial and metabolic processes. These findings are in agreement with the role of PGC-1α in other metabolic organs **[Liu and Lin 2011]**, and underscores the importance of PGC-1α as a global transcriptional regulator of mitochondrial metabolism. Ablation of PGC-1α also led to an altered transcription of several genes belonging to the solute carrier (SLC) family, and the KCN-family of potassium transporters. In addition, tubular reabsorption is a highly ATP-dependent process, and impaired mitochondrial ATP-production can inhibit transcellular transport **[Gullans et al. 1982]**. However, despite the ablation of PGC-1α in the kidney, PiNKO mice could efficiently correct their salt- and water reabsorption in response to either increased or decreased NaCl intake. Hence, PGC-1α is not necessary for the regulation of renal salt and water homeostasis. Further studies are needed to elucidate whether PGC-1α plays a regulatory role in other renal processes such as gluconeogenesis or vitamin D-activation/calcium signaling.

In our microarray data, ERRα was predicted to be one of the main transcription factors with a reduced activity in kidneys from PiNKO mice. Our data furthermore shows that PGC-1α is necessary for the transcriptional induction of PPARα and PPARα-target genes in kidney during diet-induced obesity. ERRα and PPARα are established transcriptional partners of PGC-1α in the regulation of mitochondrial and metabolic processes **[Vega et al. 2000, Huss et al. 2002]**. However, both of these transcription factors have been shown to play an important role also in salt-handling and blood pressure regulation in the kidney **[Obih and Oyekan 2008, Tremblay et al. 2010, Lee et al. 2011]**. It would thus be of importance to elucidate the full transcriptional networks of both PGC-1α/ERRα and PGC-1α/PPARα in the kidney, to determine the functional outcome of these coactivator-transcription factor interactions. Moreover, PGC-1α is known to regulate gene programs in a tissue specific manner, through interactions with certain transcription factors. One such example is the induction of hepatic gluconeogenesis through interaction with HNF4A **[Yoon et al. 2001]**. It would thus be interesting to investigate whether such tissue-specific PGC-1α-TF interactions might also exist in the kidney and which processes are accordingly regulated through these transcriptional networks. One important aspect to consider for these experiments would be to differentiate between the different compartments of the nephron, in particular since it has been shown that the kidney displays a heterogeneous TF-expression pattern in different nephron segments **[Krid et al. 2012]**. Hence, the transcriptome analysis performed in our study provides a broad overview

of the transcriptional programs regulated by PGC-1α in the kidney and its impact on renal physiology. However, future studies would benefit from focusing on the transcriptional networks regulated by PGC-1α in certain cell types or compartments of the nephron. This could be achieved by performing microarray analysis of different microdissected nephron segments such as proximal tubules, loop of henle, distal tubules and collecting ducts. For further molecular analysis and target validation, *in vitro* models of the specific cell types contained within these renal compartments could be utilized. Transgenic mice with *Cre*-expression targeted to a specific tubular cell type or compartment could also be utilized to knock out PGC-1α in specific nephron segments. While microdissection would still be needed to analyze the molecular events occurring in one specific subcompartment of the nephron, such mouse models would aid in interpreting the functional outcome of a PGC-1α deletion in a specific renal cell type.

A causal link has been suggested between aberrant mitochondrial function and renal dysfunction **[Niaudet 1998, Rahman and Hall 2013, Che et al. 2014]**. This connection is underscored by the prevalence of renal tubular dysfunction in patients suffering from mitochondrial cytopathies **[Emma et al. 2011]**. Moreover, sepsis-induced renal dysfunction has been associated with reduced mitochondrial function, and this phenotype was furthermore exacerbated in the absence of renal PGC-1α **[Tran et al. 2011]**. Our data demonstrate an essential role for PGC-1α in mitochondrial transcription and PGC-1α could therefore be an important therapeutic target to ameliorate renal disorders. However, further studies are needed to elucidate the therapeutic potential of PGC-1α activation in a renal setting. Generation of a transgenic mouse model overexpressing PGC-1α in the kidney would be an important tool to study the protective effects of PGC-1α in different renal disorders and in the context of aging. Characterization of such a transgenic mouse model would also give a broader insight into which transcriptional programs and renal functions are directly regulated by PGC-1α in the kidney, especially when compared directly to the renal transcriptional profile of PiNKO mice generated in our current study. While transgenic mouse models are important tools to elucidate the therapeutic potential of a protein, it is also essential to find ways to modulate these pathways through administration of pharmacological compounds or through dietary means. The SIRT1-activating compounds resveratrol and SRT1720 have both been shown to elicit protective effects in renal disorders **[Funk and Schnellmann 2013, Pan et al. 2014]** and ketogenic diet feeding has also been reported to ameliorate diabetic nephropathy in mice **[Poplawski et al. 2011]**. These interventions have all been suggested to increase PGC-1α transcription and activity. Treatment of PiNKO mice with either resveratrol or SRT1720 would allow to evaluate the specific requirement of tubular PGC-1α for the protective effects of these compounds in association with experimental renal pathologies, such

as ischemia/reperfusion injury, endotoxemic injury or diabetic nephropathy, amongst others. A similar setup could be used to test the requirements of PGC-1α in the protection against diabetic nephropathy elicited by ketogenic diet feeding. These experiments will help to elucidate the role of PGC-1α as a molecular effector of these interventions and the eligibility of using PGC-1α as a therapeutic target to treat renal pathological alterations in human patients.

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THE ROLE OF PGC-1α IN SKELETAL MUSCLE

6. Introduction to skeletal muscle physiology

6.1 - Skeletal muscle and PGC-1α

Skeletal muscle is the largest organ in the body (~40% of body weight) and in a sedentary state it accounts for 20-30% of the resting energy expenditure **[Summermatter and Handschin 2012, Egan and Zierath 2013]**. Skeletal muscle thus contributes less to the resting energy expenditure than brain, kidney and heart **[Muller et al. 2013]**. However, during maximal physical activity, skeletal muscle can account for up to 90% of whole body energy expenditure **[Zurlo et al. 1990]**, and ATP-turnover rates within the muscle can rise up to 100-fold over basal levels **[Egan and Zierath 2013]**. Skeletal muscle thus displays a high degree of metabolic plasticity, and exerts a significant impact on whole body metabolism in response to exercise. Hence, increased physical activity is acknowledged as an important approach to prevent and treat obesity and related metabolic disorders **[Booth et al. 2012]**. However, 30% of the global adult population are estimated to be physically inactive **[Hallal et al. 2012]** and a sedentary life-style is associated with a higher incidence of chronic non-communicable diseases such as cardiovascular diseases, diabetes and cancer **[Booth et al. 2012, Egan and Zierath 2013]**.

Skeletal muscle fibers are categorized as either oxidative/glycolytic or slow-twitch/fast-twitch based on their metabolic and contractile properties. This is however an over-simplification considering the heterogeneous nature and plasticity of the mammalian skeletal muscle. Human skeletal muscle contains 3 major fiber types; type I, type IIa and type IIx, while mouse skeletal muscle also contains a fourth sub-category, type IIb. This categorization is based on the predominant expression pattern of myosin heavy-chains (MyHC)-I, MyHC-IIa, MyHC IIx or MyHC-IIb in the respective muscle fibers **[Schiaffino and Reggiani 2011]**. Type I fibers are generally found in postural muscles like the soleus, and are characterized as slow-twitch fatigue-resistant oxidative fibers, which are also often found in elite endurance athletes. These fibers are red in appearance due to their high myoglobin content, and muscles rich in type I fibers display a high mitochondrial content and high basal expression of PGC-1α **[Schiaffino and Reggiani 2011, Yan et al. 2011, Wilson et al. 2012]**. Also type IIa fibers have a high mitochondrial content, however, these fibers are characterized as fast-twitch due to their contractile properties. Type IIx and IIb fibers are considered to be glycolytic fast-twitch fibers, with a lower expression of PGC-1α, and a fatigue-prone phenotype compared to type I and IIa fibers **[Lira et al. 2010, Schiaffino and Reggiani 2011]**. One debated aspect of skeletal muscle plasticity is the concept of true fiber-type switching **[Booth et al. 2010, Wilson et al. 2012]**, which is the transformation of type II fibers into type I fibers in response

to exercise or other stimuli. However, irrespective of any changes in MyHC composition, it is well established that skeletal muscle can attune its oxidative and functional capacity in response to different exercise modalities. Endurance exercise is characterized by high-repetition, low-resistance muscle contractions, while resistance exercise denotes a low-repetition, high-resistance load on the muscle **[Egan and Zierath 2013]**. Resistance exercise elicits a shift from type IIb to IIa fibers, and results in a hypertrophic response characterized by increased protein synthesis rates, increased muscle fiber cross-sectional area and enhanced force generation **[Booth and Thomason 1991, Fry 2004]**. Endurance exercise on the other hand leads to a higher proportion of type IIa fibers in the muscle bed compared to type IIX/IIb fibers **[Schiaffino and Reggiani 2011]**, and increases mitochondrial content, neovascularization and oxidative metabolism in the muscle without eliciting any significant hypertrophic effect on the muscle **[van Wessel et al. 2010]**. It is well known that repeated bouts of endurance exercise provokes an adaptive shift in skeletal muscle physiology towards a more oxidative, slow-twitch phenotype. These exercise adaptation includes angiogenesis, mitochondrial biogenesis, improved lactate handling and a shift in substrate utilization, leading to a higher reliance of fat oxidation during submaximal exercise **[Holloszy and Coyle 1984, Hawley 2002]**. Importantly, exercise training leads to increased PGC-1α expression and activity in skeletal muscle **[Pérez-Schindler and Handschin 2013]**, and this is an important factor for the adaptive response of skeletal muscle to exercise.

The versatile role of PGC-1α in skeletal muscle

Perturbation of skeletal muscle homeostasis during a muscle contractions induces a range of mechanical, neurological, metabolic and endocrine signals, which elicit an adaptive response to exercise within the muscle, but also in other organs. Importantly, many of these signaling pathways converge on PGC-1α (Figure 1A-B), making this coactivator an important transcriptional regulator of skeletal muscle adaptation **[Svensson and Handschin 2014]**. Induction of PGC-1α in skeletal muscle in response to exercise is postulated to depend on both exercise intensity and modality. It was recently shown that resistance exercise leads to an induction of the truncated PGC-1α-isoform, PGC-1α4 in human skeletal muscle. In addition, overexpression of PGC-1α4 in skeletal muscle elicits a hypertrophic response in transgenic mice **[Ruas et al. 2012]**. Contrariwise, full length PGC-1α does not influence the hypertrophic response in skeletal muscle **[Perez-Schindler et al. 2013]**. However, a recent study by Ydfors et al. could demonstrate that both PGC-1α4 and full length PGC-1α were induced in human skeletal muscle in response to an acute bout of either endurance or resistance exercise **[Ydfors et al. 2013]**. Hence, more research is needed to elucidate the exact contribution of the various PGC-1α isoforms and splice variants

to skeletal muscle adaptation during different exercise modalities. Both resistance and endurance exercise elicits strong transcriptional responses in human skeletal muscle **[Timmons et al. 2005, Stepto et al. 2009, Catoire et al. 2012]**. Global expression profiling furthermore reveal an induction of gene programs coding for mitochondrial function and fat oxidation specifically in response to endurance exercise training **[Stepto et al. 2009]**. Importantly, both of these genetic programs are known to be activated by PGC-1α in skeletal muscle. PGC-1α have been shown to be involved in the regulation of several gene programs involved in metabolic as well as functional adaptation of skeletal muscle. Overexpression of PGC-1α specifically in skeletal muscle leads to a shift towards an oxidative fiber type **[Lin et al. 2002]** and improved skeletal muscle refueling (Figure 1A) **[Wende et al. 2007, Summermatter et al. 2010]**. Moreover PGC-1α have been shown to regulate several processes important for exercise adaptation in skeletal muscle, such as angiogenesis **[Arany et al. 2008]**, fatty acid transport and β-oxidation **[Gerhart-Hines et al. 2007, Summermatter et al. 2011]**, mitochondrial biogenesis and function **[Lin et al. 2002, Handschin et al. 2007b]**, autophagy **[Lira et al. 2013]**, calcium signaling **[Summermatter et al. 2012]**, lactate metabolism **[Summermatter et al. 2013a]**, neuromuscular junction reprogramming **[Handschin et al. 2007c, Arnold et al. 2014]**, and myokines secretion **[Bostrom et al. 2012, Rao et al. 2014]**. Activation of PGC-1α in the context of exercise is thus important for the transcriptional activation of several gene programs necessary for adaptation of skeletal muscle to endurance exercise training. Hence, it is important to understand how PGC-1α is activated in skeletal muscle in response to exercise.

PGC-1α and exercise adaptation

PGC-1α transcription in skeletal muscle is activated through similar signaling pathways responsible for PGC-1α-activation in other metabolic organs (as discussed in chapter 1). One important signaling pathway responsible for induction of PGC-1α during muscle contractions is p38 MAPK signaling. Specifically, the p38γ MAPK isoform was shown to be important for the increased PGC-1α transcription and mitochondrial function in response to exercise **[Pogozelski et al. 2009]**. Constitutive activation of p38 MAPK in skeletal muscle is associated with enhanced PGC-1α and mitochondrial protein levels, and ATF2 was shown to be an important mediator of this effect **[Akimoto et al. 2005]**. P38 MAPK phosphorylates and activates ATF2 and MEF2 in response to exercise, thereby increasing PGC-1α transcription **[Zhao et al. 1999, McGee and Hargreaves 2004, Akimoto et al. 2005]**. Importantly, enhanced p38 MAPK activity in response to exercise is dependent on the generation of reactive oxygen species (ROS), since pharmacological inhibition of xanthine oxidase in rats blocked the activation of p38 MAPK in response to exercise **[Gomez-Cabrera et al. 2005]**. In line with this, antioxidant treatment in mice and humans was

sufficient to block induction of PGC-1α in skeletal muscle during exercise **[Ristow et al. 2009, Meier et al. 2013]**, and ROS-treatment in cultured myotubes led to an increased transcription of PGC-1α **[Silveira et al. 2006]**. ROS generation furthermore activates the TF; hypoxia-inducible factor 1 alpha (HIF-1α), which is also activated in response to reduced intracellular oxygen levels **[Shoag and Arany 2010]**. Once activated, HIF-1α binds to hypoxia response elements in the nucleus and regulates transcription of gene programs involved in angiogenesis and glycolysis. **[Egan and Zierath 2013]**. However, also PGC-1α regulates angiogenesis during exercise, and this was shown to occur through a HIF-1α-independent, ERRα-dependent mechanism **[Arany et al. 2008]**. PGC-1α was also recently shown to regulate transcription of hypoxic response genes in muscle cells through the TF activator protein 1 (AP1) **[Baresic et al. 2014]**.

During muscle contractions, oscillations in intracellular calcium levels are essential for myofibrillar contraction. Calcium also activates PGC-1α transcription through activation of calcium/calmodulin-dependent protein kinases (CaMK) and calcineurin A (CnA) signaling. CnA activation enhances PGC-1α -transcription through dephosphorylation and activation of MEF2 **[Handschin et al. 2003]**, and

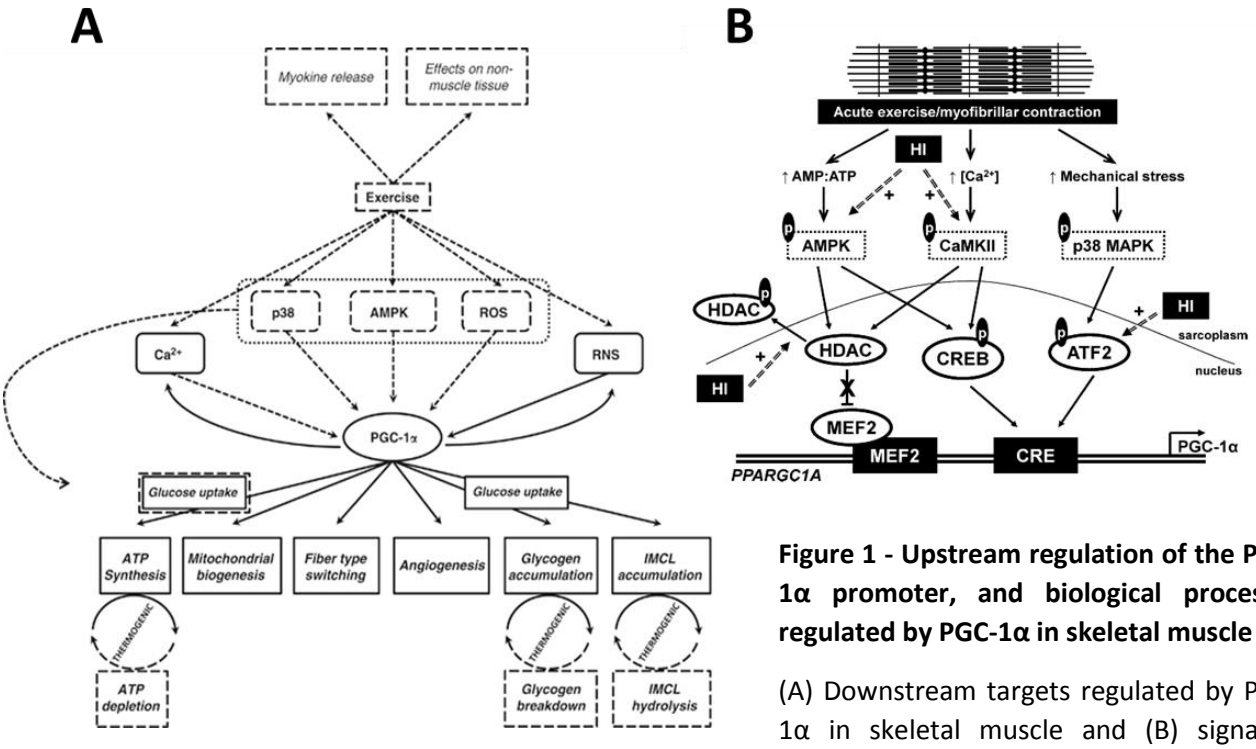

Figure 1 - Upstream regulation of the PGC-1α promoter, and biological processes regulated by PGC-1α in skeletal muscle

(A) Downstream targets regulated by PGC-1α in skeletal muscle and (B) signaling mechanisms responsible for activation of PGC-1α transcription in response to exercise. Adapted from (A) **[Summermatter and Handschin 2012]** and (B) **[Egan et al. 2010]**

overexpression of CnA in skeletal muscle is associated with increased PGC-1α transcription **[Ryder et al. 2003]**. Also overexpression of CaMKIV in skeletal muscle leads to an enhanced transcription of PGC-1α and mitochondrial genes **[Wu et al. 2002]**. However, neither inhibition of CnA activity nor knockout of CaMKIV influenced the induction of PGC-1α or mitochondrial proteins in response to exercise **[Akimoto et al. 2004, Garcia-Roves et al. 2006]**, indicating that these proteins are not essential for exercise-induced PGC-1α transcription. CaMKII is the predominant CaMK-isoform in skeletal muscle, and during exercise it is activated in an intensity-dependent manner **[Egan and Zierath 2013]**. CaMKII regulates PGC-1α transcription through modulation of transcriptional regulators such as HDACs, CREB and MEF2 **[Egan et al. 2010]**. In resting muscle, MEF2 transcriptional activity is repressed through interaction with HDACs. However, upon induction of CaMKII activation, this kinase phosphorylates HDAC4 which sequesters it in the cytoplasm, leading to transcriptional activation of the PGC-1α promoter by MEF2 (Figure 1B) **[Liu et al. 2005, Egan et al. 2010]**. Moreover, CaMKs phosphorylate and activate CREB, which enhances PGC-1α transcription by binding to CRE-elements in the PGC-1α promoter (Figure 1B) **[Sun et al. 1994, Handschin et al. 2003, Egan et al. 2010]**. Increased calcium signaling also activates ATF2-mediated transcription of PGC-1α in muscle cells through a CaMKII- and p38 MAPK-dependent mechanism **[Wright et al. 2007]**. This indicates a potential cross-talk between CaMK- and p38 MAPK-signaling in the activation of PGC-1α transcription in response to exercise.

As mentioned earlier, exercise induces prominent changes in skeletal muscle metabolism, resulting in alteration in intracellular redox status and ATP/ADP ratio. These alteration lead to activation of energy stress sensor such as AMPK and SIRT1 in skeletal muscle. AMPK is activated in skeletal muscle in response to exercise **[Wojtaszewski et al. 2000]** and has been shown to influence PGC-1α activity through several mechanisms. AMPK phosphorylates HDAC5 and CREB, thereby resulting in increased transcription of PGC-1α (Figure 1B) **[Thomson et al. 2008, McGee et al. 2009]**. Moreover, AMPK directly phosphorylates PGC-1α and increases its transcriptional activity **[Jager et al. 2007]**. In line with this, muscle-specific gain-of-function mutations of AMPK or treatment with the pharmacological AMPK-activator; 5-aminoimidazole-4-carboxamide-1-beta-D-ribofuranoside (AICAR) leads to enhanced PGC-1α expression and increased mitochondrial biogenesis in skeletal muscle **[Winder et al. 2000, Garcia-Roves et al. 2008, Narkar et al. 2008]**. However, knockout of the AMPK subunits α1 or α2 did not prevent the induction of PGC-1α transcription in response to endurance exercise **[Jorgensen et al. 2005]**. Muscle specific ablation of the upstream AMPK-kinase; liver kinase B1 (LKB1) leads to reduced AMPK-activation and consequently reduced PGC-1α expression in skeletal muscle **[Koh et al. 2006]**. Interestingly, muscle-

specific LKB1-knockout mice were not able to induce PGC-1α expression in response to an acute exercise bout **[Tanner et al. 2013]**. AMPK is furthermore important for the deacetylation of PGC-1α in response to exercise, since AMPK was shown to regulate the activity of the NAD^+-sensitive protein deacetylase SIRT1 through modulation of the intracellular NAD^+/NADH ratio (Figure 2) **[Canto et al. 2009, Canto et al. 2010]**. Exercise modulates the intracellular redox status and leads to a shift in the NAD^+/NADH ratio in muscle fibers **[White and Schenk 2012]**. NAD^+ levels were shown to peak 3 hours post-exercise in mice, and coincide with a strong deacetylation of PGC-1α **[Canto et al. 2009]**. This has been postulated to be mediated by SIRT1, since PGC-1α is a known deacetylation target of SIRT1 **[Rodgers et al. 2005]**. However, a recent study could demonstrate enhanced deacetylation of PGC-1α in response to exercise, even in the absence of SIRT1 deacetylase activity **[Philp et al. 2011]**. Interestingly, the authors could detect a decreased interaction between PGC-1α and the acetyltransferase GCN5 in response to exercise **[Philp et al. 2011]**, and this could potentially explain the altered PGC-1α acetylation status, since PGC-1α is a known acetylation-target for GCN5 **[Lerin et al. 2006]**. There are however questions being raised regarding the connection between SIRT1 and increased PGC-1α activity and mitochondrial function *in vivo* **[Gurd et al. 2012, White and Schenk 2012]**. For example, mice with a muscle-specific overexpression of SIRT1 displayed slightly reduced levels of PGC-1α in muscle **[Gurd et al. 2009]**. It was also recently shown that overexpression of SIRT1 in C2C12 myotubes led to a decreased PGC-1α activity and reduced mitochondrial protein levels **[Higashida et al. 2013]**. Hence, further research is needed to determine the exact

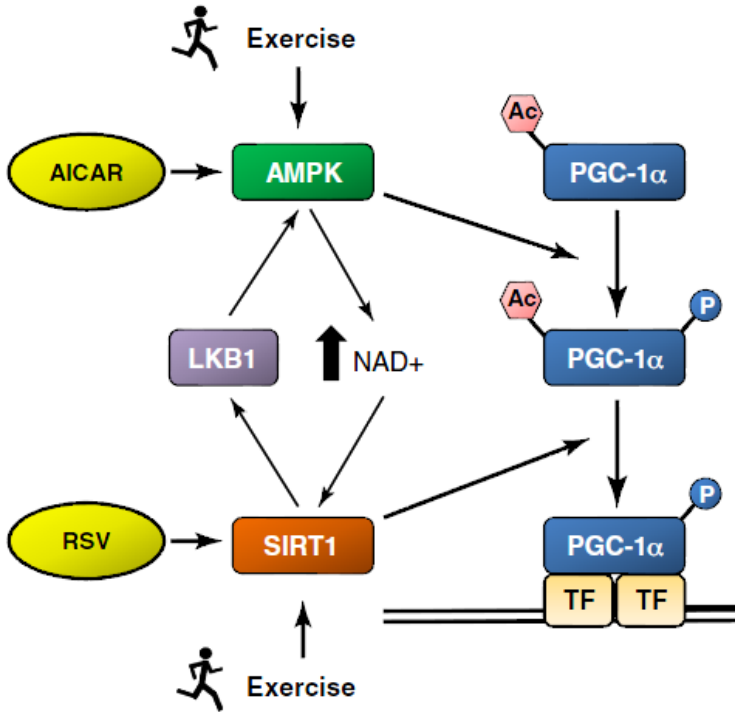

Figure 2 - Reciprocal regulation of AMPK- and SIRT1-activity, and subsequent activation of PGC-1α

Sequential activation of PGC-1α through concurrent deacetylation and phosphorylation of the PGC-1α protein by SIRT1 and AMPK respectively. AMPK activates SIRT1 by increasing the intracellular NAD^+/NADH ratio. SIRT1 deacetylates the AMPK-kinase liver kinase B1 (LKB1), resulting in increased activation of AMPK.

Adapted from: **[Svensson and Handschin 2014]**

mechanism behind PGC-1α deacetylation in response to exercise, and to determine the role of SIRT1 in this process.

Several exercise induced signals, both mechanical and metabolic, converge on PGC-1α to regulate its activity in skeletal muscle. Moreover, PGC-1α is important for the regulation of several processes (*vide supra*) necessary for the adaptation to exercise in skeletal muscle. A pertinent question is thus whether PGC-1α is necessary for the adaptation of skeletal muscle in response to endurance exercise. Muscle-specific PGC-1α knockout mice display an exercise intolerant phenotype **[Handschin et al. 2007a]**, which is exacerbated when PGC-1β is concomitantly knocked-out **[Zechner et al. 2010]**. Moreover, PGC-1α was shown to be necessary for the increased angiogenesis and mitochondrial biogenesis in response to exercise **[Chinsomboon et al. 2009, Geng et al. 2010]**. Conversely, in a recent study utilizing muscle-specific PGC-1α knockout animals, Rowe et al. could show that in response to 2 weeks of endurance exercise, transcription of mitochondrial proteins as well as total mitochondrial density were enhanced even in the absence of PGC-1α **[Rowe et al. 2012]**. PGC-1α was also shown to be dispensable for the type IIb-to-IIa fiber type transformation in response to exercise **[Geng et al. 2010]**. Interestingly, also in the PGC-1α/β double-knockout model published by Zechner et al. the authors claim that neither PGC-1α nor PGC-1β are necessary for fiber type determination in skeletal muscle **[Zechner et al. 2010]**, while a type IIa-to-IIx/IIb fiber-type switch was demonstrated in the original muscle-specific PGC-1α knockout model published by Handschin et al. **[Handschin et al. 2007a]**. A recently published double-knockout model of PGC-1α/β by Rowe et al. **[Rowe et al. 2013]** could confirm the findings by Zechner et al, that ablation of both PGC-1-isoforms in muscle did not result in a fiber type switch. However, in stark contrast to the findings by Zechner et al, the PGC-1α/β double-knockout mice published by Rowe et al. display no significant changes in either exercise performance or in the absolute mitochondrial content in skeletal muscle. Both models however display a strong reduction in transcription of mitochondrial proteins and a reduced oxidative capacity in the absence of both PGC-1-isoforms **[Zechner et al. 2010, Rowe et al. 2013]**. Hence, more research is needed to elucidate whether PGC-1α is really necessary for the adaptation of skeletal muscle to exercise, and accordingly which specific processes are PGC-1α-dependent. Nonetheless, regardless of the role of PGC-1α in the physiological adaptation to exercise, overexpression of PGC-1α in skeletal muscle has been demonstrated to elicit a range of beneficial effects in rodent models of metabolic and muscle-related disorders **[Svensson and Handschin 2014]**. Hence, activation of PGC-1α in skeletal muscle could be a promising therapeutic strategy to ameliorate metabolic and muscle-related disorders.

6.2 – PGC-1α activation and exercise mimetics

Obesity and diabetes in human patients is associated with mitochondrial impairments, glucose intolerance and exacerbated intramuscular lipid accumulation **[Roden 2005]**. Conversely, exercise is associated with increased mitochondrial biogenesis in skeletal muscle **[Holloszy 1967]**, improved glucose uptake **[Richter and Hargreaves 2013]** and enhanced capacity for fat oxidation **[Holloszy and Coyle 1984]**. Hence, increased physical activity is acknowledged as an important therapeutic strategy to prevent and treat metabolic dysfunction associated with obesity and diabetes in human patients **[Perseghin et al. 1996, Knowler et al. 2002, Strasser 2013]**. Physical inactivity is a widespread across the global population **[Hallal et al. 2012]** and a sedentary lifestyle is closely linked to a higher incidence of chronic diseases such as diabetes, cancer and neurodegenerative disorders **[Handschin and Spiegelman 2008, Booth et al. 2012]**. Physical inactivity in bed rest patients has been shown to induce a shift towards glycolytic type IIx fibers, to reduce the capacity for fat oxidation in muscle and to result in an increased intramuscular lipid content **[Bergouignan et al. 2011]**. While the cause for metabolic dysfunction in obese and diabetic patients is multifactorial, skeletal muscle plays an important role in the etiology of these diseases due to its central role in regulating systemic metabolic rate and glucose uptake. Obesity and diabetes is associated with impaired mitochondrial and metabolic function in skeletal muscle. This is illustrated by the reduced number of oxidative type I fibers **[Hickey et al. 1995]** in obese diabetic patients, and a reduced expression of PGC-1α-responsive genes in muscle **[Mootha et al. 2003]**. Moreover, reduced PGC-1α expression has been associated with increased incidence of type 2 diabetes and an increased BMI in diabetes-prone humans **[Benton et al. 2008]**. Intriguingly, diet-induced obesity in both mice and humans is associated with reduced levels of PGC-1α in skeletal muscle and concurrent mitochondrial dysfunction **[Sparks et al. 2005]**. Considering the many beneficial effects of PGC-1α on mitochondrial and metabolic processes in muscle, elevation of PGC-1α levels in skeletal muscle has been suggested as a potential therapeutic strategy to ameliorate metabolic dysfunction in obese and diabetic patients. However, overexpression of PGC-1α in skeletal muscle was shown to have a detrimental effect on insulin-stimulated glucose uptake in sedentary mice fed a HFD **[Choi et al. 2008]**. This was suggested to occur in response to an increased fatty acid uptake and *de novo* lipogenesis, leading to exacerbated intramyocellular lipid accumulation in these mice. While this phenotype could be ameliorated by concomitant exercise training **[Summermatter et al. 2013b]**, these findings indicate that activation of PGC-1α as a monotherapy in sedentary obese patients could lead to potentially detrimental effects. On the other hand, overexpression/activation of PGC-1α in skeletal muscle has been demonstrated to elicit several beneficial

effects in rodent models of various muscle-related pathologies such as cancer-associated cachexia, Duchenne muscular dystrophy, amyotrophic lateral sclerosis and disuse atrophy, to name a few **[Svensson and Handschin 2014]**. The positive effects of exercise or PGC-1α activation in metabolic and muscle-related disorders have sparked an interest in developing pharmacological compounds that activate signaling pathways normally induced in skeletal muscle during exercise. These compounds are postulated to ameliorate metabolic dysfunction in obese patients or to mimic the beneficial effects of exercise in patients suffering from muscle-related disorders. In line with the postulated use of these compounds, they have aptly been termed "exercise-mimetics" and are suggested work either as a monotherapy or as an adjuvant treatment in combination with exercise **[Fan et al. 2013]**. The term "exercise-mimetic" has emerged during the last decade **[Booth and Laye 2009]** and the discovery of a potential exercise-mimetic drug is by some considered to be the long-elusive goal of exercise science **[Narkar et al. 2008, Fan et al. 2013]**. However, the concept of mimicking exercise through the administration of a single drug has been extensively critiqued **[Goodyear 2008, Warden and Fuchs 2008, Booth and Laye 2009, Carey and Kingwell 2009, Hawley and Holloszy 2009]**. The systemic adaptations of physical exercise are polygenic and depend on integration of endocrine, neuronal, mechanical and metabolic stimuli. Moreover, exercise adaptation is the result of a concurrent activation of several distinct pathways during muscle contraction, which are unlikely to be mimicked by a monotherapeutic approach **[Booth and Laye 2009, Hawley and Holloszy 2009]**. The true therapeutic potential of exercise mimetic compounds probably lies somewhere in between these two extreme standpoints. While exercise mimetics might not be useful as a proxy for physical activity, these compounds could potentially be combined to elicit activation of complementary signaling pathways in muscle and act as adjuvant therapies to augment the beneficial effects of physical exercise.

Several studies have investigated the therapeutic potential of different pharmacological compounds on skeletal muscle metabolism and function, as well as their impact on whole body metabolism. Amongst these we find the well-known SIRT1-activating compounds RSV and SRT1720. However, also other compounds known to modulate the activity of PPARβ/δ, ERRβ/γ, AMPK and REV-ERBα/β have been suggested as potential exercise mimetic compounds **[Fan et al. 2013]**, and in this paragraph we will take a closer look at these postulated exercise mimetics. PPARβ/δ is highly expressed in skeletal muscle, and has been shown to be able to directly regulate PGC-1α expression **[Schuler et al. 2006, Hondares et al. 2007]**. PGC-1α in turn has been demonstrated to act as a coactivator of PPARβ/δ in muscle cells **[Dressel et al. 2003]**. In line with this, mice with a muscle-specific overexpression of

PPARβ/δ display a similar phenotype to PGC-1α transgenic mice, mainly characterized by an enhanced oxidative capacity in skeletal muscle **[Lin et al. 2002, Wang et al. 2004]**. Several aspects of the phenotype of PPARβ/δ muscle-transgenic mice was also mimicked through treatment of mice with the isoform-selective PPARβ/δ agonist GW501516 **[Wang et al. 2004]**. These findings were expanded upon in a study by Narkar et al. from 2008, were the authors demonstrated that GW501516 treatment enhanced exercise performance and augmented exercise-induced mitochondrial biogenesis in skeletal muscle **[Narkar et al. 2008]**. Intriguingly, the authors showed that concurrent administration of GW501516 and the AMPK-agonist AICAR led to synergistic effects on skeletal muscle transcription that partially overlapped with the transcriptional profile of GW501516 in combination with exercise **[Narkar et al. 2008]**. Hence, these data illustrate the potential benefits of using PPARβ/δ agonists in combination with other pharmacological activators, or as an adjuvant therapy together with physical activity. Interestingly, Narkar et al. also showed that administration of AICAR was sufficient to enhance exercise performance in mice and to induce PGC-1α expression in myotubes in a PPARβ/δ-dependent fashion **[Narkar et al. 2008]**. Additionally, GW501516 treatment has been show to increase fatty acid oxidation in human skeletal muscle cells **[Kramer et al. 2007]** and to enhance whole body fat oxidation in human subjects **[Riserus et al. 2008]**. These findings indicate that GW501516 could have a therapeutic potential in obese humans. Other potential exercise mimetic compounds that have been suggested are the ERRβ/γ-agonist GSK4716 and the REV-ERBα/β agonists SR9009 and SR9011 **[Fan et al. 2013]**. Treatment with GSK4716 led to an induction of PGC-1α/β and all ERR isoforms in primary mouse myotubes, together with an increase of several mitochondrial genes **[Rangwala et al. 2010]**. However, further studies are needed to evaluate the efficiency of this agonist in an *in vivo* setting. On the other hand, treatment of mice with the REV-ERBα/β agonist SR9009 has been shown to enhance exercise performance in mice and to increase mitochondrial biogenesis in C2C12 cells **[Woldt et al. 2013]**. Both GSK4716 and SR9009/9011 elicit beneficial effects in skeletal muscle, however, further research is needed to evaluate their efficiency as potential exercise mimetic compounds in rodents, and more importantly, in human subjects. The efficiency of a compound to elicit beneficial effects in obese subjects is not only connected to the effects of these compounds in skeletal muscle, but their effects on all major metabolic organs in the body. Two compounds which have demonstrated pleiotropic beneficial metabolic effects in obese rodents are the calorie restriction (CR)/exercise mimetics RSV and SRT1720.

Resveratrol and SRT1720

RSV was initially identified in the 1940s as a plant polyphenol in the roots of white hellebore **[Baur and Sinclair 2006]**. However, not until a connection was made between the RSV content in red wine, and the lipid lowering effects of red wine consumption in 1992 **[Siemann and Creasy 1992]** did the interest in the therapeutic potential of RSV take off (Figure 3A). Over the years, many studies have investigated the therapeutic potential of RSV, however due to the pleiotropic nature of this compound, several open questions still remain regarding its mode of action. Initially, this compound was shown to have beneficial effects for a range of disease states such as cancer as well as cardiovascular and inflammatory disorders **[Baur and Sinclair 2006]**. The mechanisms responsible for the pleiotropic effects of RSV are debated. Intriguingly, RSV has been shown to be an inhibitor of several enzymes such as phosphoinositide 3-kinase (PI3K) **[Frojdo et al. 2007]**, cyclo- and lipooxygenase (COX, LOX), mitogen-activated protein kinases (MAPK) **[Pirola and Frojdo 2008]** and phosphodiesterases (PDE) **[Park et al. 2012]**. The best studied aspect of RSV function is however its ability to activate the enzymes SIRT1 **[Howitz et al. 2003]** and AMPK **[Pirola and Frojdo 2008]**. Whether RSV directly activates SIRT1 is debated. Several studies have postulated that SIRT1-activation by RSV is an *in vitro* artefact **[Kaeberlein et al. 2005, Beher et al. 2009, Pacholec et al. 2010]** while a more recent study could demonstrate a direct allosteric activation of SIRT1 by RSV **[Hubbard et al. 2013]**. Nonetheless, RSV undoubtedly displays pleiotropic effects *in vivo*, and has been shown to act as a PDE-inhibitor, thereby increasing AMPK and SIRT1 activation downstream of cyclic AMP (cAMP) and exchange protein directly activated by cAMP 1 (epac1) signaling **[Park et al. 2012]**.

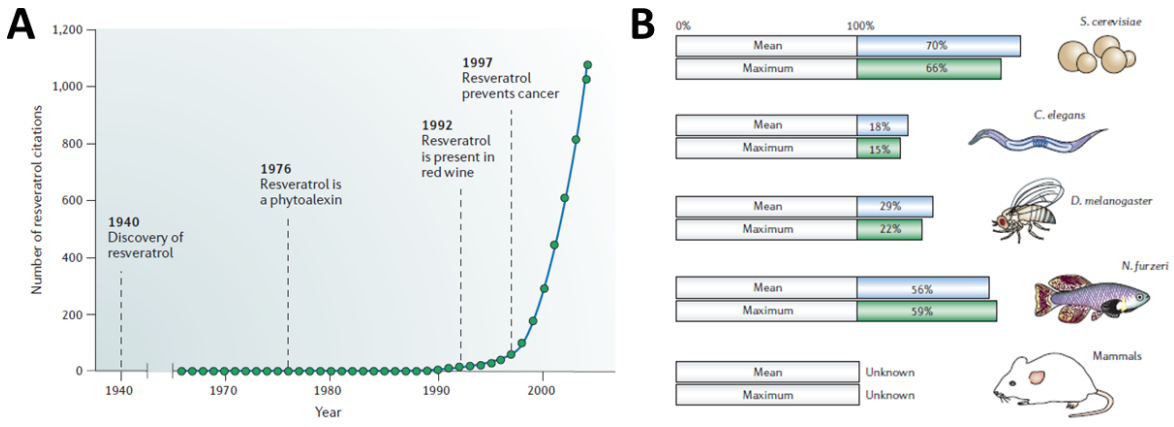

Figure 3 – Timeline of research related to resveratrol / Effects of resveratrol administration on lifespan of model organisms.

(A) Timeline of resveratrol-related research as a function of number of resveratrol citations over time (1940 – 2006). (B) Mean and maximum lifespan extension with resveratrol-treatment in *S.cerevisae*, *C.elegans*, *D.melanogaster*, *N.furzeri* and in mammals. Adapted from: **[Baur and Sinclair 2006]**.

Moreover, RSV can also act as an inhibitor of mitochondrial respiration **[Zini et al. 1999]**, which has been demonstrated to lead to reduced ATP-production and consequently AMPK activation in skeletal muscle cells **[Price et al. 2012]**. Interestingly, RSV failed to protect AMPK-null mice against HFD-induced obesity **[Um et al. 2010]**, which implies a role for AMPK in mediating the effects of RSV. RSV was furthermore shown to increase the NAD^+-to-NADH ratio in an AMPK-dependent manner **[Um et al. 2010]** and SIRT1 has been demonstrated to deacetylate the AMPK-kinase liver kinase B1 (LKB1) **[Lan et al. 2008]**, thereby forming a reciprocal link between AMPK- and SIRT1 activation (Figure 2).

The role of RSV as a SIRT1 activator was initially shown in yeast, and was connected to an extension of the life-span of *S.cerevisae* **[Howitz et al. 2003]**. The effect of RSV on organismal life-span was later demonstrated also in *C.elegans* **[Wood et al. 2004]**, *D.melanogaster* **[Bauer et al. 2004]** and *N.furzeri* **[Valenzano et al. 2006]** (Figure 3B). However, this extension of life-span could not be replicated in other studies using a similar setup **[Kaeberlein et al. 2005, Bass et al. 2007]** and has not been demonstrated in mammalian models **[Mercken et al. 2012]**. Nonetheless, RSV mimics the effects of CR at least on the transcriptional level in several organs **[Barger et al. 2008, Pearson et al. 2008]** and was shown to extend life-span of mice fed a HFD **[Pearson et al. 2008]**. Treatment with RSV is associated with several beneficial effects in obese mice such as an improved glucose tolerance, enhance energy expenditure, increased weight loss and ameliorated dyslipidemia **[Baur et al. 2006, Lagouge et al. 2006, Milne et al. 2007, Um et al. 2010]**, which could explain the enhanced life-span in these mice **[Pearson et al. 2008]**. While the beneficial effects of RSV treatment in obese mice are well established, clinical trials have demonstrated potential caveats of using RSV as a monodrug therapy in humans **[Tome-Carneiro et al. 2013]**. This includes a low bioavailability of RSV in humans and potentially harmful synergism with our drugs due to inhibition of hepatic drug-metabolism by RSV **[Smoliga et al. 2011, Tome-Carneiro et al. 2013]**. Moreover, in a recent clinical trial in patients with multiple myeloma, several study participants developed nephrotoxicity in response to SRT501 (formulated RSV) treatment **[Popat et al. 2013]**. Despite this, beneficial effects of RSV treatment have been demonstrated in clinical studies, such as a reduced metabolic rate, decreased inflammatory markers and reduced hepatic steatosis in obese men **[Timmers et al. 2011]**. These effect could however not be replicated in a related clinical study, using a longer treatment time and higher dose of RSV **[Poulsen et al. 2013]**. Intriguingly, RSV administration in obese humans leads to alteration in the transcriptional profile of both skeletal muscle and adipose tissue **[Timmers et al. 2011, Konings et al. 2014]**. Especially in skeletal muscle, RSV treatment was associated with increased PGC-1α levels, and an enhanced transcription of mitochondrial genes **[Timmers et al.**

2011], mirroring the effects of RSV treatment in mice **[Lagouge et al. 2006]**. The activation of PGC-1α in skeletal muscle and the concomitant increase in mitochondrial biogenesis with RSV treatment has led to the hypothesis that PGC-1α is a major downstream mediator of the effects of RSV. This has however not been conclusively evaluated. Considering the concurrent activation of AMPK, SIRT1 and PGC-1α in skeletal muscle during RSV treatment **[Lagouge et al. 2006, Timmers et al. 2011]**, this compound has been considered as a potential exercise mimetic drug. In keeping with this, several recent studies have focused on the potential additive effects of RSV administration and physical exercise in both mice and humans. In rodents, RSV administration enhanced endurance capacity and muscle force production **[Lagouge et al. 2006, Murase et al. 2009, Um et al. 2010, Dolinsky et al. 2012, Wu et al. 2013]** and acted synergistically with exercise training to enhance skeletal muscle cytochrome c oxidase protein content and enzymatic activity **[Menzies et al. 2013]**. Two recent studies from the group of Henriette Pilegaard focus on the potential synergism between RSV administration and lifelong exercise training on aging-related parameters. The authors could however detect no additive or synergistic effects of RSV supplementation on exercise-mediated mitochondrial biogenesis or reduction in systemic inflammatory parameters **[Olesen et al. 2013, Ringholm et al. 2013]**. In human studies, RSV administration is not associated with any improvement in exercise performance **[Voduc et al. 2014]**. Interestingly, a recent study by Gliemann et al. showed that concomitant RSV administration actually blunted oxygen uptake during exercise in aged men **[Gliemann et al. 2013]**. The interpretations of these data have however caused controversy and debate within the scientific community **[Smoliga and Blanchard 2013, Buford and Anton 2014, Hartmann and Forbes 2014]**. However, the discrepancy between the effects of RSV in rodents and humans indicate that further studies are needed to elucidate the potential role of RSV as an adjuvant therapy to endurance exercise training.

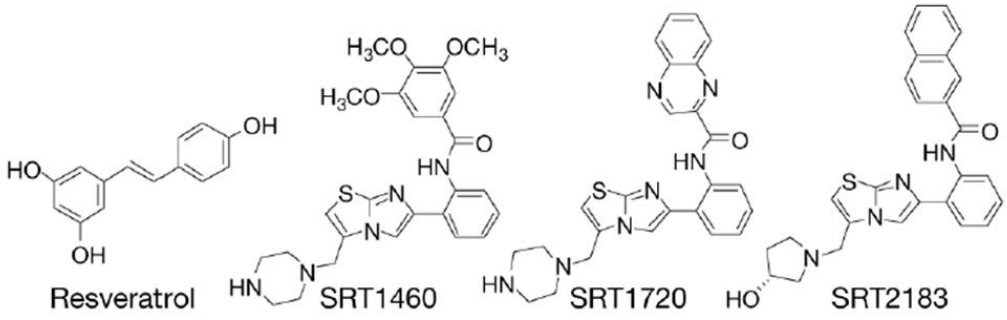

Figure 4 – SIRT1-activators

Chemical structures of resveratrol and the novel SIRT1-activators SRT1460, SRT1720 and SRT2183. Adapted from: **[Milne et al. 2007]**.

In order to develop more potent SIRT1-activating compounds than RSV, Milne et al. identified and characterized several SIRT1-activating compounds, structurally distinct from RSV, namely SRT1460, SRT1720 and SRT2183 (Figure 4). Out of these compounds, SRT1720 was shown to possess the strongest ability to activate SIRT1, approximately a 1000-fold more potent than RSV in an *in vitro* assay **[Milne et al. 2007]**. SRT1720 was further demonstrated to improve glucose tolerance, enhance exercise performance and reduce hepatic steatosis in obese rodent models **[Milne et al. 2007, Feige et al. 2008, Minor et al. 2011]**. In line with the beneficial effects of SRT1720 on systemic metabolism, this compound was also shown to increase the lifespan of both HFD-fed and chow-fed mice **[Minor et al. 2011, Mitchell et al. 2014]**. In *C.elegans* however, the lifespan-extending effect of RSV could not be replicated by SRT1720-administration **[Zarse et al. 2010]**, indicating a difference in the effects elicited by these compounds. Similar to RSV, the ability of SRT1720 to activate SIRT1 *in vivo* has been questioned **[Pacholec et al. 2010]**. Moreover, the ability of this compound to elicit deacetylation of the SIRT1-substrate p53 have been attributed to the ability of SRT1720 to inhibit p300 histone acetyltransferase activity, rather than any direct effect on SIRT1 **[Huber et al. 2010]**. SRT1720 treatment has however been shown to induced AMPK activity and lead to deacetylation of SIRT1-substrates such as p53 and PGC-1α *in vivo* **[Feige et al. 2008]**. Nevertheless, little is known regarding which would be the direct target(s) of this compound *in vivo*. Similar to RSV, it is important for the future use of these compounds in a clinical setting to elucidate which are the main targets of these compounds *in vivo*, and which of these targets are responsible for eliciting the beneficial effects of RSV and SRT1720. In this context, PGC-1α is postulated to be an important downstream mediator of the effects of both RSV and SRT1720, in particular since increased PGC-1α transcription and activity have been detected in obese mice treated with RSV or SRT1720 and this was associated with an enhanced transcription of mitochondrial genes in skeletal muscle **[Lagouge et al. 2006, Feige et al. 2008]**.

6.3 - Ketone body metabolism

In order to fully understand the complexity of skeletal muscle function and metabolism, it is essential to understand how fuel metabolism is regulated in this organ, both in the context of exercise, but also in the transition from a fed to a fasted state. During prolonged starvation, the body has developed an intricate system to cope with a reduced nutrient availability, while maintaining normal function of vital organs such as brain, kidney, heart and skeletal muscle. During prolonged starvation, the body readily mobilizes its

endogenous energy stores such as glycogen, triacylglycerols and eventually, muscle proteins to maintain energy homeostasis. The liver is an important organ in the adaptation to a fasted state, since this organ maintains blood glucose levels during fasting, initially through glycogenolysis and subsequently through gluconeogenesis using either glycerol, lactate, pyruvate, TCA-cycle intermediates or gluconeogenic amino acids as substrate (Figure 5A) **[Nuttall et al. 2008]**.

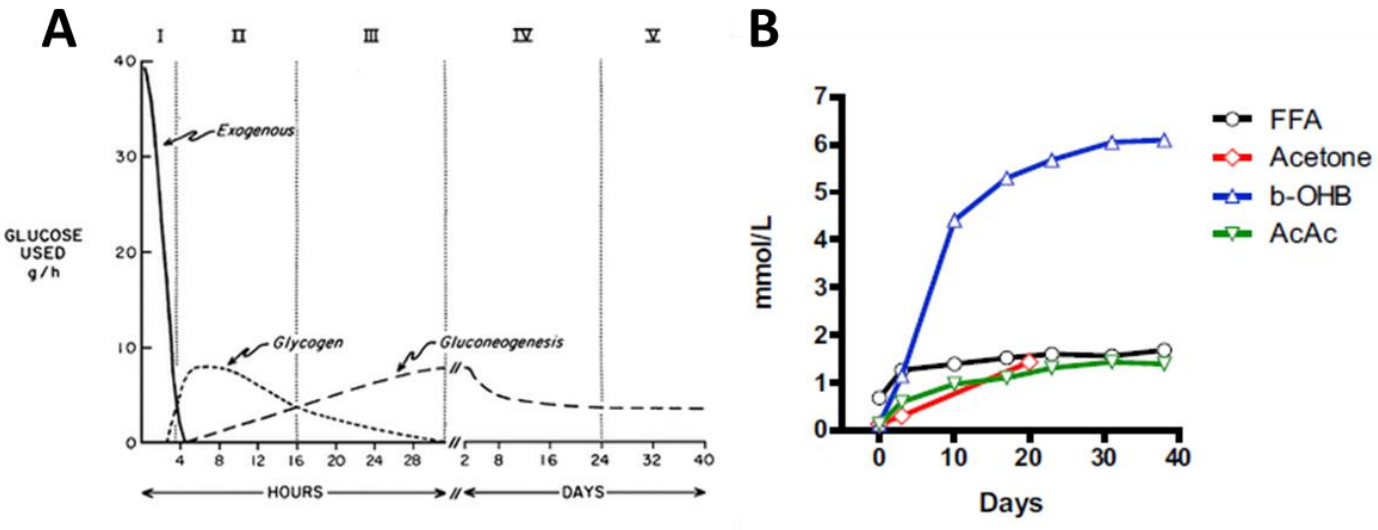

Figure 5 – Alterations of glucose, fatty acid and ketone body levels with prolonged starvation

(A) Glucose utilization is reduced in humans during prolonged starvation. Glucose originates mainly from gluconeogenesis after 24 hours starvation. Adapted from: **[Cahill 2006]**. (B) Concentrations of free fatty acids (FFA), acetone, beta-Hydroxybutyrate (b-OHB) and acetoacetate (AcAc) in blood of humans fasted for 40 days. Adapted from: **[Longo and Mattson 2014]**.

During continuous starvation, lipolysis in white adipose tissue depots is increased as a result of reduced circulating insulin levels. Fatty acids are used as a metabolic fuel in organs such as heart and skeletal muscle, but they are also efficiently oxidized within the liver. However, while the TCA-cycle intermediate oxaloacetate is diverted into hepatic gluconeogenesis, TCA-cycle activity is reduced, and acetyl-CoA accumulates in hepatocytes. At this point, acetyl-CoA is shunted into hepatic ketone body production and contributes to the production of ketone bodies, which are released into the bloodstream and utilized as alternative energy substrates in extra-hepatic organs **[Hashim and Vanitallie 2014]**. Accordingly, as glucose and insulin levels diminish during long-term fasting, concentrations of ketone bodies, beta-hydroxybutyrate (βOHB), acetoacetate (AcAc) and acetone rise in the circulation, resulting

in a state of ketosis (Figure 5B). Ketone body production, termed ketogenesis, is driven by increased delivery of fatty acids to liver during fasting, coupled to an enhanced gluconeogenesis. Moreover, hepatic ketogenesis is regulated by hormonal mechanisms as well as the intracellular redox status. Ketogenesis is catalyzed by three distinct enzymes, namely 3-hydroxy-3-methylglutaryl-CoA synthase 2 (HMGCS2), 3-hydroxymethyl-3-methylglutaryl-CoA lyase (HMGCL) and 3-hydroxybutyrate dehydrogenase, type 1 (BDH1) (Figure 6). HMGCS2 is a rate-limiting enzyme for hepatic ketogenesis, and its expression is regulated by two important nutrient sensing pathways. Insulin signaling inhibits HMGCS2-transcription through inhibition of forkhead box protein A2 (FOXA2) **[Wolfrum et al. 2003]**, while glucagon on the other hand increases FOXA2 transcriptional activity via acetylation by p300 **[von Meyenn et al. 2013]**. Moreover, mammalian target of rapamycin complex 1 (mTORC1) represses ketogenesis by increasing the activity of NCoR1 as a transcriptional repressor towards PPARα, thereby reducing hepatic ketogenesis **[Sengupta et al. 2010]**. HMGCS2 activity is furthermore regulated through direct acetylation as well as succinylation. BDH1 activity is also known to be regulated through the intracellular redox status (NAD^+/NADH-ratio) (Figure 6) **[Newman and Verdin 2014]**.

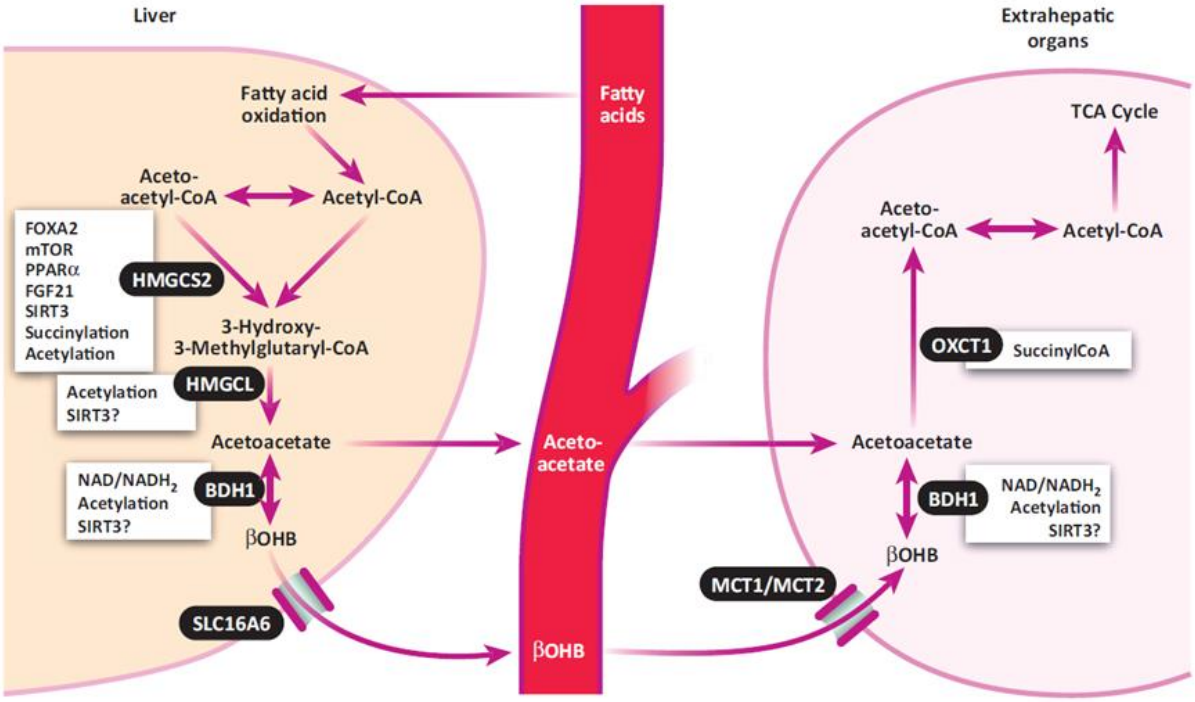

Figure 6 – Outline of ketone body metabolism.

Ketone bodies (acetoacetate, βOHB) are exclusively produced in the liver from an acetyl-CoA backbone, through the enzymes HMGCS2, HMGCL and BDH1. In extra-hepatic organs, ketone bodies are taken up through ketone body transporters MCT1/MCT2 and oxidized to acetyl-CoA, which subsequently enters the TCA-cycle to generate ATP. Adapted from: **[Newman and Verdin 2014]**

While the regulation of ketone body production in liver is well studied, the control of ketone body oxidation in target organs is not yet fully understood. Ketone body oxidation occurs in all extra-hepatic organs in a concentration-dependent manner. Ketolysis is catalyzed by three important enzymes localized to the mitochondrial matrix, namely BDH1, 3-oxoacid CoA-transferase (OXCT1) and acetyl-CoA acetyltransferase 1 (ACAT1) (Figure 6) **[Newman and Verdin 2014]**. These enzymes oxidize βOHB and AcAc into acetyl-CoA, which subsequently enters the TCA-cycle for terminal oxidation. OXCT1 is the rate-limiting ketolytic enzyme and its expression is an important determinant of the ketolytic capacity of an organ. While OXCT1 is not expressed in liver, it is highly expressed in oxidative organs such as kidney, heart, brain and skeletal muscle. Global ablation of OXCT1 in mice is lethal within 48 hours after birth **[Cotter et al. 2011]**. Mice with either muscle-, neuron- or heart-specific deletion of OXCT1 are viable, but display a maladapted fasting response characterized by fasting-induced hyperketonemia **[Cotter et al. 2013]**. Despite the apparent importance of ketone body oxidation, relatively little is known about how ketone body oxidation is regulated. In diabetic rodents, skeletal muscle uptake of βOHB is impaired **[Ikeda et al. 1991, Okuda et al. 1991]** and OXCT1 and ACAT1 activity is furthermore reduced in heart of diabetic rats **[Grinblat et al. 1986, Turko et al. 2001]**. Contrariwise, endurance exercise training enhances the activity of ketolytic enzymes in skeletal muscle **[Winder et al. 1974, Askew et al. 1975]**. Hence, ketolytic capacity in skeletal muscle is influenced by both pathological and physiological signals. Enhanced ketolytic capacity in skeletal muscle is furthermore suggested to be responsible for the reduced post-exercise ketosis in exercise trained rodents and humans **[Johnson et al. 1969, Adams and Koeslag 1988]**. Heart, kidney and brain have a higher basal OXCT1-activity than skeletal muscle and normalized to organ weight, the heart has the highest ketolytic capacity in the body (20-fold higher than skeletal muscle) **[Fukao et al. 2004]**. However, due to the size of skeletal muscle, this organ still has a large impact on the systemic ketolytic capacity. In line with this, ablation of ketolytic capacity specifically in skeletal muscle leads to fasting-induced hyperketonemia **[Cotter et al. 2013]**, indicating that this organ constitutes a large part of the systemic ketolytic capacity. Intriguingly, administration of ketone bodies to a working heart has been shown to increase heart work efficiency by 25% (work/O_2 consumed) **[Kashiwaya et al. 1994]** and has been suggested to improve exercise performance of both rodents and humans **[Hashim and Vanitallie 2014]**. Hence, ketone bodies seem to have a positive effect on muscle function and energy metabolism.

Ketone body oxidation is an important process to maintain energy homeostasis in the brain during prolonged starvation, since this organ cannot efficiently utilize long-chain fatty acids as an energy substrate **[Schonfeld and Reiser 2013]**. Hence, during prolonged starvation in humans, ketone bodies

account for up to 60% of the energy consumed in brain tissue **[Cahill 2006]**. Interestingly, ketone bodies have also been suggested to have therapeutic effects in the context of neurological disorders, such as Alzheimer's disease, Parkinson's disease and epilepsy **[Paoli et al. 2013, Hashim and Vanitallie 2014]**. While the exact mechanism behind the beneficial effects of ketonemia in these disorders is still unknown, there are several hypotheses how this could be achieved. For instance, it has been suggested that while glucose uptake and oxidation are impaired in several neurological disorders, ketone body uptake is unaltered and inducing of a state of ketosis could thus be used to rescue the metabolic dysfunction associated with diseases such as Alzheimer's disease **[Hashim and Vanitallie 2014]**. On the contrary, several types of cancers have been shown to possess ketolytic deficiencies and a chronic state of ketosis could thus be used to induce metabolic deficiencies specifically in cancer cells **[Seyfried et al. 2014]**. Ketogenic diets have been used as anticonvulsant therapies since 1920 and is together with drug therapy considered an established method to reduce epileptic seizures. However, the exact mechanism behind these effects is still unknown **[Paoli et al. 2013]**. A prolonged state of ketosis has been suggested to induce mitochondrial biogenesis in the hypothalamus, thus enhancing ATP-production leading to stabilization of the resting membrane potential **[Bough et al. 2006]**. Intriguingly, enhanced mitochondrial protein levels have also been detected in BAT in response to ketogenic diet feeding **[Srivastava et al. 2013]**. A connection between chronic ketosis and improved mitochondrial function have also been demonstrated in the context of mitochondrial dysfunction. Ketogenic diet feeding was able to ameliorate mitochondrial protein levels and function in both heart and skeletal muscle of mice with mitochondrial myopathies **[Ahola-Erkkila et al. 2010, Krebs et al. 2011]**. This effect was associated with a normalization of PGC-1α levels in the heart, which was suggested to be a mechanism how a ketogenic diet could increase mitochondrial transcription **[Krebs et al. 2011, Benit and Rustin 2012]**. Nevertheless, the exact mechanism how a ketogenic diet affects PGC-1α transcription and mitochondrial function is still unknown. Intriguingly, a recent study by Shimazu et al. demonstrated that βOHB acts as an HDAC inhibitor *in vivo,* and was thus able to inhibit the activity of HDAC1, HDAC3 and HDAC4 **[Shimazu et al. 2013]**. Importantly, treatment of mice with HDAC1-specific inhibitors was also recently demonstrated to increase PGC-1α transcription and mitochondrial biogenesis in skeletal muscle and BAT **[Galmozzi et al. 2013]**. However, the role of βOHB as an HDAC inhibitor in skeletal muscle and BAT is so far not investigated and future studies are needed to elucidate whether this effect of βOHB could account for the increased mitochondrial biogenesis in response to ketogenic diet feeding. While the therapeutic role of a ketogenic diet in the context of diabetes, cancer and neurodegenerative disease is currently being explored **[Paoli et al. 2013, Hashim and Vanitallie 2014]**, little is known about how a chronic state of ketosis affects

skeletal muscle biology. Atrophic stimuli such as fasting, uremia, diabetes and cancer cachexia have been associated with reduced expression of the main ketolytic enzyme OXCT1 in rodent skeletal muscle **[Lecker et al. 2004]**. Intriguingly, patients suffering from Duchenne muscular dystrophy have been shown to exhibit hyperketonemia, which could indicate a reduced ketolytic capacity in muscle **[Honke et al. 1997]**. While ketone bodies are important for skeletal muscle health and metabolism, more research is needed to elucidate the exact link between ketone body oxidation, and skeletal muscle function, both in physiological and pathophysiological states.

6.4 - The role of PGC-1α in skeletal muscle – Aims

It is well established that obesity and metabolic dysfunction are closely associated with a sedentary lifestyle and an excess of calorie intake. Hence, exercise and CR would be efficient ways to ameliorate metabolic dysfunction in humans. However, it is not easy to maintain these diet- and exercise regimens. Thus, it has been suggested that administration of compounds, which could mimic the effects of either exercise or CR would have a great therapeutic potential. RSV and SRT1720 are two such compounds which have been shown to activate cAMP-Epac1 signaling, AMPK and SIRT1 as well as PGC-1α in obese rodent models. This is furthermore associated with an improved metabolic phenotype in obese mice treated with these compounds. We were thus interested in whether PGC-1α is the molecular effector of the beneficial effects of resveratrol and SRT1720 in skeletal muscle and on whole body metabolism in obese mice. Moreover, we were interested in whether the structurally distinct compounds resveratrol and SRT1720 would elicit analogous or differential effects in major metabolic organs such as skeletal muscle, liver and WAT. These finding are presented in chapter 7/manuscript 2 of this thesis, and can be summarized with these two aims:

1. **Define the role of skeletal muscle PGC-1α as a potential mediator of the beneficial effects of resveratrol and SRT1720 in skeletal muscle, as well as on systemic metabolic parameters.**

2. **Compare the effects elicited by resveratrol and SRT1720 treatment in diet-induced obese mice; are the effects on organ-specific and systemic metabolic parameters analogous?**

PGC-1α is an important regulator of mitochondrial and metabolic pathways and modulates several metabolic processes involved in glucose and fatty acid oxidation in skeletal muscle. Another important metabolic fuel in skeletal muscle, especially in nutrient-deprived states, are ketone bodies. However, despite the high basal ketolytic capacity of oxidative muscles, the role of PGC-1α in the regulation of ketone body oxidation has to our knowledge so far not been investigated. Our findings regarding the role of PGC-1α in the regulation of ketone body oxidation will be presented in chapter 8/manuscript 3 of this current thesis. The fourth aim of this thesis can thus be summarized:

3. **Investigate the role of PGC-1α in the regulation of skeletal muscle ketone body oxidation and its impact on systemic ketone body metabolism.**

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Manuscript 2:

Modulation of Fat and Liver Metabolism Mediates Systemic Effects of Resveratrol and SRT1720 Independently of Skeletal Muscle PGC-1α

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7. Manuscript 2: Modulation of Fat and Liver Metabolism Mediates Systemic Effects of Resveratrol and SRT1720 Independently of Skeletal Muscle PGC-1α

Abstract

Resveratrol (RSV) and SRT1720 (SRT) elicit metabolic effects similar to caloric restriction in mice, and are postulated to ameliorate obesity and related metabolic complications in humans. While the primary target for these compounds remains controversial, peroxisome proliferator-activated receptor γ co-activator 1α (PGC-1α) has emerged as a major downstream effector responsible for metabolic remodeling of muscle and other metabolic tissues in response to RSV or SRT treatment. However, the requirement of intact PGC-1α in skeletal muscle for the effects of these compounds on systemic metabolism has so far not been demonstrated. Using muscle-specific PGC-1α knockout mice, we show that PGC-1α is necessary for transcriptional induction of mitochondrial genes in muscle with both RSV and SRT treatment. Nevertheless, the beneficial effects of these compounds on body composition and glucose homeostasis occur even in the absence of muscle PGC-1α. We furthermore investigated whether RSV- and SRT-treatment would induce comparable metabolic effects in target organs. Intriguingly, RSV and SRT treatment lead to several analogous but also differential effects on lipid metabolism and mitochondrial biogenesis in skeletal muscle, liver and white adipose tissue. Our results provide important insights into the mechanism, effects and organ specificity of caloric restriction mimetics such as RSV and SRT. These findings will prove important for the design of future therapeutic interventions aimed at ameliorating obesity and obesity-related metabolic dysfunction in patients.

Introduction

In moderation, caloric restriction (CR) elicits various beneficial metabolic effects in humans **[Speakman and Mitchell 2011]**. Thus, inducing a similar metabolic state through pharmacological interventions has been proposed as an approach to ameliorate obesity, type 2 diabetes and other metabolic complications in patients. Resveratrol (RSV) is a naturally occurring compound which mimics both metabolic and transcriptional effects of CR in mice **[Pearson et al. 2008, Smith et al. 2009]**. Moreover, RSV improves metabolic dysfunction and prolongs lifespan in high fat diet (HFD) fed rodents **[Baur et al. 2006, Lagouge et al. 2006]**. The exact molecular mechanism how RSV promotes these effects is not fully elucidated. AMP-activated protein kinase (AMPK) **[Um et al. 2010]**, sirtuin 1 (SIRT1) **[Price et al. 2012]** and cAMP-degrading phosphodiesterases (PDEs) **[Park et al. 2012]** are all proposed to be primary targets for this polyphenolic compound. It is however known that besides activation of these targets, RSV has additional properties and can act as an antioxidant, a phytoestrogen and as an anti-inflammatory agent **[Baur 2010]**. Thus, in search for new, more specific pharmacological activators of SIRT1, several synthetic compounds have been identified, including SRT1720 (SRT) **[Milne et al. 2007]**. SRT shows a higher efficacy and selectivity for activating SIRT1 compared to RSV **[Milne et al. 2007]**, and mimics many of the transcriptional changes observed during both CR and RSV treatment in mice **[Smith et al. 2009]**. SRT treatment can furthermore ameliorate metabolic defects in both HFD-fed **[Feige et al. 2008]** and aged mice **[Minor et al. 2011]**, but as for RSV, it is controversial whether SRT directly activates SIRT1 to mediate these metabolic changes **[Pacholec et al. 2010]**. For both compounds however, peroxisome proliferator-activated receptor γ co-activator 1α (PGC-1α) has been proposed as the common downstream effector in control of metabolic plasticity **[Lagouge et al. 2006, Feige et al. 2008, Martin-Montalvo and de Cabo 2013]**. This is regardless of the putative direct target of these compounds, since PGC-1α is activated by AMPK-mediated phosphorylation, SIRT1-induced deacetylation as well as increased cAMP levels **[Herzig et al. 2001, Rodgers et al. 2005, Jager et al. 2007]**. In metabolic organs, PGC-1α is an important transcriptional co-activator that controls several integrated metabolic programs such as mitochondrial biogenesis, oxidative phosphorylation (OXPHOS) and fatty acid (FA) β-oxidation **[Handschin 2009]**. These processes are furthermore postulated to be induced by CR **[Martin-Montalvo and de Cabo 2013]** and upon administration of RSV and SRT in mice **[Lagouge et al. 2006, Feige et al. 2008]**. Accordingly, RSV and SRT treatment have been reported to activate PGC-1α and lead to metabolic remodeling in several organs, especially in skeletal muscle (SKM) **[Lagouge et al. 2006, Feige et al. 2008]**. However, the requirement for PGC-1α in mediating the effects of RSV and SRT *in vivo* has so

far not been conclusively validated. Using mice with a SKM-specific deletion of PGC-1α, we now demonstrate that while functional PGC-1α is required for transcriptional induction of mitochondrial genes in muscle, this co-activator in muscle is dispensable for the systemic metabolic effects of both RSV- and SRT-treatment. Another important question is whether structurally distinct compounds such as RSV and SRT would be able to induce similar effects in major metabolic organs. To answer this, we performed a comparative analysis of these two CR-mimetic compounds on metabolic processes in SKM, liver and white adipose tissue (WAT), and could reveal several important analogous but also differential effects between RSV and SRT on lipid metabolism and mitochondrial biogenesis.

Material and methods

Animals and diets **-** PGC-1α muscle-specific knockout (MKO) mice were generated by crossing PGC-1α$^{loxP/loxP}$ mice with HSA-Cre transgenic mice, as previously described **[Perez-Schindler et al. 2013]**. Mice were housed in a conventional facility with a 12-h light/12-h dark cycle with free access to food and water. All experiments were performed in accordance with federal guidelines and were approved by the Kantonales Veterinäramt of Kanton Basel-Stadt. The high fat diet (HFD) used for this study contains 60 kcal% fat derived mainly from lard (D12492, Research Diets Inc.), and either RSV (4 g/kg diet; Cayman Chemicals) or SRT1720 (1 g/kg diet; Sanofi Aventis) was added to the diet during production. Initially, mice were fed a HFD for two weeks, and then half of the groups were switched to a diet containing either RSV for a period of 4 weeks (RSV-S), RSV for 13 weeks (RSV-L), or SRT1720 for 4 weeks (SRT). The respective control groups received a similar HFD without any additions for the remainder of the study.

Metabolic measurements - Body composition was measured using an EchoMRI-100™ analyzer (EchoMRI Medical Systems). Intraperitoneal glucose tolerance test (GTT) was performed in overnight (16h) fasted mice. Mice were injected with a bolus of 2 g glucose/kg body weight, and blood glucose was measured in tail vein blood at 0, 15, 30, 45, 60, 90, 120 and 180 minutes after injection using a handheld glucose meter (Accu-Chek, Roche). To assess whole body metabolism through indirect calorimetry, mice were individually housed in metabolic chambers (CLAMS, Columbus instruments) over a time period of 48 h after an initial acclimatization period. During the measurement period, oxygen consumption, food intake and spontaneous activity was measured in 30 minute intervals.

Blood analysis - Blood glucose was measured in a tail vein blood sample from ad libitum fed and overnight (16h) fasted mice using a handheld glucose meter (Accu-Chek, Roche). Whole tail-vein blood was collected in Microvette tubes (Sarstedt) and plasma was isolated. Levels of cholesterol, high-density lipoprotein cholesterol (HDL-c) and low-density lipoprotein cholesterol (LDL-c) in plasma were determined using an automated biochemical analyzer (Cobas c 111 analyzer; Roche). Free fatty acids in plasma were determined using a colorimetric commercial test (HR Series NEFA-HR(2); Wako Diagnostics) according to manufacturer's instructions .

Microscopy - NADH and SDH staining were performed on 10 µm cryo-sections from tibialis anterior (TA) by exposing the sections to either NADH (Sigma) or SDH (Sigma) in the presence of nitroblue tetrazolium (Sigma). Staining intensity was quantified using Image-J software on pictures taken from the same region of the muscle for all groups. Liver specimens were snap-frozen in 2-methyl butane pre-cooled in liquid nitrogen. Tissues were cut into 5 µm thick cryo-sections and stained with Oil Red O (ORO, Sigma) for lipid content. Oil Red O staining was evaluated by an expert pathologist. For studies of mitochondrial number, pieces of TA muscle were fixed in 3% paraformaldehyde/ 0.5% glutaraldehyde, further fixed in 1% osmiumtetroxid and embedded in epon. 60-70 nm sections were cut and pictures were taken with a Morgagni 268(D) TEM (FEI). Number of mitochondria in these pictures were quantified using Image-J software.

RNA extraction and RT-PCR - Snap-frozen gastrocnemius, liver or white adipose tissue was homogenized and total RNA was extracted using TRIzol reagent (Invitrogen). RNA concentration was adjusted and 1 µg of total RNA was used for cDNA synthesis. Semi-quantitative Real-time PCR analysis was performed using Power SYBR Green master mix (Applied Biosystems) on a StepOnePlus Real-Time PCR System (Applied Biosystems). Relative expression levels for each gene of interest were calculated with the ΔΔCt method, using *PolR2A*, *HPRT* or *RPL0* as normalization control.

Nuclear isolation and immunoprecipitation - For nuclear isolation, liver was homogenized in hypotonic lysis buffer (HLB) (10 mM NaCL, 1.5 mM MgCl2, 20 mM HEPES pH 7.4, 20% glycerol, 0.1% Triton-X, 1 mM DTT) supplemented with protease inhibitors (C0mplete, Roche), phosphatase inhibitors (Sigma), 10 mM Nicotinamide A (Sigma) and 10 mM Trichostatin A (Sigma), using a Dounce homogenizer. Intact nuclei were isolated through differential centrifugation at 880g for 3 minutes, and nuclear pellet was washed 3 times in HLB. Isolated nuclei were lysed using RIPA buffer supplemented with protease inhibitors (C0mplete, Roche), phosphatase inhibitors (Sigma), 10 mM Nicotinamide A (Sigma) and 10 mM

Trichostatin A (Sigma). For immunoprecipitation, 500 µg nuclear proteins were pre-cleared with protein-A beads (Protein A –Sepharose 4B, Invitrogen). Pre-cleared supernatant was incubated with 2 µg pan-Acetyl antibody (sc8649; Santa Cruz Biotechnology) for 2 hours, followed by overnight incubation with 50 µL 50% slurry of protein-A beads. Immunoprecipitated proteins were denatured in 2x Laemmli sample buffer (Sigma), and prepared for immunoblotting.

Immunoblotting - Tissues were homogenized in RIPA buffer supplemented with protease inhibitors (C0mplete, Roche), phosphatase inhibitors (Sigma), 10 mM Nicotinamide A (Sigma) and 10 mM Trichostatin A (Sigma). Equal amounts of proteins were separated on SDS-PAGE under reducing conditions, and transferred to a nitrocellulose membrane (Whatman). Proteins of interest were detected using the following antibodies: Mitoprofile (MS604; MitoSciences), AMPKα (2532; Cell signaling), p-AMPKα T172 (2531; Cell signaling), eEF2 (2332; Cell signaling), Creb (9197; Cell signaling), p-Creb S133 (9196; Cell signaling), Insulin receptor β (IRβ) (3025; Cell signaling), p-IRβ Y1146 (3021; Cell signaling), Akt (9271; Cell signaling), p-Akt S473 (9271; Cell signaling), p-Akt T308 (4056; Cell signaling), GSK3β (9315; Cell signaling), p-GSK3β (5558P; Cell signaling), PGC-1 (AB3242; Millipore), Polyclonal Swine Anti-Rabbit Immunoglobulin/HRP (P0399, Dako), Polyclonal Rabbit Anti-Mouse Immunoglobulins/HRP (P0260, Dako). Densitometric analysis of immunoblots was performed on 6-7 individual samples using Image-J software, and a representative selection is presented in each figure.

Hepatic lipid extraction - Liver tissue was homogenized in 1 mL of 2:1 chloroform:methanol mixture, and transferred to a glass tube together with 1 mL distilled water. The sample was then vigorously mixed and centrifuged 10 minutes at 2000 rpm. The upper aqueous phase was removed and the lower organic phase was transferred to a new glass tube, and dried under N_2 at 50°C. The sample was then resuspended in chloroform and added to a solid-phase extraction column (UPTI-CLEAN NH2-S 100mg/1mL SPE Columns, Interchim). Samples were eluted with chloroform, dried under N_2 at 50°C, and subsequently resuspended in chloroform containing 1% Triton-X. Triglycerides were measured using a commercial enzymatic kit (TG PAP 150, BioMérieux), and normalized to the initial weight of the tissue used for extraction.

Statistical analysis - All data are presented as means ± SEM. Unpaired student two-tailed t test was used to determine differences between groups.

Results

To investigate the requirement for SKM PGC-1α in the metabolic effects of RSV and SRT, we used mice carrying a muscle-specific knockout of PGC-1α (MKO) **[Perez-Schindler et al. 2013]** and their floxed littermates with intact PGC-1α expression as control group (CTRL). These mice were fed a HFD for two weeks and subsequently switched to a diet containing RSV either for four weeks (short-term treatment; RSV-S), or for thirteen weeks (long-term treatment; RSV-L). A third group received a HFD for two weeks, and was then switched to a diet containing SRT1720 (SRT) for four weeks. Control groups were fed a HFD for a total of 6 weeks (RSV-S and SRT) or 15 weeks (RSV-L).

In line with the suggested role of RSV as a CR-mimetic **[Pearson et al. 2008]**, RSV-treated mice displayed a reduced body weight (Fig. 1A) and fat mass (Fig. 1B) at the end of the treatment compared to HFD fed control mice. In contrast to the RSV-mediated effects, SRT treatment affected neither body weight (Fig. 1A) nor relative fat mass (Fig. 1B). The reduction in fat mass in RSV treated mice could not be attributed to any changes in either food intake (Suppl. Fig. S1A) or spontaneous activity (Suppl. Fig. S1B). The lean phenotype in the RSV groups could however be due to increased basal energy expenditure. We thus assessed oxygen consumption in our mice as a measure of energy expenditure, and could indeed detect an increased metabolic rate with RSV-treatment at both time-points (Fig. 1C). Interestingly, SRT-treated mice also exhibited an enhanced metabolic rate based on their increased oxygen consumption (Fig. 1C), which however did not correlate with a reduction in fat mass (Fig. 1B). Surprisingly, none of these parameters where affected by the ablation of PGC-1α in muscle, since PGC-1α MKO mice displayed an equivalent change in body weight (Fig. 1A), fat mass (Fig. 1B) and energy expenditure (Fig. 1C) compared to CTRL mice upon RSV- and SRT-treatment. Hence, these data demonstrate that SKM PGC-1α is dispensable for the effect of RSV and SRT on body composition and whole body energy expenditure. SKM is the major site for insulin-induced glucose uptake, and therefore an important organ in the regulation of whole body glucose homeostasis. Moreover, both RSV and SRT have been shown to improve glucose homeostasis in obese mice **[Baur et al. 2006, Lagouge et al. 2006, Milne et al. 2007, Feige et al. 2008, Smith et al. 2009, Minor et al. 2011]** and we wanted to investigate whether this effect was mediated through SKM PGC-1α. Through an intraperitoneal glucose tolerance test we could confirm that RSV- and SRT-treatment led to improved glucose excursion rates in HFD fed mice (Fig. 1D, Fig. 1E). This correlated with a reduction in both fed and fasted blood glucose levels in SRT treated mice, which could not be observed with RSV treatment (Suppl. Fig. S1C, Fig. 1F). Ablation of SKM PGC-1α led to an

improvement in glucose excursion rates (Fig. 1D, Fig. 1E) as well as reduced fasted blood glucose levels (Fig. 1F) compared to CTRL mice after 15 weeks of HFD feeding. However, ablation of PGC-1α in muscle did not prevent the effects of either RSV or SRT treatment on glucose homeostasis in HFD fed mice (Fig. 1D-F, Suppl. Fig. S1C). Taken together, these data indicate that SKM PGC-1α is dispensable for the effects of RSV and SRT on whole body glucose homeostasis.

PGC-1α is required for induction of mitochondrial gene transcription, but not for enhanced oxidative capacity in skeletal muscle of RSV- or SRT-treated mice

Since the systemic effects of RSV and SRT were unaffected by the ablation of PGC-1α in muscle, we were interested in how these compounds would affect SKM metabolism in our mice. We focused on the aspect of mitochondrial function and biogenesis since RSV and SRT have been postulated to affect these parameters via activation of PGC-1α **[Martin-Montalvo and de Cabo 2013]**. As previously shown **[Handschin et al. 2007]**, ablation of PGC-1α led to an impaired oxidative phenotype in SKM. This was evident in our mice by the reduced transcript levels of several mitochondrial genes, namely cytochrome c (*Cytc*), cytochrome c oxidase subunit 5b (*Cox5b*), citrate synthase (*Cs*) and NADH dehydrogenase (ubiquinone) 1 beta subcomplex 8 (*Ndufb8*) in SKM (Fig. 2A). PGC-1α MKO mice furthermore displayed reduced levels of proteins involved in mitochondrial OXPHOS (Fig. 2B). In the RSV-S group, RSV treatment induced no changes in mRNA expression of mitochondrial genes (Fig. 2A), OXPHOS protein content (Fig. 2B), or staining intensity for oxidative function (Fig. 2C, Suppl. Fig. S2A and S2B) in SKM. In the RSV-L group however, we detected a trend towards increased PGC-1α gene transcription (Fig. 2A), and a concomitant mild induction of some mitochondrial PGC-1α target genes (*CytC*, *Cox5b*, *Cs*) (Fig. 2A). Importantly, these transcriptional effects were dependent on PGC-1α, since the effect of RSV treatment on these genes was abolished in PGC-1α MKO mice (Fig. 2A). Surprisingly, RSV treatment affected neither OXPHOS protein levels (Fig. 2B) nor oxidative enzymatic staining in the muscle (Fig. 2C, Suppl. Fig. S2A and S2B). SRT treatment led to an increased mRNA (Fig. 2A) and protein level (Fig. 2B) of *Ndfub8* in skeletal muscle. Apart from *Ndufb8*, no other mitochondrial gene tested was found to be induced by SRT treatment (Fig. 2A, Suppl. Fig. S2C). Importantly, the effect on *Ndufb8* transcription was dependent on PGC-1α, as both induction of mRNA (Fig. 2A) and protein levels (Fig. 2B) of *Ndufb8* with SRT treatment were blunted in PGC-1α MKO mice. Despite the mild effect on mitochondrial gene transcription, SRT treatment resulted in a more intense NADH (Fig. 2B, Suppl. Fig. S2A) and SDH (Suppl. Fig. S2B) staining in SKM, indicating an enhanced oxidative capacity in this tissue. Surprisingly, this effect occurred to a

certain extent also in PGC-1α MKO mice (Fig. 2C, Suppl. Fig. S2A and S2B) implying that this effect of SRT treatment was independent of SKM PGC-1α. Increased oxidative capacity with SRT treatment could be a consequence of a higher mitochondrial number in skeletal muscle. We therefore quantified mitochondria in electron microscopy pictures from SKM, and could detect an increased number of intramyofibrillar mitochondria with SRT treatment in both genotypes, although it did not reach statistical significance for PGC-1α MKO mice (Fig. 2D). Thus, the increase in oxidative capacity with SRT treatment might be a consequence of a higher mitochondrial number in skeletal muscle.

RSV- and SRT-treatment can affect transcription of several processes in SKM besides mitochondrial function, for example glycolysis, β-oxidation and myosin heavy chain isoforms **[Lagouge et al. 2006, Feige et al. 2008]**. In our experimental cohorts however, RSV-S mice displayed no difference in the mRNA levels of genes involved in glucose uptake/glycolysis (Fig. 3A) or myosin heavy chain (MHC) subtypes in SKM (Fig. 3B). Intriguingly, we observed a PGC-1α-independent induction of uncoupling protein 3 (*UCP3*) mRNA expression (Fig. 3C) in RSV-S mice, while other genes involved in FA β-oxidation such as medium-chain acyl-CoA dehydrogenase (*MCAD*), carnitine palmitoyltransferase 1B (*Cpt1b*) and pyruvate dehydrogenase kinase isozyme 4 (*PDK4*) were unchanged (Fig. 3C). Interestingly, the increase in *UCP3* expression with RSV treatment was transient, since this effect was no longer present in the RSV-L group (Fig. 3C). In contrast, a mild, PGC-1α-dependent induction of *MHCIIA* and *MHCIIB* mRNA expression was observed in RSV-L mice (Fig. 3B), which was not present in RSV-S animals. SRT treatment promoted an induction of both hexokinase 2 (*HK2*) (Fig. 3A) and *MCAD* (Fig. 3C), while the mRNA expression of other genes involved in glycolysis, FA β-oxidation and MHC subtypes was unchanged (Fig. 3A-C). In the PGC-1α MKO group, induction of *HK2* and *MCAD* by SRT did not reach statistical significance (Fig. 3A and Fig. 3C). Due to the mild effects of RSV on gene expression in SKM, we assessed AMPK phosphorylation as readout of RSV-induced signaling in SKM. In line with the mild transcriptional effects of RSV treatment in SKM, we could detect no significant induction of AMPK phosphorylation (T172) (Fig. 3D) for either the RSV-S or RSV-L groups.

SRT induces transcription of PGC-1α target genes in liver

Since PGC-1α MKO mice displayed a similar systemic response to both RSV- and SRT-treatment as CTRL mice, this suggests only a minor role of skeletal muscle PGC-1α in the regulation of systemic metabolism by these compounds. We therefore continued to analyze the effects of RSV- and SRT-treatment in other major metabolic organs, such as liver and white adipose tissue (WAT). To avoid confounding metabolic

crosstalk due to ablation of PGC-1α in muscle, the following experiments were performed in mice with intact SKM PGC-1α expression. In contrast to the mild effects seen in SKM, SRT treatment boosted the transcription of several mitochondrial genes (*Cox5b*, *CytC*, *CS*, ubiquinol-cytochrome c reductase core protein 2 *Uqcrc2*) in liver (Fig. 4A), along with a small but significant increase in protein levels of *Uqcrc2* and *ATP synthase subunit alpha 5* (*ATP5A*) (Fig. 4B). SRT treatment furthermore led to a minor elevation in PGC-1α protein levels (Fig. 4B), as well as to the transcriptional induction of genes involved in ketogenesis (acetyl-coenzyme A acetyltransferase 1 *Acat1*, 3-hydroxymethyl-3-methylglutaryl-CoA lyase *Hmgcl*, 3-hydroxy-3-methylglutaryl-CoA synthase 2 *Hmgcs2*) and gluconeogenesis (phosphoenolpyruvate carboxykinase *PEPCK*, glucose-6-phosphatase *G6pc*) (Fig. 4C). All of these metabolic processes are regulated by hepatic PGC-1α **[Rhee et al. 2003]**. Our results therefore indicate that SRT treatment results in an increased transcriptional activity of PGC-1α in liver. Since SRT is an activator of SIRT1 **[Milne et al. 2007]**, which can deacetylates and thereby activates PGC-1α **[Rodgers et al. 2005]**, we determined the acetylation status of PGC-1α in the livers of CTRL and SRT-treated animals on a HFD. In these animals, no difference in PGC-1α acetylation status between HFD-fed CTRL and SRT-treated mice could be detected (Fig. 4D). In contrast, we observed a strong trend towards increased hepatic AMPK phosphorylation with SRT treatment (Fig. 4E, Suppl. Fig. S3A), correlating with an increased phosphorylation of the AMPK-sensitive phosphorylation site (S79) on acetyl-CoA-carboxylase (*ACC*) (Fig. 4E, Suppl. Fig. S3A). Since AMPK phosphorylates and thereby increases the transcriptional activity of PGC-1α **[Jager et al. 2007]**, AMPK activation upon SRT treatment could account for the increased hepatic PGC-1α activity in SRT-treated mice. These animals accordingly displayed an induction of several PGC-1α target genes involved in mitochondrial FA β-oxidation (*MCAD*, long-chain acyl-CoA dehydrogenase *LCAD*), mitochondrial FA import (*Cpt1b*, carnitine-acylcarnitine translocase *CACT*) and FA uptake (lipoprotein lipase *LPL*, cluster of differentiation 36 *CD36*) in the liver (Fig. 4F). Importantly, altered transcriptional activation of PGC-1α-regulated processes in liver was exclusively found in SRT-, but not in RSV-treated animals. In fact, administration of RSV even reduced transcript levels of hepatic PGC-1α (Fig. 4A, Suppl. Fig. S3B) at both time-points and did not alter transcription of mitochondrial (Fig. 4A, Suppl. Fig. S3B), ketogenic or gluconeogenic genes (Fig. 4C, Suppl. Fig. S3C). Likewise in contrast to SRT-treated mice, RSV administration resulted in no transcriptional changes on either FA β-oxidation or mitochondrial FA import (Fig. 4F, Suppl. Fig. S3D) apart from an induction of *Cpt1b* in the RSV-S group (Fig. 4F).

RSV and SRT differentially regulate hepatic lipogenesis

Due to the observed metabolic remodeling of the liver by SRT, we also measured the expression of genes important for hepatic lipogenesis in the SRT cohort. SRT treatment resulted in an induction of the lipogenic transcription factor sterol regulatory element binding protein-1c (*Srebp1c*), as well as its target genes involved in lipogenesis (acetyl-CoA carboxylase 2 *ACC2*, Stearoyl-CoA desaturase-1 *SCD1*, FA synthase *Fasn*) and FA re-esterification (glycerophosphate acyltransferase *GPAT*) (Fig. 4G). Since insulin is a known regulator of Srebp1c transcription and processing **[Horton et al. 2002]**, we determined whether increased hepatic insulin signaling could account for the increased lipogenic gene transcription upon SRT treatment. In the SRT group, we detected an elevated phosphorylation of AKT (T308), while auto-phosphorylation of insulin receptor β (IRβ) (Y1146) as well as phosphorylation of the AKT-target glycogen synthase kinase 3 beta (GSK3β) (S9) were reduced (Suppl. Fig. S3E). This would indicate reduced insulin signaling in liver with SRT treatment, and could therefore not account for the increased transcriptional activation of lipogenesis seen with this compound. Interestingly, RSV treatment resulted in a significant reduction of genes involved in lipogenesis (*ACC2*, *SCD1*, *Fasn*) and FA re-esterification (*GPAT*), as well as a statistically non-significant trend (RSV-S p=0.061 / RSV-L p=0.062) towards reduced *Srebp1c* transcription (Fig. 4G, Suppl. Fig. S3F), thereby showing an opposite regulation of hepatic lipogenesis compared to SRT treatment. We next investigated whether these strikingly opposite transcriptional responses to RSV and SRT treatment would affect hepatic triglyceride (TG) content. In the RSV-S group, a trend (p=0.056) towards reduced TG content was observed (Fig. 4H, Suppl. Fig S3H). Surprisingly, this effect was not present in the RSV-L group (Suppl. Fig. S3G, Suppl. Fig S3H), even though lipogenic gene transcription was still reduced at this time-point (Suppl. Fig. S3F). SRT treatment did not affect hepatic TG content (Fig. 4H, Suppl. Fig S3H). Next, we also studied the effect of these two compounds on hepatic cholesterol biosynthesis. In line with the increased transcription of anabolic processes, such as hepatic lipid and ketone body production, SRT treatment also increased the transcription of genes involved in cholesterol biosynthesis (acetyl-CoA acetyltransferase 2 *Acat2*, farnesyl-diphosphate farnesyltransferase 1 *Fdft1*, 7-dehydrocholesterol reductase *Dhcr7*) in the liver (Fig. 4I). Consequently, we could detect increased levels of low-density lipoprotein cholesterol (LDL-C) and high-density lipoprotein cholesterol (HDL-C) in plasma from SRT treated mice (Fig. 4J). In contrast, RSV-treatment did not affect the transcription of genes involved in cholesterol synthesis (Fig. 4I, Suppl. Fig. S3I). Nevertheless, RSV-S mice exhibited reduced levels of LDL-C in the blood (Fig. 4J), while HDL-C (Fig. 4J) levels remained unaltered. Moreover, the

reduction in circulating LDL-C levels is most likely a transient effect of RSV treatment, since no differences in either LDL or HDL cholesterol was observed in the RSV-L group (Suppl. Fig. S3J).

Long-term RSV treatment leads to an induction of genes involved in lipogenesis and lipolysis in white adipose tissue

Our data demonstrate a high selectivity for SRT over RSV to affect liver metabolism. We therefore assessed the consequence of SRT and RSV administration on WAT metabolism to investigate whether this tissue can account for the metabolic remodeling promoted by RSV. In contrast to the effects in liver, neither SRT nor RSV-S groups displayed any alterations in lipogenic gene transcription, with the exception of a down-regulation of *Fasn* transcript levels in the RSV-S mice (Fig. 5A). RSV-L mice however displayed a robust induction of a number of lipogenic genes (*Srebp1c*, *ACC1*, *Scd1*, *Fasn*) as well as genes involved in FA re-esterification (*GPAT*, diglyceride acyltransferase 1, 2 *DGAT1*, *DGAT2*) in WAT (Fig. 5A). Interestingly, these lipogenic genes were down-regulated with RSV treatment in liver at the same time-point (Fig. 4G, Suppl. Fig S3F), indicating that RSV exerts opposing effects on lipogenic gene transcription in WAT compared to liver. In contrast, SRT did not influence lipogenesis in WAT whereas a strong effect was observed in hepatic tissue. To elucidate the mechanism behind the induction of lipogenic genes in WAT with long-term RSV treatment, we analyzed the insulin signaling pathway in WAT from these mice. Protein levels of IRβ, AKT and GSK3β were all increased in WAT from the RSV-L group, together with increased auto-phosphorylation of IRβ (Y1146), as well as an increased phosphorylation of both AKT (S473) and GSK3β (S9) (Fig. 5B). Since enhanced insulin signaling in adipocytes is linked to increased lipogenesis **[Kersten 2001]**, the upregulation of IRβ protein levels and increased AKT (S473) phosphorylation in WAT from RSV-L mice could provide an explanation for the increased transcription of lipogenic genes observed in this group. Increased lipogenesis and FA re-esterification is indicative of an increased adipogenesis in WAT. However, diametrically opposite to the elevation of lipogenic gene expression, short- and long-term RSV treatment led to a reduced fat mass in our mice (Fig. 1B). Hence, we speculated that not only lipogenesis, but also other aspects of triglyceride handling might be affected in WAT of RSV-treated animals. Indeed, in addition to the transcriptional activation of lipogenic processes in the RSV-L group, we also found an induction of genes involved in lipolysis (adipose triglyceride lipase *ATGL*, hormone-sensitive lipase *HSL*) (Fig. 5C). However, despite the induction of lipolytic genes with RSV-treatment, no alterations in plasma non-esterified FA levels in the RSV-L group were observed (Suppl. Fig. S4A). These data, together with the concurrent induction of lipolysis and FA

re-esterification, would point towards an increased FA recycling in the RSV-L mice. Since the availability of glycerol-3-phosphate (G3P) is essential for FA re-esterification, we investigated transcriptional regulation of genes involved in G3P synthesis. Interestingly, we could observe an induction of genes involved in two processes important for G3P generation, namely glucose uptake/glycolysis (glucose transporter type 4 *GLUT4*, *HK2*) and glyceroneogenesis (*PEPCK*) in the RSV-L group (Fig. 5C). No differences in these genes could be detected for the RSV-S and SRT groups, apart from a significant upregulation of *HKII* in SRT treated mice (Fig. 5C).

RSV improves mitochondrial biogenesis and reduces inflammation in white adipose tissue

Since SRT treatment affected transcription of mitochondrial genes in liver, we also investigated this aspect in adipose tissue. In contrast to the effects in liver, SRT treatment only resulted in a trend towards increased PGC-1α transcription in WAT (Fig. 6A), but had no further effect on mitochondrial transcript (Fig. 6A) or protein levels (Fig. 6B, Suppl. Fig. S4B). In contrast, RSV-S mice had significantly increased PGC-1α transcript levels in WAT (Fig. 6A). RSV-L mice showed no significant induction of PGC-1α mRNA levels (Fig. 6A), while PGC-1α protein levels for this time-point were significantly induced (Fig. 6B, Suppl. Fig. S4B). In line with the induction of PGC-1α, RSV treatment resulted in a significant induction of mitochondrial genes (*Uqcrc2*, succinate dehydrogenase complex, subunit A *SDHA*) at both time-points (Fig. 6A), while only RSV-L mice displayed enhanced mitochondrial protein levels of SDHB (Fig. 6B, Suppl. Fig. S4B). To determine whether the transcriptional induction of mitochondrial genes in WAT could be mediated by increased AMPK activity, we determined AMPK phosphorylation (T172) in RSV-S and RSV-L mice. In the RSV-S group, relative AMPK phosphorylation displayed a trend towards being up-regulated ($p=0.07$) (Suppl. Fig. S4C). In RSV-L mice however, both AMPK protein levels and relative phosphorylation of AMPK (T172) were unchanged (Suppl. Fig. S4C). These data suggest that increased AMPK activity might not be the main mechanism by which RSV-treatment increases mitochondrial transcript and protein levels in WAT. Another possible explanation for the increased mitochondrial gene transcription is through down-regulation of activating transcription factor 3 (*ATF3*), a transcriptional repressor that reduces transcription of PGC-1α and mitochondrial genes in WAT **[Jang et al. 2013]**. Interestingly, we could detect a significant reduction in ATF3 transcript levels in WAT of RSV-L mice, and a strong trend ($p=0.06$) towards diminished ATF3 transcription in RSV-S mice (Fig. 6C). To substantiate these findings, we also studied the expression of adiponectin (*Adipoq*), a gene known to be repressed by ATF3 in adipocytes **[Kim et al. 2006]**. In line with the reduced ATF3 mRNA levels, expression of adiponectin was

significantly increased specifically in RSV-treated mice (Fig. 6C), indicating a reduced transcriptional repression by ATF3. In SRT treated mice however, no alterations in either ATF3 or adiponectin expression were found (Fig. 6C). This finding correlates well with the unchanged mitochondrial gene transcription with SRT treatment (Fig. 6A), and suggests that repression of ATF3 is an RSV-specific effect. To gain more insight into the mechanisms that underlie the repression of ATF3 expression by RSV, we assessed the activity of cAMP response element-binding protein (CREB) since increased CREB-activity has been associated with increased ATF3 transcription in WAT in obese conditions **[Qi et al. 2009]**. In line with the lower transcript levels of ATF3 in RSV-treated mice, we could also detect reduced levels of CREB phosphorylation (S133) (Fig. 6D, Suppl. Fig. S4D), correlating with a reduced activity of this transcription factor. Besides various beneficial systemic effects of adiponectin on whole body metabolism, this hormone also possesses anti-inflammatory properties in fat tissue **[Tilg and Wolf 2005]**. We therefore measured gene expression of inflammatory markers in WAT of our mice. In line with the repression of ATF3 and induction of adiponectin, inflammatory gene transcription (tumor necrosis factor α *TNF*, monocyte chemotactic protein-1 *MCP-1*, cluster of differentiation 68 *CD68*) was decreased in RSV treated mice (Fig. 6E). However, SRT treatment did not result in any changes in pro-inflammatory gene expression similar to the lack of effect of SRT on mitochondrial OXPHOS gene transcription, adiponectin levels or CREB activity in WAT.

Discussion

Caloric restriction (CR) mediates several beneficial metabolic effects in organisms ranging from yeast to rodents and humans **[Speakman and Mitchell 2011]** and has even been linked to an increased life- and healthspan. Therefore, it is not surprising that the concept of using pharmacological CR-mimetics, including RSV and SRT, to ameliorate metabolic disorders has received a lot of attention in recent years **[Ingram et al. 2006, Speakman and Mitchell 2011]**. In agreement with other studies **[Baur et al. 2006, Lagouge et al. 2006, Milne et al. 2007, Feige et al. 2008, Smith et al. 2009, Minor et al. 2011]**, we demonstrate that both RSV and SRT exert beneficial metabolic effects in HFD-fed mice *in vivo*, such as an increased metabolic rate, improved glucose homeostasis, and at least for RSV-treated mice, reduced adiposity. However, in contrast to previous hypotheses implying muscle PGC-1α as the common molecular effector of these CR mimetics, we now show that SKM PGC-1α is dispensable for the whole-body metabolic effects of RSV and SRT *in vivo*, since metabolic changes occurred to the same extent in

CTRL as in PGC-1α MKO mice. Importantly, PGC-1α was required for the transcriptional effects on mitochondrial genes induced by RSV and SRT treatment in SKM. In contrast to the regulation of gene expression, muscle PGC-1α was dispensable for non-transcriptional changes in SKM with SRT treatment, such as enhanced oxidative capacity, which could explain why whole body oxygen consumption was unaffected by the genetic ablation of SKM PGC-1α. Interestingly, a similar observation was made in a study by Finley et al. in regard to the requirement for muscle PGC-1α to induce the systemic effects of CR **[Finley et al. 2012]**. Analogous to our data, this study showed that during CR, PGC-1α was required for the enhanced transcription of mitochondrial genes in SKM, while basal oxygen consumption increased to the same extent even in the absence of SKM PGC-1α **[Finley et al. 2012]**. These data and our findings collectively indicate that enhanced oxidative capacity in SKM during CR, or during treatment with CR-mimetic compounds, seems to be dissociated from the induction of mitochondrial genes seen in this context, and would therefore be independent of muscle PGC-1α. However, all of these results were obtained in sedentary animals and thus do not preclude a more prominent role for muscle PGC-1α in the regulation of the metabolic phenotype of physically active mice in which muscle contributes much more significantly to the global metabolic rate.

Despite the structural difference between RSV and SRT, the effects of these compounds are considered to be analogous and ultimately mediated through the same signaling pathways. For example, the transcriptional response to these two compounds overlaps in the liver **[Minor et al. 2011]** and is similar to the hepatic transcriptional profile during CR **[Pearson et al. 2008, Smith et al. 2009]**. Importantly however, this overlap is small in comparison to the total number of regulated genes **[Minor et al. 2011]** suggesting that many effects of these two postulated CR-mimetic compounds on metabolic processes in the liver are in fact unique. In line with this, we identified several metabolic processes in this organ that were activated by SRT administration, but not altered with RSV treatment, including cholesterol metabolism, β-oxidation, ketogenesis and gluconeogenesis. Intriguingly, we also found that RSV and SRT differentially regulate the transcription of lipogenic genes in liver. In agreement with other studies **[Baur et al. 2006, Gomez-Zorita et al. 2012, Jin et al. 2013]**, we observed that RSV treatment leads to a reduction in hepatic lipogenic gene transcription, and that this results in a mild protection against hepatic steatosis. Importantly, this effect also occurs in humans that receive RSV treatment **[Timmers et al. 2011]**. Conversely, SRT treatment led to an enhanced transcription of lipogenic genes in liver, and in contrast to other studies **[Feige et al. 2008, Yamazaki et al. 2009, Minor et al. 2011]**, SRT administration did not protect against hepatic steatosis. At least in one of these studies **[Feige et al.**

2008], the SRT-linked reduction in hepatic lipid accumulation was only observed at a dose that was five times higher than that used in our study. Importantly, this high dose of SRT completely abrogated any weight gain in the HFD-fed mice **[Feige et al. 2008]** and the reduced hepatic lipid accumulation could therefore be interpreted as an extension of the lean phenotype of these mice rather than a direct hepatic effect of SRT. Importantly, in the mice receiving a similar dose of SRT (100 mg/kg/day) as that used in our study, Feige et al. **[Feige et al. 2008]** could not detect any protective effect of SRT against hepatic steatosis. Taken together, the effect of SRT on hepatic gene expression and modulation of metabolic pathways in our setup was much stronger than that of RSV. For example, SRT promoted a transcriptional induction of cholesterol biosynthesis genes in the liver. This correlated with increased circulating levels of HDL and LDL cholesterol, which might constitute a caveat for using this compound as a treatment for obesity in patients. In contrast, RSV has a more beneficial effect on whole body lipid parameters in our mice, since we could demonstrate a trend towards reduced hepatic lipid accumulation as well as significantly lower LDL-cholesterol levels in the RSV-S group. Importantly, this reduction in LDL cholesterol levels with RSV treatment has also been reported in human patients **[Bhatt et al. 2012, Tome-Carneiro et al. 2012]**.

Another important difference we could observe between SRT and RSV is the reduction in adiposity seen only with RSV administration. This reduction in fat mass in RSV-, but not SRT-treated mice was associated with robust transcriptional effects in WAT in the former cohort. The resulting gene expression profile indicates improved adipose tissue health, for example due to increased mitochondrial function, enhanced adiponectin expression and reduced adipose tissue inflammation upon RSV treatment. These effects have further been corroborated by other studies using RSV in both rodents **[Rivera et al. 2009, Beaudoin et al. 2013, Gomez-Zorita et al. 2013]** and non-human primates **[Jimenez-Gomez et al. 2013]**. In search for a mechanism how RSV mediates these effects in WAT, we could demonstrate that RSV leads to a reduction in the transcriptional repressor ATF3, a negative regulator of mitochondrial metabolism **[Jang et al. 2013]**, GLUT4 **[Qi et al. 2009]**, as well as of adiponectin expression **[Kim et al. 2006]**. CREB is a positive transcriptional regulator of ATF3 during obesity **[Qi et al. 2009]**. In line with this, we found that RSV treatment resulted in reduced phosphorylation of CREB in WAT, indicating a reduced activity of this transcription factor. Importantly, mice expressing a dominant negative form of CREB in WAT **[Qi et al. 2009]** display similar phenotypic changes as seen with RSV treatment in our study, including reduced inflammation, improved GLUT4 expression and improved whole body glucose tolerance. Moreover, in a transcription factor analysis performed in WAT from

humans receiving RSV treatment, CREB1-activity was predicted to be inhibited by RSV. This is in agreement with our findings, and gives credence that the repression of CREB-ATF3 axis by RSV treatment in WAT is also relevant in humans **[Konings et al. 2013]**.

The efficacy of CR on longevity in different species is somewhat controversial, and the extent of the effect of RSV in particular has been questioned in recent studies **[Higashida et al. 2013]**. Similarly, we could not observe an increase in mitochondrial oxidative capacity that was reported in the study by Lagouge et al. **[Lagouge et al. 2006]** even though the same dose of RSV (4 g/kg diet) was used. In contrast, two recent studies found no effect of RSV treatment (4 g/kg diet) on oxidative protein content **[Higashida et al. 2013, Ringholm et al. 2013]** and mitochondrial DNA content **[Ringholm et al. 2013]** in SKM, similar to our results. One important difference between the Lagouge and our study is the choice of HFD. The diet our mice received (D12492, Research diet) is mainly composed of lard, and thus contains a high percentage of long-chain FAs (LCFA). The HFD used by Lagouge et al. (D12327, Research diets) contains mainly coconut oil, which in comparison to lard primarily consists of medium-chain FAs (MCFA). HFDs rich in LCFAs have a more detrimental effect on adiposity and glucose tolerance in mice than MCFA-based diets **[Montgomery et al. 2013]**. In addition, MCFA-rich diets might even induce mitochondrial metabolism and oxidative capacity in SKM **[Turner et al. 2009, Montgomery et al. 2013]**. Thus, the different composition of FAs in the HFDs used by us and by Lagouge et al. might have differentially influenced the basal metabolic state of SKM and as a consequence, the response to RSV. Higashida et al. **[Higashida et al. 2013]** speculate that the lack of effect of RSV treatment in their study could be due to the low bioavailability of the compound in blood. However, using the same dose of RSV, we and others could clearly demonstrate systemic metabolic effects in mice, metabolic adaptation of SKM as well as modulation of signaling pathways in both liver and WAT. Taken together, these findings indicate that the effects of RSV on SKM metabolism and function seem to be complex and highly dependent on the specific experimental context.

In summary, we show that PGC-1α is required for the transcriptional effects of both RSV and SRT on mitochondrial gene expression in SKM, while muscle PGC-1α is dispensable for the systemic metabolic effects mediated by RSV and SRT. However, our findings suggest PGC-1α to be a potential mediator of RSV- and SRT-treatment in other tissues such as liver and WAT. Importantly, at least in sedentary, HFD-fed mice, treatment with RSV and SRT resulted in more prominent metabolic changes in WAT and liver as compared to SKM. It might therefore be of importance to adapt a broader, less muscle-

centric view on the metabolic effects of RSV- and SRT-treatment, in particular in the context of a potential therapy of sedentary, obese patients. Furthermore, the comparison of short- and long-term administration of RSV highlights some potential caveats for the design of such a therapy: for example, increased lipogenesis in WAT of the long-term RSV-treated animals could indicate that administration of this compound in patients should be time-limited. Intriguingly, despite the apparently similar systemic effects of RSV and SRT, these compounds exert differential effects on key metabolic organs including SKM, liver and WAT (Fig. 6F). Hence, our findings reveal important insights into the specific effects of these structurally distinct compounds. Importantly, this indicates that increased hepatic lipogenesis and LDL levels should be considered when designing SRT-based therapeutic approaches. It is currently unclear whether these differences between RSV and SRT are caused by variable bioavailability in these tissues, activation of different molecular targets and signaling pathways or due to non-SIRT1-mediated effects of RSV, e.g. its phytoestrogenic, anti-inflammatory or antioxidant properties. In either case, it is of importance to consider the differential effects of RSV and SRT treatment for future study design, and even more significantly for the use of these compounds in a clinical context.

Acknowledgements

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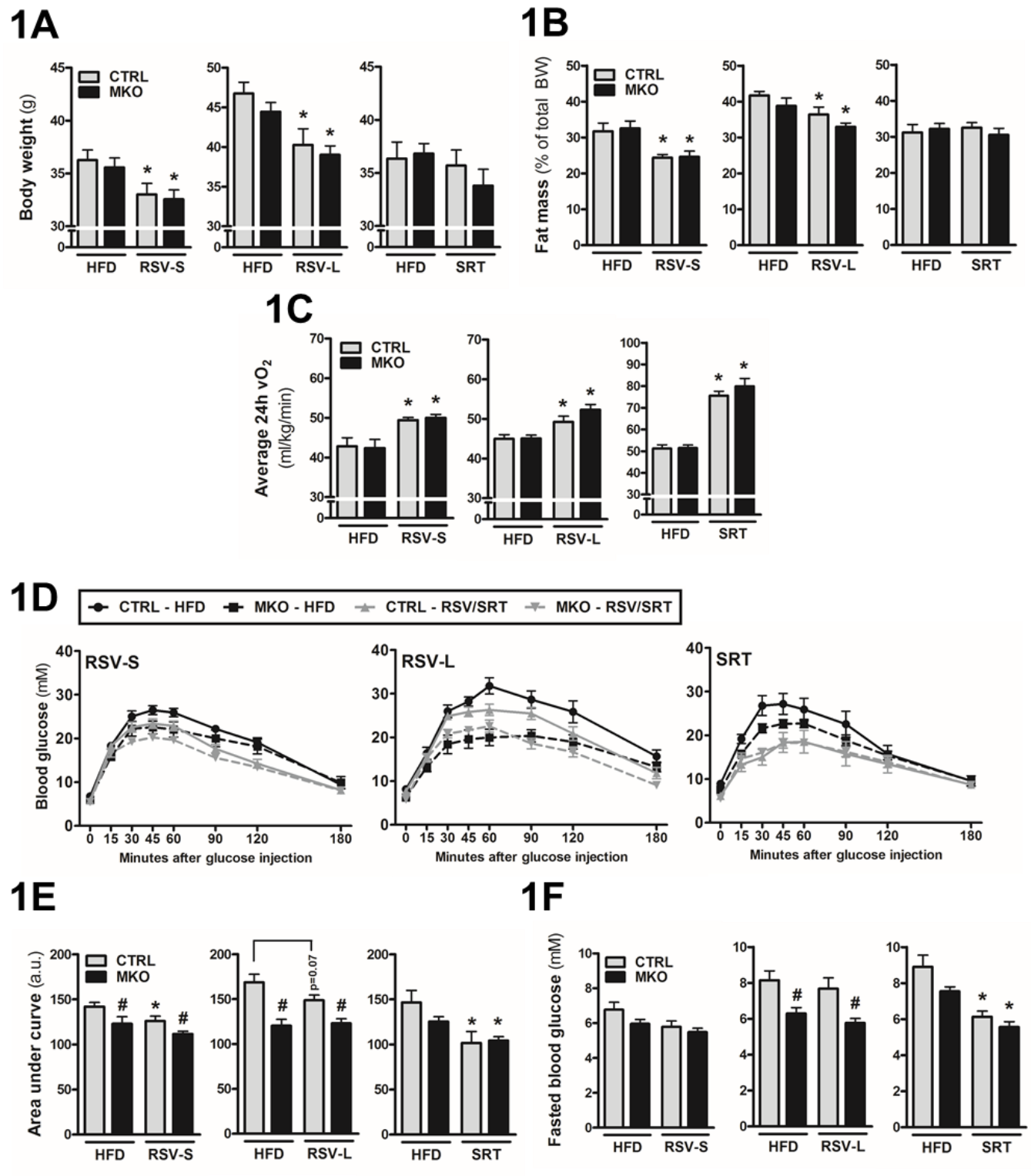

1A
Body weight (g)
CTRL
MKO
HFD
RSV-S
RSV-L
SRT
1B
Fat mass (% of total BW)
1C
Average 24h vO2 (ml/kg/min)
1D
CTRL - HFD
MKO - HFD
CTRL - RSV/SRT
MKO - RSV/SRT
RSV-S
RSV-L
SRT
Blood glucose (mM)
Minutes after glucose injection
1E
Area under curve (a.u.)
p=0.07
1F
Fasted blood glucose (mM)

Figure 1 - Systemic effects of RSV and SRT are independent of skeletal muscle PGC-1α

CTRL and PGC-1α MKO mice were fed a high fat diet (HFD) for 2 weeks, followed by resveratrol (RSV) treatment (4 g/kg diet) for 4 weeks (RSV-S), 13 weeks (RSV-L) or SRT1720 administration (1 g/kg diet) for 4 weeks (SRT). (A) Body weight at the end of the treatment period (n= 8-15 per group). (B) Fat mass expressed as percentage of total body weight at the end of the treatment period (n= 5-9 per group). (C) Basal energy expenditure as measured by 24 hour average oxygen consumption (vO_2) (n= 5-9 per group). (D) Intraperitoneal glucose tolerance test. Mice were injected with a bolus of 2 g glucose/kg body weight after a 16 hours fasting period (n= 8-10 per group). (E) Area under curve for intraperitoneal glucose tolerance test (n= 8-10 per group). (F) Blood glucose levels measured in tail vein blood after a 16 hours fasting period (n= 8-10 per group). Error bars represent mean ±SEM. Significant differences (p value < 0.05) between CTRL and PGC-1α MKO mice are indicated by a number sign (#) and between untreated and treated groups by an asterisk (*).

7. Manuscript 2: Modulation of Fat and Liver Metabolism Mediates Systemic Effects of Resveratrol and SRT1720 Independently of Skeletal Muscle PGC-1α

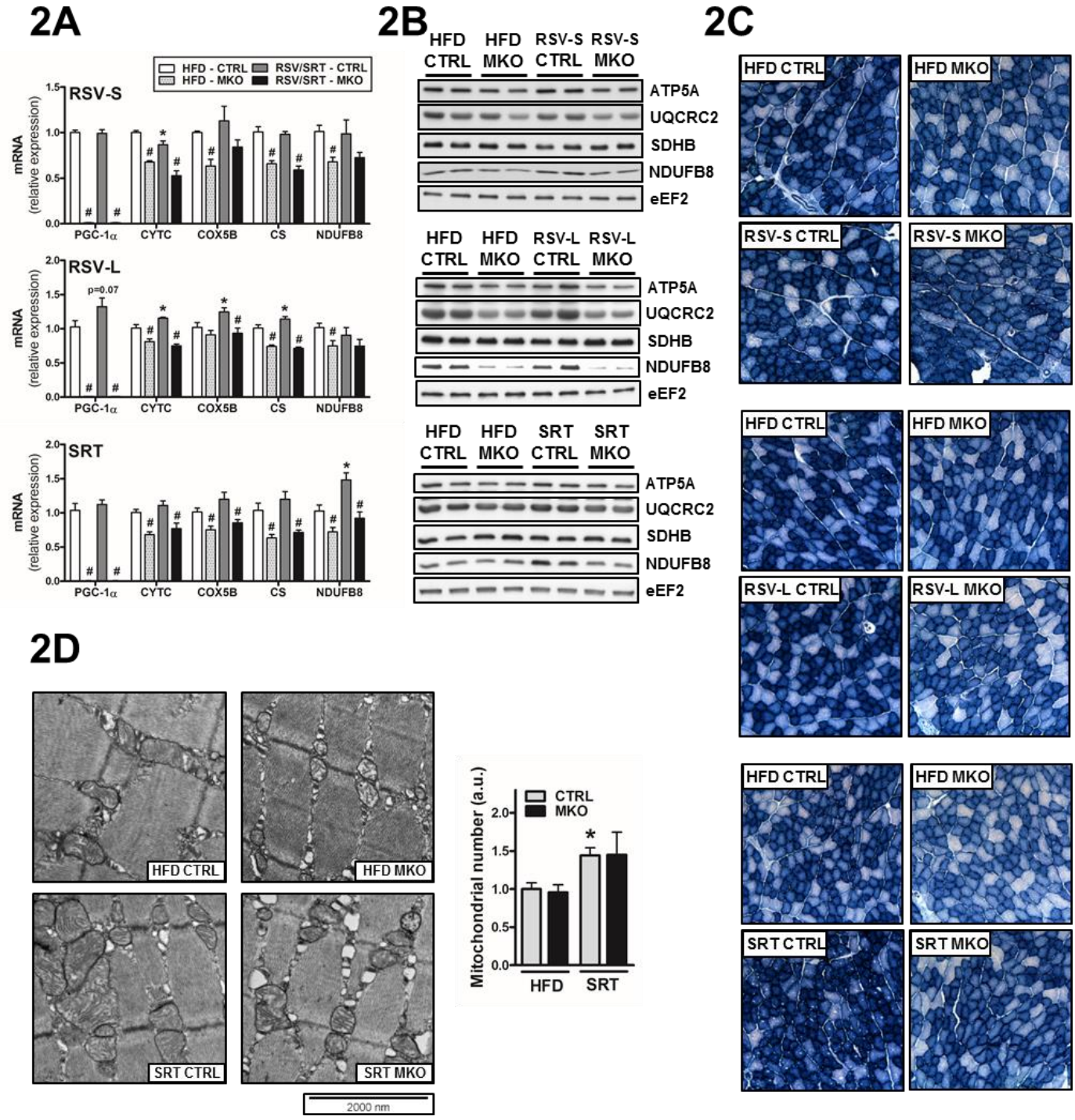
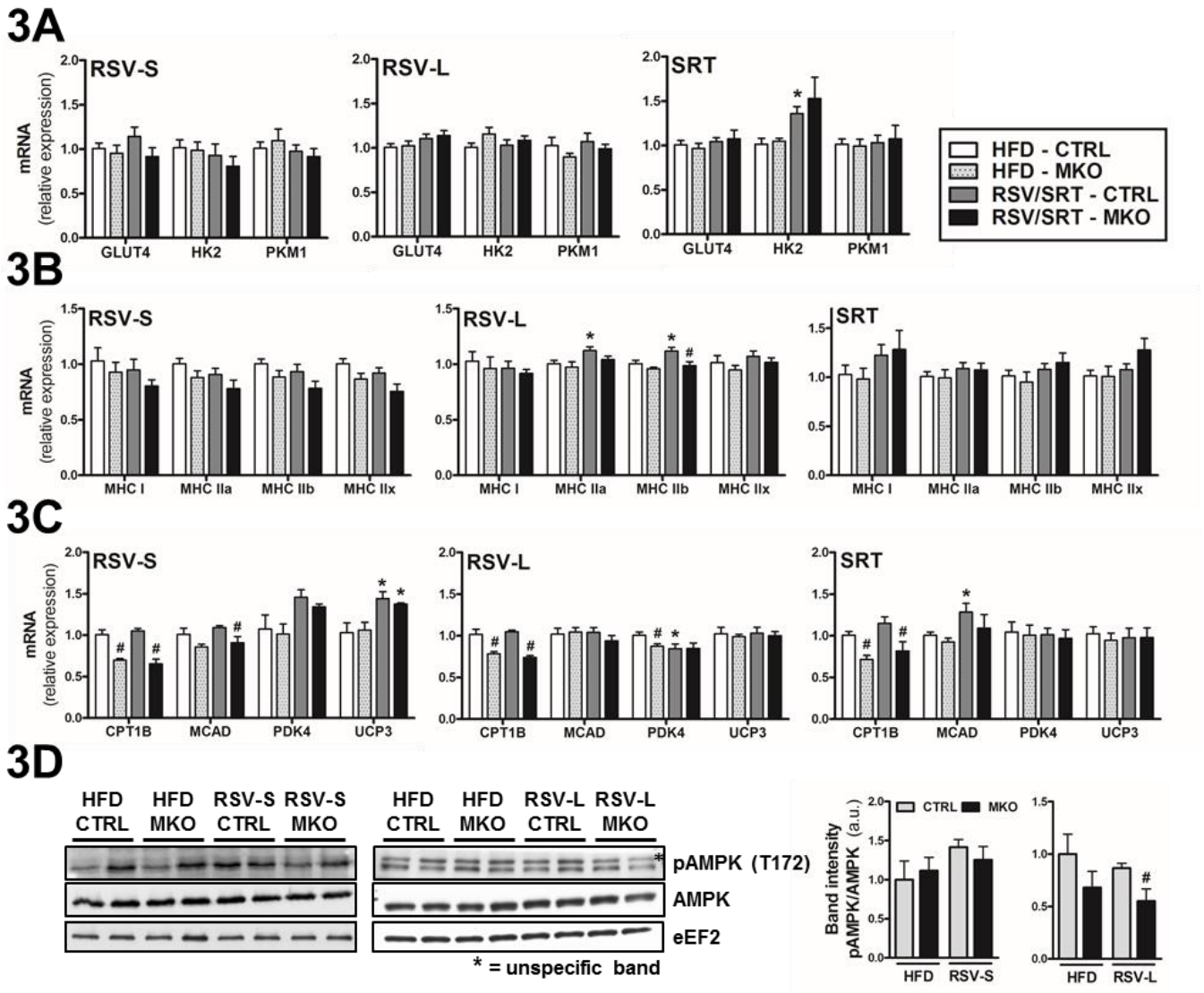


Figure 2 - PGC-1α is required for the transcriptional induction of mitochondrial genes in skeletal muscle

(A) mRNA levels of *PGC-1α* and mitochondrial genes in skeletal muscle (SKM) relative to *PolR2A* (RSV-S, RSV-L) or *HPRT* (SRT) (n= 5-8 per group). (B) Representative immunoblots of mitochondrial proteins in SKM (n= 6 per group). (C) Representative pictures of nicotinamide adenine dinucleotide (NADH) stainings of SKM sections (n= 3 per group). (D) Representative electron micrographs from SKM. Bar graph shows average mitochondrial number per image normalized to HFD CTRL, and expressed in arbitrary units (a.u.) (n= 3 per group). Error bars represent mean ±SEM. Significant differences (p value < 0.05) between CTRL and PGC-1α MKO mice are indicated by a number sign (#) and between untreated and treated groups by an asterisk (*). Abbreviations not mentioned earlier: ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit 1 *ATP5A;* ubiquinol cytochrome c reductase core protein 2 *Uqcrc2*; succinate dehydrogenase complex, subunit B *SDHB*; eukaryotic elongation factor 2 *eEF2*.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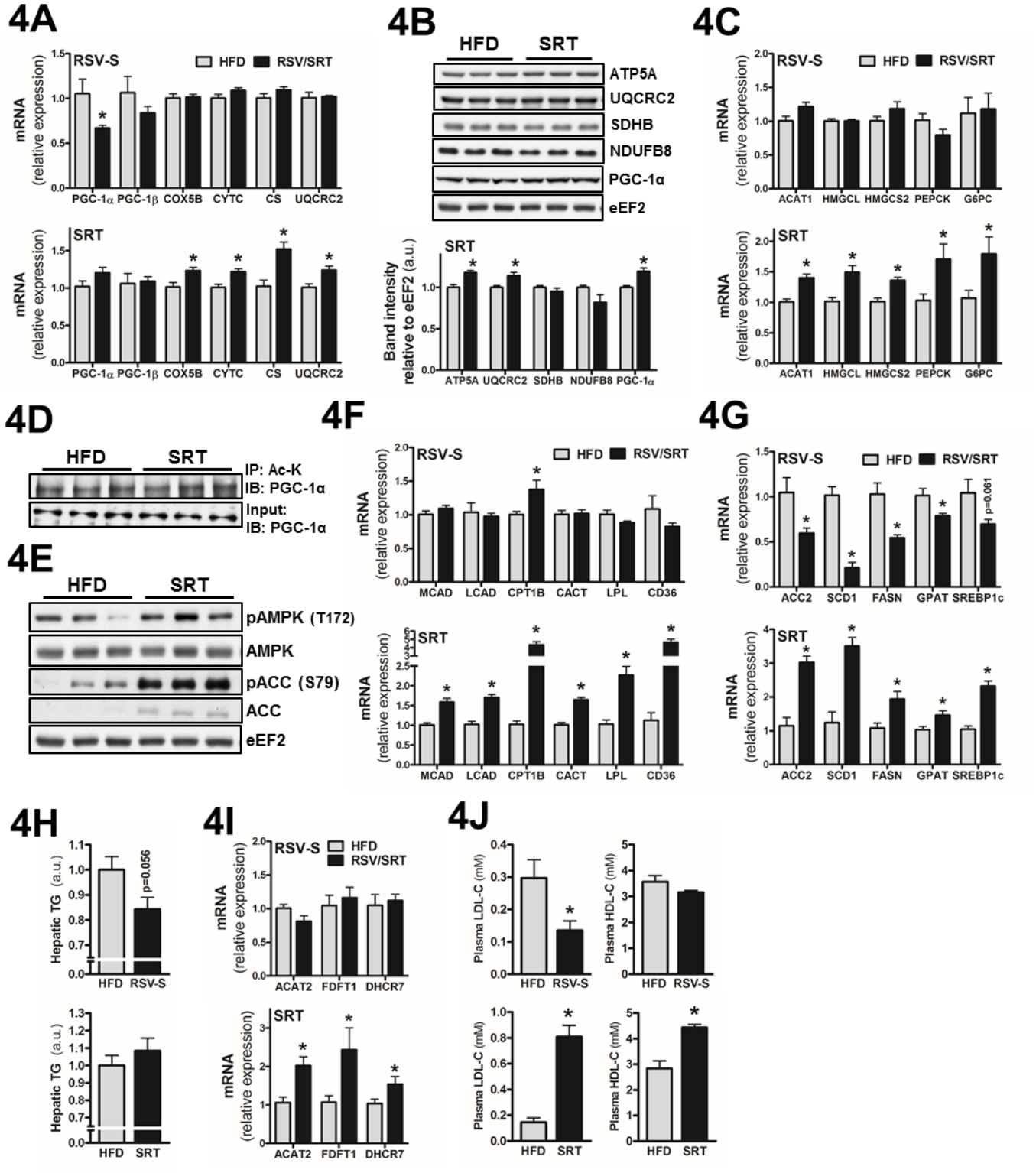
RSV-S
RSV-L
SRT
mRNA (relative expression)
GLUT4
HK2
PKM1
HFD - CTRL
HFD - MKO
RSV/SRT - CTRL
RSV/SRT - MKO
3B
MHC I
MHC IIa
MHC IIb
MHC IIx
3C
CPT1B
MCAD
PDK4
UCP3
3D
HFD CTRL
HFD MKO
RSV-S CTRL
RSV-S MKO
RSV-L CTRL
RSV-L MKO
pAMPK (T172)
AMPK
eEF2
* = unspecific band
Band intensity pAMPK/AMPK (a.u.)
CTRL
MKO
HFD
RSV-S
RSV-L

Figure 3 - RSV and SRT treatment lead to minor transcriptional effects in skeletal muscle

(A-C) mRNA levels of genes involved in (A) glucose uptake and glycolysis, (B) myosin heavy chain (MHC) subtypes and (C) fatty acid (FA) β-oxidation in skeletal muscle (SKM) relative to *PolR2A* (RSV-S, RSV-L) or *HPRT* (SRT) (n= 5-8 per group). (D) Representative immunoblots of AMPK total protein and AMPK phosphorylation (T172) in SKM. Bar graphs show quantification of AMPK phosphorylation relative to total AMPK protein content (n= 6 per group). Error bars represent mean ±SEM. Significant differences (p value < 0.05) between CTRL and PGC-1α MKO mice are indicated by a number sign (#) and between untreated and treated groups by an asterisk (*). Abbreviations not mentioned earlier: pyruvate kinase muscle isozyme 1 *PKM1.*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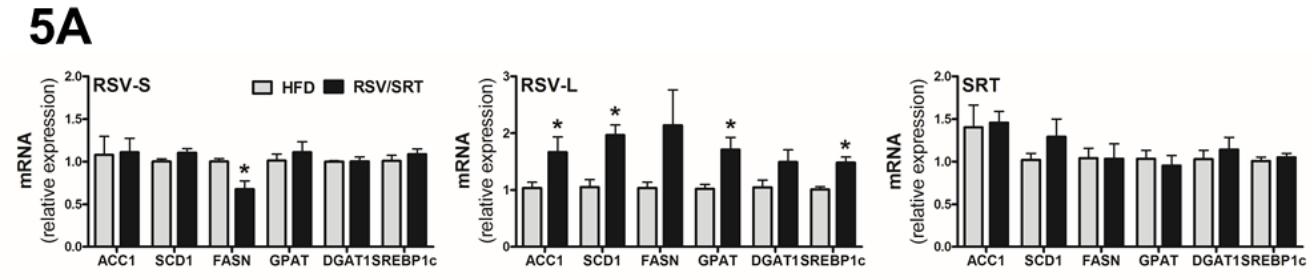
RSV-S
HFD
RSV/SRT
mRNA (relative expression)
PGC-1α PGC-1β COX5B CYTC CS UQCRC2
SRT
4B
HFD
SRT
ATP5A
UQCRC2
SDHB
NDUFB8
PGC-1α
eEF2
Band intensity relative to eEF2 (a.u.)
ATP5A UQCRC2 SDHB NDUFB8 PGC-1α
4C
ACAT1 HMGCL HMGCS2 PEPCK G6PC
4D
IP: Ac-K
IB: PGC-1α
Input:
IB: PGC-1α
4E
pAMPK (T172)
AMPK
pACC (S79)
ACC
eEF2
4F
MCAD LCAD CPT1B CACT LPL CD36
4G
ACC2 SCD1 FASN GPAT SREBP1c
p=0.061
4H
Hepatic TG (a.u.)
p=0.056
HFD RSV-S
HFD SRT
4I
ACAT2 FDFT1 DHCR7
4J
Plasma LDL-C (mM)
Plasma HDL-C (mM)

Figure 4 - RSV and SRT treatment promote differential effects on metabolic processes in the liver

(A) mRNA levels of *PGC-1α/β* and mitochondrial genes in liver, relative to *RPL0* (RSV-S) or *PolR2A* (SRT) (n= 5-8 per group). (B) Representative immunoblots of PGC-1α and mitochondrial proteins in liver. Bar graphs show quantification of PGC-1α and mitochondrial protein content relative to eEF2 (n=6 per group). (C) mRNA levels of genes involved in ketogenesis and gluconeogenesis in liver, relative to *RPL0* (RSV-S) or *PolR2A* (SRT) (n= 5-8 per group). (D) Immunoprecipitation of acetylated lysines from hepatic nuclear extracts, followed by immunoblotting against PGC-1α. For the input, an immunoblot against PGC-1α from hepatic nuclear extracts is shown (n= 3 per group). (E) Representative immunoblot of AMPK total protein and AMPK phosphorylation (T172), as well as ACC total protein and ACC phosphorylation (S79) in liver (n=6 per group). (F-G) mRNA levels of genes involved in (F) fatty acid β-oxidation, fatty acid uptake and (G) lipogenesis in liver, relative to *RPL0* (RSV-S) or *PolR2A* (SRT) (n= 5-8 per group). (H) Triglyceride content in liver expressed in arbitrary units (a.u.), relative to high fat diet (HFD) fed groups (n= 5-8 per group). (I) mRNA levels of genes involved in cholesterol biogenesis in liver, relative to *RPL0* (RSV-S) or *PolR2A* (SRT) (n= 5-8 per group). (J) LDL- and HDL cholesterol levels measured in plasma (n=8-10 per group). Error bars represent mean ±SEM. Significant differences (p value < 0.05) between untreated and treated groups are indicated by an asterisk (*).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mRNA (relative expression)
ACC1
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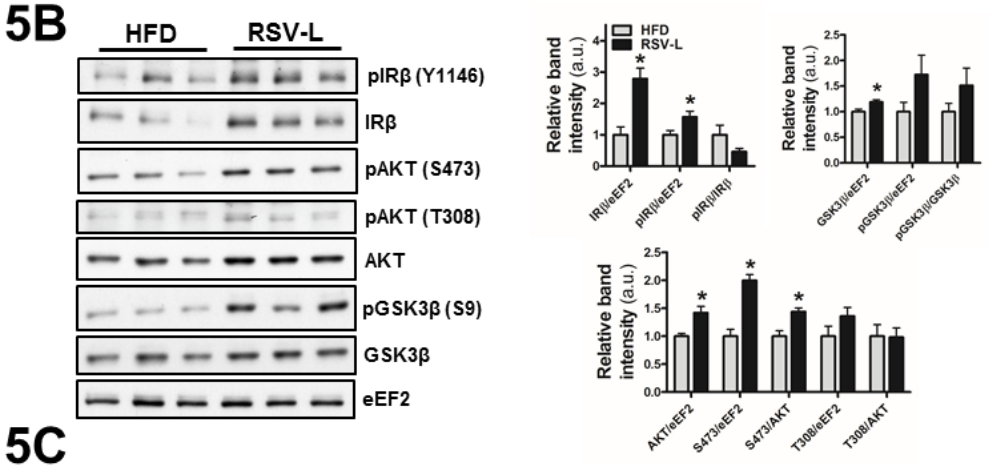
SRT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RSV-L
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IRβ
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pAKT (T308)
AKT
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eEF2
Relative band intensity (a.u.)
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pIRβ/eEF2
pIRβ/IRβ
GSK3β/eEF2
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S473/eEF2
S473/AKT
T308/eEF2
T308/AKT

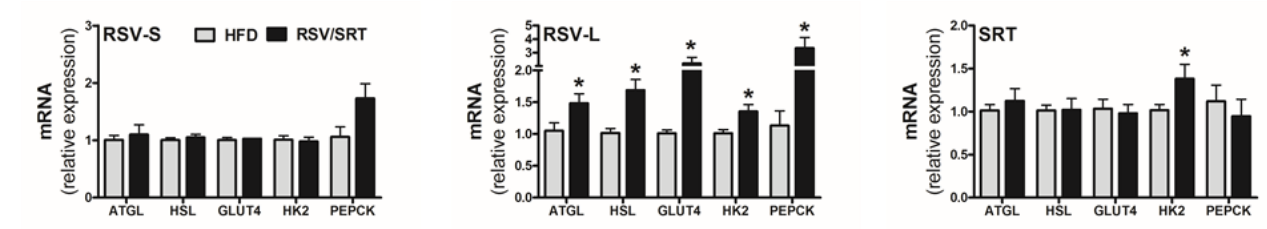

5C
RSV-S
HFD
RSV/SRT
mRNA (relative expression)
ATGL
HSL
GLUT4
HK2
PEPCK
RSV-L
SRT

Figure 5 - Long-term RSV treatment induces genes involved in lipogenesis and lipolysis in white adipose tissue

(A) mRNA levels of genes involved in lipogenesis in white adipose tissue (WAT), relative to *PolR2A* (RSV-S, RSV-L) or *HPRT* (SRT) (n= 5-8 per group). (B) Representative immunoblots of IRβ, Akt and GSK3β as well as their respective phosphorylations in WAT. Bar graphs to the right represents quantification of IRβ, Akt and GSK3β protein levels as well as their respective phosphorylations relative to eEF2, as well as phosphorylation relative to total protein level in white adipose tissue (WAT) (n=6 per group). (C) mRNA levels of genes involved in lipolysis and glycerol-3-phosphate generation in WAT, relative to *PolR2A* (RSV-S, RSV-L) or *HPRT* (SRT) (n= 5-8 per group). Error bars represent mean ±SEM. Significant differences (p value < 0.05) between untreated and treated groups are indicated by an asterisk (*).

7. Manuscript 2: Modulation of Fat and Liver Metabolism Mediates Systemic Effects of Resveratrol and SRT1720 Independently of Skeletal Muscle PGC-1α

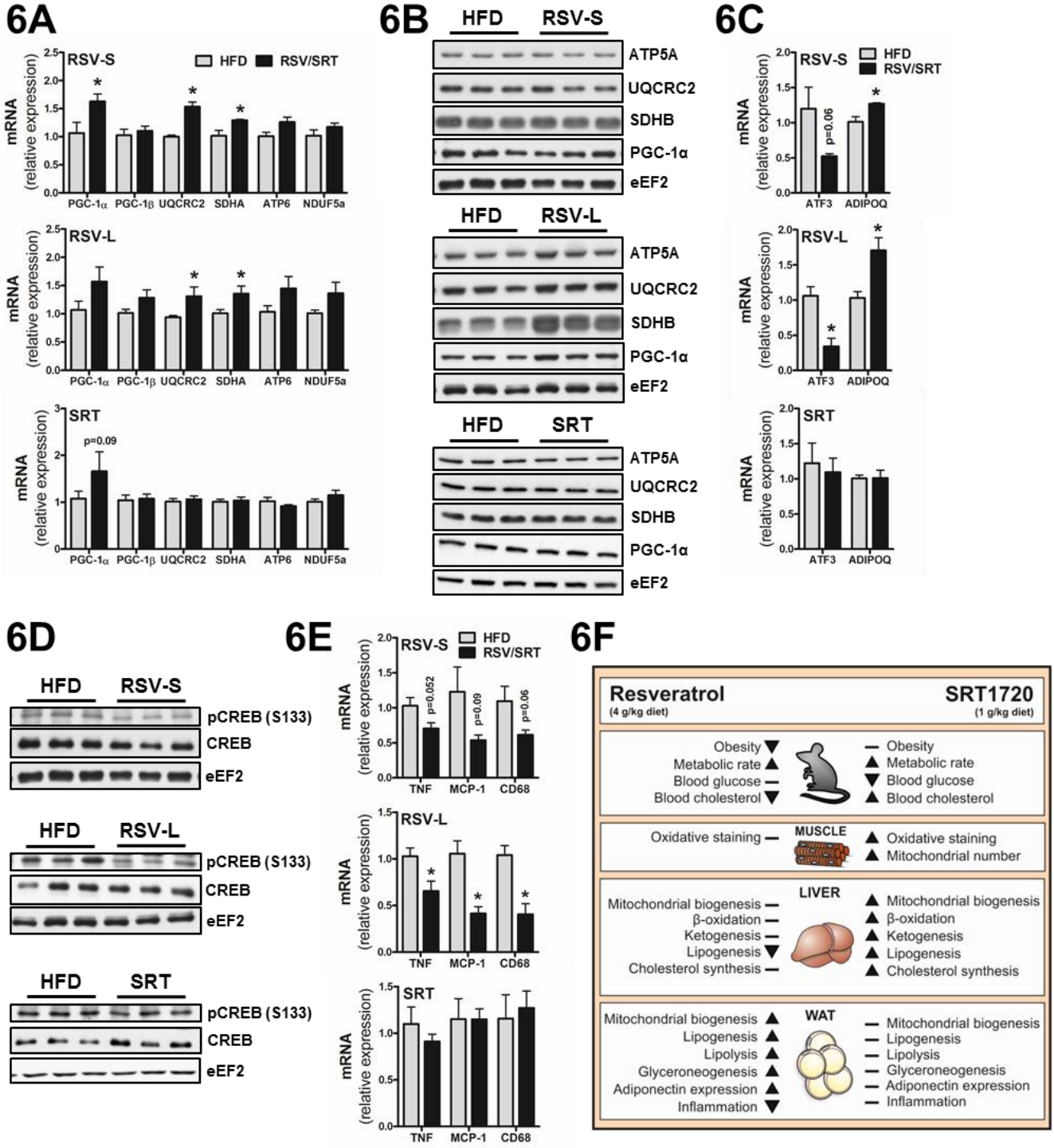
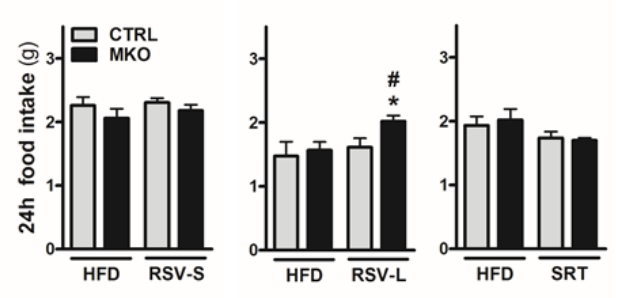


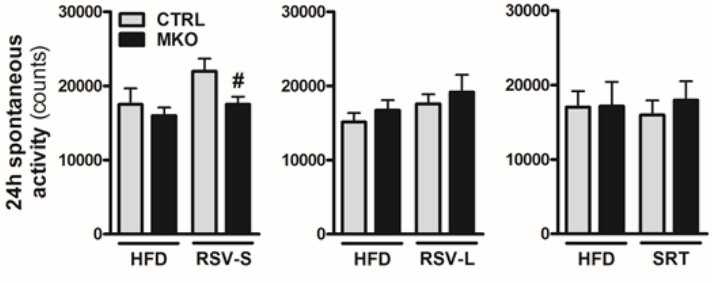
Figure 6 - RSV improves mitochondrial biogenesis and reduces inflammation in white adipose tissue

(A) mRNA levels of *PGC-1α/β* and mitochondrial genes in white adipose tissue (WAT), relative to *PolR2A* (RSV-S, RSV-L) or *HPRT* (SRT) (n= 5-8 per group). (B) Representative immunoblots of PGC-1α and mitochondrial proteins in WAT (n=6 per group). (C) mRNA levels of *ATF3* and *Adipoq* in WAT, relative to *PolR2A* (RSV-S, RSV-L) or *HPRT* (SRT) (n= 5-8 per group). (D) Representative immunoblots of CREB total protein and CREB phosphorylation (S133) in WAT (n=6 per group). (E) mRNA levels of genes associated with inflammation in WAT, relative to *PolR2A* (RSV-S, RSV-L) or *HPRT* (SRT) (n= 5-8 per group). Error bars represent mean ±SEM. Significant differences (p value < 0.05) between untreated and treated groups are indicated by an asterisk (*). (F) Schematic representation of the effects of resveratrol and SRT1720 on whole body level, as well as relevant gene programs affected in skeletal muscle, liver and white adipose tissue. Abbreviations not mentioned earlier: ATP synthase F0 subunit 6 *ATP6.*

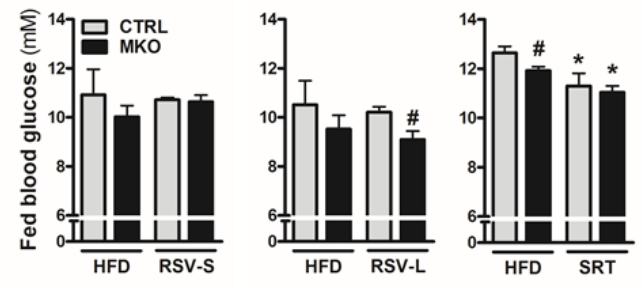
S1A


CTRL
MKO
24h food intake (g)
HFD
RSV-S
HFD
RSV-L
HFD
SRT

S1B


CTRL
MKO
24h spontaneous activity (counts)
HFD
RSV-S
HFD
RSV-L
HFD
SRT

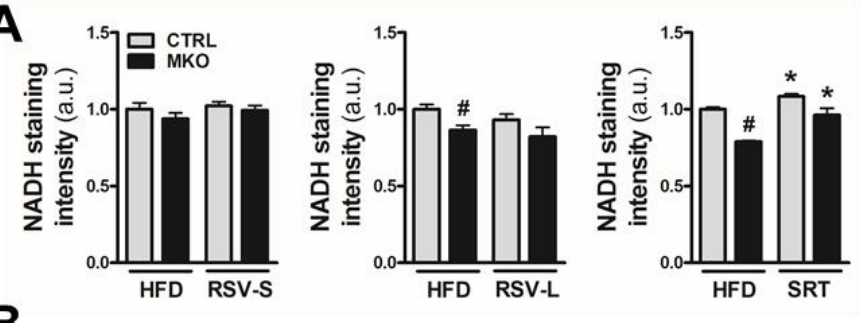
S1C


CTRL
MKO
Fed blood glucose (mM)
HFD
RSV-S
HFD
RSV-L
HFD
SRT

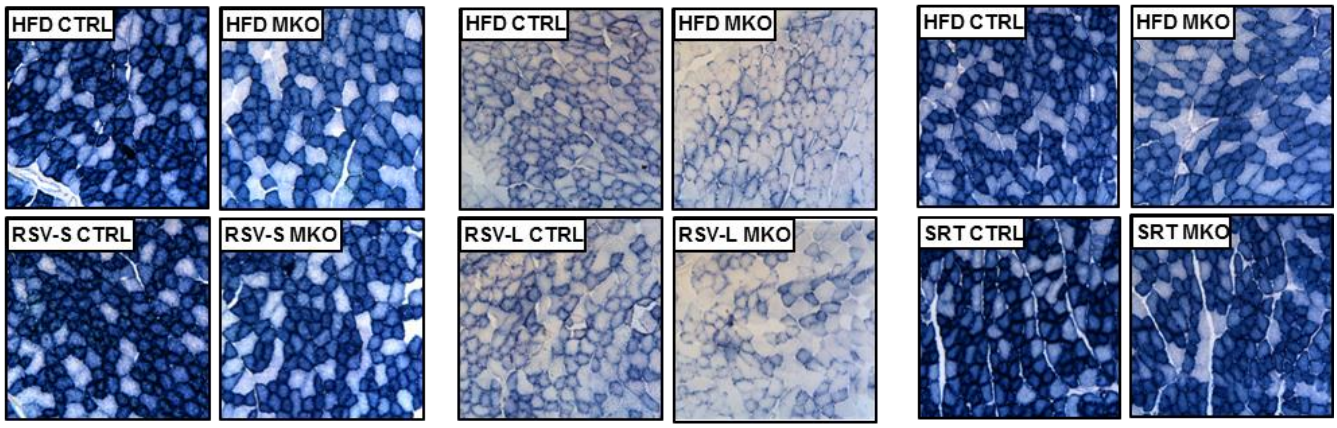
SI Figure 1

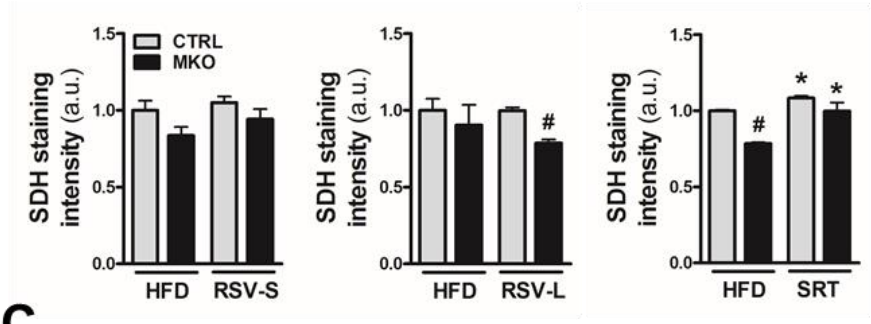
CTRL and PGC-1α MKO mice were fed a high fat diet (HFD) for 2 weeks, followed by resveratrol (RSV) treatment (4 g/kg diet) for 4 weeks (RSV-S), 13 weeks (RSV-L) or SRT1720 administration (1 g/kg diet) for 4 weeks (SRT). Average (A) food intake and (B) spontaneous ambulatory activity over 24 hours (n=5-9 per group). (C) Blood glucose measured in tail vein blood from ad libitum fed mice (n=8-10 per group). Error bars represent mean ±SEM. Significant differences (p value < 0.05) between CTRL and PGC-1α MKO mice are indicated by a number sign (#) and between untreated and treated groups by an asterisk (*).

S2A


CTRL
MKO
NADH staining intensity (a.u.)
1.5
1.0
0.5
0.0
#
*
HFD
RSV-S
RSV-L
SRT

S2B


HFD CTRL
HFD MKO
RSV-S CTRL
RSV-S MKO
RSV-L CTRL
RSV-L MKO
SRT CTRL
SRT MKO


CTRL
MKO
SDH staining intensity (a.u.)
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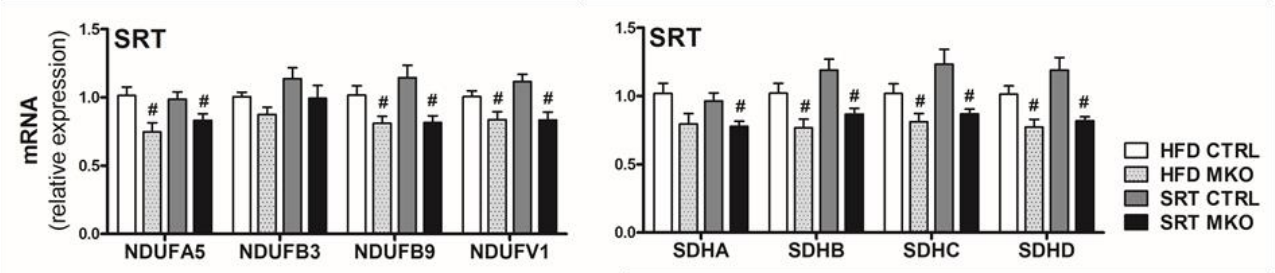
1.0
0.5
0.0
#
*
HFD
RSV-S
RSV-L
SRT

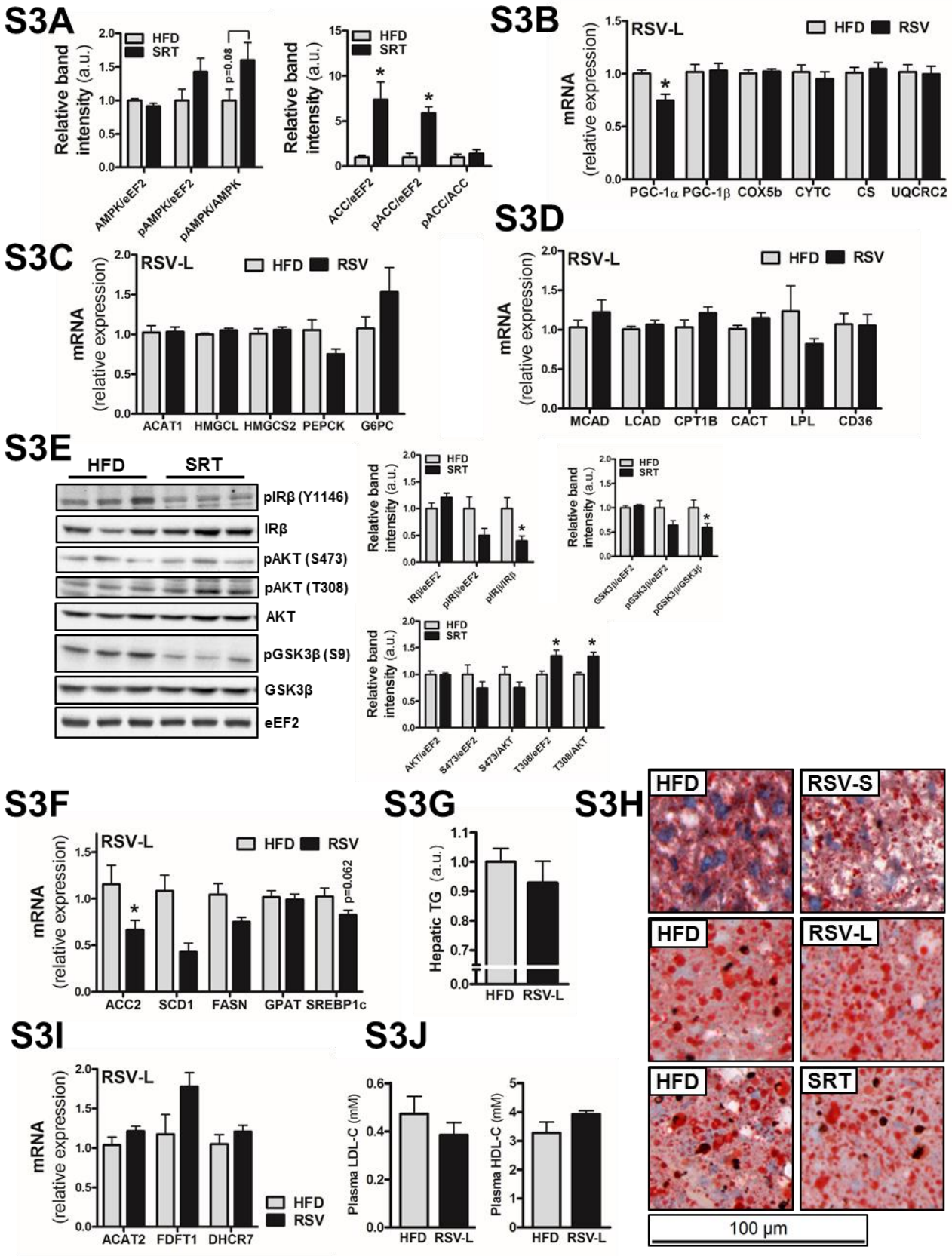
S2C


SRT
mRNA (relative expression)
1.5
1.0
0.5
0.0
#
NDUFA5
NDUFB3
NDUFB9
NDUFV1
SDHA
SDHB
SDHC
SDHD
HFD CTRL
HFD MKO
SRT CTRL
SRT MKO

SI Figure 2

(A) Quantification of nicotinamide adenine dinucleotide (NADH) stainings of skeletal muscle (SKM) sections, expressed in arbitrary units (a.u.) normalized to HFD fed CTRL mice (n=3 per group). (B) Representative pictures of enzymatic succinic dehydrogenase (SDH) stainings of SKM sections. Bar graphs show quantification of SDH staining, expressed in arbitrary units (a.u.) normalized to HFD fed CTRL mice (n=3 per group). (C) mRNA levels of mitochondrial genes in SKM relative to *HPRT* (n=5-8 per group). Error bars represent mean ±SEM. Significant differences (p value < 0.05) between CTRL and PGC-1α MKO mice are indicated by a number sign (#) and between untreated and treated groups by an asterisk (*). Abbreviations not mentioned earlier: NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 5 *Ndufa5*; NADH Dehydrogenase (Ubiquinone) 1 Beta Subcomplex, 3 *Ndufb3*; NADH Dehydrogenase (Ubiquinone) 1 Beta Subcomplex, 9 *Ndufb9*; NADH Dehydrogenase (Ubiquinone) Flavoprotein 1 *Ndufv1*.

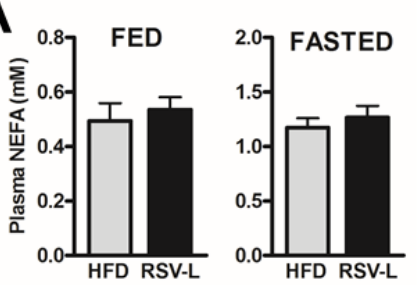
7. Manuscript 2: Modulation of Fat and Liver Metabolism Mediates Systemic Effects of Resveratrol and SRT1720 Independently of Skeletal Muscle PGC-1α



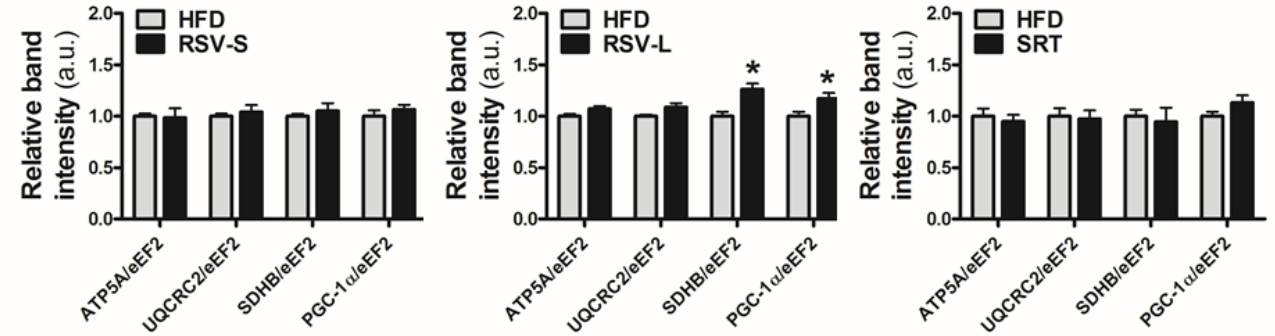
SI Figure 3

(A) Quantification of immunoblots of AMPK and ACC and their respective phosphorylations relative to eEF2, as well as phosphorylation relative to total protein level of AMPK and ACC in liver (n=6 per group). (B) mRNA levels of *PGC-1α/β* and mitochondrial genes in liver, relative to *PolR2A* (n=8 per group). (C) mRNA levels of genes involved in ketogenesis and gluconeogenesis in liver, relative to *PolR2A* (n=8 per group). (D) mRNA levels of genes involved in fatty acid β-oxidation and fatty acid uptake in liver, relative to *PolR2A* (n=8 per group). (E) Representative immunoblot of IRβ, Akt and GSK3β as well as their respective phosphorylations. Bar graphs show quantification of total protein level and phosphorylation relative to eEF2, as well as phosphorylation relative to total protein level of IRβ, Akt and GSK3β (n=6 per group). (F) mRNA levels of genes involved in de novo lipogenesis in liver relative to *PolR2A* (n=8 per group). (G) Triglyceride content in liver expressed in arbitrary units (a.u.), relative to high fat diet (HFD) fed group (n=8 per group). (H) Representative pictures of Oil Red O staining in liver sections (n=5 per group). (I) mRNA levels of genes involved in cholesterol biogenesis in liver, relative to *PolR2A* (n=8 per group). (J) LDL- and HDL cholesterol levels measured in plasma (n=8 per group). Error bars represent mean ±SEM. Significant differences (p value < 0.05) between untreated and treated groups are indicated by an asterisk (*).

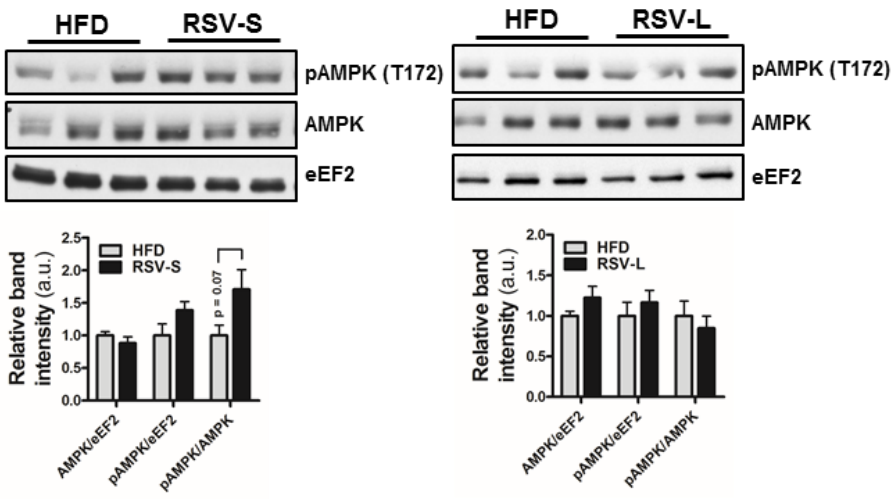
S4A



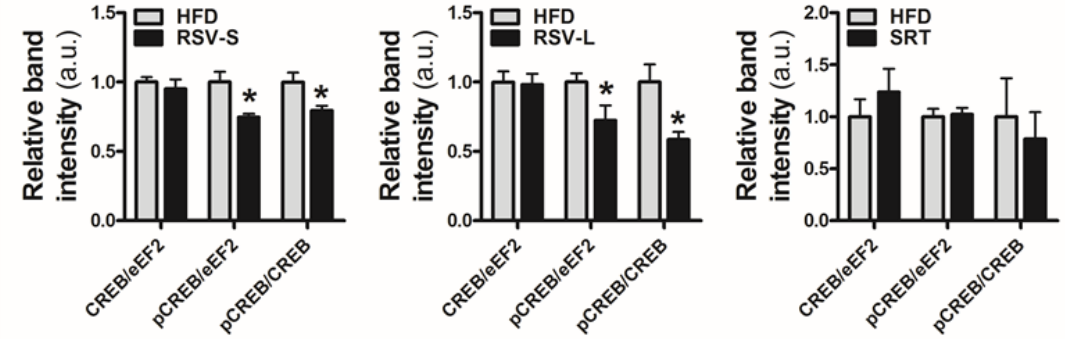
S4B



S4C



S4D



SI Figure 4

(A) Fasted and fed non-esterified fatty acid (NEFA) levels in plasma (n=8 per group). (B) Quantification of PGC-1α and mitochondrial protein content relative to eEF2 in immunoblots from white adipose tissue (WAT) (n=6 per group). (C) Representative immunoblots of AMPK total protein and AMPK phosphorylation (T172) in WAT. Bar graphs show quantification of AMPK protein levels and AMPK phosphorylation (T172) relative to eEF2, as well as AMPK phosphorylation relative to total AMPK protein content in WAT (n=6 per group). (D) Quantification of CREB and CREB phosphorylation (S133) relative to eEF2, as well as CREB phosphorylation relative to total CREB protein content in WAT (n=6 per group). Error bars represent mean ±SEM. Significant differences (p value < 0.05) between untreated and treated groups are indicated by an asterisk (*).

Table S1 - qPCR primer sequences

Target gene	Forward primer	Reverse primer
ACAT1	GTGAAGGAAGTCTACATGGGCA	TGTGGTGCATGGAGTGGAAATA
ACAT2	ATTCAAAACATGGGGATTCGGC	TCAATGGGAAACCGAGAGACAG
ACC1	AAGGCTATGTGAAGGATG	CTGTCTGAAGAGGTTAGG
ACC2	CTTGCTTCTCTTTCTGACTTG	GGCTTCCACCTTACTGTTG
ADIPOQ	AAGAAGGACAAGGCCGTTCTC	GCTATGGGTAGTTGCAGTCAG
ATF3	CTGGCTTCCTGTGCACTTCTA	GGTCAATGCAGTAGGTCACCA
ATGL	CACTTTAGCTCCAAGGATGA	TGGTTCAGTAGGCCATTCCT
ATP6	ACTATGAGCTGGAGCCGTAATTACA	TGGAAGGAAGTGGGCAAGTG
CACT	CTGCGCCCATCATTGGA	CAGACCAAACCCAAAGAAGCA
CD36	GGCAAAGAACAGCAGCAAAAT	TGGCTAGATAACGAACTCTGTATGTGT
CD68	CAGCTCCAAGCCCAAATTCAAA	GATATGCCCCAAGCCCTCTTTA
COX5B	CTTCAGGCACCAAGGAAGAC	TTCACAGATGCAGCCCACTA
CPT1B	ATCATGTATCGCCGCAAACT	CCATCTGGTAGGAGCACATGG
CS	CCCAGGATACGGTCATGCA	GCAAACTCTCGCTGACAGGAA
CYTC	AAATCTCCACGGTCTGTTCG	TATCCTCTCCCCAGGTGATG
DGAT1	TGGTGTGTGGTGATGCTGATC	GCCAGGCGCTTCTCAA
DHCR7	AGGCTGGATCTCAAGGACAATG	CAATGAATGGAGCGAAGAGCAG
FASN	GCTGCGGAAACTTCAGGAAAT	AGAGACGTGTCACTCCTGGACTT
FDFT1	GTTCGTCAAGTGTCTAGGCCA	GTGAGTCCTGGTCCATCTTGG
G6PC	GTGCTTGCATTCCTGTATGGTA	TAGGCTGAGGAGGAGAAAACTG
GLUT4	CATGGCTGTCGCTGGTTTC	AAACCCATGCCGACAATGA
GPAT	TGACAGTTGGCACAATAGAC	CGGCTCATAAGGCTCTCC
HK2	AAAACCAAGTGCAGAAGGTTGAC	GAACCGCCTAGAAATCTCCAGAA
HMGCL	CCAGCTTTGTTTCTCCCAAGTG	GATGCCGGGAAACTTCTGAATG
HMGCS2	CCACAAGGTGAACTTCTCTCCA	TGCATCTCATCCACTCGTTCAA
HPRT	ATGCCGAGGATTTGGAAAAAGTG	TGACATCTCGAGCAAGTCTTTCA
HSL	CACAGACCTCTAAATCCCACGAG	AGGGAGTAGTCGATGGAGAAGAT
LCAD	CCAGCTAATGCCTTACTTGGAGA	GCAATTAAGAGCCTTTCCTGTGG
LPL	AAGCAGCAAGATGTACCTGAAGA	CATCCTCAGTCCCAGAAAAGTGA
MCAD	AACACTTACTATGCCTCGATTGCA	CCATAGCCTCCGAAAATCTGAA
MCP-1	AGGTGTCCCAAAGAAGCTGTAG	TGTCTGGACCCATTCCTTCTTG
MHC I	CCTCCTCACATCTTCTCCATCTCT	TGGACTGATTCTCCCGATCTG
MHCIIa	CAACCTCAAAGAGCGTTATGCA	AGGGTTGACGGTGACACAGAA
MHCIIb	CAACCCATATGACTTTGCTTACGT	TCCCAGGATATCAACAGCAGTGT
MHCIIx	GGCCCCACCCCACATC	CTCCCGATCTGTCAGCATGA
NDUF5A	ACATGCAGCCTATAGAAAATACACAGA	TCCGCCTTGACCATATCCA
NDUFB3	TAC CAC AAA CGC AGC AAA CC	AAG GGA CGC CAT TAG AAA CG
NDUFB8	CAAGAAGTATAACATGCGAGTGGAA	CCATACCCCATGCCATCATC

NDUFB9	CATGGTGTATCCACAGGGACAA	GCTGGGTAGCCCTCATCATATC
NDUFV1	CTTCCCCACTGGCCTCAAG	CCAAAACCCAGTGATCCAGC
PDK4	AAAATTTCCAGGCCAACCAA	CGAAGAGCATGTGGTGAAGGT
PEPCK	CTTCTCTGCCAAGGTCATCC	TTTTGGGGATGGGCAC
PGC-1α	AGCCGTGACCACTGACAACGAG	GCTGCATGGTTCTGAGTGCTAAG
PGC-1β	CCATGCTGTTGATGTTCCAC	GACGACTGACAGCACTTGGA
PKM1	CATTATCGTGCTCACCAAGTCTG	GATTTCGAGTCACGGCAATGATA
POLR2A	AATCCGCATCATGAACAGTG	CAGCATGTTGGACTCAATGC
RPL0	CTGCTGAACATGCTGAACATCTC	CTTCAGGGTTATAAATGCTGCCG
SCD1	GTCAGGAGGGCAGGTTTC	GAGCGTGGACTTCGGTTC
SDHA	GCTGGTGTGGATGTCACTAAGG	CCCACCCATGTTGTAATGCA
SDHB	TGACGTCAGGAGCCAAAATGG	CCTCGACAGGCCTGAAACTG
SDHC	AGTTCAAACCGTCCTCTGTCTC	CCTCCACTCAAGGCTATTCCAG
SDHD	TTCTCTTAAAGCTGGGCGTTCT	GAAATGCTGACACATAAGCGGG
SREBP1C	GGAGCCATGGATTGCACATT	GGCCCGGGAAGTCACTGT
TNF	GCCTATGTCTCAGCCTCTTCTC	TGGGAACTTCTCATCCCTTTGG
UCP3	TTTTGCGGACCTCCTCACTT	TGGATCTGCAGACGGACCTT
UQCRC2	CCCATCTTGCTTTGCTGTCTG	AATAAAATCTCGAGAAGGACCCG

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Manuscript 3

A Novel Role for PGC-1α in the Regulation of Skeletal Muscle Ketolytic Capacity and Systemic Ketone Body Homeostasis

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8. Manuscript 3: A Novel Role for PGC-1α in the Regulation of Skeletal Muscle Ketolytic Capacity and Systemic Ketone Body Homeostasis

Abstract

In response to a reduced nutrient intake, the body readily mobilizes its energy stores to maintain systemic energy homeostasis. In a low-carbohydrate environment, such as during fasting or adherence to a high-fat/low-carbohydrate diet, ketone bodies become an important energy substrate in extra-hepatic organs. This is especially pertinent in organs with a high energy demand such as brain, kidney, heart and skeletal muscle. Here we show that the transcriptional coactivator peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC-1α) is an important transcriptional regulator of ketolytic enzymes in these organs. Consequently, overexpression or knockout of PGC-1α specifically in skeletal muscle is sufficient to modulate systemic ketone body homeostasis in response to fasting, high-fat/low-carbohydrate diet feeding and exercise. PGC-1α is furthermore necessary for the transcriptional induction of ketolytic enzymes and improved ketolytic capacity in response to endurance exercise training. Importantly, overexpression of PGC-1α was sufficient to ameliorate diabetic hyperketonemia in mice. These results demonstrate a novel role of skeletal muscle PGC-1α as a transcriptional regulator of systemic ketolytic capacity, and a potential therapeutic target to ameliorate diabetic ketoacidosis.

8. Manuscript 3: A Novel Role for PGC-1α in the Regulation of Skeletal Muscle Ketolytic Capacity and Systemic Ketone Body Homeostasis

Introduction

To maintain energy homeostasis, the body has developed several mechanisms to adjust for variations in nutrient availability. During nutrient-deprivation, such as fasting, the body can mobilize endogenous energy stores to compensate for a reduced energy intake. Moreover, this is accompanied by a switch in fuel-preference in many organs. During prolonged starvation, ketone bodies become an important alternative fuel to help maintain energy homeostasis. **[Cahill 2006]**. This is especially important in tissues with a high energy demand, such as kidney, heart, brain and skeletal muscle **[Robinson and Williamson 1980, Newman and Verdin 2014]**. The collective term ketone body encompasses β-hydroxybutyrate (βOHB), acetoacetate (AcAc) and acetone. βOHB and AcAc are short-chain derivatives of acetyl-CoA, almost exclusively produced in the liver through oxidation of fatty acids and subsequent ketogenesis, while acetone is formed through spontaneous degradation of AcAc **[Robinson and Williamson 1980, Newman and Verdin 2014]**. Hepatic ketogenesis is induced during states of reduced carbohydrate intake, such as fasting **[Cahill 2006]** and low-carbohydrate ketogenic diets (LCKD) **[Wheless 2008]** but also after exercise **[Johnson et al. 1969]**. The ketogenic rate of the liver is dictated by the availability of circulating fatty acids and the intracellular redox status, but also through direct hormonal control by insulin and glucagon **[Laffel 1999, Newman and Verdin 2014]**. In extra-hepatic organs, βOHB and AcAc are oxidized to acetyl-CoA through an enzymatic process termed ketolysis, and subsequently oxidized in the TCA-cycle. Ketolysis occurs in the mitochondrial matrix and is catalyzed by 3-hydroxybutyrate dehydrogenase, type 1 (BDH1), succinyl-CoA:3-ketoacid-coenzyme A transferase 1 (SCOT; OXCT1) and acetyl-CoA acetyltransferase 1 (ACAT1) **[Robinson and Williamson 1980, Newman and Verdin 2014]**. Mutations within genes encoding these enzymes are associated with exacerbated ketosis during starvation in humans **[Saudubray et al. 1987, Fukao et al. 2011]** and whole-body knockout of the rate-limiting enzyme OXCT1 leads to severe hyperketonemia and lethality in mice **[Cotter et al. 2011]**. Hyperketonemia is also a common complication in diabetic patients and can lead to severe and possibly lethal ketoacidosis **[Laffel 1999]**. Diabetic ketoacidosis has been attributed to an exacerbated lipolysis, coupled to increased hepatic ketogenesis **[McGarry and Foster 1980, Laffel 1999]**. However, there is also evidence for reduced ketone body uptake **[Ikeda et al. 1991, Okuda et al. 1991]** and impaired OXCT1 activity **[Grinblat et al. 1986, Turko et al. 2001]** in heart and skeletal muscle of diabetic rodents. These data suggest a possible involvement of impaired ketolysis in the etiology of diabetic ketoacidosis. However, relatively little is known about how ketolytic enzymes are regulated **[Newman and Verdin 2014]**. An important metabolic

regulator in oxidative organs is the transcriptional coactivator peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC-1α) **[Lin et al. 2005]**. PGC-1α is known to control several gene programs associated with mitochondrial and metabolic processes in oxidative organs **[Lin et al. 2005]**. Moreover, PGC-1α has also been shown to regulate transcription of the main lactate and ketone body transporter monocarboxylate transporter 1 (MCT1) in skeletal muscle **[Summermatter et al. 2013a]**. Interestingly, exercise training, a stimulus known to increase PGC-1α activity in muscle **[Baar et al. 2002, Pilegaard et al. 2003, Mathai et al. 2008]**, enhances ketolytic capacity in skeletal muscle of both healthy and diabetic rodents **[Adams and Koeslag 1988, El Midaoui et al. 2005]**. PGC-1α might therefore be involved in the regulation of ketone body oxidation. In this study, we demonstrate that PGC-1α is an important transcriptional regulator of ketolytic enzymes. Moreover, we show that modulation of PGC-1α in skeletal muscle affects systemic ketosis in response to various stimuli such as fasting, LCKD feeding and exercise. In line with this, muscle-specific overexpression of PGC-1α was sufficient to reduce hyperketonemia in diabetic mice, and would thus constitute a novel therapeutic target to ameliorate diabetic ketoacidosis.

Material and methods

Animals and diets **-** Animals were housed in a conventional facility with a 12-h light/12-h dark cycle with free access to food and water. All experiments were performed in accordance with federal guidelines and were approved by the Kantonales Veterinäramt of Kanton Basel-Stadt. PGC-1α muscle specific knockout mice (mKO), PGC-1α muscle specific transgenic mice (mTG) and whole-body PGC-1α-knockout mice (gKO) used in this study have been described elsewhere **[Lin et al. 2002, Lin et al. 2004, Perez-Schindler et al. 2013]**. The diets used for this study were either a chow diet (CHOW) (AIN-93G, Provimi Kliba Nafag AG) containing 7% fat, 58.5% carbohydrates and 18% proteins, or a low-carbohydrate ketogenic diet (LCKD) (XL75:XP10, Provimi Kliba Nafag AG), containing 74.4% fat, 3% carbohydrates and 9.9% proteins.

Blood analysis **-** Blood glucose and blood βOHB were measured in a tail vein blood sample using either a handheld glucose meter (Accu-Chek, Roche) or βOHB-meter (Precision Xtra, Abbott). For non-esterified fatty acid (NEFA) levels, whole tail-vein blood was collected in Microvette tubes (Sarstedt) and plasma was isolated. NEFA levels in plasma were determined using a colorimetric commercial test (HR Series NEFA-HR(2); Wako Diagnostics) according to manufacturer's instructions. Plasma acetoacetate (AcAc)

levels were determined using a colorimetric commercial test (Acetoacetate Assay Kit; Abcam) according to manufacturer's instructions.

βOHB tolerance test **-** Animals were fasted for 5 hours in the morning, and then received a bolus intraperitoneal (I.P.) injection of 1.5 g/kg bodyweight Na-βOHB (Sigma). Blood βOHB levels were consecutively measured in tail vein blood at 0, 15, 30, 45, 60 and 90 minutes after injection.

Acute and chronic exercise **-** Chronic exercise training was performed by allowing mice free access to running wheels (Columbus instruments) in their home cages, starting from the age of 10-12 weeks, and lasting for a total of 8 weeks. Sedentary control groups were housed under similar conditions in cages without running wheels. Twenty hours prior to sacrifice, mice were moved from their wheel cages to cages without running wheels. For post-exercise ketosis tests, animals were acclimatized to treadmill running 2 days before the start of the experiment, for 5 minutes at 8 meters/minute (m/m) followed by 5 minutes at 10 m/m, at an incline of 5°. For the actual experiment, treadmill (Columbus instruments) was kept at an incline of 5°. The program started at 5 m/m for 5 minutes, followed by 8 m/m for 10 minutes. The speed of the treadmill was then increased by 2 m/m every 15 minutes. Before the run, basal blood βOHB and glucose of the mice was assessed in a tail-vein blood sample. Mice were removed from the treadmill after 80 minutes of running, before any of the mice reached terminal exhaustion. Blood βOHB and glucose levels were consecutively measured in tail vein blood at 0, 30 and 180 minutes after termination of treadmill running.

Streptozotocin-administration **–** Mice were fasted for 10 hours previous to injection, and subsequently injected with Streptozotocin (STZ) (Sigma) I.P. at a dose of 150 mg/kg. 10% sucrose was administered to the mice during the first 24 hours after injection. Successful induction of hyperglycemia was confirmed at 3 days after injection. Blood βOHB and glucose levels were measured in tail vein blood at day 5, and mice were sacrificed for organ collection.

In vitro experiments **-** C2C12 myoblasts were grown to confluence in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal calf serum (FCS) and 1% penicillin/streptomycin (P/S). Confluent myoblasts were differentiated into myotubes through addition of DMEM containing 2% horse serum and 1% P/S. All subsequent experiments were conducted on myotubes that were differentiated for 4 days. For overexpression of PGC-1α, myoblasts were transduced with adenovirus containing either GFP-PGC-1α or GFP (control). After 24 hours, virus was removed, and fresh differentiation medium was added. 48 hours

after initial infection, cells were washed with PBS and harvested on ice. For inhibition of ERRα, myotubes were co-incubated with 10 µM XCT-790 (Sigma) or 0.2% DMSO (vehicle) for 48 hours. Three independent experiments were performed in triplicates.

RNA extraction and qRT-PCR **-** Frozen tissue or cells were homogenized and total RNA was extracted using TRIzol reagent (Invitrogen). RNA concentration was adjusted and cDNA synthesis was performed using 1 ug of total RNA. Semi-quantitative Real-time PCR analysis was performed using Fast SYBR Green master mix (Applied Biosystems) on a StepOnePlus Real-Time PCR System (Applied Biosystems). Relative expression levels for each gene of interest were calculated with the $\Delta\Delta C_t$ method, using 18s as normalization control.

Immunoblotting **-** Tissues were homogenized in RIPA buffer, and equal amounts of proteins were separated on SDS-PAGE under reducing conditions, and transferred to a nitrocellulose membrane (Whatman). Proteins of interest were detected using the following antibodies: OXCT1 (ab105320; Abcam), ACAT1 (HPA004428; Sigma), eEF2 (2332; Cell signaling), Polyclonal Swine Anti-Rabbit Immunoglobulins/HRP (P0399, Dako), Polyclonal Rabbit Anti-Mouse Immunoglobulins/HRP (P0260, Dako). Densitometric analysis of immunoblots was performed on 6 – 7 individual samples using Image-J software, and a representative selection from this group is presented in each figure.

Statistical analysis - All data are presented as means ± SEM. Unpaired student two-tailed t test or one-way ANOVA with Bonferroni's post-hoc test was used to determine differences between groups. Significance was considered with $p<0.05$.

Results

To study the role of PGC-1α in systemic ketone body homeostasis, we used mice with a whole body knockout of PGC-1α (global KO; gKO) and measured circulating βOHB levels after 24 hours of fasting. Fasting induced a state of ketosis in CTRL mice, as indicated by the increased levels of βOHB in blood (FIG 1A). Interestingly, gKO mice displayed a significant hyperketonemia compared to CTRL mice in response to fasting (FIG 1A). To investigate whether this phenotype could be explained by an increased ketone body production, we measured mRNA levels of genes involved in hepatic ketogenesis. We could detect a complete ablation of PGC-1α mRNA in liver in gKO mice (FIG 1B). However, the loss of PGC-1α in liver did

not impair the induction of ketogenic genes, such as *Acat1*, 3-hydroxy-3-methylglutaryl-CoA synthase 2 (*Hmgcs2*) and 3-Hydroxymethyl-3-Methylglutaryl-CoA lyase (*Hmgcl*) with fasting (FIG 1B). This finding is surprising considering the supposed role of PGC-1α as a transcriptional coactivator of hepatic ketogenic genes **[Rhee et al. 2003, Tabata et al. 2014]** but could be connected to the aberrant systemic metabolic phenotype of global PGC-1α knockout mice **[Lin et al. 2004, Leone et al. 2005]**. Nonetheless, changes in hepatic ketogenesis are not likely to explain the fasting-induced hyperketonemia in gKO mice. Interestingly, mice with a global reduction in ketolytic capacity such as whole-body OXCT1 knockout mice **[Cotter et al. 2011]** display increased circulating ketone body levels due to impaired extra-hepatic ketone body oxidation. We therefore hypothesized that impaired ketolysis could account for the fasting-induced hyperketonemia in gKO mice. Hence, we measured expression of PGC-1α and genes important for ketone body oxidation in major ketolytic organs such as kidney (FIG 1C), brain (FIG 1D), heart (FIG 1E) and skeletal muscle (FIG 1F). As observed in liver, gKO mice displayed a complete ablation of PGC-1α mRNA in all ketolytic organs investigated (FIG 1C-F). Interestingly, this was associated with a reduced transcription of the ketolytic genes *Bdh1*, *Oxct1* and *Acat1* in kidney, brain and heart (FIG 1C-E). Hence, PGC-1α is a global transcriptional regulator of ketolytic enzymes. Surprisingly, only *Oxct1* transcription was affected in skeletal muscle of gKO mice, while no significant differences could be detected for *Bdh1* and *Acat1* (FIG 1F).

PGC-1α knockout in skeletal muscle leads to reduced transcription of ketolytic genes

Since skeletal muscle has been demonstrated to contribute significantly to the removal of ketone bodies from the blood after fasting **[Cotter et al. 2013]**, and since *Oxct1* transcription was significantly reduced in skeletal muscle from gKO mice (FIG 1F), we decided to further investigate the role of PGC-1α in the regulation of skeletal muscle ketone body oxidation. We thus utilized muscle-specific PGC-1α knockout (mKO) mice, which allows us to circumvent the aberrant metabolic phenotype associated with global deletion of PGC-1α. PGC-1α mKO mice displayed an ablation of PGC-1α in skeletal muscle (FIG 1G-H) and in contrast to gKO mice (FIG 1F), this was accompanied by reduced transcription of all ketolytic genes (*Bdh1*, *Oxct1*, and *Acat1*) (FIG 1G-H). Importantly, in line with the reduced *Oxct1* and *Acat1* mRNA levels, also protein levels of both OXCT1 and ACAT1 were reduced in gastrocnemius muscle of mKO mice (FIG 1I). Moreover, mRNA levels of the main ketone body transporter *Mct1,* together with mRNA levels of TCA cycle enzymes such as citrate synthase (*Cs*), aconitase 2 (*Aco2*) and isocitrate dehydrogenase [NAD] subunit alpha (*Idh3a*) were decreased in muscle from mKO mice (FIG 1J). Collectively, these data

demonstrate that PGC-1α is important for the transcriptional regulation of ketone body transporters, ketolytic enzymes as well as TCA-cycle enzymes. PGC-1α thus affects transcriptional regulation of the components necessary for terminal oxidation of ketone bodies in skeletal muscle.

PGC-1α muscle-specific knockout mice display fasting-induced hyperketonemia

To investigate whether muscle-specific ablation of PGC-1α could recapitulate the hyperketonemia seen with global ablation of PGC-1α, we fasted the mice for 24 hours and measured circulating βOHB levels in blood. PGC-1α mKO mice displayed a significant hyperketonemia with increased circulating levels of βOHB (FIG 2A) and AcAc (FIG 2B) compared to CTRL mice. To confirm that the increased ketosis in mKO mice was not due to an altered fasting response in liver, we measured transcription of genes important for hepatic ketogenesis. There was no difference between CTRL and mKO mice in the induction of genes involved in hepatic fatty acid uptake (cluster of differentiation 36, *CD36*) β-oxidation (carnitine palmitoyltransferase 1B, *Cpt1b*; long-chain acyl-CoA dehydrogenase, *Lcad*) (FIG 2C) or ketogenesis (*Acat1*, *Hmgcl*, *Bdh1*) (FIG 2D) with fasting. Moreover, circulating levels of non-esterified fatty acids (NEFA) in blood were comparable between CTRL and mKO mice upon fasting (FIG 2E). These findings indicate that neither fatty acid availability nor maladaptation of hepatic ketogenesis in response to fasting could account for the fasting-induced hyperketonemia in mKO mice. To assess systemic ketolytic capacity in mice without contribution from hepatic ketogenesis, we measured βOHB-clearance in blood after a bolus injection of βOHB. In line with the reduced levels of ketolytic enzymes in skeletal muscle, mKO mice had an impaired ability to clear βOHB from the blood, as indicated by the increased retention of βOHB in blood after the injection compared to CTRL mice (FIG 2F). Taken together, these data demonstrate that muscle PGC-1α plays an important role in the regulation of systemic ketone body homeostasis. To further characterize the impact of skeletal muscle-specific ablation of PGC-1α on ketone body homeostasis, we fed CTRL and mKO mice a low-carbohydrate ketogenic diet (LCKD) to induce a chronic state of ketosis. In line with our results from the fasting experiments, also after three weeks on a LCKD, mKO mice displayed elevated βOHB levels in blood compared to CTRL mice (FIG 2G). However, in contrast to fasting induced ketosis, plasma AcAc levels were not significantly altered between CTRL and mKO mice with LCKD feeding (FIG 2H). This effect could however be explained by an increased interconversion between AcAc and βOHB during LCKD feeding. Again, we could not detect any differences in the transcriptional induction of ketogenic genes in liver between CTRL and mKO mice after 3 weeks LCKD feeding (FIG 2I), suggesting that an impaired ketolysis accounts for the LCKD-induced hyperketonemia in mKO mice. Collectively, these

data demonstrate that ablation of PGC-1α in skeletal muscle leads to a reduction in ketolytic enzymes in muscle, and exacerbates systemic hyperketonemia in response to fasting or LCKD feeding.

PGC-1α is necessary for the transcriptional induction of ketolytic genes with exercise

Elevated ketone body levels can also be observed after an acute exercise bout, a phenomenon termed post-exercise ketosis. To investigate whether muscle PGC-1α also affects post-exercise ketosis, CTRL and mKO mice were subjected to an acute exercise bout on a treadmill, and we measured βOHB levels in blood. While no significant difference in βOHB levels were detected between CTRL and mKO mice at either 0 minutes or 30 minutes after exercise cessation, mKO mice displayed an exacerbated post-exercise ketosis compared to CTRL mice at 180 minutes after exercise (FIG 3A). It has been shown that exercise modulates skeletal muscle ketone body metabolism, since muscles from trained rats display enhanced ketolytic enzyme activity **[Askew et al. 1975]**. We were therefore interested in whether PGC-1α was important for exercise-induced changes in ketone body homeostasis in skeletal muscle. To this end, mice were given free access to running wheels in their home cages for 8 consecutive weeks to induce an exercised state. After these 8 weeks, we measured βOHB clearance from the blood following a βOHB bolus injection. Exercised CTRL mice displayed a faster clearance of βOHB from the blood as compared to sedentary CTRL mice (FIG 3B). Interestingly, trained mKO mice did not show any improvement in βOHB clearance from the blood (FIG 3B). Hence, muscle PGC-1α is required to improve ketolytic capacity with exercise. To investigate the molecular changes in muscle that could account for the improved ketolytic capacity, we measured gene expression of ketolytic enzymes. In agreement with the improved βOHB clearance in exercise trained CTRL mice, we observed a significant increase in transcript levels of ketolytic genes (*Bdh1*, *Oxct1*, and *Acat1*) in muscle of trained CTRL mice (FIG 3C). Importantly, mKO mice did not display an induction of these genes in muscle in a trained state (FIG 3C). The induction of *Oxct1* and *Acat1* mRNA in CTRL mice did however not result in increased protein levels of these enzymes in muscle (FIG 3D-E). We could furthermore detect a normalization of OXCT1 protein levels in trained mKO mice to similar levels as trained CTRL mice (FIG 3D-E). Intriguingly, this occurred despite no induction of *Oxct1* transcription in trained mKO mice (FIG 3C). These data suggest that exercise training can normalize the levels of OXCT1 protein in mKO mice through a transcription-independent mechanism. However, despite induction of OXCT1 protein levels in trained mKO mice, this did not result in a significant improvement in βOHB clearance. A possible reason for the improved βOHB clearance in trained CTRL mice, despite no changes in OXCT1 or ACAT1 protein levels, could be due to an increased ketolytic enzyme activity, which

has been shown to occur in trained rats **[Askew et al. 1975]**. A possible explanation for the inability of mKO mice to improve their βOHB clearance rates with exercise training could be due to the reduced levels of ACAT1 protein levels observed in trained mKO mice (FIG 3D-E). Moreover, mRNA levels of *Mct1* and components of the TCA cycle were still down-regulated due to the loss of PGC-1α in mKO mice (FIG 3F, and could thus contribute to the reduced ability to remove βOHB from the blood.

Overexpression of PGC-1α in skeletal muscle improves ketolytic capacity

Muscle-specific ablation of PGC-1α affects several processes involved in ketone body homeostasis and leads to hyperketonemia in response to fasting, LCKD feeding and exercise. We were therefore interested in whether overexpression of PGC-1α in skeletal muscle could enhance ketolytic capacity in this tissue, and improve systemic ketone body homeostasis. To investigate this, we used mice with a muscle-specific overexpression of PGC-1α, muscle transgenic (mTG) mice. PGC-1α mTG mice show a strong induction of PGC-1α transcript levels in skeletal muscle (FIG 4A). In addition, mTG mice display increased mRNA levels of ketolytic enzymes (*Bdh1*, *Oxct1*, and *Acat1*) (FIG 4A), the main ketone body transporter (*Mct1*) and TCA cycle enzymes (*Cs*, *Aco2*, and *Idh3α*) (FIG 4B) in muscle. Importantly, the increase in *Oxct1* and *Acat1* mRNA (FIG 4A) led to significantly increased protein levels of these enzymes in muscle of mTG mice (FIG 4C). To elucidate whether the increased levels of ketolytic enzyme in skeletal muscle would result in an enhanced ketolytic capacity in mTG mice, we performed a βOHB clearance test. In line with the increased levels of ketolytic enzymes in muscle, mTG mice displayed a faster βOHB clearance from blood after a bolus βOHB injection when compared to CTRL mice (FIG 4D). Next we assessed whether overexpression of PGC-1α in skeletal muscle would also affect systemic ketone body homeostasis during either fasting, LCKD feeding or exercise. In response to 24 hours fasting or 3 weeks LCKD feeding, we detected significantly reduced circulating βOHB (FIG 4E) and AcAc (FIG 4F) levels in mTG mice compared to CTRL mice. Moreover, mTG mice displayed a significant reduction in post-exercise ketosis at both 30 and 180 minutes after exercise (FIG 4G). Collectively, these data demonstrate that enhanced ketolytic capacity through overexpression of PGC-1α in skeletal muscle is sufficient to reduce circulating ketone body levels during physiological states of ketosis.

ERRα is a transcriptional partner of PGC-1α in the regulation of ketolytic enzymes

Since we have demonstrated that PGC-1α is a novel transcriptional regulator of ketolytic enzymes, we were interested in elucidating which is the transcriptional partner of PGC-1α in the regulation of these genes. We used differentiated C2C12 myotubes to study the molecular mechanisms behind the

transcriptional regulation of ketolytic enzymes by PGC-1α. By overexpressing PGC-1α in myotubes using adenoviral vectors, we could confirm that overexpression of PGC-1α led to a significant increase in *Bdh1*, *Oxct1* and *Acat1* mRNA levels (FIG 5A) compared to GFP-transduced cells. Estrogen-related receptor alpha (ERRα) is an important transcriptional partner of PGC-1α in the regulation of mitochondrial and metabolic gene transcription **[Mootha et al. 2004]**. *ERRα* also displayed a matching transcriptional pattern to that of ketolytic enzymes in skeletal muscle, since ERRα mRNA levels were down-regulated in mKO mice (FIG 5B) and accordingly up-regulated in mTG skeletal muscle (FIG 5C). We thus hypothesized that ERRα might be a transcriptional partner of PGC-1α in the regulation of ketolytic enzymes in muscle. To test this, we overexpressed PGC-1α in C2C12 myotubes in the absence or presence of the inverse ERRα agonist XCT-790. Inhibition of ERRα did not affect *PGC-1α* transcript levels, but it efficiently prevented the increase of *ERRα* and cytochrome c oxidase subunit Vb (*Cox5b*) mRNA levels induced by PGC-1α overexpression (FIG 5D). Importantly, inhibition of ERRα significantly blunted the induction of *Bdh1*, *Oxct1* and *Acat1* mRNA levels induced by PGC-1α overexpression (FIG 5E). This indicates that ERRα is an important transcriptional partner of PGC-1α in the regulation of ketolytic gene transcription. Moreover, ERRα was also required for the induction of *Cs*, *Aco2* and *Idh3a* with PGC-1α overexpression (FIG 5F). Conclusively, these findings indicate that ERRα together with PGC-1α regulates the expression of ketolytic- and TCA-cycle enzymes necessary for the complete oxidation of ketone bodies in skeletal muscle.

Overexpression of PGC-1α in skeletal muscle ameliorates diabetic hyperketonemia

As shown earlier, modulation of PGC-1α levels in skeletal muscle is sufficient to affect systemic ketone body homeostasis. We were therefore interested in whether overexpression of PGC-1α in muscle could ameliorate pathological states of hyperketonemia. An elevation of circulating ketone body levels is often encountered in patients suffering from diabetes **[Jain et al. 2006]**. Without intervention, this can progress to diabetic ketoacidosis, a potentially life-threatening state **[Barski et al. 2012]**. To study the role of muscle PGC-1α in the modulation of ketone body homeostasis during diabetic ketoacidosis, we injected mTG mice with streptozotocin (STZ) to induce an insulinopenic diabetic state in our mice. At day 3 post-injection, CTRL and mTG mice developed a significant hyperglycemia (FIG 5G), indicating that overexpression of PGC-1α does not affect the induction of a hyperglycemic diabetic state. Moreover, at this time point, diabetic CTRL mice displayed a significant induction of circulating βOHB levels (FIG 5H). Importantly, in line with the enhanced ketolytic capacity in SKM, PGC-1α mTG mice displayed reduced circulating levels of βOHB during STZ-induced diabetes (FIG 5H). Interestingly, in concurrence with the

reduced hyperketonemia in mTG mice after a STZ-injection, this group displayed a higher survival rate compared to STZ-injected CTRL mice post-injection (FIG 5I). In conclusion, these data demonstrate that overexpression of PGC-1α in skeletal muscle is sufficient to reduced systemic hyperketonemia in diabetic mice, and can potently affect the survival rate of mice after a STZ-injection.

Discussion

It is well known that βOHB and AcAc serve as vital metabolic fuels during states of reduced nutrient availability **[Robinson and Williamson 1980, Cahill 2006]**. Induction of ketosis is also postulated to exert beneficial effects in several diseases characterized by metabolic impairments, such as cancer **[Poff et al. 2013, Poff et al. 2014]** and neurodegenerative diseases **[Prins 2008]**. Despite the important role of ketone bodies as alternative energy substrates, relatively little is known as to how their oxidation in target organs is regulated. We have now shown that the pivotal metabolic regulator PGC-1α is an important transcriptional regulator of ketolytic enzymes. PGC-1α null mice are thus characterized by a reduced transcription of ketolytic enzymes in several organs. Elevated ketone body levels is a sign of impaired ketolytic capacity in both rodents **[Cotter et al. 2011, Cotter et al. 2013]** and humans **[Fukao et al. 2011, Shafqat et al. 2013]**, and PGC-1α null mice accordingly display an exacerbated hyperketonemia in response to fasting. Ketone body oxidation is especially important during neonatal development. This is evident in OXCT1 null mice, which die within 48 hours after birth due to an inability to generate energy through oxidation of ketone bodies **[Cotter et al. 2011]**. Interestingly, also PGC-1α null mice display increased neonatal mortality rates **[Lin et al. 2004]**. Based on our finding that PGC-1α is an important regulator of ketone body oxidation, we thus hypothesize that the reduced neonatal survival rate of PGC-1α null mice could be due to the systemic ketolytic impairment in these mice.

Skeletal muscle is one of the major ketolytic organs in the body **[Fukao et al. 1997]** and genetic ablation of the key ketolytic enzyme OXCT1 in muscle leads to development of fasting-induced hyperketonemia **[Cotter et al. 2013]**. Hence, in this study we focused on the role of PGC-1α in the regulation of skeletal muscle ketone body oxidation. We could confirm the crucial role of skeletal muscle in determining whole body ketolytic capacity, since muscle-specific PGC-1α knockout mice displayed a comparable increase in fasting-induced hyperketonemia as global PGC-1α knockout mice. Hence, we have identified skeletal muscle PGC-1α as a critical component in the regulation of systemic ketone body

homeostasis. Furthermore PGC-1α-ablation led to a reduced transcription of the major ketone body transporter MCT1 in muscle. It is however difficult to assess the impact of reduced MCT1 levels on the hyperketonemic phenotype of PGC-1α mKO mice, since it has been suggested that the rate of ketone body uptake is not a rate-limiting factor of ketone body oxidation in skeletal muscle **[Robinson and Williamson 1980]**. Apart from the regulation of ketone body transporters and ketolytic enzymes by PGC-1α, we and others **[Burgess et al. 2006, Summermatter et al. 2013b]** have demonstrated an important role of PGC-1α in the transcription of TCA cycle genes. Thus, in addition to the direct regulation of ketolytic enzymes, PGC-1α could indirectly affect the ketolytic capacity of skeletal muscle by modulating TCA cycle activity. Conclusively, our data demonstrates that PGC-1α is a transcriptional regulator of a genetic program responsible for terminal oxidation of ketone bodies within skeletal muscle mitochondria. Importantly, the orphan nuclear receptor ERRα is required for the transcriptional induction of ketolytic- and TCA-cycle enzymes by PGC-1α in muscle cells. It is well established that ERRα and PGC-1α mediate the transcription of several other metabolic processes, such as oxidative phosphorylation **[Mootha et al. 2004]** and β-oxidation **[Huss et al. 2004]**. Hence, our data expand the metabolic transcriptional network regulated by PGC-1α/ERRα, further accentuating the central role of PGC-1α/ERRα in the regulation of oxidative metabolism. Apart from skeletal muscle, the brain is a major contributor to systemic ketolytic capacity, since ablation of OXCT1 specifically in neurons results in a fasting-induced hyperketonemia in the range of what could be seen for OXCT1 muscle-knockout mice **[Cotter et al. 2013]**. It would thus be interesting in future studies to evaluate the role of neuronal PGC-1α in the regulation of energy homeostasis in the brain, especially in the context of long-term starvation, where ketone body oxidation is an important process for maintenance of energy homeostasis in the brain **[Cahill 2006]**.

Exercise is a potent stimulus of hepatic ketone body production. This is evident by a transient hyperketonemia after intense exercise, a phenomenon termed “post-exercise ketosis” **[Johnson et al. 1969]**. Interestingly, exercise training has been demonstrated to improve the ketolytic capacity of skeletal muscle and to reduce the extent of post-exercise ketosis in both rodents **[Adams and Koeslag 1988]** and humans **[Johnson et al. 1969]**. In our study, we demonstrate a strong correlation between skeletal muscle PGC-1α levels, and the extent of post-exercise ketosis after an acute exercise bout. Importantly, in the absence of PGC-1α in muscle, mice could not increase their systemic ketolytic capacity in response to endurance exercise training. Collectively, these data show that PGC-1α plays an important role in the adaptation of ketolytic capacity during exercise training. It has been demonstrated that PGC-1α is

increased in human skeletal muscle in response to exercise training **[Pilegaard et al. 2003, Mathai et al. 2008]**. Hence, PGC-1α could play an important role in the reduced post-exercise ketosis observed in trained humans **[Johnson et al. 1969]**. In line with this, muscle-specific overexpression of PGC-1α resulted in reduced circulating ketone body levels in response to fasting, LCKD feeding and exercise. Importantly, overexpression of PGC-1α in skeletal muscle was sufficient to ameliorate STZ-induced diabetic hyperketonemia in mice. The blunted hyperketonemia in mTG mice was not associated with any amelioration of the hyperglycemic diabetic phenotype, since CTRL and mTG mice displayed similar blood glucose levels after STZ injection. Intriguingly, overexpression of PGC-1α in skeletal muscle was sufficient to improve the survival rate of mice after a single STZ-injection. A worsened health status of the mice and a moribund state was, when examined, associated with highly elevated βOHB levels (4.5 – 6.5 mM) (unpublished observation). Hence, these data demonstrate that increased skeletal muscle PGC-1α can reverse pathological accumulation of ketone bodies in the circulation. Moreover, this could provide a likely explanation for the increased survival of mTG mice after a STZ injection compared to CTRL mice. Activation of PGC-1α in skeletal muscle could thus be an important therapeutic strategy to ameliorate ketoacidosis in patients suffering from type 1 or type 2 diabetes.

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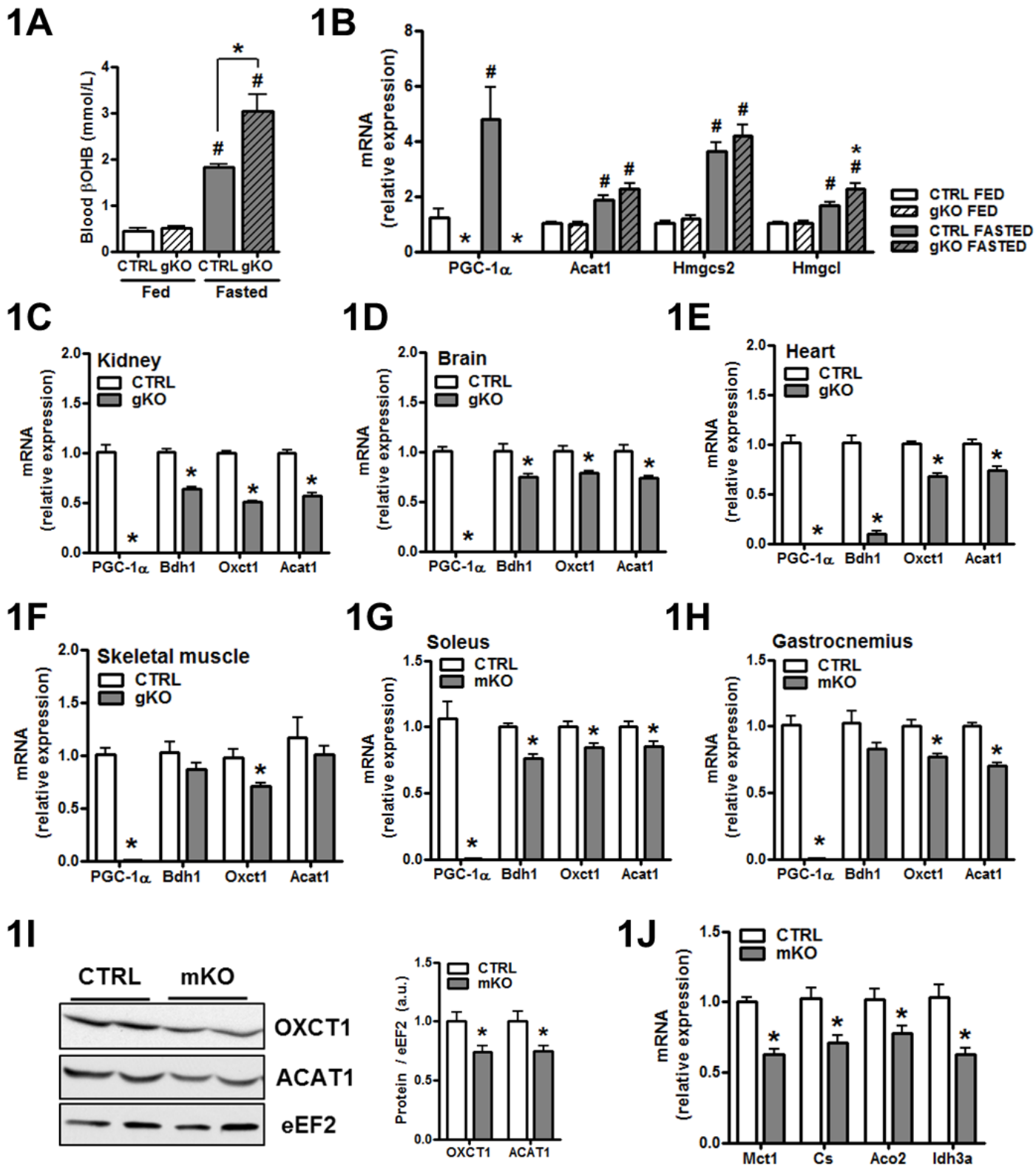

Figure 1 – Global PGC-1α knockout mice display fasting-induced hyperketonemia

(A) Blood βOHB levels in fed and 24 hour fasted CTRL and PGC-1α global knockout (gKO) mice (n=4-5/group). (B) mRNA levels of *PGC-1α*, *Acat1*, *Hmgcs2* and *Hmgcl* in liver from fed and 24 hour fasted CTRL and PGC-1α gKO mice (n=5/group). mRNA levels of *PGC-1α*, *Bdh1*, *Oxct1* and *Acat1* in kidney (C), brain (D), heart (E) and gastrocnemius muscle (F) of CTRL and PGC-1α gKO mice (n=6/group). mRNA levels of *PGC-1α*, *Bdh1*, *Oxct1* and *Acat1* in soleus (G) and gastrocnemius (H) muscles of CTRL and PGC-1α muscle-knockout (mKO) mice (n=8/group). (I) Representative immunoblots of OXCT1, ACAT1 and eEF2 protein levels in gastrocnemius muscle of CTRL and PGC-1α mKO mice. Bar graph represents the band intensities of OXCT1 and ACAT1 normalized to eEF2 (n=6/group). (J) mRNA levels of *Mct1*, *Cs*, *Aco2* and *Idh3α* in gastrocnemius muscle of CTRL and PGC-1α mKO mice (n=8/group). All mRNA levels normalized to 18s rRNA. Error bars represent mean ±SEM. Significant differences (p-value <0.05) between CTRL and PGC-1α mKO mice are indicated by an asterisk (*) and between fed/fasted groups with a number sign (#).

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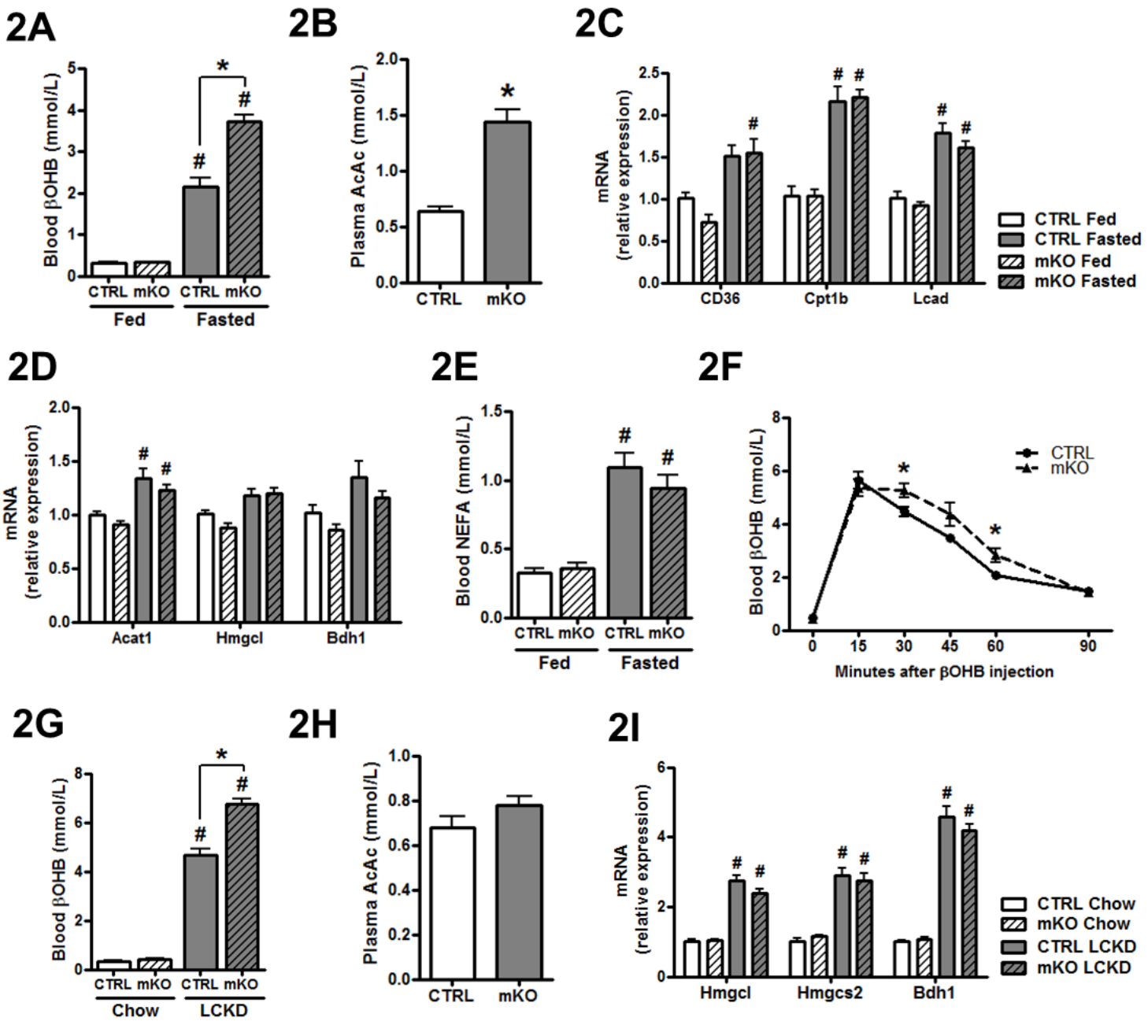

Figure 2 - PGC-1α muscle-specific knockout mice display fasting-induced hyperketonemia

(A) Blood βOHB levels in fed and 24 hour fasted CTRL and PGC-1α mKO mice (n=8/group). (B) Plasma acetoacetate (AcAc) levels in 24 hour fasted CTRL and PGC-1α mKO mice (n=7-8/group). (C) mRNA levels of *CD36*, *Cpt1b* and *Lcad* in liver from fed and 24 hour fasted CTRL and PGC-1α mKO mice (n=6-9/group). (D) mRNA levels of *Acat1*, *Hmgcl* and *Bdh1* in liver from fed and 24 hour fasted CTRL and PGC-1α mKO mice (n=6-9/group). (E) Plasma non-esterified fatty acid (NEFA) levels in fed and 24 hour fasted CTRL and PGC-1α mKO mice (n=6-9/group). (F) Blood βOHB levels at given time-points after a bolus intraperitoneal (I.P.) injection of 1.5 g/kg body weight βOHB in CTRL and PGC-1α mKO mice (n=6/group). (G) Blood βOHB levels in CTRL and PGC-1α mKO mice after 3 weeks on a low-carbohydrate ketogenic diet (LCKD) (n=7-13/group). (H) Plasma acetoacetate (AcAc) levels in CTRL and PGC-1α mKO mice after 3 weeks on a LCKD (n=7-9/group). (I) mRNA levels of *Hmgcl*, *Hmgcs2* and *Bdh1* in liver from CTRL and PGC-1α mKO mice after 3 weeks on a LCKD (n=7-8/group). All mRNA levels normalized to 18s rRNA. Error bars represent mean ±SEM. Significant differences (p-value <0.05) between CTRL and PGC-1α mKO mice are indicated by an asterisk (*) and between fed/fasted or chow/LCKD groups with a number sign (#).

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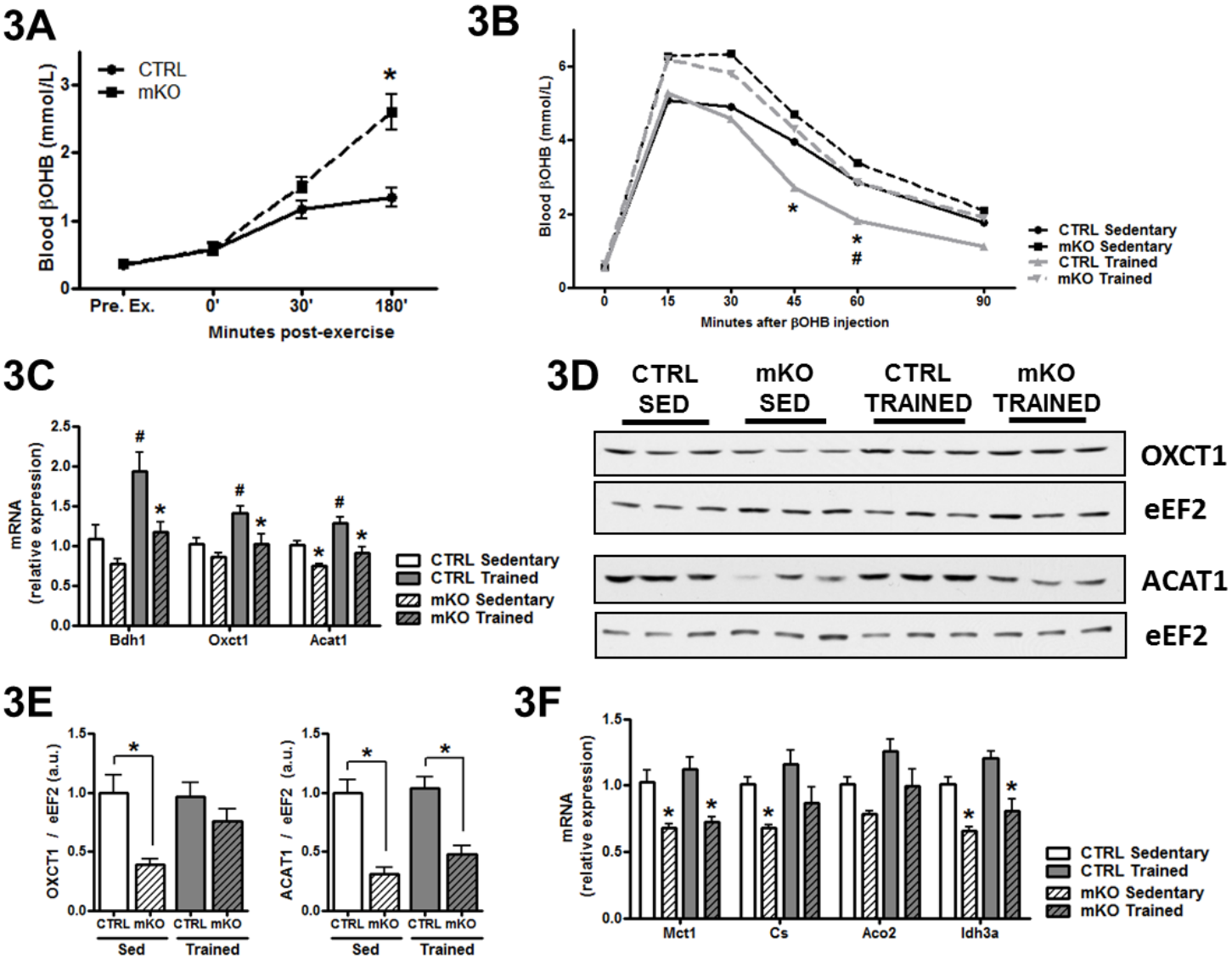

Figure 3 - PGC-1α is necessary for the transcriptional induction of ketolytic genes with exercise

(A) Blood βOHB levels in CTRL and PGC-1α mKO mice pre-exercise and at 0, 30 and 180 minutes post-exercise. (n=5/group). (B) Blood βOHB levels at given time-points after a bolus intraperitoneal (I.P.) injection of 1.5 g/kg bodyweight βOHB in either sedentary or trained CTRL and PGC-1α mKO mice (n=6/group). (C) mRNA levels of *Bdh1*, *Oxct1* and *Acat1* in skeletal muscle from either sedentary or trained CTRL and PGC-1α mKO mice (n=6-8/group). (D) Representative immunoblots of OXCT1, ACAT1 and eEF2 protein levels in skeletal muscle of either sedentary or trained CTRL and PGC-1α mKO mice. (E) Bar graph represents the band intensities of OXCT1 and ACAT1 normalized to eEF2 (n=6/group). (F) mRNA levels of *Mct1*, *Cs*, *Aco2* and *Idh3a* in skeletal muscle from either sedentary or trained CTRL and PGC-1α mKO mice (n=6-8/group). All mRNA levels normalized to 18s rRNA. Error bars represent mean ±SEM. Significant differences (p-value <0.05) between CTRL and PGC-1α mKO mice are indicated by an asterisk (*) and between sedentary or trained groups with a number sign (#).

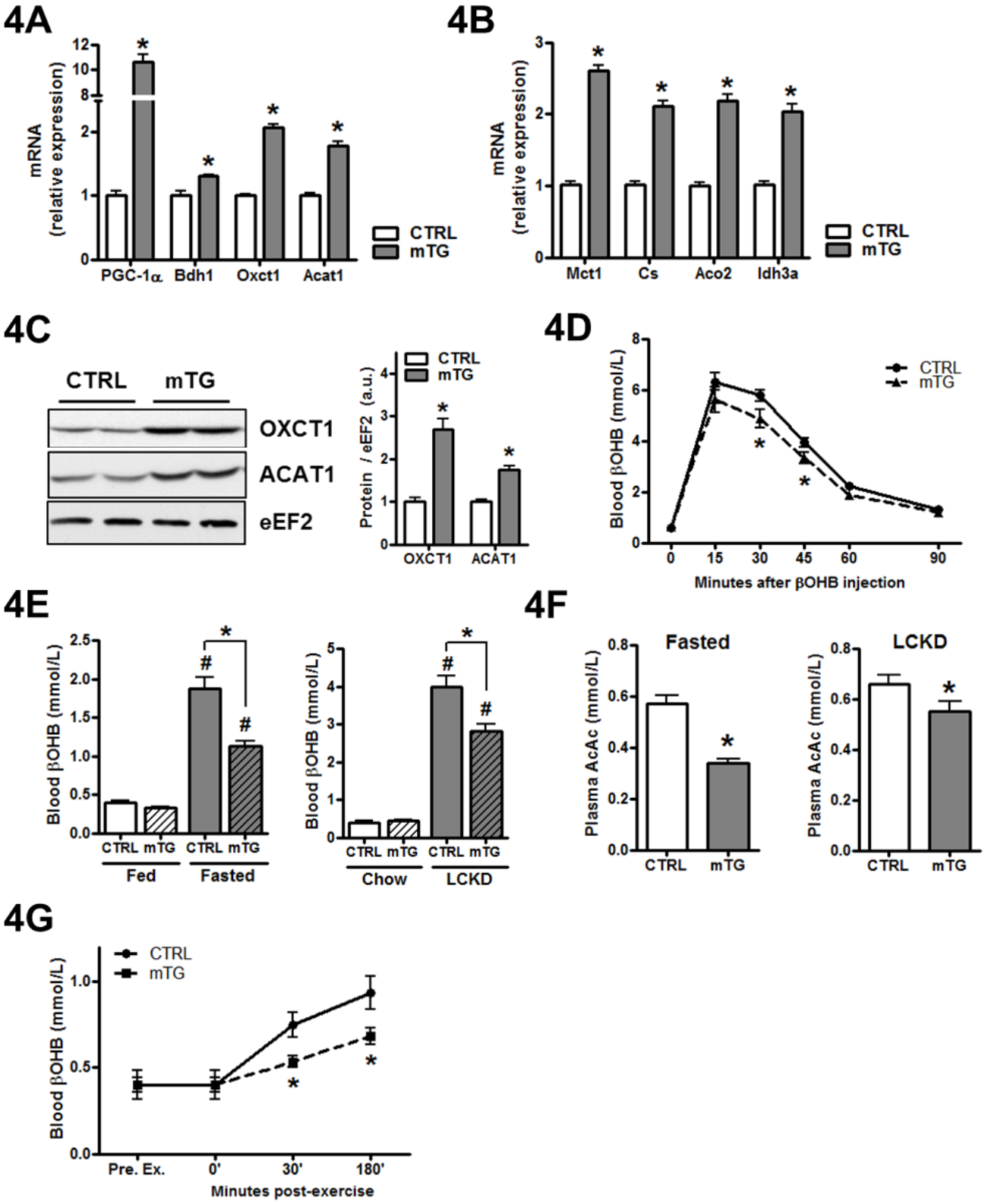
4A
mRNA (relative expression)
PGC-1α
Bdh1
Oxct1
Acat1
CTRL
mTG
4B
mRNA (relative expression)
Mct1
Cs
Aco2
Idh3a
CTRL
mTG
4C
CTRL
mTG
OXCT1
ACAT1
eEF2
Protein / eEF2 (a.u.)
OXCT1
ACAT1
4D
Blood βOHB (mmol/L)
Minutes after βOHB injection
CTRL
mTG
4E
Blood βOHB (mmol/L)
Fed
Fasted
Chow
LCKD
4F
Fasted
LCKD
Plasma AcAc (mmol/L)
CTRL
mTG
4G
Blood βOHB (mmol/L)
Pre. Ex.
0'
30'
180'
Minutes post-exercise
CTRL
mTG

Figure 4 - Overexpression of PGC-1α in skeletal muscle increases ketolytic capacity

(A) mRNA levels of *PGC-1α*, *Bdh1*, *Oxct1* and *Acat1*, and (B) *Mct1*, *Cs*, *Aco2* and *Idh3a* in skeletal muscle of CTRL and PGC-1α muscle-transgenic (mTG) mice (n=8/group). (C) Representative immunoblots of OXCT1, ACAT1 and eEF2 protein levels in skeletal muscle of CTRL and PGC-1α mTG mice. Bar graph represents the band intensities of OXCT1 and ACAT1 normalized to eEF2 (n=6/group). (D) Blood βOHB levels at given time-points after a bolus intraperitoneal (I.P.) injection of 1.5 g/kg body weight βOHB in CTRL and PGC-1α mTG mice (n=6/group). (E) Blood βOHB levels in CTRL and PGC-1α mTG mice in either fed state or after 24 hours fasting, or after 3 weeks of either chow- or low-carbohydrate ketogenic diet (LCKD) feeding (n=6-10/group). (F) Plasma acetoacetate (AcAc) levels in CTRL and PGC-1α mKO mice after 24 hour fasting or after 3 weeks on a LCKD (n=7/group). (G) Blood βOHB levels in CTRL and PGC-1α mTG mice pre-exercise and at 0, 30 and 180 minutes post-exercise. (n=6/group). All mRNA levels normalized to 18s rRNA. Significant differences (p-value <0.05) between CTRL and PGC-1α mKO mice are indicated by an asterisk (*) and between fed/fasted or chow/LCKD groups with a number sign (#).

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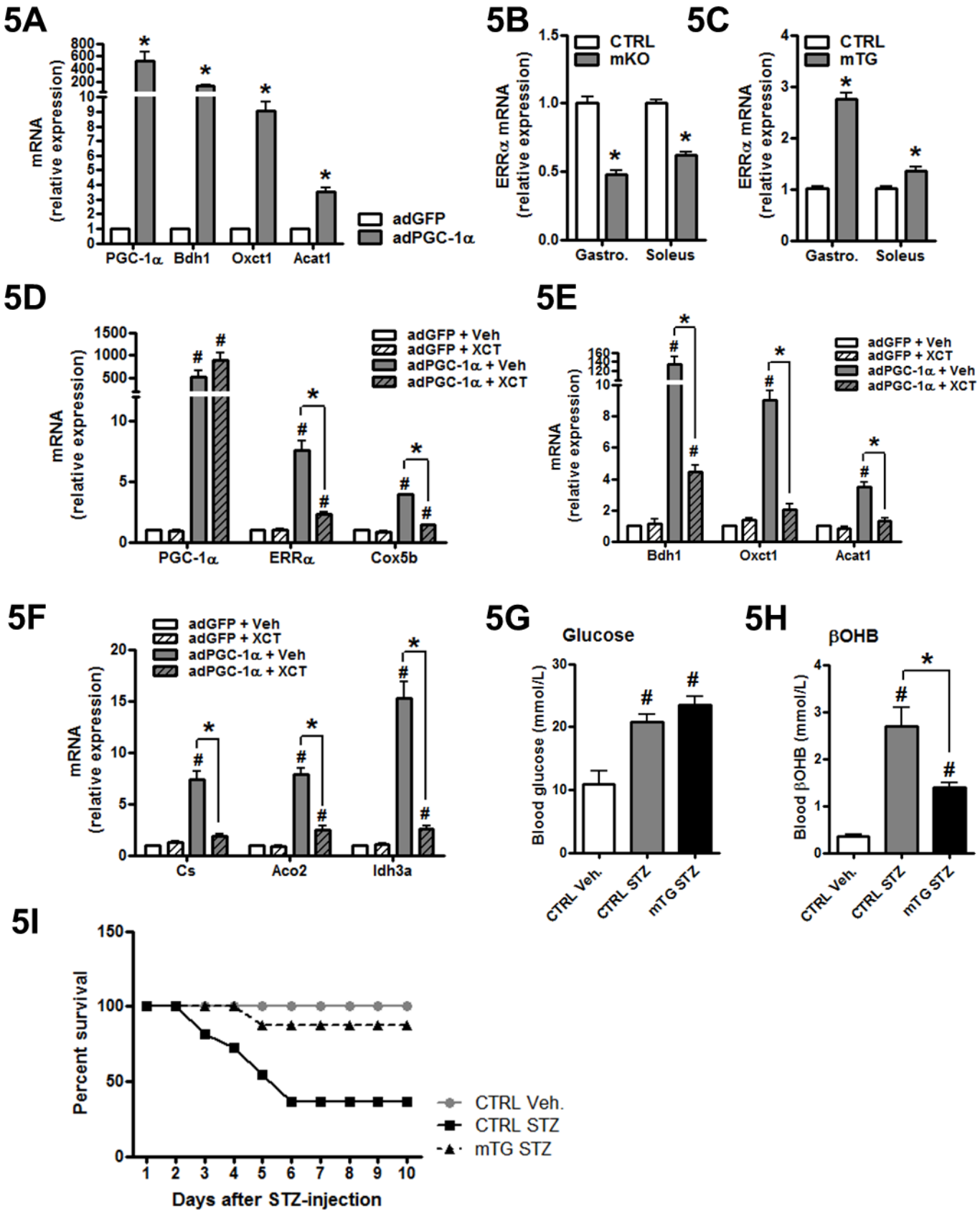

Figure 5 - ERRα is a transcriptional partner of PGC-1α in the regulation of ketolytic genes

(A) mRNA levels of *PGC-1α*, *Bdh1*, *Oxct1* and *Acat1* in C2C12 myotubes transduced by adenovirus expressing either GFP or bicistronic GFP-PGC-1α (n=3/experiment, performed in triplicates). (B) mRNA levels of *ERRα* in gastrocnemius and soleus muscles of CTRL and PGC-1α mKO mice (n=8/group). (C) mRNA levels of *ERRα* in gastrocnemius and soleus muscles of CTRL and PGC-1α mTG mice (n=6-8/group). mRNA levels of (D) *PGC-1α*, *ERRα*, *Cox5b*, (E) *Bdh1*, *Oxct1*, *Acat1* and (F) *Cs*, *Aco2*, *Idh3α* in C2C12 myotubes transduced by adenovirus expressing either GFP or bicistronic GFP-PGC-1α, and co-treated with either 0.2% DMSO (Veh) or 10 μM XCT-790 (XCT). Blood glucose (G) and βOHB (H) levels in mice 3 days after injection with either citrate buffer (Veh, n=2) or 150 mg/kg streptozotocin (STZ, n=9-11). (I) Percent survival of mice injected with either citrate buffer (Veh, n=2) or 150 mg/kg streptozotocin (STZ, n=9-11). All mRNA levels normalized to 18s rRNA. Error bars represent mean ±SEM. Significant differences (p-value <0.05) between adGFP or adPGC-1α infected cells or CTRL and PGC-1α mTG mice are indicated by an asterisk (*) and between Veh/XCT or Veh/STZ groups with a number sign (#).

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SI Table I. qPCR Primer sequences.

Target gene	Forward primer	Reverse primer
18s	AGTCCCTGCCCTTTGTACACA	CGATCCGAGGGCCTCACTA
Acat1	GTGAAGGAAGTCTACATGGGCA	TGTGGTGCATGGAGTGGAAATA
Aco2	ACATTGTCCGTAAACGGTTGAA	TATGTCTTTCCCCGCTCGATCT
Bdh1	TTTGCTGGCTGTTTGATGAAGG	TTGAGCTGGATGGTTCTCAGTC
Cd36	GGCAAAGAACAGCAGCAAAAT	TGGCTAGATAACGAACTCTGTATGTGT
Cox5b	CTTCAGGCACCAAGGAAGAC	TTCACAGATGCAGCCCACTA
Cpt1b	ATCATGTATCGCCGCAAACT	CCATCTGGTAGGAGCACATGG
Cs	CCCAGGATACGGTCATGCA	GCAAACTCTCGCTGACAGGAA
Hmgcl	CCAGCTTTGTTTCTCCCAAGTG	GATGCCGGGAAACTTCTGAATG
Hmgcs2	CCACAAGGTGAACTTCTCTCCA	TGCATCTCATCCACTCGTTCAA
Idh3a	GCTGGTGGTGTTCAGACAGTAA	CACTGAATAGGTGCTTTGGCAG
Lcad	CCAGCTAATGCCTTACTTGGAGA	GCAATTAAGAGCCTTTCCTGTGG
Mct1	TGCAACGACCAGTGAAGTATCA	ACAACCACCAGCGATCATTACT
Oxct1	CCCATACCCACTGAAAGACGAA	CTGGAGAAGAAAGAGGCTCCTG
PGC-1α	AGCCGTGACCACTGACAACGAG	GCTGCATGGTTCTGAGTGCTAAG

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9. The role of PGC-1α in skeletal muscle – Discussion and future perspectives

9.1 – Discussion

Skeletal muscle displays a high metabolic plasticity and its oxidative capacity can be modulated through changes in physical activity **[Egan and Zierath 2013]**. The ability to modulate systemic energy expenditure through increased physical activity is considered an important therapeutic tool in the treatment of obesity and metabolic dysfunction in human patients **[Strasser 2013]**. Also dietary interventions, such as CR enhance the metabolic activity of skeletal muscle. CR has been shown to enhance mitochondrial biogenesis and respiratory function of individual mitochondria in skeletal muscle of humans and mice **[Civitarese et al. 2007, Gouspillou and Hepple 2013]**. While exercise and CR elicit beneficial effects on whole body metabolism, increased calorie intake and reduced physical activity are the two major predisposing factors for obesity in humans and they are related to an increased prevalence of related metabolic comorbidities such as diabetes, cardiovascular disease and cancer **[Handschin and Spiegelman 2008, Booth et al. 2012]**. The positive effects of exercise and CR on whole body metabolism have sparked an interest in developing pharmacological compounds that stimulate signaling pathways normally activated in skeletal muscle during either exercise or CR. These compounds are envisioned to ameliorate metabolic dysfunction in obese human patients, either as a monotherapy, or as an adjuvant treatment in combination with exercise or CR. In line with the postulated use of these compounds, they have aptly been termed exercise-mimetics or CR-mimetics **[Ingram et al. 2006, Fan et al. 2013]**. The distinction between exercise- and CR-mimetics is however in many cases superfluous. For instance, activation of SIRT1 by RSV would fall under both categories, considering that this pathway is activated in skeletal muscle during both exercise and CR. RSV, and the SIRT1-activating compound SRT1720, are accordingly suggested to be potential CR/exercise mimetic compounds and have received a lot of attention due to their ability to elicit beneficial metabolic effects in obese rodents **[Baur et al. 2006, Lagouge et al. 2006, Milne et al. 2007, Feige et al. 2008, Um et al. 2010, Park et al. 2012, Price et al. 2012]**. An open question, however, is whether RSV and SRT1720 exert their effects through direct activation of SIRT1, or whether other signaling pathways are involved in mediating the effects of these compounds **[Huber et al. 2010, Pacholec et al. 2010, Villalba et al. 2012]**. Several recent studies have elegantly demonstrated that the effects of RSV are indeed pleiotropic and depend on more than one single signaling pathway. For instance, the dependence of SIRT1 for the effects of RSV was dose-dependent in C2C12 myotubes. High doses of RSV disrupted mitochondrial respiration and reduced ATP levels, thereby activating AMPK independently of SIRT1 **[Um et al. 2010, Price et al. 2012]**. Importantly, with a lower dose of RSV, AMPK was activated in

skeletal muscle in a SIRT1-dependent manner **[Price et al. 2012]**. RSV was recently shown to act as a PDE-inhibitor and thereby activate AMPK and SIRT1 downstream of cAMP signaling and Epac1 in skeletal muscle cells **[Park et al. 2012]**. While the effects of RSV are pleiotropic, SRT1720 has been postulated to mediate its effects mainly through specific activation of SIRT1. This idea however has been questioned **[Huber et al. 2010]**. Hence, in order to fully understand the therapeutic potential of RSV and SRT1720, it is important to elucidate which signaling pathways are mediating the effects of these compounds. Since RSV and SRT1720 have been shown to increase PGC-1α activity and transcription of PGC-1α target genes in skeletal muscle **[Lagouge et al. 2006, Feige et al. 2008]**, this coactivator has accordingly been postulated to be a major downstream mediator of these CR/exercise mimetic compounds. Indeed, PGC-1α can be activated by AMPK-, SIRT1- and cAMP-signaling **[Herzig et al. 2001, Rodgers et al. 2005, Jager et al. 2007]**, and would thus likely be activated in response to RSV and SRT1720 treatment regardless of the main upstream signaling pathway activated by these compounds. Interestingly, despite the general consensus in the field that PGC-1α mediates the effects of RSV and SRT1720, this has not been conclusively demonstrated.

Accordingly, in the second manuscript of this thesis we investigated the requirements for skeletal muscle PGC-1α in mediating the beneficial metabolic effects elicited by RSV and SRT1720 treatment in obese mice, both in skeletal muscle and on a whole body level. To answer this question, we used mice with a skeletal-muscle specific knockout of PGC-1α, which were fed a HFD supplemented with either RSV or SRT1720. This setup allowed us to study the role of skeletal muscle PGC-1α as a molecular effector of RSV and SRT1720 in a therapeutically relevant model, considering the ultimate use of RSV and SRT1720 as treatments for obesity and metabolic dysfunction in humans. Importantly, we demonstrated that RSV and SRT1720 elicited beneficial systemic metabolic effects in HFD-fed mice, such as an increased energy expenditure, improved glucose tolerance and a reduced fat mass. Intriguingly, these effects occurred independently of skeletal muscle PGC-1α, thus indicating that skeletal muscle PGC-1α is not necessary for the systemic metabolic effects of RSV and SRT1720 treatment. However, molecular analysis revealed that PGC-1α was required for the transcriptional induction of mitochondrial genes in skeletal muscle with both RSV and SRT1720 treatment. Interestingly, our findings are analogous to those from a study by Finley et al. **[Finley et al. 2012]**. In this study, the authors showed that PGC-1α was necessary for the induction of mitochondrial genes in skeletal muscle during CR. However, in analogy with our findings, skeletal muscle PGC-1α was dispensable for the alterations in energy expenditure and improved glucose tolerance in mice during CR **[Finley et al. 2012]**. The enhanced energy expenditure with SRT1720 treatment in our study

could be attributed to the increased mitochondrial number in skeletal muscle of mice treated with this compound, an effect which was independent of PGC-1α. However, the enhanced metabolic rate during CR or in response to RSV/SRT1720 treatment could also be due to effects on other organs, and thus completely independent of any effect in skeletal muscle. This hypothesis is supported by the fact that mice with a muscle-specific overexpression of PGC-1α, which have increased mitochondrial number and levels of oxidative enzymes in skeletal muscle, surprisingly do not display an enhanced metabolic rate **[Choi et al. 2008]**.

The minor effects of RSV and SRT1720 on skeletal muscle metabolism in our study were surprising, particularly in relation to other studies using RSV and SRT1720 in mice. In these studies the transcriptional profile of skeletal muscle was altered and consequently oxidative capacity and exercise performance was increased **[Lagouge et al. 2006, Feige et al. 2008]**. However, two recent studies by Higashida et al. and Ringholm et al. could corroborate our findings, since neither of these studies found any increase in mitochondrial metabolism in skeletal muscle using the same dose of RSV as that used in our study (4g RSV/kg diet) **[Higashida et al. 2013, Ringholm et al. 2013]**. Interestingly, these doses are similar to those used by both Lagouge et al. and Um et al. in their studies **[Lagouge et al. 2006, Um et al. 2010]**. It is difficult to explain why our findings, together with the studies by Ringholm et al. and Higashida et al. show no major effects on mitochondrial protein levels in skeletal muscle with RSV treatment, especially when considering the clear effects in earlier studies **[Lagouge et al. 2006, Um et al. 2010]**. One reason for these disparate results could be due to differences in the batch as well as manufacturer of RSV when comparing our study and the study by Higashida et al. **[Higashida et al. 2013]** to earlier studies using RSV in mice **[Baur et al. 2006, Lagouge et al. 2006, Um et al. 2010]**. Another reason could be due to the type of HFD used in these initial studies, since both the studies by Baur et al. and Lagouge et al. used a high percentage of coconut oil in their diets **[Baur et al. 2006, Lagouge et al. 2006]**, which in comparison to the long-chain fatty acid (LCFA) rich lard-based HFD used in our study, primarily consists of medium-chain fatty acids (MCFA). MCFA-rich diets have been shown to induce mitochondrial metabolism and oxidative capacity in skeletal muscle **[Turner et al. 2009, Montgomery et al. 2013]** and could thus help boost the effect of RSV on mitochondrial transcription in these studies. However, if we focus solely on our study, one likely explanation for the minor effects of RSV in muscle compared to other organs could be due to RSV bioavailability, since this compound has been shown to preferentially accumulate in liver and WAT of rats **[Andres-Lacueva et al. 2012]**. Indeed, in line with this, we found more prominent effects of RSV and SRT1720 in both WAT and liver. Interestingly, we detected enhanced transcription of several PGC-1α-

regulated gene programs in liver and WAT with SRT1720 and RSV treatment, respectively. This would imply that PGC-1α could indeed play an important role in mediating the effects of RSV and SRT1720 in other organs than skeletal muscle. This would however have to be confirmed in future studies, preferentially employing a similar setup as our study, but using mice with a liver or WAT-specific deletion of PGC-1α (see below). In conclusion, we demonstrate improved systemic metabolic parameters with RSV and SRT1720 treatment, despite only a minor effect of these compound in skeletal muscle. These findings would warrant a less muscle-centric view on the effects of RSV and SRT1720 and future research would thus benefit from evaluating the effects of exercise/CR-mimetic compounds such as RSV and SRT1720 also in other metabolic organs.

Accordingly, in the second part of this study, we were interested in comparing the organ-specific effects of RSV and SRT1720 treatment in liver and WAT. This is a pertinent question, since RSV has been shown to possess several effects apart from its ability to activate SIRT1, as it can act as an antioxidant, a phytoestrogen and also as an anti-inflammatory agent **[Baur et al. 2006]**. Furthermore, RSV inhibits the activity of a range of enzymes (reviewed in **[Pirola and Frojdo 2008]**), out of which PDE-inhibition was recently shown to be important for the metabolic effects of RSV **[Park et al. 2012]**. SRT1720 on the other hand has been suggested to be a more potent SIRT1 activator than RSV **[Milne et al. 2007]**. However, the direct effect of SRT1720 on SIRT1-activity has been questioned **[Huber et al. 2010]** and it is not yet clear whether RSV and SRT1720 can be considered to be analogous compounds concerning their effects in target organs. Hence, in order to avoid potential caveats when using RSV or SRT1720 to treat humans, it is necessary to have a complete overview of the effects elicited by these compounds in all major metabolic organs. However, to our knowledge, no extensive comparison between the *in vivo* effects of these two compounds has so far been made. RSV and SRT1720 elicit similar changes on whole body metabolism, since both compounds improved glucose tolerance and enhanced metabolic rate in mice, both in our study and in others **[Lagouge et al. 2006, Feige et al. 2008]**. Despite similar effects on whole body metabolism, we could however demonstrate distinct organ-specific effects of both RSV and SRT1720 in HFD fed mice. For instance, in our study, RSV treatment led to a reduced transcription of genes involved in lipogenesis in liver, together with a reduced hepatic triglyceride content. Interestingly, SRT1720 treatment resulted in the opposite effect, leading to enhanced transcription of lipogenic genes in liver. This however did not lead to an increase in hepatic triglyceride accumulation, which could be due to the concurrent induction of both hepatic β-oxidation and ketogenesis by this compound. SRT1720 treatment also enhanced hepatic transcription of genes involved in cholesterol biosynthesis, which was associated

with an increase in circulating LDL and HDL cholesterol levels. Contrariwise, RSV treatment led to a reduction in circulating LDL-cholesterol levels. Hence, our findings indicate that RSV and SRT1720 exert differential effects on hepatic metabolism. Importantly, our findings are corroborated in a study by Minor et al. **[Minor et al. 2011]**, where the hepatic transcriptional profiles of RSV- and SRT1720-treated mice were compared. Interestingly, the authors found only a small overlap between the hepatic transcriptional profiles of RSV- and SRT1720-treated mice **[Minor et al. 2011]**. To elucidate the main mechanism behind the effects of SRT1720 treatment on hepatic mitochondrial and metabolic processes, we assessed PGC-1α acetylation as a readout of SIRT1 activity, but could detect no alterations in PGC-1α acetylation status. However, AMPK activity was increased in liver from SRT1720-treated mice. AMPK is important for PGC-1α expression and transcription of mitochondrial genes in liver **[Guigas et al. 2007]**. Enhanced AMPK-activity could thus explain the improved hepatic mitochondrial transcription with SRT1720 treatment in our study. Hepatic AMPK activation has however been associated with a reduced activity of hepatic gluconeogenesis, lipogenesis and cholesterol synthesis **[Viollet et al. 2009]**, which are processes that were induced at a transcriptional levels with SRT1720 treatment. Hence, AMPK activation could only partially explain the hepatic phenotype of SRT1720-treated mice, and the concomitant activation of both anabolic and catabolic processes in liver would strongly indicate that more than one signaling pathways would be involved in this regulation. However, further studies are needed to elucidate the exact mechanism behind the effects of SRT1720 on mitochondrial and metabolic processes in liver, and whether these effects are cell-autonomous or could be attributed to inter-organ cross-talk. To answer these open questions, it would be imperative to perform further experiments to elucidate the molecular effects of SRT1720 treatment in liver cells *in vitro*. Stimulation of cultured hepatocytes with SRT1720 and subsequent transcriptional analysis would help to delineate the transcriptional networks regulated by SRT1720 in liver cells. These *in vitro* findings could then be compared to the transcriptional changes induced by SRT1720 in liver *in vivo*. This comparison would help to elucidate which processes are regulated directly by SRT1720, and which effects could be attributed to inter-organ cross-talk due to the alteration of metabolic processes in other organs. Considering that many genes induced by SRT1720 in liver in our study were known PGC-1α target genes, it would be interesting to knock down PGC-1α in hepatocytes to directly investigate the role of this coactivator as a down-stream molecular effector of SRT1720 in this cell type. These findings could later be expanded to an *in vivo* setting, were mice with a liver-specific ablation of PGC-1α would be administered SRT1720. This would allow us to validate the findings from our *in vitro* setup, but would also help characterize the functional outcome of hepatic PGC-1α deletion on the systemic metabolic effects of SRT1720 in mice. While SRT1720 had a strong effects on liver metabolism

in our study, RSV treatment led to more prominent changes in WAT. RSV improved the adipose tissue phenotype in HFD fed mice, which included increased mitochondrial protein levels, enhanced adiponectin transcription and reduced inflammation. While AMPK activity in adipose tissue was unaltered with RSV treatment, we detected a significantly reduced cAMP response element-binding protein (CREB) phosphorylation in WAT in response to both short-term and long-term RSV treatment. Increased CREB activity has been associated with transcriptional activation of ATF3 in adipose tissue, and an insulin resistant phenotype **[Qi et al. 2009]**, and in line with this we could detect reduced ATF3 transcription and increased insulin-signaling in WAT with RSV treatment. Importantly, overexpression of a dominant-negative CREB in adipose tissue of obese mice improves whole body insulin sensitivity and reduces adipose tissue inflammation **[Qi et al. 2009]**. Hence, the inhibition of CREB-signaling by RSV specifically in adipose tissue depots could have a beneficial effect on whole body metabolism, and could thus contribute to the improved systemic phenotype of RSV-treated mice. Intriguingly, CREB activity was also predicted to be reduced in adipose tissue depots of humans receiving RSV-treatment **[Konings et al. 2013]**. These findings would warrant a closer examination of the beneficial effects of RSV-treatment on adipose tissue metabolism and inflammation during obesity, and the contribution of these effects on whole body glucose tolerance in rodents as well as humans patients. In analogy with the above-mentioned *in vitro* experiments for SRT1720 treatment in hepatocytes, a similar setup could be used to elucidate the molecular effects elicited by RSV treatment in adipocytes. Also in this case, knockdown of PGC-1α in cultured adipocytes and subsequent transcriptional analysis would help define the exact role of PGC-1α as a molecular effector of RSV treatment in this cell type. These experiments could also be extended to an *in vivo* setup, using mice with an adipocyte-specific ablation of PGC-1α. Treatment of these mice with RSV would help determine whether adipose tissue PGC-1α is required to mediate the beneficial systemic effects of RSV treatment, such as enhanced oxygen consumption or improved glucose tolerance. In extension, it would be important to treat additional cell types (i.e. myocytes, adipocytes, hepatocytes) with both RSV and SRT1720 *in vitro*. This setup would help explain whether the differential effects of RSV and SRT1720 in different organs *in vivo* are due to cell-autonomous effects or whether it could be attributed to different *in vivo* bioavailability of these compounds in various organs. In line with this, it would also be necessary to measure the concentrations of RSV and SRT1720 in the different tissues from mice treated with these compounds. In conclusion, our data demonstrates that the effects of RSV and SRT1720 are non-analogous. Moreover, these compounds exert differential effects on mitochondrial and metabolic processes in both liver and WAT, which is important to take into consideration when using these compounds in a clinical setting. It also leaves us with the open question of how these compounds

exert their effects in target organs. Both RSV and SRT1720 have been shown to activate SIRT1, however, considering the opposing effects of RSV and SRT1720 on hepatic metabolism, it is unlikely that one unique signaling pathway is responsible for these differential effects. Thus, based on our findings, this warrant a closer examination of the signaling pathways activated by RSV and SRT1720 in liver and WAT. Molecular dissection of the signaling pathways activated by these compounds will aid in our understanding of the therapeutic potential of RSV and SRT1720. Moreover, it will also help with the design of new, more efficient CR/exercise-mimetic compounds, which could lead to novel treatments for obesity and related metabolic comorbidities.

In the third study of this thesis, we were interested in elucidating the regulation of ketone body oxidation by PGC-1α, and the impact of this regulation on systemic ketone body homeostasis. Ketone bodies are important energy substrates during nutrient-deprived states, and are oxidized in most organs in a concentration-dependent manner. Ketone body levels in the circulation are determined by the rate of ketogenesis in the liver, which in turn is regulated by the availability of fatty acids, which is the main ketogenic substrate. Circulating ketone body levels thus rise in response to stimuli that increase circulating fatty acid levels, such as fasting, exercise, or in a pathological context, diabetes. However, utilization of ketone bodies through oxidation is also an important determinant of circulating ketone body levels. For example, reduced ketolytic capacity specifically in brain or skeletal muscle leads to increased circulating ketone body levels in mice **[Cotter et al. 2013]**. Moreover, ketone body levels are increased in humans with hereditary ketolytic deficiencies **[Shafqat et al. 2013]**. Hence, systemic ketone body homeostasis is dictated by both hepatic ketogenesis and extra-hepatic ketolysis. Ketone bodies play a critical role in whole body energy homeostasis, since during starvation in humans, ketone bodies account for up to 50% of the oxygen consumption of most organs, and to an even higher degree in the brain **[Cahill 2006]**. The importance of ketone body oxidation is further underscored in mice with a complete ketolytic deficiency, which die within 48 hours after birth **[Cotter et al. 2011]**. Interestingly, it was recently demonstrated that ketone bodies are more than just passive energy substrates. The most abundant circulating ketone body, βOHB, was shown to act as an HDAC1-inhibitor, and thus influences transcriptional regulation and oxidative stress protection in kidney **[Shimazu et al. 2013]**. Intriguingly, HDAC1-specific inhibitors were also recently demonstrated to enhance oxidative capacity in skeletal muscle cells **[Galmozzi et al. 2013]**. In line with this, induction of a chronic state of ketosis by feeding rodents a ketogenic diet has been demonstrated to improve mitochondrial biogenesis in both brain **[Bough et al. 2006]**, brown adipose tissue **[Srivastava et al. 2013]** and skeletal muscle **[Ahola-Erkkila et**

al. 2010]. The exact mechanism how mitochondrial biogenesis is enhanced during ketogenic diet feeding however is still unknown. Dietary induction of ketosis has been suggested as a promising therapeutic strategy to treat various disorders such as diabetes, neurodegenerative disorders and cancer **[Paoli et al. 2013, Hashim and Vanitallie 2014]**. Due to the therapeutic potential of dietary ketosis, and the importance of ketone bodies as metabolic fuels, it is essential to understand how ketone body oxidation is regulated in target organs. This is especially pertinent in the context of skeletal muscle, which has a high capacity to oxidize ketone bodies. PGC-1α has been shown to play an important role in the regulation of fatty acid oxidation in muscle, and it is known to regulate uptake of both glucose and fatty acids into muscle **[Choi et al. 2008, Summermatter et al. 2013]**. However, the role of PGC-1α in the regulation of ketone body oxidation has to our knowledge not been conclusively elucidated. Thus, in the third manuscript of this thesis, we investigated the role of PGC-1α in the regulation of ketone body oxidation.

In this study, we demonstrated that PGC-1α is a transcriptional regulator of the rate-limiting ketolytic enzymes BDH1, OXCT1 and ACAT1. Moreover, in C2C12 myotubes we demonstrated that ERRα is an important transcriptional partner of PGC-1α in the regulation of these genes. However, further experiments using ChIP are needed to elucidate whether this is due to direct interaction of PGC-1α and ERRα on the promoters of these genes, or whether ERRα regulates these genes independently of PGC-1α. Importantly, in global PGC-1α null mice, ablation of PGC-1α was associated with a reduced transcription of ketolytic enzymes in kidney, heart, brain and skeletal muscle. This is an important finding, since this demonstrates a global role of PGC-1α in the regulation of ketolytic capacity. In line with this, global ablation of PGC-1α led to increased circulating βOHB-levels after 24 hours fasting, which is in line with a reduced ketolytic capacity in the absence of PGC-1α. As mentioned earlier, mice with a global deletion of OXCT1, and thus a complete ablation of ketolysis, develop a severe hyperketonemia. Due to the importance of ketone body oxidation in maintaining energy homeostasis during early extrauterine development **[Platt and Deshpande 2005]**, OXCT1 knockout mice die during this neonatal period **[Cotter et al. 2011]**. Intriguingly, also PGC-1α null mice display an increased neonatal mortality **[Lin et al. 2004]**, however not with the same frequency as OXCT1 null mice **[Cotter et al. 2011]**. Hence, we speculate that the increased neonatal mortality of PGC-1α null mice, could be linked to the systemic impairment in ketolytic capacity in these mice. Closer examination of the ketone body levels in neonatal PGC-1α null mice would help confirm this hypothesis. Next, we focused on the role of PGC-1α in the regulation of skeletal muscle ketolytic capacity. Using mice with a muscle specific ablation or overexpression of PGC-1α, we identified PGC-1α as an important regulator of the ketolytic capacity in this organ. In skeletal

muscle, PGC-1α regulates the transcription, not only of ketolytic enzymes, but also of the main ketone body transporter MCT1 as well as TCA-cycle enzymes. Hence, PGC-1α modulates the entire transcriptional program responsible for complete oxidation of ketone bodies in skeletal muscle. Intriguingly, ablation of PGC-1α in skeletal muscle led to a fasting-induced hyperketonemia in the same range as that seen in global PGC-1α null mice, indicating that skeletal muscle is a major contributor to systemic ketolytic capacity. Systemic hyperketonemia in mKO mice was also detected during ketogenic diet feeding and exercise. Exercise training can reduce post-exercise ketosis in both rodents **[Adams and Koeslag 1988]** and humans **[Johnson et al. 1969]**, and PGC-1α overexpression in skeletal muscle could mimic this effect in our mice, since mTG mice had significantly reduced post-exercise βOHB levels. Accordingly, we were interested in whether PGC-1α was involved in the adaptation of skeletal muscle ketolytic capacity during exercise. Whole body ketolytic capacity was enhanced in mice after being kept for 8 weeks in running-wheel cages. Importantly, this induction could not be seen in PGC-1α mKO mice, indicating that skeletal muscle PGC-1α plays an important role in this adaptation. In line with this, transcription of ketolytic genes in skeletal muscle were induced in a PGC-1α-dependent manner in response to exercise. However, we could detect no induction in protein levels of ketolytic enzymes in trained mice. This suggests that long-term exercise alters ketolytic enzyme activity rather than absolute protein levels of these enzymes in skeletal muscle. In line with this, it has been shown that exercise training in rats led to an increase in enzymatic activity of both OXCT1 and ACAT1 in skeletal muscle **[Askew et al. 1975]**. An important future experiment would thus be to measure enzymatic activity of OXCT1 and ACAT1 in sedentary and trained PGC-1α mKO and control mice. This would help to elucidate whether PGC-1α in skeletal muscle is required for an induction of ketolytic enzyme activity with exercise. In the next part of this study, we were interested in how a basal increase in PGC-1α levels in skeletal muscle would impact systemic ketosis. Interestingly, increased PGC-1α levels specifically in skeletal muscle of mice led to a reduced systemic ketosis in response to fasting, ketogenic diet feeding and exercise. Moreover, mice with a muscle-specific overexpression of PGC-1α displayed a significantly reduced hyperketonemia in response to experimentally induced diabetes. Diabetic ketoacidosis is a potentially lethal complication found in diabetic patients **[Laffel 1999]**. Hence, based on our findings, activation of PGC-1α in skeletal muscle could be used as a therapeutic strategy to ameliorate diabetic ketoacidosis in patients suffering from type 1 diabetes. However, PGC-1α levels are shown to be increased in liver of type 1 and type 2 diabetic mouse models **[Puigserver and Spiegelman 2003]**, and could thus contribute to both hyperglycemia and hyperketonemia during diabetes. Hence PGC-1α activation in type 1 diabetic patients would have to be restricted to skeletal muscle to have a beneficial effect in this context.

As an extension of this study, we also worked on a side-project aimed at investigating the adaptation of skeletal muscle to a chronic state of ketosis, and the role of PGC-1α therein. The role of PGC-1α in skeletal muscle adaptation to a ketogenic diet is important considering that a chronic ketogenic environment have been show to improve mitochondrial biogenesis and function in several organs. For example, ketogenic diet feeding was shown to increase mitochondrial biogenesis in both brain **[Bough et al. 2006]** and BAT **[Srivastava et al. 2013]**, and to improve mitochondrial function in skeletal muscle **[Ahola-Erkkila et al. 2010]** and heart **[Krebs et al. 2011]** of mice suffering from mitochondrial disorders. In this context, it was suggested that PGC-1α would act as an important down-stream mediator of these effects induced by ketogenic diet feeding. Hence, we were interested in elucidating whether ketogenic diet feeding would improve mitochondrial number and function in skeletal muscle, and whether PGC-1α would be required to mediate these effects. Since PGC-1α is an important regulator of skeletal muscle ketolytic capacity, we were also interested in whether a muscle-specific ablation of PGC-1α would influence the systemic adaptation to a ketogenic diet. To this end we used mice with a muscle-specific deletion of PGC-1α (mKO), and fed these mice a ketogenic diet (95 kcal% fat, 5 kcal% protein, <0.1% carbohydrates) or a normal chow-diet as control, for a period of 12 weeks. After 12 weeks on a ketogenic diet, we observed no differences between CTRL and PGC-1α mKO mice in either body weight (FIG 1A), calorie intake (FIG 1B) or relative fat mass (FIG 1C). However, a ketogenic diet slightly reduced absolute body weight in both groups (FIG 1A) and led to a significant increase in their relative fat mass (FIG 1C). Interestingly, even after 12 weeks on a ketogenic diet, mKO mice still displayed a significant hyperketonemia (FIG 1D). Moreover, both CTRL and mKO mice displayed reduced blood glucose levels (FIG 1E), which reflects the low carbohydrate content of the ketogenic diet. Importantly, we detected increased circulating levels of cell damage markers such as alanine transaminase (ALT) (FIG 1F) and lactate dehydrogenase (LDH) (FIG 1G) in plasma of CTRL and mKO mice. ALT is a liver-specific damage marker and indicates that the liver is damaged upon ketogenic diet feeding in our mice. This finding is in line with other studies which also reported increased hepatic steatosis and liver inflammation in mice fed a ketogenic diet **[Garbow et al. 2011]**. Taken together, these findings suggest that a ketogenic diet leads to detrimental effects in liver, most likely as a result of the high fat content of the diet. Importantly, increased incidence of hepatic steatosis is also associated with ketogenic diet feeding in humans **[Schugar and Crawford 2012]**. While the hyperketonemic phenotype of PGC-1α mKO mice was evident even after 12 weeks of ketogenic diet feeding, no other differences were detected between CTRL and mKO mice. Next we were interested in whether long-term ketogenic diet feeding would affect transcription of mitochondrial genes. In line with the known function of PGC-1α as a regulator of mitochondrial function,

we detected reduced transcript (FIG 2A) and protein (FIG 2B) levels of mitochondrial enzymes involved in oxidative phosphorylation. Moreover ADP-induced respiration in mitochondria isolated from skeletal muscle was reduced in the absence of PGC-1α (FIG 2C-D). However, in contrast to the results shown with ketogenic diet feeding in other studies **[Bough et al. 2006, Ahola-Erkkila et al. 2010, Krebs et al. 2011, Srivastava et al. 2013]**, we could not find any differences in either transcript (FIG 2A) or protein (FIG 2B) levels of mitochondrial genes. Moreover, ADP-induced complex I and II respiration in isolated mitochondria (FIG 2C-D) was not affected by ketogenic diet feeding. Complex I activity was even significantly reduced in both CTRL and mKO mice fed a ketogenic diet (FIG 2C). These data indicate that long-term feeding of a ketogenic diet does not induce protein levels of mitochondrial enzymes or mitochondrial function in skeletal muscle.

We were furthermore interested in how skeletal muscle metabolism adapts to a high-fat/low-carbohydrate diet such as a ketogenic diet. Since we showed earlier in our study that PGC-1α is important for ketolytic enzyme expression in skeletal muscle, we accordingly measured transcript levels of the ketone body transporter MCT1 and the ketolytic enzymes BDH1, OXCT1 and ACAT1 in skeletal muscle. As shown earlier, these genes were reduced in the absence of PGC-1α in skeletal muscle (FIG 2E). Moreover, both BDH1 and OXCT1 transcript levels were significantly reduced in skeletal muscle by ketogenic diet feeding (FIG 2E). Interestingly, at the protein level we detected the opposite effect, since OXCT1 protein levels were significantly increased in skeletal muscle with a ketogenic diet in both CTRL and mKO mice (FIG 2F). This indicates that the induction of OXCT1 with ketogenic diet feeding is likely mediated through a transcription-independent mechanism, which could also explain that the increase in OXCT1 occurred to the same extent in PGC-1α mKO mice as in CTRL mice (FIG 2F). Also ACAT1 protein levels were induced in skeletal muscle during ketogenic diet feeding, however this induction was slightly blunted in PGC-1α mKO mice (FIG 2F). Conclusively, this indicates that skeletal muscle ketolytic capacity is induced in skeletal muscle during long term ketogenic diet feeding. This increase is however not directly dependent on the transcriptional regulation of OXCT1 and ACAT1, and would warrant a closer examination of the mechanisms behind the increased levels of ketolytic enzymes in skeletal muscle. Further investigation demonstrated a significant reduction in transcript levels of glucose transporter 4 (*GLUT4*), hexokinase 2 (*HKII*), and pyruvate kinase, muscle isoform (*PKM1*) in both CTRL and mKO mice (FIG 2G), which reflects an adaptation to the reduced dietary carbohydrate intake. Moreover, the strong induction of pyruvate dehydrogenase lipoamide kinase isozyme 4 (*PDK4*) transcription (FIG 2G) and enhanced transcript levels of proteins involved in fatty acid uptake (cluster of differentiation 36, *CD36*) and β-oxidation (carnitine

palmitoyltransferase 1B, *Cpt1b;* long chain acyl-CoA dehydrogenase, *Lcad*) (FIG 2H) indicate a shift in skeletal muscle substrate utilization towards a higher reliance on lipids as an energy substrate. Interestingly, this adaptation did not occur to the same extent in PGC-1α mKO mice, since the induction of *CD36*, *Cpt1b* and *Lcad* were blunted in the absence of PGC-1α (FIG 2H). In conclusion, we demonstrate that PGC-1α is important for the induction of fatty acid transporters and β-oxidation enzymes in skeletal muscle during ketogenic diet feeding. This is most likely an adaptation in skeletal muscle to the high lipid content of the ketogenic diet. In contrast, induction of ketolytic proteins with ketogenic diet feeding occurred through a transcription-independent mechanism, and was accordingly independent of PGC-1α. Moreover, we did not detect any improvement of the mitochondrial phenotype in skeletal muscle during ketogenic diet feeding. In light of this, and considering the negative systemic effects of a high-fat/low-carbohydrate diet on accumulation of fat mass, and increased liver damage markers, we decided to discontinue this part of the study. It would however in future studies be interesting to elucidated whether increased levels of ketone bodies could exert beneficial effects on mitochondrial metabolism in skeletal muscle when disconnected from the negative effects of the high fat content of a ketogenic diet. Preferably, this would be performed as an extension of our current study. We could thus administer βOHB or ketone body esters **[Hashim and Vanitallie 2014]** to our mice through an implantable osmotic pump. We would try to match the ketone body levels in the circulation to those achieved in the mice fed a ketogenic diet and to perform the treatment for the same period of time. Subsequently we would perform the same metabolic tests and molecular analyses as performed in LCKD-fed mice. This would allow us to differentiate between the effects of increased βOHB levels and those elicited by the high lipid content of the ketogenic diet. One important question would be to discern whether increased βOHB levels enhance skeletal muscle mitochondrial biogenesis in chow-fed mice. As a positive control, we could use BAT where mitochondrial content was enhanced by ketogenic diet feeding in a recent study **[Srivastava et al. 2013]**. This would allow us to confirm the findings of this particular study and to conclusively answer whether a similar effect also occurs in skeletal muscle *in vivo*. A careful mapping of the metabolic changes elicited by LCKD-feeding compared to βOHB administration in mice would also help elucidate any potential caveats of using these interventions as a therapeutic strategy. These findings would provide important information that could be used to design future treatments using either ketogenic diets or βOHB-administration in humans.

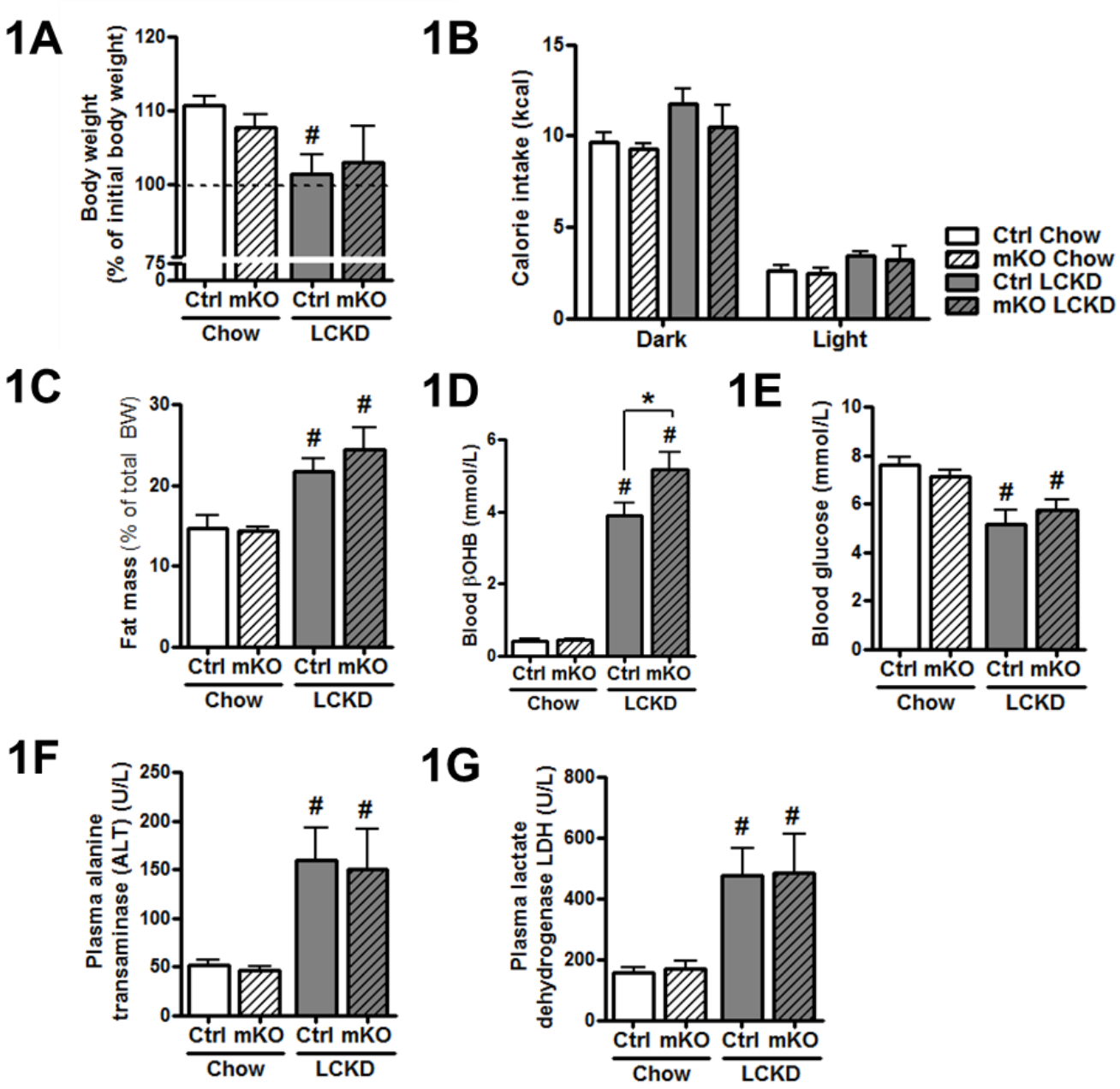

Figure 1 - Low-carbohydrate ketogenic diet (LCKD) feeding results in enhanced adiposity and increased circulating liver damage markers.

(A) Body weight of mice after 12 weeks feeding of either a chow diet (CHOW) or a low-carbohydrate ketogenic diet (LCKD) (n=13-16/group). (B) Calorie intake of either chow-fed or LCKD-fed mice after 10 weeks on the respective diets (n=7-8/group). (C) Fat mass normalized to absolute body weight. (D) Blood βOHB levels in the fed state (n=7-8/group). (E) Blood glucose levels in the fed state (n=7-9/group). (F) Plasma alanine transaminase (ALT) levels or (G) Plasma lactate dehydrogenase (LDH) levels after 6 weeks on either a chow- or LCKD-diet (n=8/group). Error bars represent mean ±SEM. Significant differences (p value < 0.05) between chow-fed and LCKD-fed groups are indicated by a number sign (#), and between CTRL mice and mKO mice by an asterisk (*).

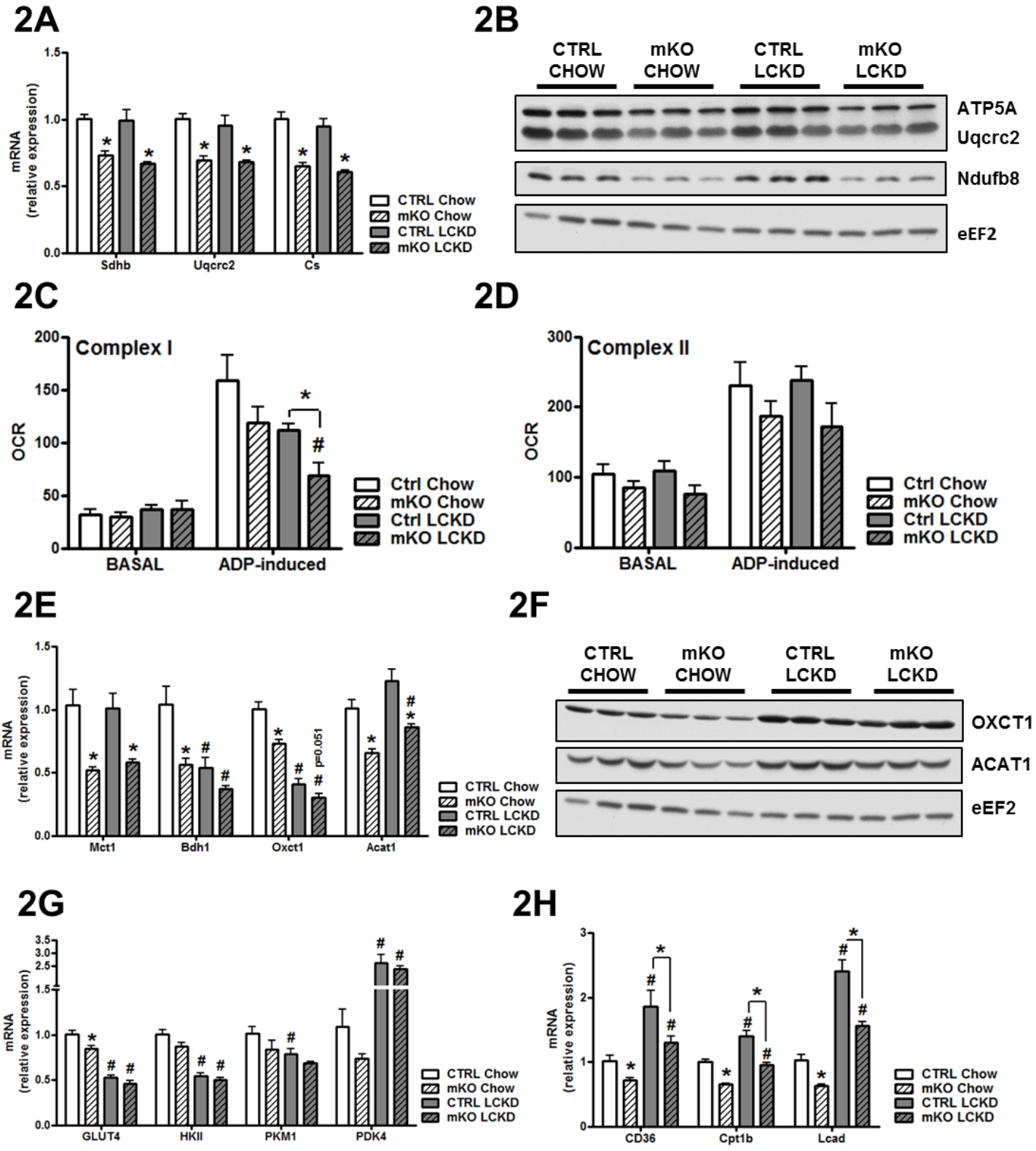
2A
mRNA (relative expression)
Sdhb
Uqcrc2
Cs
CTRL Chow
mKO Chow
CTRL LCKD
mKO LCKD
2B
CTRL CHOW
mKO CHOW
CTRL LCKD
mKO LCKD
ATP5A
Uqcrc2
Ndufb8
eEF2
2C
Complex I
OCR
BASAL
ADP-induced
Ctrl Chow
mKO Chow
Ctrl LCKD
mKO LCKD
2D
Complex II
OCR
BASAL
ADP-induced
Ctrl Chow
mKO Chow
Ctrl LCKD
mKO LCKD
2E
mRNA (relative expression)
Mct1
Bdh1
Oxct1
Acat1
p=0.051
CTRL Chow
mKO Chow
CTRL LCKD
mKO LCKD
2F
CTRL CHOW
mKO CHOW
CTRL LCKD
mKO LCKD
OXCT1
ACAT1
eEF2
2G
mRNA (relative expression)
GLUT4
HKII
PKM1
PDK4
CTRL Chow
mKO Chow
CTRL LCKD
mKO LCKD
2H
mRNA (relative expression)
CD36
Cpt1b
Lcad
CTRL Chow
mKO Chow
CTRL LCKD
mKO LCKD

Figure 2 – LCKD feeding does not alter mitochondrial parameters in skeletal muscle, but leads to a shift in substrate utilization.

(A) mRNA levels of the mitochondrial proteins *Sdhb*, *Uqcrc2* and *Cs* in skeletal muscle from CTRL and PGC-1α mKO mice after 12 weeks on a chow- or a LCKD (n=6-8/group). (B) Representative immunoblots of ATP5A, Uqcrc2, Ndufb8 and eEF2 in skeletal muscle from CTRL and PGC-1α mKO mice after 12 weeks on a chow- or a LCKD. Basal and ADP-induced oxygen consumption rate (OCR) of complex I (C) and complex II (D) of mitochondria isolated from skeletal muscle of CTRL and PGC-1α mKO mice after 12 weeks on a chow- or a LCKD (n=4-6/group). (E) mRNA levels of *Mct1*, *Bdh1*, *Oxct1* and *Acat1* in skeletal muscle from CTRL and PGC-1α mKO mice after 12 weeks on a chow- or a LCKD (n=6-8/group). (F) Representative immunoblots of OXCT1, ACAT1 and eEF2 in skeletal muscle from CTRL and PGC-1α mKO mice after 12 weeks on a chow- or a LCKD. mRNA levels of (G) *GLUT4*, *HKII*, *PKM1*, *PDK4* and (H) *CD36*, *Cpt1b*, *Lcad* in skeletal muscle from CTRL and PGC-1α mKO mice after 12 weeks on a chow- or a LCKD (n=6-8/group). All mRNA levels normalized to 18s rRNA. Error bars represent mean ±SEM. Significant differences (p-value <0.05) between chow-fed and LCKD-fed groups are indicated by a number sign (#), and between CTRL mice and mKO mice by an asterisk (*).

9.2 – Future perspectives

Obesity is a risk factor for the development of several severe diseases, such as diabetes, cardiovascular disease and cancer, amongst others. Exercise and CR would be efficient ways to ameliorate metabolic dysfunction in humans. However, it is not easy to maintain these diet- and exercise regimens. Thus, it has been suggested that administration of compounds, which could mimic the effects of either exercise **[Fan et al. 2013]** or CR **[Ingram et al. 2006]** would elicit beneficial metabolic effects in obese human patients. In this context, RSV and SRT1720 have been suggested to be potential candidates, since these compounds have been shown to activate SIRT1 **[Milne et al. 2007]**, and to be able to ameliorate metabolic dysfunction in obese mice **[Lagouge et al. 2006, Feige et al. 2008]**. The effects of RSV are dependent on several down-stream effectors, since AMPK, SIRT1 and cAMP-signaling were required to mediate the full effects of RSV in skeletal muscle **[Um et al. 2010, Park et al. 2012, Price et al. 2012]**. Downstream of AMPK, SIRT1 and cAMP signaling, PGC-1α has been suggested to be an important mediator of the beneficial effects of both RSV and SRT1720 in skeletal muscle **[Lagouge et al. 2006, Feige et al. 2008]**. However, it is not known whether the effects of these compounds are dependent on activation of PGC-1α in skeletal muscle. Accordingly, in the second manuscript of this thesis, we investigated whether skeletal muscle PGC-1α is an important down-stream mediator of the effects of RSV and SRT1720 in obese mice. Surprisingly, while treatment with these compounds resulted in beneficial metabolic effects in obese mice, such as increased energy expenditure, reduced adiposity and improved glucose tolerance, neither of these effects were dependent on skeletal muscle PGC-1α. Intriguingly, RSV and SRT1720 exerted only mild effects on skeletal muscle metabolism, while the effects of these compounds were more prominent in liver and adipose tissue, resulting in profound metabolic alterations in these organs. Hence, these findings indicate that modulation of PGC-1α activity, and in extension skeletal muscle metabolism, is not necessary for the ability of RSV and SRT1720 to ameliorate metabolic dysfunction in obese mice. However, the induction of several PGC-1α target genes in liver with SRT1720 treatment, and in adipose tissue with RSV treatment, indicates that PGC-1α might be an important mediator of the effects of RSV and SRT1720 in these organs. Hence, further studies are needed to elucidate the role of PGC-1α in response to RSV/SRT1720 treatment in liver and adipose tissue. As mentioned earlier, this could be achieved through the use of liver- and adipose tissue-specific PGC-1α knockout models and by employing a similar setup as used in our current study. This experimental setup would allow the dissection of the transcriptional networks affected by RSV or SRT1720 treatment in liver and adipose tissue. It would furthermore help elucidate whether PGC-1α is an important molecular effector of RSV and SRT1720 in

these organs. Studies on the organ-specific effects of RSV and SRT1720 treatment in obese mice would thus help delineate the contribution of different organs to the improved metabolic rate and enhanced glucose tolerance with these compounds and would help guide the design of more potent and specific therapeutic drugs. In line with this, to avoid potential caveats during RSV and SRT1720 treatment in humans, it is essential to identify the down-stream signaling pathways, which are affected by these compounds. Since RSV and SRT1720 have been demonstrated to activate SIRT1 **[Milne et al. 2007]** and since they elicit similar effects on whole body metabolism **[Lagouge et al. 2006, Feige et al. 2008]**, these compounds have been suggested to act through the same signaling pathways. Contrariwise, our study clearly demonstrates that RSV and SRT1720 do not exert analogous effects on mitochondrial and metabolic processes in adipose tissue and liver. Hence, RSV and SRT1720 do not seem to cause their effect through modulation of similar signaling pathways. Future studies should thus be aimed at elucidating the exact molecular mechanisms how RSV and SRT1720 exert their effects in target organs. These experiments would preferably be performed using either adipocyte and hepatocyte cell lines or primary cells. While such models cannot completely mimic the *in vivo* environment in a HFD-fed mouse, these experiments would be important to elucidate the molecular mechanisms how RSV and SRT1720 elicit their effects in these cell types. To allow an unbiased approach to elucidating the molecular effectors of RSV and SRT1720 in liver or adipose tissue, high-throughput techniques such as microarrays, proteome analysis as well as phosphoproteomics and acetylomics could be utilized. Bioinformatic approaches would then play an important role in confirming the predicted molecular effectors of these compounds (i.e. SIRT1, AMPK, PGC-1α) and to facilitate the discovery of any novel molecular effectors or target genes regulated by either RSV or SRT1720. Finally, the requirement for these novel targets can then be tested in an *in vivo* model by deleting the gene of interest in the organ of interest, and to treat these mice with either RSV or SRT1720. Due to the promising results obtained in rodent studies, there have been several clinical trials using both formulated RSV (SRT501) or other small-molecule activators of SIRT1, such as SRT2104 and SRT2379 in human subjects **[Villalba et al. 2012]**. However, in contrast to the unequivocal beneficial effects on metabolism in rodents, treatment of human patients did not result in clear beneficial effects. These findings suggest that the effects of RSV and SIRT1 activators on whole body metabolism do not seem to be as straight forward as initially thought. In line with this, activation of SIRT1 seems to be important to ameliorate metabolic dysfunction associated with obesity, but this is most likely not the only signaling pathway altered during exercise or caloric restriction. Hence, to successfully design CR/exercise mimetic compounds, a complete knowledge of the metabolic alterations occurring during exercise or CR must be obtained. Hence, it is important to build on the findings regarding RSV and SRT1720 from our

current study and through further research gain novel insight into the mode of action of these compounds. This will facilitate our understanding of the therapeutic potential of pharmacological CR/exercise mimetics and aid in the development of better and more efficient therapeutic strategies.

In the third study of this thesis, we focused on the role of PGC-1α in the transcriptional regulation of ketolytic enzymes, and its impact on systemic ketone body homeostasis. We found that PGC-1α is an important regulator of the ketolytic capacity in skeletal muscle, through its role together with ERRα as transcriptional regulators of the rate-limiting ketolytic enzymes, BDH1, OXCT1 and ACAT1. Importantly, we demonstrated that skeletal muscle ketolytic capacity is an important determining factor of circulating ketone body levels during states of ketosis. Interestingly, an increase in PGC-1α levels in skeletal muscle was sufficient to ameliorate hyperketonemia in diabetic mice. Hence, our findings have implications for the management of hyperketonemia in patients suffering from either diabetic or alcoholic ketoacidosis. Importantly, PGC-1α was also required for the increase in ketolytic capacity with exercise training. Hence, physical exercise could be beneficial not only for the management of glucose levels in diabetic patients **[Lopes Souto and Paes de Miranda 2011]**, but also for ameliorating diabetic hyperketonemia. Since PGC-1α regulates transcription of ketolytic enzymes in several organs, an open question is whether modulation of the ketolytic capacity in these organs would have an equally significant impact on systemic ketosis as in skeletal muscle. Cotter et al. showed that knockout of OXCT1 in neurons had a greater impact on circulating ketone body levels after 48 hours fasting in mice than ablation of OXCT1 in skeletal muscle **[Cotter et al. 2013]**. Hence, it would be interesting to study the adaptation of brain-specific PGC-1α knockout mice to long-term fasting. However, based on our findings that global PGC-1α null mice do not develop a more severe hyperketonemia than skeletal muscle specific knockouts, this would point towards a more prominent role of skeletal muscle in this context. However, these studies were only performed using a 24-hour fasting regime, and the individual contribution of different organs to the systemic ketolytic capacity could indeed be altered after 48 hours fasting. Interestingly, ablation of PGC-1α in skeletal muscle did not affect the adaptation to long term ketogenic diet feeding. A likely reason for this could be that the induction of OXCT1 protein in skeletal muscle during this chronic ketotic state occurred through non-transcriptional mechanisms. In contrast to this, in a study by Wentz et al. the authors observed a decrease in both transcript and protein levels of OXCT1 in heart during a ketogenic diet **[Wentz et al. 2010]**. Considering our findings, there might be fundamental differences in the adaptation to a chronic ketotic state between heart and skeletal muscle and understanding this regulation would be an important subject for future studies. This is crucial, especially in the context of the widespread use of ketogenic diets

for both weight loss and anticonvulsant therapies **[Paoli et al. 2013]**. Recently, Schugar et al. published a study where the metabolic profile of mice with a cardiac-specific deletion of OXCT1 was characterized **[Schugar et al. 2014]**. It would be interesting in future experiments to replicate this experiment in OXCT1-deficient skeletal muscle and thereby determine the metabolic profile in skeletal muscle in the absence of ketolytic capacity. Surprisingly, we did not observe any increase in either mitochondrial protein or function in skeletal muscle with ketogenic diet feeding. This is an interesting finding, considering that such an effect has been shown to occur for both BAT and brain **[Bough et al. 2006, Srivastava et al. 2013]**. This could once again represent a differential adaptation to a chronic ketogenic state between different organs and would need to be studied in more detail. It would also be interesting to investigate the role of chronic ketosis on mitochondrial capacity of skeletal muscle disconnected from the administration of a ketogenic diet. This could be achieved through the administration of ketone body esters **[Hashim and Vanitallie 2014]** as discussed earlier in this chapter. Such an approach would increase circulating ketone body levels without any negative impact on blood pH, or any negative metabolic impact from the elevated fat content of a ketogenic diet. This is an intriguing question, since βOHB has been shown to function *in vivo* as an HDAC1-inhibitor **[Shimazu et al. 2013]** and treatment with other HDAC1-inhibitors was shown to increase mitochondrial biogenesis in C2C12 myotubes in a PGC-1α-dependent manner **[Galmozzi et al. 2013]**. Hence, further studies are needed to elucidate whether βOHB can act as an HDAC-inhibitor also in skeletal muscle *in vivo,* and if disconnected from the negative effects of a high fat diet, whether it could lead to an activation of PGC-1α and mitochondrial biogenesis. In line with this, it would be interesting to study the effects of βOHB administration in C2C12 myotubes. Subsequent transcriptional analysis would provide important insight into the effects on gene expression elicited by βOHB. Hence, this *in vitro* setup would allow the characterization of the molecular effects elicited by βOHB administration in skeletal muscle cells, devoid of any precluding effects due to the high lipid content of a ketogenic diet. Moreover, this setup would enable us to characterize mitochondrial content and oxidative function of myotubes in the context of βOHB administration. To this end we would use methods such as immunoblotting and transcriptional analysis to quantify mitochondrial transcript and protein levels. Moreover, oxidative capacity and metabolic flux in βOHB-treated myotubes would be quantified using am extracellular flux analyzer such as the Seahorse system. Would these experiments yield promising results, βOHB administration could also be performed in mice to study both the acute and the long-term effects of ketone bodies on skeletal muscle physiology and function. In this context, whole body metabolic parameters such as metabolic rate, fat content and glucose tolerance would be assessed. Moreover, mitochondrial content and function in skeletal muscle would be analyzed using similar methods as in our

in vitro setup mentioned above. Conclusively, as far as we can tell, our study is the first to elucidate the transcriptional regulation of ketone body oxidation in skeletal muscle. While previous studies have demonstrate the importance of skeletal muscle as a determinant of whole body ketolytic capacity **[Cotter et al. 2013]**, we can show that by enhancing the ketolytic capacity in skeletal muscle through overexpression of PGC-1α, we can ameliorate diabetic hyperketonemia. This provides important insight into the molecular regulation of ketolysis. However, further studies are needed to identify additional molecular effectors which are important for both transcriptional and transcription-independent regulation of ketone body oxidation. Together this will improve our understanding of systemic ketone body homeostasis and the therapeutic potential of a state of ketosis, especially in the context of metabolic and muscle-related disorders.

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10. Thesis – Conclusions and future perspectives

PGC-1α is a versatile transcriptional coactivator which is important for transcriptional adaptation of cellular metabolism in response to various environmental cues, such as fasting, cold or exercise. Since the first cloning of PGC-1α in 1998 and the subsequent characterization of its role as an important thermogenic regulator in BAT **[Puigserver et al. 1998]**, a plethora of studies have expanded our understanding of PGC-1α biology and its role in physiological as well as pathophysiological states. During the work on this thesis, several studies have been published that have discovered new aspects of PGC-1α biology, such as its role in regulation of myokine crosstalk between skeletal muscle and adipose tissue **[Bostrom et al. 2012, Rao et al. 2014]** or its novel roles in retinal and intestinal energy metabolism **[D'Errico et al. 2011, Egger et al. 2012, Saint-Geniez et al. 2013]**, to name a few. Moreover, through the use of high-throughput sequencing techniques, the global transcriptional network of PGC-1α has been mapped in both liver and muscle cells **[Charos et al. 2012, Baresic et al. 2014]**. Collectively, these and many other studies have increased our knowledge of the ubiquitous role of PGC-1α as a metabolic regulator in different organs. However, these studies also reveal that there are still many aspects of PGC-1α biology which are not fully elucidated. Hence, in the scope of this thesis we have investigated several novel aspects of PGC-1α function.

PGC-1α is an established transcriptional regulator of thermogenesis, gluconeogenesis and exercise adaptation. Hence, the role of PGC-1α is best understood in metabolic organs such as BAT, liver and skeletal muscle. In contrast, relatively little is known regarding the role of PGC-1α in the kidney. While the kidney is a highly metabolic organ with a high baseline PGC-1α expression, it does not contribute extensively to systemic metabolic homeostasis. This could be one explanation for the lack of information on PGC-1α in this organ. Nonetheless, mitochondrial and metabolic dysfunction have emerged as important components in the pathogenesis of renal disorders **[Niaudet 1998, Sharma et al. 2013, Che et al. 2014]**. Accordingly, this has sparked an interest in finding ways to ameliorate mitochondrial dysfunction in the kidney, potentially through activation of PGC-1α. However, to recognize the full therapeutic potential of PGC-1α activation in the kidney, it is important to understand the role of this coactivator in basal renal physiology. In this thesis we demonstrate that PGC-1α is an important regulator of mitochondrial and metabolic gene programs in the kidney. Moreover, our data indicate that ERRα and PPARα are important transcriptional partners of PGC-1α in the kidney, which links PGC-1α to the regulation of renal mitochondrial biogenesis and fatty acid oxidation. Intriguingly, several nuclear receptors display a heterogeneous expression pattern in different nephron segments **[Krid et al. 2012]**.

This would in extension suggest that the transcriptional networks regulated by PGC-1α differ between different parts of the kidney, depending on the local transcription factor expression profile. Accordingly, future studies should be aimed at dissecting the transcriptional networks of PGC-1α in each individual nephron segment or cell type. This is of importance when considering the differential function, as well as mitochondrial and metabolic heterogeneity of different nephron segments **[Valtin 1977, Kurokawa 1981]**. Hence, the transcriptional analysis from our study represents the combined effects of PGC-1α deletion in all nephron segments and thus the complete renal transcriptional program. For a more detailed functional analysis, these transcriptional alterations would have to be mapped to a specific region along the renal nephron. As discussed in chapter 5, this could be accomplished through microdissection of specific nephron segments. Preferably, these experiments would be performed in PiNKO mice and subsequent microarray analysis could reveal the distinct transcriptional networks of PGC-1α in different segments of the nephron. Such an approach would increase the resolution of the renal transcriptional networks regulated by PGC-1α, and would provide a more comprehensive overview of the effects of this coactivator in the kidney. Surprisingly, despite the strong impact of PGC-1α deletion on metabolic and mitochondrial gene transcription networks, deletion of PGC-1α in tubular epithelial cells did not interfere with the ability of the kidney to regulate systemic ion, water or nutrient homeostasis. However, we noticed that aged PiNKO mice had difficulties to adapt to a low-sodium environment. Aging is related to a decline in renal mitochondrial function, combined with increased inflammation and reduced renal function **[Weinstein and Anderson 2010, Schmitt and Melk 2012]**. It has been shown that PGC-1α activation ameliorates mitochondrial dysfunction and confers therapeutic benefits in both skeletal muscle and heart during aging **[Dillon et al. 2012]**. Hence, absence of PGC-1α in the kidney might increase the risk of developing aging-related renal dysfunctions. So far it has not been investigated how PGC-1α influences aging-related renal dysfunctions and an important future direction would thus be to elucidate the precise role of PGC-1α in this process (as discussed in chapter 5). In our study we have focused on elucidating the role of PGC-1α in the basal function of tubular salt and water reabsorption. However, future studies should be aimed at investigating whether PGC-1α is involved in regulation of other renal functions, for example gluconeogenesis. Intriguingly, fasting has been shown to induce PGC-1α expression in the kidney **[Teng et al. 2011]** and several TFs known to interact with PGC-1α in the regulation of hepatic gluconeogenesis (i.e. HNF4a and CREB) are also expressed in the kidney **[Mutel et al. 2011, Krid et al. 2012]**. Considering that the kidney can match the gluconeogenic potential of liver in the post-prandial state **[Mitrakou 2011]**, it will be important to elucidate whether PGC-1α also regulates this process in the kidney. To evaluate the role of PGC-1α in renal gluconeogenesis, it is important to localize the main site of *de novo* glucose

production in the kidney. Since the proximal tubules express high levels of gluconeogenic genes **[Mather and Pollock 2011, Krid et al. 2012]**, this would be a good cell type to begin investigating. The next step would be to assess whether HFD- or fasting-induced gluconeogenic gene transcription is altered in this cell type and whether the absence of PGC-1α would blunt the induction of the renal gluconeogenic gene program, preferably using the PiNKO mouse model. While both liver and kidney use pyruvate as a gluconeogenic substrate, glutamine is preferentially used by the kidney. Hence, glutamine administration and measurement of *de novo* glucose synthesis allows measuring the renal gluconeogenic potential in PiNKO mice. If a connection between PGC-1α and renal gluconeogenesis would be apparent, the next step would be to confirm with ChIP experiments whether PGC-1α directly binds to the promoter region of gluconeogenic genes in the kidney and whether this binding can be enhanced by fasting. These experiments would help widen our knowledge of how renal gluconeogenesis is regulated and the role of PGC-1α therein. Taken together, we have demonstrated that PGC-1α has a central role as a regulator of metabolic and mitochondrial transcriptional programs in the kidney. Despite the fact that PGC-1α is dispensable for basal renal function, it could still be a promising therapeutic target to ameliorate renal metabolic disorders associated with mitochondrial dysfunction and lipotoxicity. Hence, future studies should be aimed at determining the therapeutic potential of PGC-1α activation in the kidney (as discussed in chapter 5).

Exercise and CR elicit several beneficial metabolic effects on skeletal muscle metabolism, such as improved mitochondrial function, reduced insulin resistance and increased fat oxidation **[Schenk et al. 2011, Lanza et al. 2012, Egan and Zierath 2013]**. In line with this, overexpression of PGC-1α in skeletal muscle has been demonstrated to ameliorate mitochondrial and metabolic dysfunction in several rodent disease models **[Svensson and Handschin 2014]**. PGC-1α has a central role in metabolic regulation and is regulated by a multitude of upstream signaling pathways (see chapter 1). Hence, PGC-1α is postulated to be the molecular effector of several drugs, which modulate mitochondrial and metabolic properties in oxidative organs. Relevant examples are RSV and SRT1720, which are both SIRT1-activating compounds and in turn have been shown to activate PGC-1α in skeletal muscle **[Lagouge et al. 2006, Feige et al. 2008]**. In the scope of this thesis we wanted to validate the role of PGC-1α as a molecular effector of the beneficial metabolic effects elicited by RSV and SRT1720 in skeletal muscle. While we observed only mild effects on transcription of mitochondrial genes in skeletal muscle with RSV and SRT1720 treatment, these effects were dependent on PGC-1α. Intriguingly, SRT1720 treatment could improve the oxidative phenotype of skeletal muscle and increase mitochondrial density even in the absence of PGC-1α and

without any major alterations in mitochondrial gene transcription. Unfortunately, due to the mild effects of RSV and SRT1720 on skeletal muscle metabolism and mitochondrial content, we cannot draw any strong conclusions regarding the role of PGC-1α as a molecular effector of RSV and SRT1720 in skeletal muscle. We can however conclude that skeletal muscle PGC-1α is not necessary to confer the systemic metabolic effects of RSV and SRT1720, since both systemic metabolic rate and glucose tolerance were improved in the absence of skeletal muscle PGC-1α. Importantly, the effects of RSV and SRT1720 were more prominent in liver and adipose tissue, resulting in profound metabolic alterations in these organs. The induction of several PGC-1α target genes in liver with SRT1720 and in adipose tissue with RSV treatment indicates that PGC-1α might be an important mediator of the effects of RSV and SRT1720 in these organs. Hence, further studies are required to elucidate the role of PGC-1α in response to RSV/SRT1720 treatment in liver and adipose tissue (as discussed in chapter 9). Since the results in this study were obtained in sedentary mice, this could preclude a more prominent role for muscle PGC-1α in the regulation of the metabolic phenotype of physically active mice. Hence, it will be important to study the efficiency of RSV and SRT1720 as adjuvant therapies together with either exercise or CR in mice. So far, RSV supplementation during exercise has not resulted in any conclusive positive results in neither mice nor humans **[Gliemann et al. 2013, Olesen et al. 2013, Ringholm et al. 2013, Voduc et al. 2014]**. However, further studies should be aimed at investigating the beneficial effects of SRT1720 supplementation on exercise performance and metabolic health. Based on our findings, SRT1720 elicited a positive effect on skeletal muscle oxidative capacity and mitochondrial number that was not seen with RSV treatment. Hence, SRT1720 could potentially boost the beneficial effects of exercise training on oxidative metabolism in skeletal muscle to a greater extent than RSV. A similar setup as that used in our current study would provide a good basis for studying the effects of SRT1720 as an adjuvant therapy to exercise for the treatment of obesity and metabolic dysfunction. Mice would be administered SRT1720 in a HFD and kept either sedentary in their home cage or exercised by treadmill running 3 times a week for a duration of one month. This specific setup has yielded significantly improved endurance capacity in mice in previous experiments (unpublished observation). It would also be interesting to assess the ability of SRT1720 to boost the endurance exercise performance of chow-fed mice in combination with continuous exercise training. In such as setup, the potential of SRT1720 to act as an exercise mimetic would be assessed without any confounding effects imparted by HFD-feeding. In another direction, neither RSV nor SRT1720 have been extensively studied as adjuvant therapies together with CR. This would be an interesting direction for future research, especially considering the beneficial effects of reduced calorie intake on metabolic health in both mice and humans **[Mercken et al. 2012]**. As indicated

by our study and by other recent studies investigating the effects of RSV in skeletal muscle **[Higashida et al. 2013, Olesen et al. 2013, Ringholm et al. 2013]**, it is evident that skeletal muscle is not the main organ targeted by these SIRT1-activating compounds. Hence, future studies should be aimed at elucidating the target-organs and molecular effectors of the beneficial metabolic effects elicited by CR/exercise mimetics such as RSV and SRT1720. As mentioned earlier, these studies should be performed with an unbiased approach based on microarray analysis or proteomics/phosphoproteomics to map the molecular events occurring in liver, adipose tissue and skeletal muscle during treatment with either RSV or SRT1720. Any novel molecular targets of these compounds identified by such a study could subsequently be characterized in more detail. Initially this should be done in an *in vitro* setup. However, at a later stage a similar setup as we have employed in this thesis could be used, where the protein of interest is knocked out and the metabolic effects of either RSV or SRT1720 are investigated on this genetic background.

PGC-1α is an important transcriptional regulator of mitochondrial ATP-production and skeletal muscle metabolism. PGC-1α increases the capacity of skeletal muscle to oxidize lipids and sustain oxidative metabolism during submaximal exercise. Moreover, it is closely involved in muscle refueling through regulation of glucose uptake, glycogenesis and lipogenesis **[Chan and Arany 2014]**. Despite its central role in the regulation of mitochondrial metabolic processes, it is not known to which extent PGC-1α contributes to skeletal muscle ketone body oxidation. In this thesis we demonstrate that PGC-1α is an important regulator of skeletal muscle ketolytic capacity and that modulation of PGC-1α specifically in muscle is sufficient to affect systemic ketone body homeostasis in response to fasting, LCKD feeding or exercise. Moreover, overexpression of PGC-1α in skeletal muscle was sufficient to ameliorate diabetic ketoacidosis in mice. We furthermore show that PGC-1α is necessary for the transcriptional induction of ketolytic enzymes in skeletal muscle and the increased systemic ketolytic capacity with exercise. Importantly, ERRα is an important partner of PGC-1α in the transcriptional regulation of ketolytic enzymes. Hence, in this thesis we have for the first time investigated the transcriptional regulation of ketolytic enzymes in muscle. Further studies are now needed to map the complete transcriptional network responsible for regulation of skeletal muscle ketolytic capacity. We have been able to link both NCoR1- and PGC-1β to the regulation of skeletal muscle ketolysis (unpublished data), indicating that PGC-1α is not a unique regulator of this metabolic process. Moreover, analysis of ChIP-sequencing data from the promoter regions of the ketolytic genes *Acat1*, *Oxct1* and *Bdh1* reveal that PGC-1α and ERRα bind only within the *Oxct1*-gene (unpublished observation). Hence, these data point towards an indirect mechanism how PGC-1α affects the transcription of *Acat1* and *Bdh1*. However, these findings need to be

confirmed and the transcription factors relevant for the regulation of these genes need to be identified. This could be accomplished on one hand through bioinformatic-based *de novo* motif search, where TF binding sites are predicted based on the sequence of the promoter region of the gene of interest. Additionally, reverse ChIP could be used to extract a stretch of genomic DNA to assess which proteins interact with this specific region, for example the promoter region of the ketolytic genes *Acat1*, *Oxct1* and *Bdh1*. These methods could reveal novel putative transcriptional regulators of ketolytic gene transcription, and would subsequently have to be tested in an *in vitro* setup. Considering the extensive network of transcriptional regulators known to interact with PGC-1α, it would be interesting to use either knockdown or pharmacological inhibition of the predicted TFs in combination with overexpression of PGC-1α. In analogy with the experiments using ERRα inhibition in our current setup, these experiments could reveal novel transcriptional partners of PGC-1α in the regulation of ketolytic gene transcription.

Intriguingly, during long term LCKD-feeding, OXCT1 protein levels were increased in skeletal muscle despite a strong reduction in *Oxct1* mRNA levels. This divergence indicates that OXCT1 protein levels are also regulated through non-transcriptional mechanisms. In order to understand how the ketolytic capacity in skeletal muscle is controlled, it will be interesting to elucidate the molecular mechanisms responsible for the non-transcriptional regulation of OXCT1 protein levels. Considering that enhanced OXCT1 protein levels during long-term LCKD feeding could be due to increased protein stability, it would be interesting to assess whether PTMs, such as phosphorylation or acetylation of OXCT1 differ between chow- and LCKD-fed mice. To make a connection between OXCT1 protein stability and proteolytic breakdown, it would also be interesting to assess polyubiquitination of the OXCT1 protein. The initial step would be to perform immunoprecipitation of OXCT1 and evaluate these individual PTMs using specific antibodies. Additional steps would be to map the specific residues of OXCT1 carrying PTMs *in vivo* by using a mass spectrometry approach. Finally, mutations of one or several of these residue could help to evaluate the effect these PTMs would confer on OXCT1 protein stability. Another interesting aspect would be to study the role of ketone bodies as a metabolic fuel in the context of exercise. Administration of ketone body-precursors has been postulated to enhance exercise capacity **[Hashim and Vanitallie 2014]** and the increase in circulating ketone bodies after exercise could potentially contribute to post-exercise muscle refueling. Understanding ketone body metabolism in the context of exercise might thus provide novel insights into exercise physiology. To study the impact of ketolytic capacity on exercise performance in mice, it would be interesting to use a skeletal muscle-specific OXCT1-knockout mouse model. In these mice, the ketolytic capacity is ablated and it would thus be possible to specifically

evaluate the importance of ketone bodies as an energy substrate, both during and after endurance exercise. It would furthermore be interesting to assess substrate utilization in skeletal muscle during exercise. This could be accomplished by measuring parameters such as glucose, fatty acids, lactate and ketone bodies in the blood and in skeletal muscle before and after an acute endurance exercise bout. More importantly, mice could be exercised in a closed treadmill system, where oxygen consumption rates as well as respiratory quotient could be measured. When combined with molecular analysis in skeletal muscle of different metabolic enzymes and transporter, and quantification of intramuscular energy stores and metabolic intermediates, this would give a comprehensive overview on how skeletal muscle metabolism is altered in the absence of ketolytic capacity. In extension, muscle-specific OXCT1 knockout mice could be trained by either being kept in running wheel cages, or through treadmill running, as mentioned earlier. By conducting similar experiments as mentioned above, this would help elucidate the importance of ketolytic capacity for the metabolic adaptation of skeletal muscle during long-term endurance exercise training. Finally, future studies should be aimed at determining the impact of ketone bodies on PGC-1α activation and mitochondrial biogenesis in skeletal muscle. While we could not observe any positive effects on these parameters in the context of ketogenic diet feeding, the beneficial effects of increased ketosis could however have been precluded by the high lipid content of the diet. Hence, administration of ketone body precursors or ketone body esters would be a more suitable approach. Since βOHB was recently shown to act as an HDAC-inhibitor *in vivo* **[Shimazu et al. 2013]**, it is likely that βOHB administration would confer beneficial metabolic effects in mice and humans. Especially since HDAC-inhibitors have been demonstrated to improve mitochondrial function in skeletal muscle and BAT **[Galmozzi et al. 2013]** and to protect against pathogenic alterations in both heart and brain **[Kazantsev and Thompson 2008, McKinsey 2012]**. Moreover, HDAC-inhibition has been suggested as a novel therapeutic strategy to ameliorate diabetic complications **[Christensen et al. 2011]**. Hence, future studies should be aimed at determining the therapeutic potential of βOHB administration and its ability to improve the metabolic profile of skeletal muscle as well as other organs (as discussed in chapter 9).

In the studies performed during this thesis we have revealed new insights into the role of PGC-1α in renal physiology and its potential role as a therapeutic target in kidney. Moreover, we have evaluated the role of PGC-1α as a mediator of SIRT1-activating compounds in skeletal muscle and identified PGC-1α as a novel transcriptional regulator of skeletal muscle ketolytic capacity. These studies demonstrate the importance of PGC-1α for many different biological processes, which are all ultimately connected to mitochondrial metabolism. Due to the pervasive role of PGC-1α in metabolic regulation in oxidative

organs, targeting this coactivator would be a potential therapeutic strategy to ameliorate many disorders with a metabolic or mitochondrial etiology. Hence, the data presented in this thesis increases our understanding of the complex transcriptional networks and cellular processes regulated by PGC-1α and will help in the development of more efficient therapeutic strategies against metabolic disorders.

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APPENDICES

REVIEWS Drug Discovery Today • Volume 19, Number 7 • July 2014

Reviews • POST SCREEN

Modulation of PGC-1α activity as a treatment for metabolic and muscle-related diseases

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Physical inactivity is a predisposing factor for various disease states, including obesity and cardiovascular disease, as well as for certain types of cancer. Regular endurance exercise mediates several beneficial effects, such as increased energy expenditure and improved skeletal muscle function, and has been suggested as a therapeutic strategy for both metabolic and muscle-related disorders. 'Exercise mimetic' is a collective term for compounds that can pharmacologically activate pathways that are normally induced during skeletal muscle contraction, and that could be used in the treatment of metabolic or muscle-related diseases. Two such experimental 'exercise mimetics' are 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) and resveratrol, which have both been extensively studied in the context of metabolic dysfunction and muscle wasting in rodent disease models. These compounds have been postulated to activate AMP-activated protein kinase (AMPK) and sirtuin 1 (SIRT1), respectively, in skeletal muscle, and to increase the activation of the peroxisome proliferator-activated receptor γ co-activator 1α (PGC-1α). PGC-1α can mediate several metabolic and functional adaptations in skeletal muscle in response to physical exercise and, therefore, is an interesting target for the development of new 'exercise mimetic' drugs.

Although regular exercise is important to maintain health and longevity, physical inactivity predisposes for many chronic diseases of metabolic origin, such as obesity, type 2 diabetes mellitus (T2DM) and cardiovascular pathologies, as well as certain types of cancer [1]. Importantly, lack of physical activity is also a key element in the etiology of aging-associated dysfunctions, for example sarcopenia and cognitive impairment [2]. Endurance exercise has been successfully applied or suggested as a therapy in an array of metabolic and muscle-related disorders, for example in patients who are obese or who have T2DM [3], cancer-associated cachexia [4] or muscular dystrophies [5]. Considering the physiological impact of exercise training at the whole body level and its low cost, this strategy is a preferred choice to either surgical or pharmacological interventions in patients who are obese [6]. However, this approach is not without inherent difficulties, because adherence to an exercise regime can be difficult to maintain, and the impairment of skeletal muscle function observed in patients with diseases such as sarcopenia or muscular dystrophies can limit the use of exercise as therapy [5]. Hence, in some pathological contexts, it would be advantageous to pharmacologically activate the same pathways that are activated by muscle contraction and, thus, enhance the therapeutic effects of exercise.

When the homeostasis of a resting muscle cell is perturbed during a contraction, several pathways are either activated or inhibited to mediate the adaptive response to exercise, both at a post-translational and transcriptional level (reviewed in [7]). The transcriptional response in skeletal muscle to a single bout of exercise in humans can influence the expression of more than 900 genes, resulting in adaptive changes in mitochondrial metabolism, angiogenesis, β-oxidation and inflammation [7,8]. A key mediator of these transcriptional changes and a main point of convergence for different signaling events taking place in a contracting muscle is PGC-1α [9]. Skeletal muscle PGC-1α is increased during exercise in humans [10] and can drive a slow oxidative gene program through the activation of several transcription factors, such as estrogen-related receptor α (ERRα), nuclear respiratory factor 1/2 (NRF-1/2) and transcription factor A, mitochondrial (TFAM) [7,9]. In response to an energy stress such as muscle contraction, PGC-1α can be activated by two important energy sensors: AMPK and SIRT1 [11] (Figure 1). AMPK is allosterically activated by both ADP and AMP during energy deficits, and positively regulates ATP production through phosphorylation of metabolic enzymes [12]. Interestingly, AMPK also confers long-term transcriptional adaptations in response to energy stress, by phosphorylation and activation of several transcription factors as well as transcriptional co-activators, including PGC-1α [13]. Another important energy sensor during muscle contraction is SIRT1, a member of the SIRT family of class III deacetylases. SIRT1

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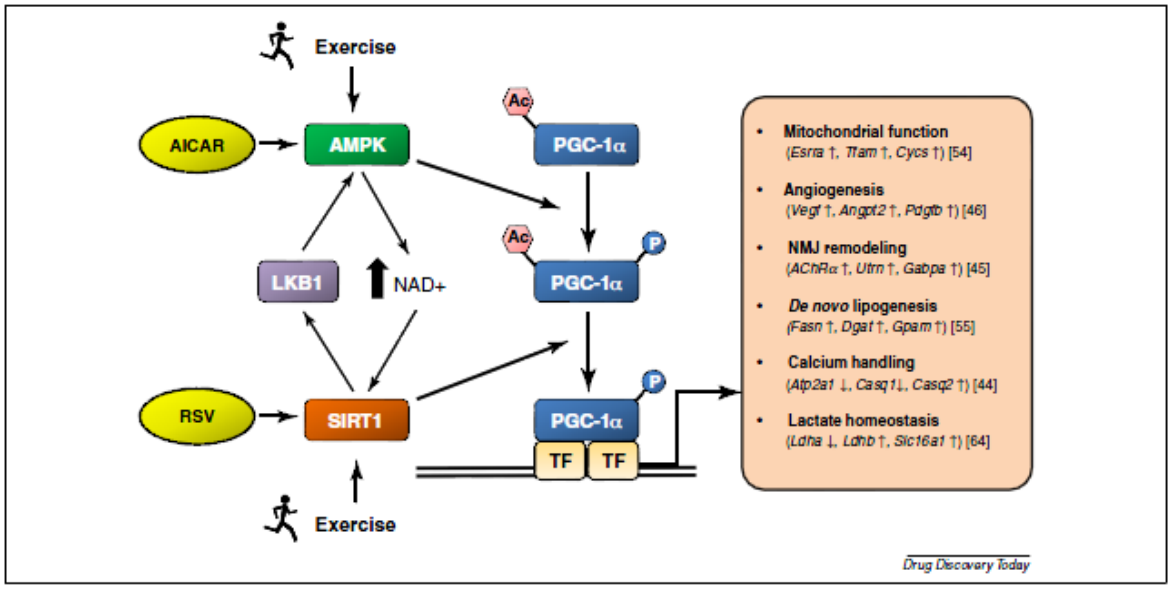

FIGURE 1

Schematic representation of the processes involved in AMP-activated protein kinase (AMPK)- and sirtuin 1 (SIRT1)-mediated activation of peroxisome proliferator-activated receptor γ coactivator 1-α (PGC-1α). During physical exercise, AMPK and SIRT1 are activated in skeletal muscle. Crosstalk between these two proteins occurs through SIRT1-mediated deacetylation of the AMPK-kinase liver kinase B1 (LKB1), and through the ability of AMPK to raise intracellular NAD+ levels to activate SIRT1 [11]. Coordinated activation of AMPK and SIRT1 leads to activation of PGC-1α through AMPK-mediated phosphorylation and subsequent deacetylation by SIRT1. PGC-1α then interacts with several transcription factors (TF), to mediate the induction of gene programs important for skeletal muscle adaptation to exercise (see box), through either direct or indirect transcriptional modulation. 5-Aminoimidazole-4-carboxamide ribonucleotide (AICAR) and resveratrol are pharmacological compounds that can activate AMPK and SIRT1, respectively, and thereby increase activation of PGC-1α in skeletal muscle. Given their ability to activate signaling pathways normally induced during physical exercise, AICAR and resveratrol have been termed 'exercise mimetics' [61]. Abbreviations: AChRα, acetylcholine receptor α; Angpt2, angiopoietin-2; Atp2a1, sarco/endoplasmic reticulum Ca^{2+}-ATPase 1; Casq1, calsequestrin 1; Casq2, calsequestrin 2; Cycs, cytochrome complex; Dgat, diglyceride acyltransferase; Esrra, estrogen-related receptor α; Fasn, fatty acid synthase; Gabpa, GA-binding protein alpha chain; Gpam, mitochondrial glycerol-3-phosphate acyltransferase; Ldha, lactate dehydrogenase a; Ldhb, lactate dehydrogenase b; NMJ, neuromuscular junction; Pdgfb, platelet-derived growth factor subunit B; Slc16a1, monocarboxylate transporter 1; Tfam, transcription factor A, mitochondrial; Utrn, utrophin; Vegf, vascular endothelial growth factor. Key: (P), phosphorylation; (Ac), acetylation; ↑, upregulation; ↓, downregulation.

senses perturbations in the NAD+:NADH ratio in muscle cells during exercise, and can mediate adaptive changes in mitochondrial function through deacetylation and activation of PGC-1α, as well as through direct deacetylation of several transcription factors [14]. The central role for AMPK and SIRT1 as energy sensors in skeletal muscle, as well as the presence of pharmacological activators of these two enzymes, makes them attractive targets for possible 'exercise mimetics'. In this review, we highlight the therapeutic potential of such putative 'exercise mimetics', as well as the implications of using direct PGC-1α activation as a treatment for metabolic and muscle-related disorders.

AMPK and AICAR

AMPK is activated in skeletal muscle during exercise and mediates both acute and long-term metabolic adaptations to cope with the increased energy demand. Interestingly, in patients who are obese and/or have T2DM, activation of skeletal muscle AMPK during exercise is blunted [15]. This reduced activity of AMPK was also present in obese mice, and was further associated with reduced exercise capacity and diminished glucose uptake into skeletal muscle cells [16]. Therefore, in this context, it would be useful to enhance the positive effects of endurance training in patients who are obese by pharmacological activation of AMPK in skeletal muscle. One potential candidate for this is AICAR, an AMP analog that can activate AMPK *in vivo* [13]. AICAR administration has been shown to reduce obesity and improve glucose homeostasis in mice [17] and to increase glucose uptake in skeletal muscle of both healthy patients and those with T2DM [18], which reflects the potential therapeutic use of AICAR as an 'exercise mimetic'. Importantly, AICAR can boost PGC-1α transcription and post-translational modifications in skeletal muscle [19]. AICAR enhances endurance performance and energy expenditure through increased transcription of mitochondrial and metabolic genes in sedentary mice [20]. PGC-1α had a central role in mediating these 'exercise mimetic' effects of AMPK activation, because at least some genes activated by AICAR administration have been demonstrated to be dependent on PGC-1α [21]. Given the beneficial effects on skeletal muscle function and metabolism, AICAR has also been studied in the context of muscular dystrophies and muscle wasting. In angiotensin II-mediated muscle wasting in mice, daily AICAR administration normalized AMPK activity, reversing the loss of skeletal muscle mass [22]. By contrast, AICAR could not attenuate the decrease in muscle mass induced by denervation in mice, even though PGC-1α expression and

mitochondrial protein content in atrophic muscles were normalized [23]. These divergent effects of AICAR on attenuation of skeletal muscle wasting could be dependent on the different etiologies of these two atrophic mouse models, and would represent a variable therapeutic effect of AICAR depending on the nature of the muscle wasting disorder.

In several recent papers, AICAR was used to treat mdx mice, a model for Duchenne muscular dystrophy (DMD). In this model, AICAR administration improved skeletal muscle function through pleiotropic effects, including increased mitochondrial activity, reduced inflammation and increased mitophagy [24–26]. Furthermore, AICAR treatment in combination with the peroxisome proliferator-activated receptor δ (PPARδ) agonist GW1516 was shown to potentiate AMPK-activity during concomitant exercise in mdx mice [27], and could prove effective in enhancing beneficial effects of exercise in patients with DMD.

Thus, in summary, AMPK activation in skeletal muscle by AICAR can lead to several beneficial effects in obese and diabetic mouse models. In atrophic and dystrophic mouse models, AICAR can improve muscle function and mitochondrial activity, although attenuation of skeletal muscle loss could not be demonstrated in all atrophic mouse models.

SIRT1 and resveratrol

Resveratrol (RSV) is a naturally occurring antioxidant that activates SIRT1 *in vivo*, and thereby promotes PGC-1α deacetylation [28,29]. In obese sedentary mice, RSV improved exercise performance, ameliorated metabolic dysfunction and extended lifespan [28,29]. RSV also boosted mitochondrial adaptations in skeletal muscle in response to exercise [30,31], and some of these additive effects of RSV and exercise were further demonstrated to be dependent on skeletal muscle SIRT1 [31]. These findings have made RSV an interesting candidate for also treating obesity and T2DM in human subjects. In a recent clinical study, oral RSV administration did not affect either body weight or insulin sensitivity in patients who were obese [32], although, in a similar study, RSV supplementation mediated mild metabolic adaptations combined with an increased transcription of mitochondrial genes in skeletal muscle [33]. By contrast, in patients with T2DM, RSV administration has shown potential to improve glycemic control and insulin sensitivity [34,35]. RSV has also been investigated as a potential treatment for muscle wasting. In a rat model of mechanical unloading, RSV administration prevented the decline in skeletal muscle oxidative capacity [36] and attenuated the loss of skeletal muscle strength induced by muscle disuse [36,37]. It was also found to attenuate skeletal muscle wasting in a mouse model for cancer-associated cachexia, an effect that was associated with a reduced nuclear factor kappa B (NFκB) activation and a reduction in muscle RING-finger protein 1 (MuRF1) expression [38]. The protective effects of RSV in these mouse models of skeletal muscle atrophy could be attributed to the activation of PGC-1α, because this co-activator has been shown to reduce NFκB-driven transcription and attenuate activation of atrophic pathways in skeletal muscle [23,39].

Similar to AICAR, RSV has also been studied in the context of muscle dystrophy. In dystrophic mdx mice, RSV attenuated the decline in skeletal muscle mass [40] and fatigue resistance [41], but did not ameliorate the ongoing inflammation or muscle injury [40]. By contrast, a different study in young mice demonstrated an ameliorating effect of RSV treatment also on the infiltration of inflammatory cells into dystrophic muscle [42].

Thus, RSV administration has shown therapeutic potential for both metabolic and muscle-wasting disorders in mice, whereas in human obese subjects, the beneficial effects of oral RSV administration so far are less promising.

PGC-1α: a central regulator in exercise

Physical exercise induces a range of signaling pathways, all contributing to the metabolic and functional adaptations of skeletal muscle. The beneficial effects of activating one of these pathways through pharmacological modulation of either AMPK or SIRT1 have been demonstrated in several rodent disease models. This approach to designing 'exercise mimetics' has yielded encouraging results, but given the complex response to exercise in skeletal muscle, these compounds could only be considered as partial 'exercise mimetics'. A more complete 'exercise mimetic' would instead directly target a central effector that could mediate both metabolic and functional adaptations to exercise in the skeletal muscle, and one such potential target would be PGC-1α.

Skeletal muscle PGC-1α drives several gene programs that are important for muscle adaptation to endurance exercise. This is evident in mice overexpressing PGC-1α specifically in skeletal muscle, which demonstrate a shift towards a slow-twitch oxidative phenotype, characterized by an increased mitochondrial biogenesis and higher fatigue resistance [43]. These changes are consistent with what has been observed in humans during acute endurance exercise, where an increase in PGC-1α was associated with a slow-type muscle fiber phenotype [10]. PGC-1α can also regulate other processes that are important for exercise adaptation, such as muscle fatigue resistance and force generation [44], adaptation of the neuromuscular junction [45], angiogenesis [46], as well as glycogen storage [47]. PGC-1α overexpression in skeletal muscle of mice resulted in an increased endurance capacity combined with a higher utilization of lipids as energy substrate during exercise [48]. The opposite was seen in mice carrying a skeletal muscle-specific knockout of PGC-1α, where exercise performance was reduced [49]. Mice deficient for skeletal muscle PGC-1α also displayed increased muscle damage after an acute exercise bout [49], demonstrating an important role for PGC-1α in maintaining muscle integrity during exercise.

PGC-1α acts as a convergence point for many signaling events taking place in a contracting skeletal muscle, and drives a pleiotropic transcriptional response resulting in improved muscle metabolism and endurance capacity. This makes modulation of PGC-1α an interesting target for the treatment of metabolic and muscle related diseases.

PGC-1α: a potential target for 'exercise mimetics'

An important factor in the prevention and treatment of obesity is to increase energy expenditure. Importantly, resting energy expenditure is increased up to 48 h after an acute exercise bout in humans [50]. Irisin, a myokine released from skeletal muscle during exercise is an obvious candidate to mediate this effect, because it has been shown to increase whole-body energy expenditure through enhanced mitochondrial uncoupling in white adipose tissue [51]. PGC-1α regulates the transcription of irisin in skeletal muscle and, in PGC-1α muscle-knockout mice, circulating plasma levels of this

myokine were reduced accordingly [51]. Interestingly, in contrast to the increased resting whole-body energy expenditure seen in mice with an ectopic overexpression of irisin in liver [51], no positive effect on this parameter was demonstrated in chow-fed mice overexpressing PGC-1α in skeletal muscle [48,52]. These results could indicate a minor role for skeletal muscle PGC-1α in the regulation of energy expenditure through irisin during physical inactivity, while still being able to modulate energy expenditure during exercise. This suggestion is further corroborated by the increased peak oxygen consumption seen in PGC-1α transgenic mice during acute exercise [48].

In mice fed a high-fat diet, increased skeletal muscle PGC-1α was not followed by increased resting energy expenditure, altered weight gain or improved glucose and insulin levels [52]. Surprisingly, overexpression of PGC-1α in muscle was instead found to have a detrimental effect on insulin-stimulated glucose uptake in sedentary mice fed a high-fat diet [52]. This increased insulin resistance was attributed to an increased uptake of fatty acids into muscle, which exceeded the capacity of mitochondrial lipid oxidation and, combined with elevated *de novo* lipogenesis, resulted in increased intramuscular lipid storage and a concomitant reduction in skeletal muscle insulin sensitivity [52]. Importantly, however, when these mice received a continuous exercise intervention during the high-fat feeding, these detrimental effects were reversed, and muscle glucose uptake as well as whole-body glucose homeostasis was improved [53], demonstrating a synergistic effect of physical exercise and PGC-1α overexpression on glucose homeostasis. By increasing PGC-1α in skeletal muscle, the metabolic flexibility in the muscle is increased, mimicking what has been demonstrated during physical exercise [64]. These changes include not only an upregulation of mitochondrial fat oxidation [54], but also shunting of glucose away from oxidation, towards glycogen synthesis [47] and lipogenesis [55]. Thus, if PGC-1α activity was increased in skeletal muscle of sedentary individuals, this could lead to an uncoupling of the anabolic refueling processes induced by exercise from the increase in energy expenditure during muscle contraction. In a sedentary obese state where circulating lipid levels are already increased, activation of PGC-1α might result in exacerbated intramuscular lipid accumulation and peripheral insulin resistance. These results suggest that using PGC-1α as an 'exercise mimetic' for the treatment of obesity and T2DM in the absence of actual physical activity could worsen the metabolic dysfunction in skeletal muscle as well as at a whole-body level.

However, activation of PGC-1α might still prove to be a valid therapeutic option in the context of metabolic dysfunctions that are not primarily associated with increased obesity. One such example is during aging, where overexpression PGC-1α in skeletal muscle of mice was shown to ameliorate aging-associated insulin resistance and improve the metabolic flexibility of aged skeletal muscles [56].

In aged mice, increased PGC-1α expression also improved muscle function and endurance capacity in conjunction with reduced sarcopenia [56]. A similar protective role of PGC-1α in skeletal muscle atrophy was also seen in fasting- and denervation-induced atrophy, where PGC-1α overexpression rescued oxidative capacity and reduced the induction of a transcriptional atrophic response in the muscle [39]. This effect of PGC-1α was proposed to occur through inhibition of forkhead box O3 (FoxO3) and NFκB-driven transcription of atrophy-specific ubiquitin ligases, leading to a reduction in proteolysis [23]. The ability of PGC-1α to inhibit NFκB-driven transcription has also recently been demonstrated for expression of pro-inflammatory cytokines in skeletal muscle [57]. In mice with cancer-induced cachexia, PGC-1α overexpression upregulated mitochondrial biogenesis, but did not prevent the reduction in body weight and muscle mass associated with the disease [58]. Interestingly, the authors also showed that increased levels of PGC-1α in skeletal muscle caused an increase in tumor size, an effect that was speculated to occur through growth-promoting myokines and could limit the potential therapeutic use of PGC-1α activation in this context [58]. By contrast, in dystrophic mdx-mice, transgenic PGC-1α overexpression reduced muscle damage and improved muscle function [45]. These effects were also demonstrated in mice subjected to PGC-1α gene transfer into dystrophic muscles, resulting in a fast-to-slow fiber-type switch, increased expression of utrophin and increased satellite cell activation [41,59]. Increased muscle PGC-1α also demonstrated a therapeutic potential in a mouse model for amyotrophic lateral sclerosis (ALS), where it improved muscle function and endurance capacity of the mice even during the later stages of the disease, unfortunately in the absence of a positive effect on lifespan of the mice [60].

These results demonstrate a strong potential for PGC-1α to attenuate muscle wasting in several etiologically distinct disease states and, furthermore, to improve both the metabolic phenotype and function of the affected muscles.

Concluding remarks

The use of AICAR and RSV to mimic exercise through activation of exercise-induced pathways in the sedentary skeletal muscle has shown therapeutic beneficial effects in rodent models of metabolic and/or muscle-related diseases (Table 1). Given that it is hard to mimic all the metabolic, neuronal as well as mechanical stimuli that occur during an actual muscle contraction, these 'exercise mimetic' compounds could instead be administered in combination with physical activity to enhance the therapeutic potential of exercise. A direct effect of such an 'exercise enhancer' would be to boost the activation of certain pathways during exercise, and thereby increase the beneficial adaptations to exercise in the muscle, a method that has proven to be successful for both RSV treatment [30,31] and PGC-1α activation [53]. By contrast, in conditions where skeletal muscle dysfunction is a central part of the disease etiology, administration of an 'exercise enhancer' to such patients could increase their mobility and ability to perform physical activities, and thereby improve life quality. The fact that PGC-1α can activate gene programs associated with both mitochondrial and functional adaptations in skeletal muscle, makes it an important potential candidate for treatment of diseases where reduced muscle function is a main part of the etiology. In line with this, increased levels of PGC-1α in skeletal muscle have been shown to ameliorate the decline in muscle function in mouse models associated with muscle-wasting diseases, such as DMD [45] and ALS [60], as well as in aging [56] (Table 1). By contrast, in metabolic diseases such as obesity and T2DM, PGC-1α activation could instead prove to be detrimental, because it activates lipid refueling in skeletal muscle without a concomitant increase in energy expenditure, which could exacerbate insulin resistance.

Therefore, activation of PGC-1α in skeletal muscle on the one hand could represent a potential way to improve muscle function

TABLE 1

Effects of AICAR, resveratrol and PGC-1α overexpression on metabolism and muscle function in rodent disease models[a]

Disease model	AICAR		Resveratrol		PGC-1α overexpression	
	Effect	Refs	Effect	Refs	Effect	Refs
Obesity and/or T2DM	Obesity ↓ Insulin sensitivity ↑	[17] [17]	Obesity ↓ Insulin sensitivity ↑ Exercise performance ↑ Life span ↑	[28,29] [28] [29]	Intramuscular lipid storage ↑ Insulin sensitivity ↓ **In combination with exercise:** Glucose homeostasis ↑ Insulin sensitivity ↑	[52] [53]
Aging	Not determined		Mitochondrial activity ↔ Muscle wasting ↔	[63]	Insulin sensitivity ↑ Exercise performance ↑ Muscle wasting ↓	[56]
Angiotensin II-induced atrophy	Muscle wasting ↓	[22]	Not determined		Not determined	
Disuse and/or denervation-induced atrophy	Mitochondrial proteins ↑ Muscle wasting ↔	[23]	Oxidative capacity ↑ Muscle force ↑	[36] [36,37]	Oxidative capacity ↑ Muscle wasting ↓	[39]
Cancer-associated cachexia	Not determined		Muscle wasting ↓	[38]	Mitochondrial activity ↑ Muscle wasting ↔ Tumor size ↑	[58]
DMD	Mitochondrial proteins ↑ Muscle weight ↑ Inflammation ↓	[24,25] [25]	Muscle wasting ↓ Inflammation ↓ Muscle function ↑	[40] [42] [41]	Muscle function ↑ Muscle damage ↓	[41,45,59]
ALS	Not determined		Not determined		Muscle function ↑ Exercise performance ↑ Life span ↔	[60]

[a] ↑, increase or ↓ decrease in the described process and/or phenotype in treated and/or transgenic mice compared with untreated and/or wild type mice; ↔, no difference between treated and/or transgenic mice compared with untreated and/or wild type mice.

in atrophic diseases, but, on the other hand, could be less suitable as a treatment for obesity in the absence of actual physical exercise. Finally, it is important to note that the experimental 'exercise mimetics' AICAR, RSV and PPARδ ligands [20] have several potential drawbacks that hinder broad application in humans, such as poor oral bioavailability and adverse effects that could complicate chronic treatment [61]. Furthermore, it is debatable whether real 'exercise mimetics' can even be developed [62]. Therefore, until such therapeutically deployable compounds are found, bona fide physical activity remains a cheap, yet effective intervention of choice for many diseases.

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MicroRNAs Emerge as Modulators of NAD$^+$-Dependent Energy Metabolism in Skeletal Muscle

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Obesity, type 2 diabetes, and related metabolic disorders are associated with reduced mitochondrial function in skeletal muscle and other organs (1). However, causality of aberrant mitochondrial activity in the etiology of these diseases and the exact mechanism(s) linking metabolic pathologies to mitochondrial dysfunction are still under debate. Therefore, a better molecular understanding of this observation is required in order to design novel therapeutic strategies aimed at modulation of mitochondrial function in these disease contexts. The silent mating type information regulation 2 homolog 1 (SIRT-1) and the peroxisome proliferator-activated receptor γ coactivators 1 (PGC-1) are important regulators of mitochondrial function in skeletal muscle (1). In response to deacetylation by SIRT-1, PGC-1α is activated and subsequently promotes an oxidative muscle fiber phenotype. In turn, SIRT-1 activity is regulated by the substrate NAD$^+$, and thus acts as an intracellular rheostat by increasing mitochondrial biogenesis to match the energy needs of the cell (2).

Interestingly, recent studies have exploited this mechanism by using the NAD$^+$ precursors nicotinamide riboside (3) and nicotinamide mononucleotide (4) to increase SIRT-1 activity and thereby the oxidative capacity of skeletal muscle. Alternatively, intracellular NAD$^+$ can be elevated through inhibition of other enzymes that use NAD$^+$. In particular, the poly(ADP-ribose) polymerases (PARPs), which metabolize NAD$^+$ to form polymers of ADP-ribose (PAR) on other proteins, are major consumers of NAD$^+$ in various cell types. PARPs are involved in regulation of several distinct cellular processes, such as DNA repair, inflammation, and differentiation (5), but they have also recently been recognized as having important roles in metabolism (6). In fact, germ-line deletion of either PARP-1 or PARP-2, or pharmacological inhibition of PARP activity by PJ-34, results in elevated intracellular NAD$^+$ levels in skeletal muscle with the expected outcome of increased SIRT-1 activity and mitochondrial oxidative function (7,8). In most tissues, PARP-1 accounts for the vast majority (>85%) of cellular PARP activity, and is therefore also the major NAD$^+$ consumer (6). Thus, PARP-1 deletion presumably affects SIRT-1 primarily due to increased NAD$^+$ levels (8). In contrast, even though PARP-2 null mice also show increased NAD$^+$ levels (7), PARP-2 also acts as a transcriptional repressor of SIRT-1 (7).

Modulation of PARP activity provides a novel therapeutic strategy to increase SIRT-1 expression and activity in skeletal muscle, and represents an alternative to pharmacological activation of SIRT-1 using resveratrol or SRT1720 (9,10). Interestingly, several studies indicate that PARP activity in murine skeletal muscle is dysregulated during high-fat diet (HFD) feeding (8) and aging (11), implying a direct involvement of PARPs in development of metabolic dysfunction in muscle. This idea is now further supported in the study by Mohamed et al. (12) published in this issue, in which the authors demonstrate that skeletal muscle PARylation was increased in HFD-fed mice concomitant with elevated expression of PARP-2. Surprisingly, in contrast to other studies that have reported elevated PARP-1 in HFD-fed mice (8), the authors detected no differences in PARP-1 expression with HFD feeding. As expected however, elevated PARP-2 expression was associated with lower NAD$^+$ levels and reduced SIRT-1 expression, together with a reduction in mitochondrial oxidative function. Depletion of intracellular NAD$^+$ through increased PARP-2 activity was therefore postulated as a major mechanism leading to mitochondrial dysfunction in these mice.

Although simple and attractive, the findings do not exclude the idea that PARPs, apart from acting as a transcriptional repressor of SIRT-1 (7), also interact with

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See accompanying article, p. 1546.

other transcriptional regulators such as forkhead box class O1 (FoxO1) (13) and peroxisome proliferator-activated receptor γ (PPARγ) (14). Further, direct PARylation of mitochondrial proteins has also been demonstrated to reduce oxidative function (15), suggesting that the connection between PARP-2 and mitochondrial dysfunction in skeletal muscle is multifaceted. In a series of elegant experiments, Mohamed et al. (12) investigated the long-elusive mechanism behind increased PARP-2 expression in HFD-fed mice by performing a microRNA (miRNA) screen in skeletal muscle. Several differentially regulated miRNAs were discovered, of which two (miR-149 and miR-712–3p) were significantly downregulated with HFD feeding, and therefore considered potential candidates for the observed regulation of PARP-2. However, only miR-149 was able to reduce PARP-2 levels in vitro, and it bound to a conserved seed region in the 3′UTR of PARP-2. In a well-designed in vitro setup, the authors could then demonstrate that overexpression of miR-149 in cultured myotubes led to a reduction in PARP-2, elevated NAD^+ levels, increased SIRT-1 activity, and concomitantly promoted mitochondrial biogenesis. These in vitro data are in many aspects opposite to the muscle phenotype observed in HFD-fed mice, and it will therefore be interesting to see whether overexpression of miR-149 in skeletal muscle in vivo will have a protective effect against obesity-induced metabolic dysfunction. Skeletal muscle–specific inhibition of PARP-2 activity would also circumvent the dichotomy present in global PARP-2 null mice, which simultaneously exhibit increased peripheral insulin sensitivity as well as β-cell dysfunction and insulinopenia (7). Furthermore, it will be important to elucidate whether the miR-149/PARP-2/SIRT-1 axis postulated here in a mouse model is also dysregulated in human obesity, and thus a valid therapeutic target in patients.

Mohamed et al. have identified a novel miRNA that modulates oxidative function in skeletal muscle by affecting the intracellular levels of the metabolite NAD^+. Besides miR-149, other miRNAs, such as miR-494 (16), miR-23 (17), and miR-696 (18), analogously affect mitochondrial biogenesis and function in skeletal muscle, however by inhibiting different targets (Fig. 1). Interestingly, all of these miRNAs are regulated by exercise (16–18). Since both exercise and caloric restriction are linked to increased SIRT-1 activity and improved mitochondrial function, it will be interesting to investigate whether miR-149, and by extension also PARP-2, is differentially regulated in these metabolically beneficial states. In conclusion, Mohamed et al. (12) provides novel evidence of dysregulated PARP-2 during obesity, and proposes an important role of miR-149 in the control of intracellular NAD^+ levels, thus providing another example of a miRNA that acts as a major regulator of cellular metabolism. The relevance of this article is underscored by the emerging role of PARPs in metabolic regulation (6), and thus provides a framework for futures studies on the connection between PARP activity and mitochondrial function. Moreover, these findings put an emphasis on the clinical relevance of PARP inhibitors, not only in the field of oncology (19), but also as an incipient therapeutic strategy for treatment of obesity and obesity-related metabolic disorders.

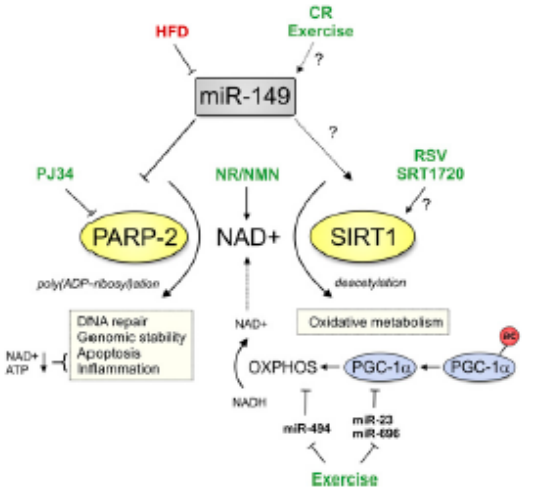

Figure 1—MiRNA-controlled regulation of metabolic reprogramming of skeletal muscle cells by the metabolite NAD^+. Expression of miR-149 is regulated by HFD feeding and potentially by caloric restriction (CR) as well as exercise. PARP-2 is inhibited by miR-149 while directly or indirectly, this miRNA increases the activity of SIRT1. Thus, by shifting the balance between two major NAD^+ consumers, miR-149 determines the metabolic fate of muscle cells and promotes SIRT-1–dependent deacetylation of the transcriptional coactivator PGC-1α. As a consequence, mitochondrial function and oxidative metabolism in muscle cells are boosted. This system has recently been shown to likewise be under exercise-controlled regulation via different miRNAs. Inversely, HFD-mediated repression of miR-149 results in increased activity of PARP-2, which, in turn, not only promotes DNA damage repair, but also apoptosis and inflammation in a cellular context of depleted NAD^+ and ATP. This dichotomy in the regulation of cellular metabolism in muscle can furthermore be modulated by pharmacological interventions aimed at PARP-2 and SIRT-1 activities, as well as elevation of cellular NAD^+ levels using PJ34, resveratrol (RSV), SRT1720, nicotinamide riboside (NR), and nicotinamide mononucleotide (NMN), respectively. OXPHOS, oxidative phosphorylation.

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ARTICLE

The coactivator PGC-1α regulates skeletal muscle oxidative metabolism independently of the nuclear receptor PPARβ/δ in sedentary mice fed a regular chow diet

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Abstract
Aims/hypothesis Physical activity improves oxidative capacity and exerts therapeutic beneficial effects, particularly in the context of metabolic diseases. The peroxisome proliferator-activated receptor (PPAR) γ 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, though their interdependence remains unclear. Hence, our aim was to determine the functional interaction between these two factors in mouse skeletal muscle in vivo.
Methods Adult male control mice, PGC-1α muscle-specific transgenic (mTg) mice, PPARβ/δ muscle-specific knockout (mKO) mice and the combination PPARβ/δ mKO + PGC-1α mTg mice were studied under basal conditions and following PPARβ/δ agonist administration and acute exercise. Whole-body metabolism was assessed by indirect calorimetry and blood analysis, while magnetic resonance was used to measure body composition. Quantitative PCR and western blot were used to determine gene expression and intracellular signalling. The proportion of oxidative muscle fibre was determined by NADH staining.
Results Agonist-induced PPARβ/δ activation was only disrupted by PPARβ/δ knockout. We also found that the disruption of the PGC-1α–PPARβ/δ axis did not affect whole-body metabolism under basal conditions. As expected, PGC-1α mTg mice exhibited higher exercise performance, peak oxygen consumption and lower blood lactate levels following exercise, though PPARβ/δ mKO + PGC-1α mTg mice showed a similar phenotype. Similarly, we found that PPARβ/δ was dispensable for PGC-1α-mediated enhancement of an oxidative phenotype in skeletal muscle.
Conclusions/interpretation Collectively, these results indicate that PPARβ/δ is not an essential partner of PGC-1α in the control of skeletal muscle energy metabolism.

Keywords Coregulators · Exercise · Nuclear receptors · Skeletal muscle metabolism

Abbreviations

AMPK	AMP-activated protein kinase
CON	Control mice
GTT	Glucose tolerance test
IMTG	Intramyocellular triacylglycerol
ITT	Insulin tolerance test
mKO	Muscle-specific knockout
mTg	Muscle-specific transgenic
PGC	PPARγ coactivator
PPAR	Peroxisome proliferator-activated receptor
qPCR	Quantitative PCR
RER	Respiratory exchange ratio

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TBP	TATA binding protein
UCP3	Uncoupling protein 3
$\dot{V}O_{2peak}$	Peak oxygen consumption

Introduction

The regulation of energy metabolism in skeletal muscle is highly controlled by the peroxisome proliferator-activated receptor (PPAR) γ coactivator-1α (PGC-1α) [1]. PGC-1α drives the expression of genes involved in catabolic processes leading to aerobic ATP synthesis [1] while concomitantly promoting anabolic processes, including de novo lipogenesis [2]. Once activated, PGC-1α boosts the activity of different transcription factors to control gene programmes resembling an endurance-trained phenotype in skeletal muscle [1, 3]. These adaptations are associated with an enhanced oxidative capacity, which contributes to an increased skeletal muscle fatigue resistance ex vivo and exercise performance in vivo [4–6]. 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 mainly based on cell culture and pharmacological studies [7]. PGC-1α acts as a coactivator of PPARβ/δ [8–10], while PPARβ/δ can directly regulate PGC-1α expression [11, 12], indicating that this nuclear receptor acts both upstream and downstream of PGC-1α. Furthermore, transgenic mouse models for PPARβ/δ exhibit a similar phenotype to their counterparts for PGC-1α [4, 5, 13, 14]. Nevertheless, although the PGC-1α–PPARβ/δ axis appears to play a key role in the regulation of energy metabolism, the epistatic interaction between these proteins is currently unclear. 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.

Methods

Animals Mice were housed in a conventional facility with a 12 h night/day cycle and had free access to food/water. 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/δ*$^{loxP/loxP}$ mice with HSA-Cre transgenic mice [11]. Finally, PGC-1α mTg and HSA-Cre positive *PPARβ/δ*$^{loxP/loxP}$mKO mice were crossed to generate PPARβ/δ mKO + PGC-1α mTg mice. *PPARβ/δ*$^{loxP/loxP}$ mice without *Cre* and *Pgc-1α* (also known as *Ppargc1a*) transgene expression were used as control (CON) mice. All mice had mixed sv129 and C57BL/6 background. Genotypes were confirmed through PCR procedures (data not shown) and quantitative PCR analysis in kidney and skeletal muscle (Fig. 1a, b).

PPARβ/δ agonist administration CON mice were subjected to an intraperitoneal injection of either 0.9% NaCl (control) or 1 mg/kg of body weight of the PPARβ/δ agonist GW0742 (Tocris No. 2229; Tocris, Bristol, UK), as previously described [15]. Muscles were collected 8 h following drug administration.

Body composition analysis Lean and fat mass were measured via magnetic resonance imaging (EchoMRI, Houston, TX, USA).

Blood and plasma analysis Blood samples were collected under basal conditions or immediately after maximal exercise from fed and/or overnight-fasted mice, as previously described [9].

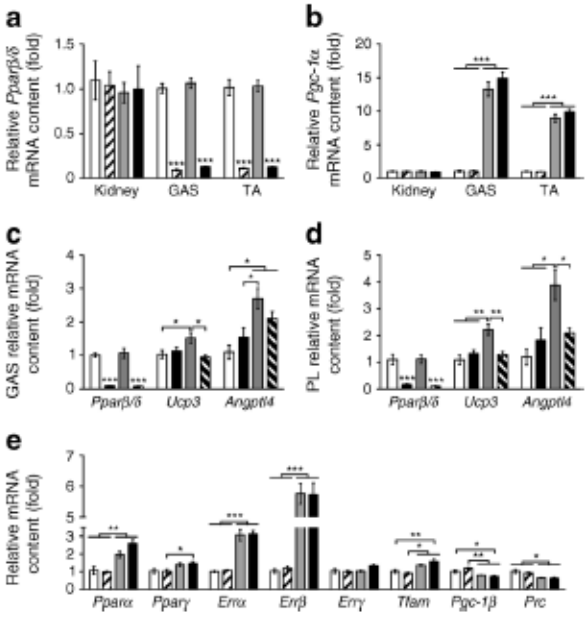

Fig. 1 PGC-1α and PPARβ/δ mouse models. (**a**, **b**) *Pparβ/δ* and *Pgc-1α* mRNA levels in kidney, gastrocnemius (GAS) and tibialis anterior (TA) (n=6 per group). (**c**, **d**) *Pparβ/δ*, *Ucp3* and *Angptl4* mRNA levels in GAS and plantaris (PL) 8 h after the injection of 0.9% NaCl (as control) or 1 mg/kg of body weight of the PPARβ/δ agonist GW0742 (n=6 per group). (**e**) mRNA level of different transcriptional regulators in GAS (n=6 per group). In (**a**), (**b**) and (**e**): white bars, CON; hatched bars, PPARβ/δ mKO; grey bars, PGC-1α mTg; black bars, PPARβ/δ mKO + PGC-1α mTg. In (**c**) and (**d**): white bars, CON + NaCl; black bars, PPARβ/δ mKO + NaCl; grey bars, CON + GW0742; hatched bars, PPARβ/δ mKO + GW0742. Values are mean ± SEM. *p<0.05, **p<0.01 and ***p<0.001 for the indicated comparisons. In (**a**), (**c**) and (**d**) ***p<0.001 vs CON and/or PGC-1α mTg for same tissue/treatment. When shown, fold changes are reported vs CON group

Glucose and insulin tolerance test Intraperitoneal glucose tolerance tests (GTTs) were carried out by injecting 2 g/kg of body weight of glucose after mice had been fasted for 16 h. Insulin tolerance tests (ITTs) were performed by injecting 0.8 U/kg of body weight of insulin (Novo Nordisk, Bagsvaerd, Denmark) after mice had been fasted for 6 h.

Indirect calorimetry Mice were individually housed in a Comprehensive Lab Animal Monitoring System (Columbus Instruments, Columbus, OH, USA) for an acclimatisation period of 48 h during which they were allowed free access to food and water. Subsequently, indirect calorimetry was performed for 48 h and data was analysed with the Oxymax software (Columbus Instruments).

Maximal exercise test Exercise tests were performed as previously described [9]. Briefly, 2 days after acclimatisation, mice performed a maximal exercise test in a closed treadmill (Columbus Instruments), allowing the measurement of peak oxygen consumption ($\dot{V}O_{2peak}$) and respiratory exchange ratio (RER; CO_2 produced divided by consumed $O_2[\dot{V}CO_2/\dot{V}O_2]$).

Histology NADH staining was performed on 10 μm cross sections from tibialis anterior by exposing the sections to 1 mg/ml NADH (Sigma, St Louis, MO, USA) in the presence of 1 mg/ml nitro blue tetrazolium (Sigma).

NEFA measurement Plasma NEFA were measured using a commercial kit (HR Series NEFA-HR(2); Wako Diagnostics, Richmond, VA, USA), according to the manufacturer's instructions. Blood samples were collected under basal conditions and following 1 h of treadmill running at 13 m/min with 5° slope.

Intramyocellular triacylglycerol extraction Quadriceps intramyocellular triacylglycerols (IMTGs) were extracted by standard procedures using a solid-phase extraction column (UPTI-CLEAN NH2-S 100 mg/1 mL SPE Columns; Interchim, Montluçon, France) and quantified with a commercial kit (Triglyceride enzymatique PAP 150; Biomérieux, Marcy-l'Etoile, France), according to the manufacturer's instructions.

RNA isolation and quantitative PCR Total RNA isolation from fed (ad libitum) mice and quantitative PCR (qPCR) analysis was performed by standard procedures [9]. Sequences of qPCR primers are depicted in electronic supplementary material (ESM) 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 between experimental conditions.

Protein isolation and western blot Protein isolation and western blot was conducted as previously described [9]. Proteins were detected with primary antibodies to Akt (Cell Signaling No.9272; Cell Signaling, Danvers, MA, USA), p-Akt^{T308} (Cell Signaling No. 4056), AMP-activated protein kinase (AMPK) α (Cell Signaling No. 2603), p-AMPKα^{T172} (Cell Signaling No. 2535), total OXPHOS (ab110413; Abcam, USA) and eEF2 (Cell Signaling No. 2332).

Statistical analysis Values are expressed as 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 $p<0.05$.

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 mKO mice with PGC-1α mTg mice, referred to as PPARβ/δ mKO + PGC-1α mTg mice. As expected, both PPARβ/δ mKO and PPARβ/δ mKO + PGC-1α mTg mice showed a reduction in *Pparβ/δ* (*Ppard*) mRNA specifically in skeletal muscle, while *Pgc-1α* mRNA was upregulated by ~12-fold in skeletal muscle of PGC-1α mTg and PPARβ/δ mKO + PGC-1α mTg mice compared with control (CON) mice (Fig. 1a, b). To validate the functional consequence of *Pparβ/δ* deletion in skeletal muscle, we assessed the effects of the PPARβ/δ agonist GW0742 on the expression levels of PPARβ/δ target genes [7, 16]. Acute treatment with GW0742 did not affect *Pparβ/δ* mRNA in gastrocnemius and plantaris muscles whereas *Ucp3* mRNA levels were induced in CON, but not in PPARβ/δ mKO mice (Fig. 1c, d). Moreover, as previously reported [16], *Angptl4* was upregulated by GW0742 in a way that was partially dependent on PPARβ/δ (Fig. 1c, d). Importantly, *Pparβ/δ* deletion did not affect the transcript levels of *Pparα* (*Ppara*) and *Pparγ* (*Pparg*) (Fig. 1e). We subsequently measured the expression levels of other transcription factors and coactivators regulating metabolism, including the oestrogen-related receptors, mitochondrial transcription factor A, Pgc-1β and PGC-1-related coactivator. The expression levels of genes encoding these factors/coactivators (*Errα* [*Esrra*], *Errβ* [*Esrrb*], *Tfam*, *Pgc-1β* [*Ppargc1b*], *Prc* [*Pprc1*]) were altered in skeletal muscle of PGC-1α mTg and PPARβ/δ mKO + PGC-1α mTg mice, thus independent of PPARβ/δ ablation (Fig. 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 PPARβ/δ mKO, PGC-1α mTg, PPARβ/δ mKO + PGC-1α mTg and CON mice (Fig. 2a). Analysis of plasma triacylglycerol, cholesterol, LDL-cholesterol and HDL-cholesterol

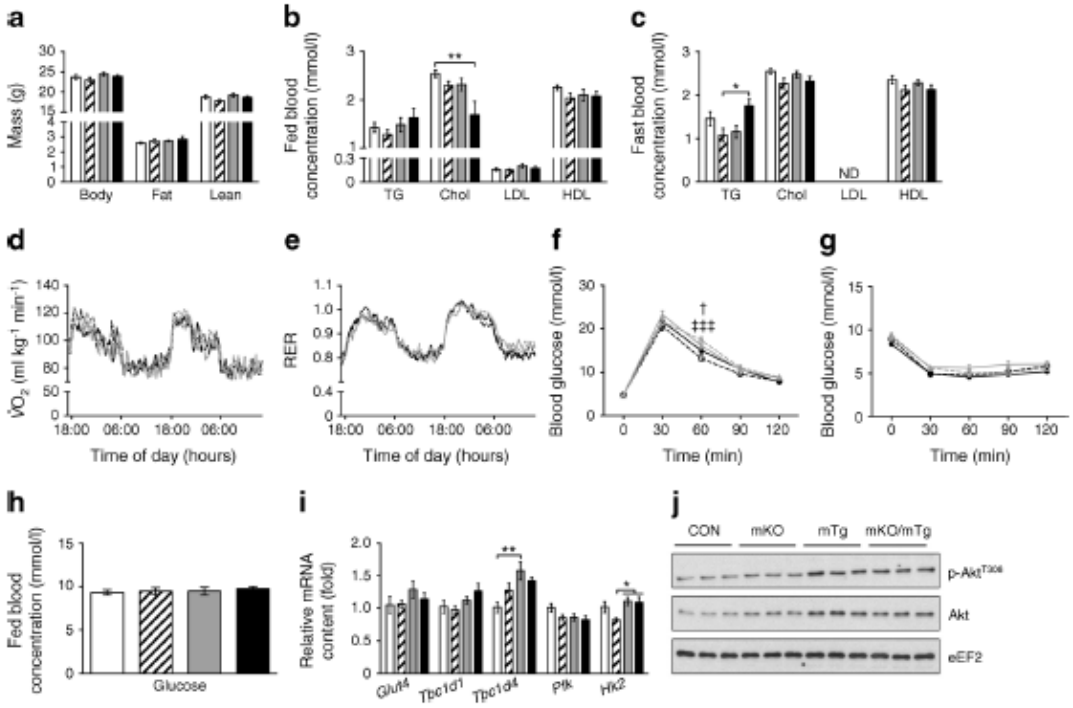

Fig. 2 Body composition, systemic variables and glucose handling. (**a**) Assessment of body composition (n=10–12 mice per group). (**b**, **c**) Plasma concentration of TG, cholesterol (Chol), LDL-cholesterol and HDL-cholesterol under fed and fasted conditions (n=10–12 mice per group). (of $\dot{V}O_2$ (**d**) and RER (**e**) over a period of 48 h (n=10–14 mice per group). (**f**, **g**) Blood glucose levels during GTTs (**f**) and ITTs (**g**). (**h**) Blood glucose levels in fed mice (n=10–12 per group). (**i**) Gastrocnemius mRNA levels of genes involved in glucose metabolism (n=6 mice per group). (**j**) Western blot assessment of Akt phosphorylation status in gastrocnemius muscle (n=6 mice per group). In (**a**–**c**), (**h**) and (**i**): white bars, CON; hatched bars, PPARβ/δ mKO; grey bars, PGC-1α mTg; black bars, PPARβ/δ mKO + PGC-1α mTg. *p<0.05 and **p<0.01 for the indicated comparisons. In (**d**–**g**): solid black line, CON; dashed black line, PPARβ/δ mKO; solid grey line, PGC-1α mTg; dashed grey line, PPARβ/δ mKO + PGC-1α mTg. †p<0.05, PPARβ/δ mKO vs PGC-1α mTg; ‡‡‡p<0.001 PPARβ/δ mKO vs PPARβ/δ mKO + PGC-1α mTg. In graphs, values are mean ± SEM. When shown, fold changes are reported vs CON group

during the fed and fasted state exhibited no differences except for a significant decrease in fed cholesterol in the PPARβ/δ mKO + PGC-1α mTg mice (Fig. 2b, c). Moreover, indirect calorimetry during 48 h revealed no differences in $\dot{V}O_2$ or RER between any of the genotypes (Fig. 2d, e and ESM Fig. 1a, b).

Pharmacological activation of PPARβ/δ attenuates the detrimental effects of obesity and type 2 diabetes on systemic glucose homeostasis [13, 17, 18]. Compared with CON mice, neither GTTs nor ITTs were affected by PGC-1α overexpression and/or *Pparβ/δ* deletion in skeletal muscle in mice fed a regular chow diet (Fig. 2f, g and ESM Fig. 1c, d). Moreover, we did not find any differences in blood glucose levels in fed mice between the four different genotypes (Fig. 2h). These findings were corroborated by unchanged expression of genes involved in glucose transport and catabolism, such as *Glut4* (*Slc2a4*), *Tbc1d1*, *Pfk* and *Hk2* (encoding glucose transporter 4, TBC domain family member 1, phosphofructokinase and hexokinase 2, respectively), in skeletal muscle of PPARβ/δ mKO, PGC-1α mTg and PPARβ/δ mKO + PGC-1α mTg mice (Fig. 2i). In contrast, *Tbc1d4* (*As160*, and which encodes Akt substrate of 160 kDa) was significantly upregulated in PGC-1α mTg mice (Fig. 2i). Finally, we observed an increase in total Akt protein levels following PGC-1α overexpression, with no substantial effect of *Pparβ/δ* deletion (Fig. 2j and ESM Fig. 1e). Consistently, PGC-1α overexpression slightly decreased relative Akt^{T308} phosphorylation levels, although this effect was not statistically significant (ESM Fig. 1f). 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 mice.

Modulation of skeletal muscle metabolism by the PGC-1α–PPARβ/δ axis Skeletal muscle PGC-1α and PPARβ/δ have been proposed to be key regulators of exercise performance and lactate metabolism [19, 20]. Consequently, we next assessed exercise performance in treadmill-based tests, which revealed a higher exercise performance in PGC-1α mTg mice as expected (Fig. 3a–c). Interestingly, *Pparβ/δ* muscle knockout did not reduce this difference when PPARβ/δ mKO + PGC-1α mTg mice were compared with CON mice (Fig. 3a–c). Moreover, $\dot{V}O_2$ was significantly enhanced in PGC-1α mTg

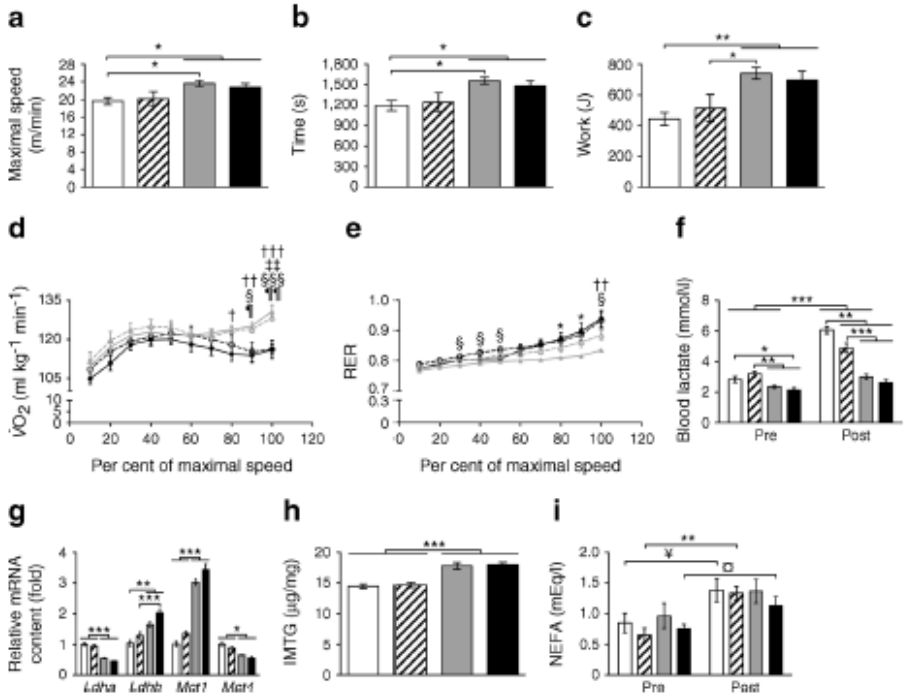

Fig. 3 Skeletal muscle PGC-1α modulates whole-body metabolism in mice during maximal exercise. (**a**–**c**) Maximal speed, time and work achieved during exercise tests to exhaustion (n=10–12 mice per group). (**d**, **e**) Measurement of $\dot{V}O_2$ and RER during the maximal exercise test (n=10–12 mice per group). (**f**) Blood lactate levels before (Pre) and after (Post) maximal exercise (n=10–12 mice per group). (**g**) mRNA levels of key genes of lactate metabolism in gastrocnemius (n=6 per group). (**h**) Quadriceps IMTG content (n=5 per group). (**i**) Plasma levels of NEFA before (Pre) and after (Post) exercise (n=4–6 mice per group). Values are mean ± SEM. In (**a**–**c**, **f**–**i**): white bars, CON; hatched bars, PPARβ/δ mKO; grey bars, PGC-1α mTg; black bars, PPARβ/δ mKO + PGC-1α mTg. In (**d**, **e**): black continuous line, CON; black discontinuous line, PPARβ/δ mKO; grey continuous line, PGC-1α mTg; grey discontinuous line, PPARβ/δ mKO + PGC-1α mTg. *p<0.05, **p<0.01 and ***p<0.001 for the indicated comparisons. In (**d**, **e**): $^{\dagger}p$<0.05, $^{\dagger\dagger}p$<0.01 and $^{\dagger\dagger\dagger}p$<0.001, CON vs PGC-1α mTg; $^{\ddagger\ddagger}p$<0.01, PPARβ/δ mKO vs PPARβ/δ mKO + PGC-1α mTg; $^{\S}p$<0.05 and $^{\S\S\S}p$<0.001, PPARβ/δ mKO vs PGC-1α mTg; $^{\P}p$<0.05 and $^{\P\P}p$<0.01, CON vs PPARβ/δ mKO + PGC-1α mTg. In (**i**) $^{¥}p$=0.067; $^{¤}p$=0.065. When shown, fold changes are reported vs CON group

and PPARβ/δ mKO + PGC-1α mTg mice during maximal exercise (Fig. 3d), thus altered by PGC-1α independent of PPARβ/δ. In contrast, the decrease in the RER in PGC-1α mTg mice was attenuated by concomitant *Pparβ/δ* deletion (Fig. 3e). Blood lactate concentration increased following maximal exercise in CON mice (Fig. 3f). This effect was attenuated in PPARβ/δ mKO mice and virtually abolished in both PGC-1α mTg and PPARβ/δ mKO + PGC-1α mTg mice (Fig. 3f). Similarly, pre-exercise blood lactate levels were reduced only in the mouse models with elevated skeletal muscle PGC-1α (Fig. 3f). Consistently, mRNA levels of genes encoding lactate dehydrogenase A (*Ldha*) and monocarboxylic acid transporter 4 (*Mct4* [*Slc16a3*]) were reduced only by PGC-1α overexpression in skeletal muscle, while in the same mice, *Ldhb* and *Mct1* (*Slc16a1*) genes were upregulated (Fig. 3g), reflecting an attenuated lactate production as well as higher catabolism. To assess substrate availability, we measured IMTG content and, consistent with the function of PGC-1α in de novo lipogenesis [2], both PGC-1α mTg and PPARβ/δ mKO + PGC-1α mTg mice showed elevated IMTG levels, though *Pparβ/δ* knockout had no effect (Fig. 3h). Finally, we measured plasma levels of NEFA before and after exercise. Exercise significantly increased plasma NEFA levels in PPARβ/δ mKO mice, while CON and PPARβ/δ mKO + PGC-1α mTg mice showed a trend toward an increase (Fig. 3i). 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 investigated the relevance of PGC-1α and PPARβ/δ interaction in the regulation of skeletal muscle metabolism. We therefore determined the mRNA levels of 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 genes involved in the tricarboxylic acid cycle, β-oxidation and electron transport chain (Fig. 4a, b). In contrast, most of these genes were strongly upregulated by PGC-1α overexpression in a PPARβ/δ-independent manner (Fig. 4a, b). Assessment of the protein content of different components of mitochondrial complexes supported the mRNA data, although the overall effects were milder

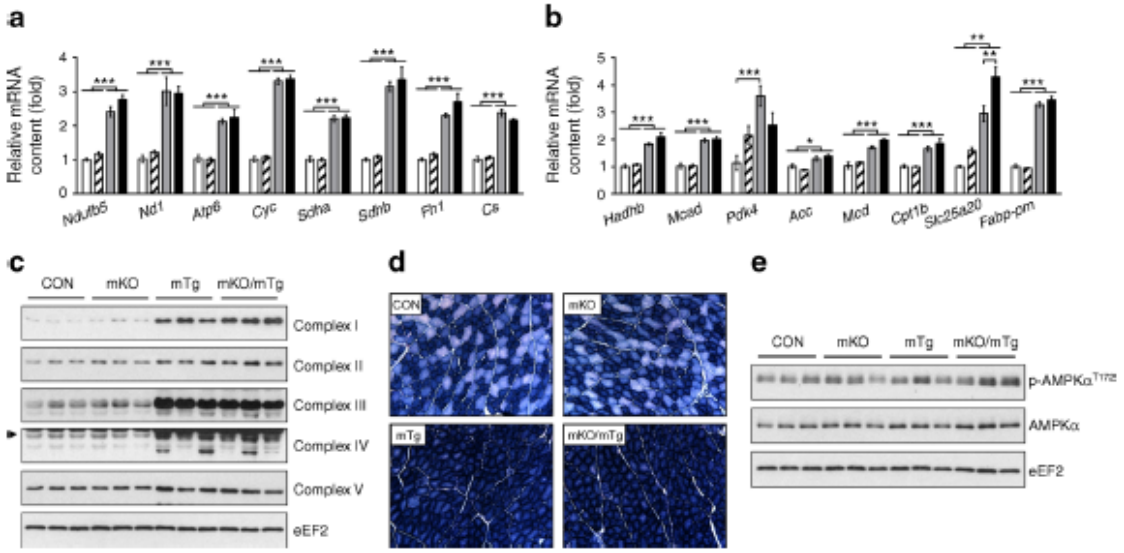

Fig. 4 Oxidative metabolism of gastrocnemius is enhanced by PGC-1α even in the absence of PPARβ/δ. (**a**, **b**) mRNA levels of genes regulating oxidative and fatty acid metabolism (n=6 per group). (**c**) Western blot analysis of key proteins regulating the electron transport chain (n=6 per group). (**d**) Assessment of oxidative muscle fibres (dark blue) via NADH staining (n=3 per group). (**e**) Western blot analysis of AMPK phosphorylation status (n=6 per group). In (**a**, **b**): white bars, CON; hatched bars, PPARβ/δ mKO; grey bars, PGC-1α mTg; black bars, PPARβ/δ mKO + PGC-1α mTg. Values are mean ± SEM. *p<0.05, **p<0.01 and ***p<0.001 for the indicated comparisons. When shown, fold changes are reported vs CON group

(Fig. 4c and ESM Fig. 2a). We then assessed the metabolic muscle phenotype by determining the proportion of oxidative fibre using NADH staining. This revealed a higher oxidative activity and proportion of oxidative fibres in PGC-1α mTg and PPARβ/δ mKO + PGC-1α mTg mice independent of a functional *Pparβ/δ* gene (Fig. 4d). The total protein content and phosphorylation levels of the key metabolic regulator AMPK did not differ between PPARβ/δ mKO, PGC-1α mTg and PPARβ/δ mKO + PGC-1α mTg mice, suggesting that there was no alteration in energy status in any of these models (Fig. 4e and ESM Fig. 2b, c). We finally explored the relevance of the PGC-1α–PPARβ/δ axis in the context of the PPARβ/δ agonist GW0742-induced gene expression. As shown in ESM Fig. 2d, GW0742 enhanced the expression of the PPARβ/δ target genes *Angptl4* and *Ucp3*, whereas it did not affect the mRNA levels of the key regulators of oxidative metabolism. Moreover, the effects of PGC-1α overexpression on gene expression were not affected by GW0742 (ESM Fig. 2d). Finally, as expected, *Pparβ/δ* gene ablation likewise abrogated any effect of the synthetic ligand (Fig. 1c, d and ESM Fig. 2d).

Discussion

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

Importantly, supporting our hypothesis, contrary to the effects observed in PGC-1α muscle-specific transgenic mice, the enhancement of skeletal muscle oxidative metabolism is 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 and 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, which 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. These observations are consistent

with cell culture-based experiments showing that PGC-1α strongly increases oxidative metabolism in the absence of PPARβ/δ in skeletal muscle cells [21]. It thus appears 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 upregulated in the skeletal muscle of both PGC-1α mTg and PPARβ/δ mKO + PGC-1α mTg mice, suggesting that these transcription factors might have a more relevant function in this context. Importantly, our results indicate that *Pparβ/δ* deletion by itself does not result in a compensatory activation of such related transcription factors. In fact, PPARβ/δ mKO mice do not exhibit an upregulation of PPARs, oestrogen-related receptors or mitochondrial transcription factor A in skeletal muscle. In addition, several target genes of these transcription factors were unaltered in PPARβ/δ mKO mice.

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 is an efficient treatment for metabolic disorders [13, 17, 18, 22], 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 mice are fed a high-fat diet [23], unless the mice are concomitantly exercised [24]. In elderly animals, however, overexpression of PGC-1α in muscle prevents age-induced insulin resistance [25]. These findings indicate that in some pathological settings, PPARβ/δ activation might be more relevant than PGC-1α, particularly in the absence of physical activity.

Surprisingly, in our study, PPARβ/δ mKO mice had a similar phenotype to CON mice, with minimal or no changes in body composition, blood variables and gene expression. In contrast, Schuler 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 [18, 26–29]. These differences in the phenotype of PPARβ/δ KO mouse models in the chow-fed sedentary condition might stem from different environmental factors (e.g. diet and temperature), which could lead to a partial PPARβ/δ activation in CON mice and thus lead to more pronounced phenotypic differences in metabolic variables when compared with knockout mice. Importantly, most of the effects of skeletal muscle *Pparβ/δ* deletion reported by Schuler et al on energy metabolism are observed following high-fat diet feeding and/or in elderly mice [11]. Moreover, in the same study, the phenotype of adult PPARβ/δ mKO mice fed chow diet is rather mild and not substantially different from our results, reflected by the magnitude and variability of the data [11].

During exercise, skeletal muscle exerts a greater impact on whole-body metabolism. Accordingly, PGC-1α mTg mice exhibit a higher $\dot{V}O_2$ 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 $\dot{V}O_2$ during exercise was maintained in the absence of a functional PPARβ/δ gene, knockout of *Pparβ/δ* attenuated the decrease in the RER in PPARβ/δ mKO + PGC-1α mTg mice. In line with our observations, it has been shown that PPARβ/δ overexpression in skeletal muscle does not affect $\dot{V}O_2$ and RER during treadmill running [20]. 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 [21]. Surprisingly, the effect of PPARβ/δ knockout on RER during maximal exercise appears to be unrelated to mRNA level of genes controlling fatty acid transport and oxidation. Interestingly, *Pparβ/δ* deletion attenuated the upregulation of *Pdk4* induced by PGC-1α overexpression. Importantly, skeletal muscle pyruvate dehydrogenase kinase 4 has been extensively shown to be a key regulator of fatty acid oxidation during exercise [30], suggesting a possible mechanism by which PPARβ/δ modulates RER and thus energy substrate use during maximal exercise. It should be noted that *Pparβ/δ* knockout induced the upregulation of *Pdk4*, an effect that supports the idea that this nuclear receptor can actively repress target genes in the absence of ligand [16, 31]. Together, these data suggest that the effects of skeletal muscle PGC-1α on $\dot{V}O_2$ are not dependent upon PPARβ/δ, even though this nuclear receptor appears to be partially involved in the PGC-1α-mediated increase in β-oxidation during exercise. In addition, our findings support previous data suggesting that PGC-1α-controlled lactate metabolism is predominantly regulated by oestrogen-related receptor α and not by PPARβ/δ [19].

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 effect of PGC-1α on skeletal muscle remodelling. Importantly, the different therapeutic effects of PPARβ/δ and PGC-1α in the context of metabolic diseases during sedentary vs exercise/ageing 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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