Function and regulation of Steroid Receptor Coactivator 2

Transcriptional regulation of cellular metabolism

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"If we fail to anticipate the unforeseen or expect the unexpected in a universe of infinite possibilities, we may find ourselves at the mercy of anyone or anything that cannot be programmed, categorized or easily referenced." – Fox Mulder

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Abbreviations

6MB-cAMP	N^6-monobutyryladenosine-3',5'-cyclic monophosphate
AD	Activation domain
AICAR	5-aminoimidazole-4-carboxamide ribonucleotide
AMP	Adenosine 3'-5'-monophosphate
AMPK	AMP-activated protein kinase
bZIP	Basic leucine zipper
Cα	PKA catalytic subunit
cAMP	Cyclic AMP
ChIP	Chromatin immunoprecipitation
CREB	cAMP response element binding protein
CRTC	CREB regulated transcription coactivator
DBD	Domain binding domain
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EMEM	Eagle's minimum essential medium
FASN	Fatty acid synthase
FBS	Fetal bovine serum
FC	Fold change
FDR	False discovery rate
G6Pase/ G6Pc	Glucose-6-phosphatase catalytic subunit
GRIP1	Glucocorticoid receptor-interacting protein 1
HMGCR	3-hydroxy-3-methylglutaryl-CoA reductase
HMGCS1	3-hydroxy-3-methylglutaryl-CoA synthase 1
HNF4α	Hepatocyte nuclear factor 4-alpha
LKB1	Liver kinase B1
mRNA	Messenger RNA
mTORC1	Mammalian target of rapamycin complex 1
NAFLD	Non-alcoholic fatty liver disease
NCOA	NR coactivator
NID	NR-interaction domain
NR	Nuclear hormone receptor
PANTHER	Protein analysis through evolutionary relationships
PCR	Polymerase chain reaction
PEPCK	Phosphoenolpyruvate carboxykinase
PEST	Penicillin-streptomycin
PGC-1α	PPARγ coactivator 1-alpha
PKA	Protein kinase A
PPARγ	Peroxisome proliferator-activated receptor gamma
PTM	Post-translation modification
qPCR	Quantitative real-time reverse transcriptase PCR
RAR	Retinoic acid receptor
RNA	Ribonucleic acid
RORα	RAR-related orphan receptor alpha
RORE	RORalpha response element
RPL4	Ribosomal protein L4
SD	Standard deviation
SEM	Standard error of the mean
siRNA	Small interfering RNA
SRC	Steroid receptor coactivator
SRE	Sterol regulatory element
SREBP	SRE-binding protein
TIF2	Transcriptional intermediary factor 2
TORC2	Transducer of regulated CREB protein 2
Ub	Ubiquitin
Veh	Vehicle treatment

Abstract

Nuclear hormone receptors (NRs) associate with coregulators to enable complex programs of gene expression. The p160 steroid receptor coactivator 2 (SRC-2/GRIP1) regulates metabolism and cellular processes in a tissue-specific manner by enhancing transcription of hormone regulated target genes. The transcriptional regulation and metabolic programming imposed by SRC-2 and its partner NRs has implications for several pathological processes. Although SRC-2 is known to facilitate lipogenesis and survival of prostate cancer cells, its role in breast cancer remains unclear. Hepatic ablation of SRC-2 impairs expression of key lipogenic and gluconeogenic enzymes, causing fasting hypoglycemia in mice. Previously, it has been shown that the cAMP/PKA pathway regulates the protein level of SRC-2. This thesis aimed to further investigate the functions of SRC-2 and characterize whether upstream regulation of SRC-2 affects its ability to promote expression of metabolic target genes.

Paper I elucidates a mechanism by which cellular SRC-2 protein is subject to inhibitory upstream regulation by the cAMP/PKA pathway. Here, we showed that the cAMP response element-binding protein (CREB) has a key role in this mechanism by interacting with and stimulating proteasomal degradation of SRC-2. We demonstrated that the CREB basic leucine zipper (bZIP) domain and two functionally independent protein domains of SRC-2 (amino acids 347-758 and 1121-1462) are required to trigger degradation of SRC-2. Furthermore, overexpression of CREB inhibited the ability of SRC-2 to modulate expression of estrogen receptor alpha (ERα) target genes. These findings suggest that the previously established inhibitory effect of PKA on SRC-2 is executed via a direct interaction between CREB and SRC-2.

Paper II reports that SRC-2 has a crucial function in regulating hepatocellular gluconeogenesis. Transcription of gluconeogenic enzymes including the rate-limiting glucose 6-phosphatase (G6Pase) is enabled by fasting hormone signaling via the cAMP/PKA pathway. Previously, it was demonstrated that SRC-2 liver knock-out

mice exhibit fasting hypoglycemia due to reduced G6Pase expression. We validated that SRC-2 coactivates the RAR-related orphan receptor alpha (RORα) at the proximal G6Pase promoter in HepG2 cells. In line with the findings from the first paper, we observed that SRC-2 protein was subject to degradation in presence of the PKA catalytic subunit (PKA-Cα). Overexpression of PKA-Cα also reduced recruitment of SRC-2 and RNA polymerase II to the G6Pase promoter and rendered SRC-2 unable to coactivate RORα. Furthermore, we found that the presence of SRC-2, via its association with RORα, is required for the transactivational effect of the master gluconeogenic regulator PPARG coactivator 1-alpha (PGC-1α) on the G6Pase promoter. These results suggest that PKA-mediated degradation of SRC-2 may represent an indirect feedback mechanism by which gluconeogenesis is suppressed throughout long-term starvation.

Paper III elucidates a novel mechanism by which the anti-diabetic drug metformin reprograms hepatocellular metabolism via SRC-2. Tissue from SRC-2 liver knock-out mouse model is characterized by impaired expression of lipogenic and gluconeogenic enzymes. Here, we showed that treatment of cells with metformin is accompanied by transcriptional repression of specifically SRC-2. Microarray analysis of FaO hepatoma cells treated with metformin revealed an overrepresentation of downregulated SRC-2 target genes involved in lipid and cholesterol biosynthesis. Promoter analyses confirmed that these genes were also transcriptional targets of the lipogenic sterol regulatory element (SRE) binding protein 1 (SREBP-1). Transactivation assays demonstrated that SRC-2 is a coactivator of SREBP-1, but not SREBP-2, on the fatty acid synthase (FASN) promoter. By repressing SRC-2 expression, metformin impeded recruitment of SRC-2 and RNA polymerase II to the G6Pc promoter and to SREs of SRC-2/SREBP-1 target gene promoters. Furthermore, metformin or knock-down of SRC-2 and SREBP-1 reduced hepatocellular fat accretion. Accordingly we propose that metformin transcriptionally suppresses hepatic gluconeogenesis and lipogenesis partly by inhibiting expression of SRC-2.

List of publications

Paper I

Nuclear Hoang T, Fenne IS, Madsen A, Bozickovic O, Johannessen M, Bergsvåg M, Lien EA, Stallcup MR, Sagen JV, Moens U, and Mellgren G. **cAMP Response Element-Binding Protein Interacts With and Stimulates the Proteasomal Degradation of the Nuclear Receptor Coactivator GRIP1.**
Endocrinology 2013, 154(4):1513–1527

Paper II

Madsen A, Bjune JI, Bjørkhaug L, Mellgren G and Sagen JV. **The cAMP-dependent protein kinase downregulates glucose-6-phosphatase expression through RORα and SRC-2 coactivator transcriptional activity.**
Revised manuscript submitted to *Molecular and Cellular Endocrinology*.

Paper III

Madsen A, Bozickovic O, Bjune JI, Mellgren G and Sagen JV. **Metformin inhibits hepatocellular glucose, lipid and cholesterol biosynthetic pathways by transcriptionally suppressing steroid receptor coactivator 2 (SRC-2).**
Revised manuscript submitted to *Scientific Reports*.

Contents

1. Introduction

1.1 Nuclear Hormone Receptors and Coregulators

1.1.1 Hormone signaling

Hormones are chemical signal molecules that induce systemic changes in gene expression and metabolism of receptive target cells. The specificity of a hormone is provided by the interaction with its cognate receptor. Whereas peptide hormones generally associate with membrane-bound receptors to trigger different cell signaling events, hydrophobic steroid hormones bind to intracellular nuclear hormone receptors, rendering them transcriptionally active.

1.1.2 Nuclear hormone receptors

Nuclear hormone receptors (NRs) are transcription factors that stimulate expression of target genes in response to activation by corresponding ligands. The NR superfamily is comprised of 48 different receptors in humans [1]. Canonical NRs include a DNA binding domain (DBD), ligand binding domain (LDB) and two activation function domains (AF1 and AF2) that enables interaction with other proteins [2]. Whereas type I NRs, also called steroid hormone receptors, are characterized by homodimerization and nuclear import in response to ligand binding, type II NRs are natively located in the cell nucleus and heterodimerize with retinoid X receptor (RXR) [3]. Several NRs have unknown endogenous ligands and are referred to as orphan NRs. Activated NR dimers are recruited to DNA sequences termed hormone response elements (HREs), which are composed of two hexameric core half-sites [4]. Such HREs position the NRs in close proximity of target gene promoters across the genome and facilitate recruitment of chromatin remodeling factors, general transcription factors and RNA polymerase II that ultimately enable gene transcription. HREs are also frequently located at distal DNA regulatory elements called enhancers that, due to the three-dimensional chromatin structure, are able to regulate target gene expression [5].

1.1.3 Coregulators

The ability of NRs to activate a target gene promoter is subject to regulation by post-translational modifications (PTMs) including phosphorylation, ubiquitylation, SUMOylation and interaction with coregulators [6]. Transcriptional coregulators (coactivators and corepressors) provide an additional layer of enhancing cellular responses to hormone signaling by forming a bridge between DNA-binding transcription factors and the basal transcriptional machinery. Coactivators and corepressors enhance or repress NR-dependent transcriptional activity, respectively. Binding of ligands to type I NRs induces conformational changes that liberate the NRs from chaperone sequestration and expose functional protein domains, allowing for nuclear import and interaction with transcriptional partner proteins [7]. Specifically, agonist ligand binding changes the conformation of the carboxy-terminal AF2 domain that serves as an interface between NRs and coactivators [3].

1.1.4 Regulation of chromatin by histone modifications

An important feature of coregulators is to directly or indirectly induce histone modifications near target gene promoters [8]. In eukaryote cells, the higher-order arrangement of histones and genomic DNA wrapped around nucleosomes is referred to as chromatin. The projecting amino-tails of multimeric histones are subject to a complex and ever-growing array of covalent PTMs referred to as the epigenetic code. In general, histone acetytransferases (HATs) serve to open up chromatin and facilitate transcription factor accessibility by neutralizing the positive charge of lysine-rich histone tails [9]. Conversely, the activity of histone deacetylases (HDACs) is associated with compacting and reduced accessibility of chromatin. Histone tail methylation is believed to be more static, and the epigenetic readout from this covalent modification is context-dependent and less understood [10]. Patterns and combinations of PTMs on histone tail residues form discrete motifs that are recognized by cognate domains of chromatin-associated factors [9]. Furthermore, methylation of adenine and cytosine residues of the genomic DNA also impacts chromatin structure and is associated with transcriptional silencing [11].

1.1.5 Steroid receptor coactivators

The structurally homologous p160 steroid receptor coactivator (SRC) family includes the ubiquitously expressed SRC-1 (NCOA1), SRC-2 (NCOA2/GRIP1/TIF2/) and SRC-3 (NCOA3/AIB1). The SRCs have three structural protein domains that facilitate interactions relating to their functions in transcriptional regulation [12]. The amino-terminal basic helix-loop-helix/Per-ARNT-Sim (bHLH/PAS) domain enables interaction with several tissue-specific transcription factors and additional coactivators, and also contains a nuclear localization signal (NLS) [8]. The central region of SRCs constitutes the nuclear receptor interaction domain (NRID) which contains three LxxLL (L is leucine and x is any amino acid) motifs. These positively charged motifs associate with the AF2 domain of activated NRs. Lastly, the carboxy-terminal activation domains (AD1 and AD2) allow for interaction with additional coactivators and chromatin-modifying enzymes [13]. The AD1 and AD2 domains are flanked by a glutamine (Q)-rich region that mediates interaction with NRs [14]. When associated with ligand-bound NRs, the SRCs recruit cofactors that facilitate transcription of target genes [8]. Several of these factors exhibit histone-modulating activity, including coactivator-associated arginine methyltransferase 1 (CARM1), protein arginine methyltransferase 1 (PRMT1), p300 and cAMP response element-binding protein (CREB)-binding protein (CBP) [15]. Additionally, SRC-1 and SRC-3 exhibit enzymatic HAT activity conferred by a component of the Q-rich protein domain [16, 17]. Although the SRCs were initially found to coactivate type I nuclear receptors, several novel properties and transcriptional interaction partners have since been characterized.

1.1.6 Steroid receptor coactivator 2 (SRC-2)

The SRC-2 protein exhibits extensive sequence homology with the SRC-1 and SRC-3 proteins, however, SRC-2 has no intrinsic HAT activity [13]. The function of SRC-2 is attributed to its functional protein domains (Figure 1). Depending on physiological context and tissue, SRC-2 has been shown to coactivate several nuclear receptors including androgen receptor (AR), progesterone receptor (PR), glucocorticoid receptor (GR), estrogen receptor (ERα), RAR-related orphan receptor alpha (RORα),

vitamin D receptor (VDR), farnesoid X receptor (FXR), and the thyroid receptor (TR) [13, 18-22]. Some studies have also reported that SRC-2 may act as a conditional corepressor on certain promoters [23, 24]. Accordingly the physiological readout of SRC-2 function depends on nuclear receptor ligand availability and also which hormone signaling pathway it is examined in conjunction with.

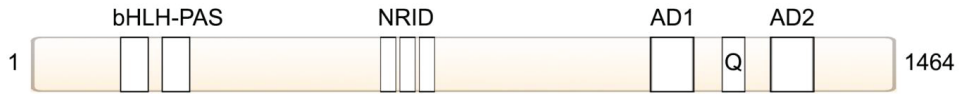

Figure 1. Functional domains of the SRC-2 protein. The basic helix-loop-helix and Per/ARNT/Sim domains (bHLH-PAS), nuclear receptor interaction domain (NRID), glutamine-rich (Q) region and activation domains AD1 and AD2 of the 1464 amino acids comprising the SRC-2 protein are indicated. Made using Adobe Photoshop.

Genetic ablation studies in mice have been instrumental for elucidating the function of SRC-2 *in vivo*. Current knowledge proposes that SRC-2 exerts important functions in development, whole-body energy homeostasis, metabolic regulation, oncogenic potential and reproduction [8]. Jeong *et al*. demonstrated that female SRC-$2^{-/-}$ knock-out mice are infertile due to impaired placental development [25]. In 2002, Picard *et al.* observed that whole-body ablation of SRC-2 in mice was associated with protection from diet-induced obesity, improved insulin sensitivity and enhanced adaptive thermogenesis [26]. This study established several important aspects of SRC-2 function in adipose tissue. Notably, white adipose tissue from SRC-2 knock-out mice was characterized by significantly lowered lipid content and primary adipocytes from these animals exhibited higher levels of lipolysis and energy expenditure compared to wild-type littermates [26]. Picard *et al.* also demonstrated that SRC-$2^{-/-}$ knock-out mice had lower fasting blood glucose levels than the wild-type animals [26]. In humans, a weak association between obesity and an SRC-2 gene single nucleotide polymorphism has been identified [27]. In 2006, Jeong *et al.* presented microarray analyses from tissue of individual SRC-$1^{-/-}$, SRC-$2^{-/-}$ or SRC-$3^{-/-}$ liver knock-out mice [28]. Interestingly, Venn diagram comparison tests revealed that there was a surprisingly low amount of common target genes for the three individual

SRCs [28]. Transcriptomics analyses confirmed that 245 out of 270 differentially expressed genes were upregulated when SRC-1 was knocked out, and only 22 genes were downregulated upon ablation of SRC-3 [28]. In contrast, hepatic ablation of SRC-2 was accompanied by downregulation 338 genes, several of which represent key regulatory enzymes pertaining to glucose, lipid and cholesterol biosynthesis [28].

These findings were revisited by Chopra *et al.* in 2008 where it was confirmed and demonstrated that ablation of SRC-$2^{-/-}$ in both a whole-body and liver-specific manner caused fasting hypoglycemia. Furthermore, knock-out animals exhibited a phenotype resembling the glycogen storage disease-1a, characterized by loss-of-function mutations in the gluconeogenic glucose-6-phosphatase catalytic subunit (G6Pc/G6Pase) [19]. The authors provided a mechanism to explain the hypoglycemic phenotype by showing that SRC-2 stimulates hepatic G6Pase expression by coactivating the nuclear receptor RORα at the G6Pase promoter [19]. An enrichment of RORα and SRC-2 was detected by chromatin immunoprecipitation at an atypical RORα response element (RORE) sequence in the proximal G6Pase promoter (-54/-49 relative to G6Pase transcription start site). Interestingly, this particular promoter binding site was previously characterized as a crucial regulatory element of G6Pase expression: In parallel in 2008, Schilling *et al.* demonstrated that the ability of PGC-1α to coactivate the hepatocyte nuclear factor 4-alpha (HNF4α) at the proximal mouse G6Pase promoter was abolished upon mutation of the RORE sequence located adjacently to the -76/-64 HNF4α binding site [29]. However, these authors were unable to confirm the affinity or binding of any one nuclear receptor to this adjacent (-54/-49) site. Although the findings from Chopra *et al*. solved one piece of this puzzle, the evident functional relationship between the adjacent HNF4α/PGC-1α and RORα/SRC-2 NR-coactivator complexes has remained unexplained (Figure 2).

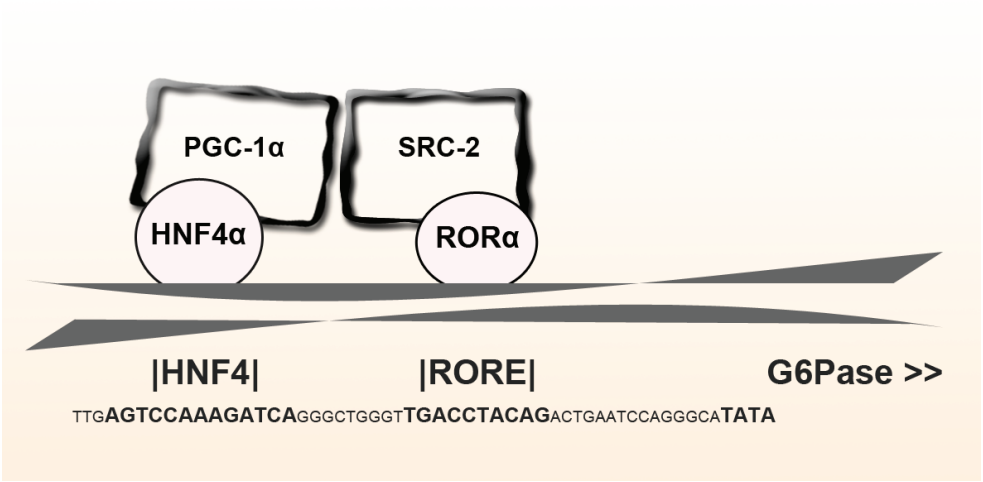

Figure 2. The proximal G6Pase promoter. Previous studies have shown that ablation of hepatic SRC-2 causes fasting hypoglycemia in mice by impairing expression of the rate-limiting gluconeogenic enzyme G6Pase. SRC-2 coactivates the orphan nuclear receptor RORα on the proximal G6Pase promoter. Additionally, the RORα binding sequence is required for the transactivational effect of PGC-1α on this promoter. Made using Adobe Photoshop.

In 2011, Chopra *et al.* further pursued the phenotype of whole-body SRC-2 ablation and reported that SRC-$2^{-/-}$ knock-out mice subjected to overnight fasting also exhibited dietary fat malabsorption and reduced plasma triglycerides [22]. This defective malabsorption was rescued upon both feeding of the mice with exogenous bile acids or adenoviral overexpression of the bile salt export pump (BSEP/ABCB11) gene [22]. These findings were reconciled with the evident transcriptional activation of the BSEP gene following activation of the AMP-activated protein kinase (AMPK) pathway, which is characteristically associated with starvation. The authors also showed that AICAR, an AMP-mimicking and thus AMPK-activating compound, increased the intrinsic transcriptional activity of SRC-2, and that the AMPK holoenzyme phosphorylated SRC-2 *in vitro* [22]. Furthermore, transfection of HepG2 cells with the AMPKα2 catalytic subunit increased recruitment of SRC-2 to the FXR response element (FXRE) at the BSEP promoter [22].

The role of SRC-2 in facilitating lipid biosynthesis was emphasized in a 2015 study conducted by Dasgupta *et al.*, based on the notion that SRC-2 is frequently

upregulated in metastatic prostate cancer and thus may represent an oncogene [30]. Interestingly, aggressive prostate cancer frequently exhibits a particularly lipid-reliant metabolic profile which is granted through dysregulation of the liver X receptors (LXR), sterol regulatory element binding proteins (SREBP-1 and SREBP-2) and lipogenic enzymes including fatty acid synthase (FASN) and acetyl-CoA carboxylase (ACC) [31-35]. The lipid metabolic component and bioenergetic basis of prostate malignancy is unique and contrasts the established "Warburg effect" paradigm whereby cancer cells primarily rely on anaerobic glucose catabolism and lactate fermentation as energy source [36]. Several studies indicate that restricting fatty acid availability and synthesis suppresses cancer progression [31]. In line with this logic, Daspugta *et al.* provided data to support a model by which SRC-2 promotes prostate cancer progression by contributing to neoplastic fat accretion by coactivating SREBP-1 and promoting expression of *de novo* lipogenic enzymes FASN and stearoyl-CoA desaturase (SCD) [30]. It was also shown that phosphorylation of SRC-2 by the nutrient-activated mammalian target of rapamycin complex 1 (mTORC1) was required for SRC-2 to coactivate SREBP-1 [30].

Whereas SRC-1 and SRC-3 (also known as Amplified in Breast Cancer 1, AIB1) unambiguously promote breast cancer, the role of SRC-2 in this pathology is not clearly defined [18, 37-39]. Transcriptional target genes of ERα include both oncogenes and tumor-suppressor genes [18, 40]. By differentially modulating the expression of ERα target genes, SRC-2 may or may not represent an accessory to the oncogenic potential of ER-positive (ER^+) breast cancer cells [18]. Whereas some studies have demonstrated that SRC-2 stimulates cell proliferation, increases cell cycle progression and decreases apoptosis in the MCF-7 breast cancer cell model [41, 42], others have concluded that SRC-2 may have antiproliferative properties in breast cancer [18]. Importantly, one article found that the incretin hormone glucagon-like peptide 1 (GLP-1), by activating the cAMP/PKA pathway via its cognate G-protein coupled receptor, inhibits growth of MCF-7 breast cancer cells [43]. The authors of this article suggested a link between reduced serum GLP-1, as observed in type 2 diabetes, and the correspondingly increased risk of breast cancer.

Our group has previously focused on the role of SRC-2 in breast cancer and in relation to the cAMP/PKA pathway. Initial findings in our group suggested that the cAMP-dependent protein kinase (cAMP/PKA) pathway had an inhibitory effect on the ability of SRC-2 to coactivate the steroidogenic factor 1 (SF-1/NR5A1) [44]. Subsequent studies in our group elaborated on this finding and demonstrated that SRC-2 is recruited to transcriptional target genes of the estrogen receptor (ERα) in response to transient stimulation of the cAMP/PKA pathway in the MCF-7 breast cancer cell line [45]. However, prolonged or pronounced activation of the cAMP/PKA pathway consistently was shown to cause degradation of SRC-2 protein [45]. It was also demonstrated that PKA actively regulates SRC-2 by inducing its degradation through the ubiquitin-proteasome pathway [46]. In this context, the first article of this thesis identifies the cAMP responsive element binding protein (CREB) as a key mediator in the mechanism by which the cAMP/PKA pathway targets SRC-2 for proteasomal destruction [47].

1.2 Liver metabolism

The liver represents a core metabolic organ that maintains homeostatic levels of circulating glucose, fat and nutrients. Glucose is an essential energy substrate for several organs and cells in the body and the liver is tasked with providing a steady glucose bloodstream supply [48]. After feeding, insulin signaling and high intracellular [ATP]/[AMP] ratio stimulate glycolysis and storage of excess glucose as hepatic glycogen. During fasting, prevailing glucagon signaling and decreasing intracellular [ATP]/[AMP] ratio promote glycogen breakdown coupled with glucose export and *de novo* glucose production via the anabolic process known as gluconeogenesis. Glycogen thus serves as a dynamic buffer and rechargeable energy source that is replenished after feeding and drained during fasting [49]. When glycogen stores are depleted during fasting, hepatic gluconeogenesis represents the primary source of glucose for export into the circulation. In terms of metabolic coordination, glycolytic and gluconeogenic enzymes are reciprocally regulated by both the insulin/glucagon ratio and allosteric modifications caused by the intracellular [ATP/AMP] ratio and glucose metabolites [50].

1.2.1 Hepatic gluconeogenesis

Gluconeogenesis is the metabolic pathway that enables endogenous glucose production on demand. In biochemical kinetics, the overall flux of metabolites through a pathway of sequential enzymatic modifications is determined by its slowest and thus determining step. In the fed state, gluconeogenesis is inhibited due to transcriptional suppression of the pathway rate-limiting enzymes phosphoenolpyruvate carboxykinase (PEPCK/PCK1) and glucose-6-phosphatase catalytic subunit (G6pc/G6Pase). Although PEPCK catalyzes the true rate-limiting step of gluconeogenesis, the generation of fructose 6-phosphate by fructose-1,6-bisphosphatase (F1,6BPase) is also an important regulatory checkpoint [50, 51]. The final conversion of glucose-6-phosphate into glucose by G6Pase is also rate-limiting in terms of hepatic glucose output, in the sense that only free glucose is eligible for export into circulation via the GLUT2 transporter (Figure 3).

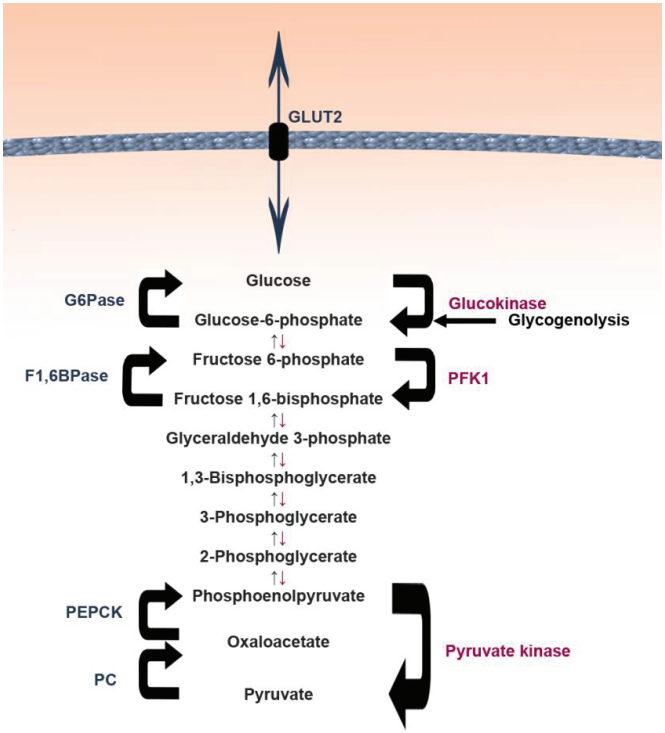

Figure 3. Enzymatic steps of glycolysis and gluconeogenesis. Glycolysis (purple) is the canonical catabolism of glucose into pyruvate. Gluconeogenesis (blue) employs a dedicated set of enzymes that enables anabolic *de novo* glucose production. G6Pase catalyzes the final step of both gluconeogenesis and glycogenolysis to generate free glucose eligible for export into the circulation via the GLUT2 transporter. PFK1, phosphofructokinase-1; PC, pyruvate kinase; PEPCK, phosphoenolpyruvate carboxykinase; F1,6BPase, fructose-1,6-bisphosphatase; G6Pase, glucose-6-phosphatase. Made using Adobe Photoshop.

Notably, G6Pase also marks the final step of both gluconeogenesis and glycogenolysis. During long-term starvation, activity of the gluconeogenic pathway is attenuated due to reduced substrate availability. Hepatocytes generate new pyruvate from lactic acid and amino acids and this serves to prolong the gluconeogenic flux [52]. Adipose tissue responds to starvation with lipolysis of stored triacylglycerol and release of non-esterified fatty acids (NEFAs) and glycerol into the circulation [53]. Whereas the glycerol can be utilized by hepatocytes, hepatic fatty acid β-oxidation does not generate substrates to sustain gluconeogenesis [53]. However, byproducts of hepatic β-oxidation include ketone bodies that in turn are exported and serve as

metabolic fuel for extrahepatic tissues during starvation [53]. During long-term starvation, glucose-dependent tissues including the brain ultimately transition into ketone metabolism [54].

1.2.2 The insulin signaling pathway

Food intake is accompanied release of insulin from pancreatic beta cells to compensate for increasing circulating glucose levels. In skeletal muscle and adipose tissue, insulin signaling triggers translocation of the glucose transporter GLUT4 from the cytoplasm to the cell membrane to facilitate glucose uptake [55]. In the liver, insulin signaling regulates several metabolic pathways aimed to store excess fuel as glycogen and lipids. Insulin signaling is physiologically associated with glucose abundance and thus suppresses gluconeogenesis (Figure 4).

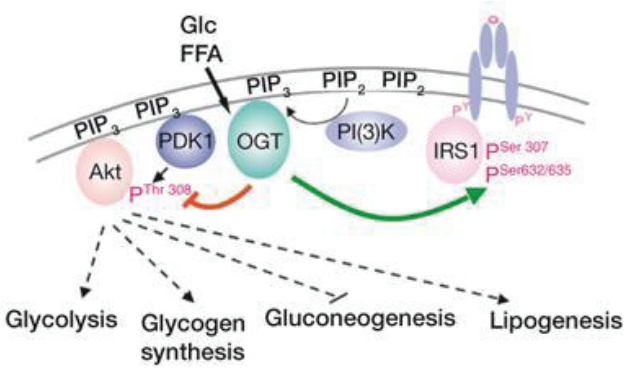

Figure 4. Metabolic regulation by the insulin signaling pathway. A central event in the insulin pathway is the activation of phosphoinositide 3-kinases (PI3Ks) and generation of phosphatidylinositol-3,4,5-triphosphate (PIP3) that serves as docking site for the phosphoinositide-dependent kinase 1 (PDK1) and Akt [60]. PDK1 phosphorylates and activates Akt. Downstream effects of hepatic Akt include inhibition of gluconeogenesis. The nutrient-sensitive OGT reversibly modifies serine/threonine residues of several components of this pathway and attenuates insulin signaling. Reprinted by permission from Macmillan Publishers Ltd: Nature [58], 2008.

Binding of insulin or insulin-like growth factor (IGF) to the cognate insulin receptor tyrosine kinase causes phosphorylation of the insulin receptor substrates IRS1 and IRS2 [56]. Both IRS1 and IRS2 are targets for *O*-linked β-N-

acetylglucosamine (*O*-GlcNAc) transferase (OGT) and it has been shown that increased OGT activity attenuates insulin signaling and downstream activation of Akt [57]. Furthermore, hepatic overexpression of OGT causes insulin resistance, dyslipidemia and impairs expression of insulin target genes [58]. Phosphorylated IRS1 serves as a docking site for the Grb2/SOS complex, which in turn activates Ras and the mitogen-activated protein kinase (MAPK) signaling pathway [59]. However, the main effector of the insulin signaling pathway is the protein kinase B (PKB/Akt). Akt phosphorylates and inhibits glycogen synthase 3 (GSK3) and this promotes activity of the GSK3 target glycogen synthase (GS). Akt is known to trigger GLUT4 vesicle mobilization and stimulate protein synthesis and lipogenesis by directly activating mTORC1 and SREBP1 [61, 62]. Hepatic insulin signaling also leads to phosphorylation of CRTC2/TORC2 at Ser171, which thus disrupts its ability to coactivate CREB and stimulate expression of PGC-1α [63].

1.2.3 The cAMP/PKA signaling pathway

Fasting is accompanied by release of glucagon from pancreatic alpha cells to compensate for decreasing circulating glucose levels. Glucagon binding to the glucagon receptor, a G protein-coupled receptor, activates adenylyl cyclase and generation of cAMP that in turn binds to and liberates the regulatory subunit from the cAMP protein kinase (PKA) holoenzyme which causes dimerization of the catalytic alpha subunits (PKA-Cα) [64]. Adrenaline also activates the cAMP/PKA pathway with similar downstream consequences [65]. Activated PKA phosphorylates several regulatory enzymes and transcription factors in order to initiate a metabolic program designed to counteract a transient and systemic energy demand. In particular, this entails activation of hepatic glycogenolysis and gluconeogenesis.

In the absence of cAMP/PKA signaling, the bifunctional enzyme phosphofructokinase-2 (PFK2) favors generation of fructose 2,6-bisphosphate, and this metabolite allosterically inhibits the activity of key regulatory gluconeogenic enzymes [50]. When phosphorylated by PKA, the PFK2 phosphatase activity is stimulated and the resulting removal of fructose 2,6-bisphosphate restores the activity of gluconeogenic enzymes, in particular F1,6BPase [50, 66].

PKA stimulates transcription of several metabolic target genes characterized by promoter cAMP response elements (CRE) by activating CRE-binding protein (CREB) [67]. In addition to PKA-mediated phosphorylation of CREB at Ser133, interaction with both CREB regulated transcription coactivator 2 (CRTC2/TORC2) and the coactivator CREB binding protein (CBP) is crucial to inducing effective target gene transcription [68]. A key transcriptional target of hepatic CREB is the master gluconeogenic coactivator PGC-1α. Notably, insulin signaling stimulates activity of salt-inducible kinase 2 (SIK2) which in turn renders CRTC2 inactive [69]. The CREB complex is only transcriptionally active in the absence of insulin [63]. Thus, insulin and fasting hormone pathways converge to regulate PGC-1α at the transcriptional level (Figure 5). Hepatic PGC-1α coactivates both HNF4α and FOXO1 to stimulate expression of target gluconeogenic genes, and it has been shown that PGC-1α knock-out mice exhibit fasting hypoglycemia [70].

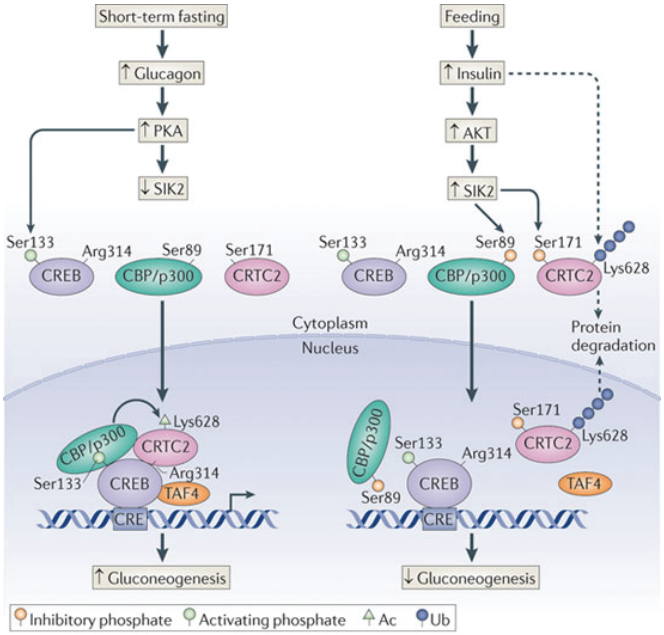

Figure 5. Control of coactivators is central to the regulation of gluconeogenesis. The CREB coactivators CRTC2 and CBP are subject to inhibitory PTMs induced by the insulin signaling pathway. As a result, transcription of the target gene PGC-1α proceeds only in the absence of insulin. In turn, PGC-1α enables transcription of rate-limiting gluconeogenic enzymes. Reprinted by permission from Macmillan Publishers Ltd: Nature [71], 2011.

1.2.4 The AMPK signaling pathway

As starvation progresses, hepatic gluconeogenesis diminishes partly due to substrate availability. Metabolic stress and prolonged glucose deprivation cripple ATP production and result in an increased intracellular [AMP]/[ATP] ratio. This condition triggers activation of the AMP-activated protein kinase (AMPK), a metabolic master regulator. The AMPK holoenzyme is a heterotrimeric complex consisting of a catalytic α subunit and regulatory subunits β and γ. Binding of AMP activates AMPK allosterically and enables phosphorylation of Thr172 of the AMPKα subunit by the upstream liver kinase B1 (LKB1) [72]. The overall mandate of the AMPK pathway is to restore energy balance by stimulating catabolic pathways to generate energy while shutting down anabolic pathways to save energy [73]. By phosphorylating key regulatory enzymes, AMPK promotes glycolysis, fatty acid beta-oxidation, GLUT4 translocation while inhibiting gluconeogenesis, lipogenesis, proliferation and biosynthesis of protein and cholesterol (Figure 6).

Notably, AMPK phosphorylates and inactivates the CREB coactivator CRTC2/TORC2 to inhibit gluconeogenic gene expression [74]. AMPK-mediated phosphorylation of hepatic SREBP-1 suppresses its proteolytic processing, nuclear translocation and lipogenic target gene expression [75]. AMPK has also been found to inhibit SREBP-1 at the transcriptional level, by inhibiting liver X receptor (LXR) ligand production and possible also LXR activity [76]. The LXR nuclear receptors are activated by cholesterol derivate ligands and stimulate hepatic lipogenesis [77]. Conversely, AMPK phosphorylates acetyl-CoA carboxylase (ACC), an important enzyme involved in *de novo* lipogenesis [78]. AMPK has also been shown to phosphorylate and inactivate the carbohydrate-responsive element-binding protein (ChREBP/MLXIPL) [79, 80].

The AMP analog compound AICAR has been used experimentally to stimulate AMPK activity. By interacting with the regulatory AMPK γ-subunit in the same manner as conventional AMP, AICAR manipulates the perceived [AMP]/[ATP] ratio and triggers the energy sensor rheostat AMPK [81].

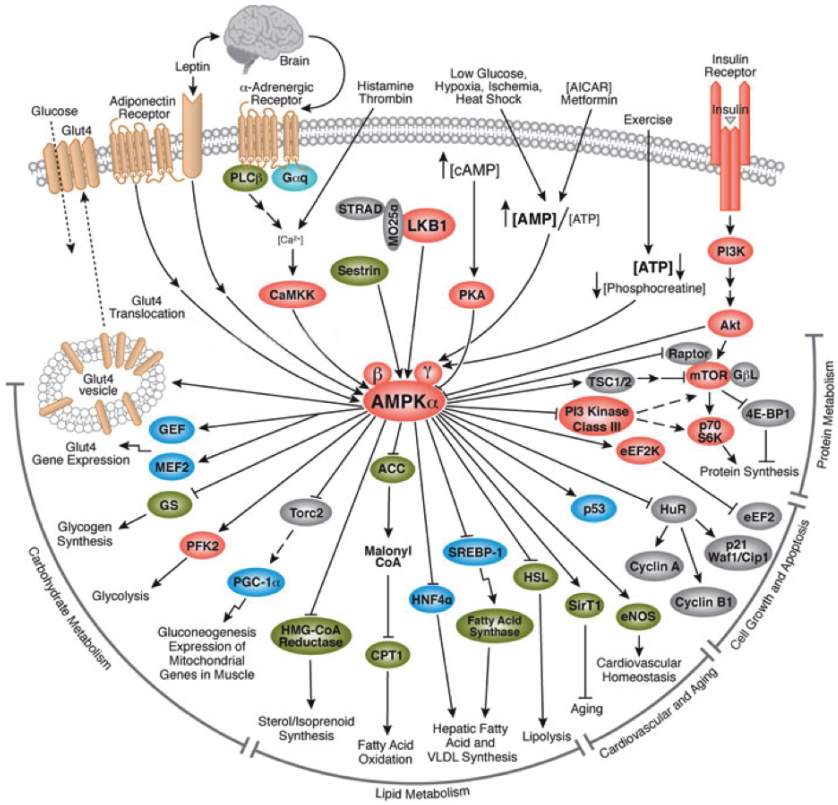

Figure 6. The AMPK pathway regulates hepatocellular energy balance. Cellular energy depletion triggers AMPK activation. The AMPK metabolic reprogramming is mediated by phosphorylation of several key downstream regulatory enzymes and transcription factors. Illustration reproduced courtesy of Cell Signaling Technology, Inc., 2015.

1.2.6 Regulation of hepatic lipid metabolism

De novo anabolism of lipids from acetyl-CoA is referred to as lipogenesis. The initial substrate modification is catalyzed by acetyl-CoA carboxylase (ACC) and generates malonyl-CoA, and this is considered to be the rate-limiting step in lipogenesis [82]. Fatty acid synthase (FASN) catalyzes iterative incorporation of acetyl-CoA to generate long-chain fatty acids at the cost of NADPH. FASN exhibits seven different catalytic sites in addition to including an acyl carrier protein [83]. Since FASN is regulated at the transcriptional level, there is now consensus that it represents a situational rate-limiting enzyme of the lipogenic pathway [82].

Regulation of lipogenesis is central to maintaining energy homeostasis, and this is largely mediated by nuclear receptors in response to upstream intracellular signaling. In the liver, insulin signaling triggers the lipogenic transcription factor triangle consisting of LXR, ChREBP and SREBP-1c [84]. LXRs are activated by oxysterols and promote lipogenesis at the transcriptional level by dimerizing with Retinoid X receptor (RXR) [85]. LXRs are also activated in response to insulin signaling and the insulin-dependent O-GlcNAc post-translational modification [86, 87]. Target genes of LXR include lipogenic enzymes as well as SREBP-1c and ChREBP [88, 89]. Lipogenic genes are also regulated by glucose and, in response to high glucose concentrations, ChREBP binds carbohydrate response elements to activate transcription of target genes including G6Pase, FASN and ACC [90, 91].

SREBP-1 is regarded as a master lipogenic regulator and its expression is reduced by fasting due to suppression of insulin and increased glucagon levels [92]. Downregulation of SREBP-1c also occurs in response to treatment with metformin and the AMPK-stimulating compound AICAR [93]. Conversely, insulin signaling associated with refeeding stimulates SREBP-1 activity [94]. SREBP-1c is the predominant hepatic SREBP isoform and stimulates expression of lipogenic enzymes in response to its own upregulation and maturing post-translational processing by the insulin signaling pathway [95]. Target genes of SREBP-1c are characterized by a sterol regulatory element (SRE) binding sequence, and this is found in the promoters of genes encoding enzymes that catalyze various steps in fatty acid and triglyceride synthesis [95]. SREs are also frequently found in promoters of enzymes pertaining to cholesterol biosynthesis. Notably, SREBP-1a and especially SREBP-2 appear to control expression of cholesterol related genes to a larger extent than SREBP-1c [92, 96]. The inactive precursor SREBP-1 is tethered to the endoplasmic reticulum membrane, and its proteolytic processing is mediated by the SREBP cleavage-activating protein (SCAP) and two site-specific proteases (S1P and S2P) [92]. The processing and nuclear import of SREBP is enabled by combination of insulin signaling and cholesterol depletion [92].

1.3 Type 2 diabetes

The recent emergence of a global obesity epidemic characterized by a sedentary lifestyle, excessive calorie intake and resulting adiposity has dramatically increased the incidence of lifestyle diseases including non-insulin-dependent type 2 diabetes (T2D) [97, 98]. The Norwegian Diabetes Association have estimated that 350 000 Norwegians have T2D, and that this form of diabetes accounts for more than 90% of diabetic patients [99]. Although the pathogenesis remains unclear and attributable to environmental factors, genome-wide association studies have explored the genetic components of T2D and shown that this is a polygenic disease with several implicated "diabetogenes" that predispose to both initiation and progression of disease [100, 101]. Essentially, T2D is characterized by insufficient pancreatic insulin production coupled with systemically impaired insulin signaling, and this is the basis for insulin resistance. The following definition is proposed by Lebovitz [102]: "Insulin resistance is defined clinically as the inability of a known quantity of exogenous or endogenous insulin to increase glucose uptake and utilization in an individual as much as it does in a normal population".

Notably, insulin resistance also negates the inhibitory effect of insulin on hepatic glucose production. Insulin inhibits hepatic gluconeogenesis indirectly by restricting pancreatic α-cell glucagon secretion and also by limiting adipocyte lipolysis that generates gluconeogenic precursors [103]. Furthermore, insulin signaling in hepatocytes directly restricts gluconeogenesis by several mechanisms including transcriptional suppression of PGC-1α and its target gluconeogenic genes by activation of PI3K/Akt [104, 105]. Insulin resistance renders insulin signaling unable to control the gluconeogenic enzymes and this contributes to increased hepatic glucose production and hyperglycemia [105]. Pharmacological management of T2D is required to mitigate the adverse effects of chronic hyperglycemia including, but not limited to retinopathy, neuropathy, nephropathy, atherosclerosis, coronary heart disease, glycation product formation and stroke [106, 107].

1.3.1 Metformin

Herbal medicine in the medieval Europe employed extract from the French lilac (*Galega officinalis*) to treat urinary symptoms that today are attributed to diabetes [108]. A guanidine compound was subsequently characterized as the active ingredient, and pharmacological optimization of this original plant-derived molecule resulted in the synthetic biguanide drug that today is known by its generic name metformin [108]. Clinically, metformin ameliorates hyperglycemia without causing hypoglycemia or weight gain [109]. Since the 1970s, metformin has generally been the mainstay treatment for T2D and currently it marks the most widely used antidiabetic drug worldwide [110]. Little progress was made to elucidate the mechanism by which metformin exerted its effects until 2001, when Zhou and co-workers established that administration of metformin caused activation of the canonical AMPK pathway in hepatocytes [111]. Although an explosion of subsequent publications has greatly increased our knowledge of how metformin causes metabolic reprogramming of target cells, its exact mechanisms of action remain elusive.

It is thought that accumulation of metformin in the mitochondrial matrix disrupts the proton gradient and transiently inhibits the electron transport respiratory chain complex I, thus indirectly leading to increased intracellular [AMP]/[ATP] ratio and successive activation of LKB1 and the energy-sensing AMPK [112-114]. In accordance with the known downstream effects of the AMPK pathway, several studies have shown that metformin inhibits gluconeogenesis and biosynthesis of lipids and cholesterol [109, 111, 112, 115, 116]. A recent study by Shulman and co-workers showed that metformin non-competitively and acutely inhibits the mitochondrial redox shuttle enzyme glycerophosphate dehydrogenase, and that the resultant modulation of cytosolic and mitochondrial redox states *per se* reduced hepatic glucose production in rats [117]. Studies have reported that metformin retains inhibition of gluconeogenesis and the mTOR pathway in absence of AMPK [109, 118, 119]. Thus, the precise mechanisms by which metformin modulates hepatic metabolism remains elusive.

As discussed previously, the pleiotropic AMPK pathway regulates cellular energy balance and metabolism in part by inhibiting the mTOR pathway [112]. In this

regard, metformin may exhibit antineoplastic properties [110, 111]. Preliminary studies have demonstrated that metformin treatment was associated with decreased mortality both in HER2-positive breast cancer patients [120] and prostate cancer patients [121].

1.4 Methodology

1.4.1 Cell lines

All cell lines were purchased from the American Type Culture Collection (ATTC) and cultured in line with the recommended instructions. Cultures were checked for mycoplasma infection prior to use.

For paper I, the COS-1 African green monkey kidney fibroblast cell line was used for transient plasmid overexpression experiments due to high transfection efficiency using an established protocol [45]. Stable knock-down of SRC-2/GRIP1 in the breast cancer adenocarcinoma MCF-7 cell line was obtained by lentiviral integration of a SRC-2 short hairpin RNA (shRNA). A corresponding empty vector (control shRNA) was introduced for the purpose of obtaining a control MCF-7 cell line.

For papers II and III, we employed the hepatocellular cell models to elucidate mechanisms by which SRC-2 regulates hepatic metabolism. FaO and HepG2 cell lines are derived from rat and human hepatomas, respectively, and represent well established cell lines for studies of hepatocellular metabolism. Both of these cell lines are insulin-sensitive and exhibit gluconeogenic potential. Due to high transfection efficiency, HepG2 cells were preferentially employed for transient plasmid overexpression assays.

1.4.2 Microarray analysis

Agilent biochip microarray was performed to characterize the effect of metformin on the combined transcriptome of FaO cells in response to treatment with metformin. Biological triplicates of cells treated with water (control vehicle) or metformin (5 mM) for 24 hours were processed for RNA purification and RNA integrity number (RIN) analysis. The microarray analysis was performed at the Norwegian Microarray Consortium core facility at the University of Bergen. The parallel quantification of a large array of mRNA transcripts is based on hybridization of the sample mRNA, cRNA or cDNA with complementary sequences across thousands of ‘spots’ of a pre-arranged microarray slide [122]. When the slide is excited with laser, the level of

hybridization for individual genes can be detected due to the presence of fluorescent dyes [122].

Digital data output from the microarray was processed using J-Express gene expression analysis software. A significance analysis of microarrays (SAM) was performed to analyze differential gene expression between control and metformin treated cells. SAM provides an algorithm that calculates the significance of gene expression through multiple comparisons, taking into account both the actual and expected signal intensity associated with each gene. Following the SAM analysis, genes are attributed a score, Delta [i], which is based on both the fold change and difference between observed and expected statistical strength. Permutations of the data set allows for determination of the false discovery rate (FDR), a measure of the expected rate of false positives (type I errors) when conducting multiple comparison [123]. The local FDR associated with a gene reflects the probability of that gene being a false positive [124]. Some studies prefer to present significance of microarray results using the q-value, which is an FDR-adjusted p-value. Refinement of the SAM data is achieved by implementing lowest acceptable cut-off points in terms of both absolute gene fold change (FC) and FDR. The set of differentially expressed genes that meet these validation criteria is next subject to a gene ontology bioinformatic analysis where information pertaining to functional classification is assigned to each gene. In accordance with the established instructions [125], we employed the PANTHER database (pantherdb.org) to generate a comprehensible and functional overview of statistically overrepresented gene categories. Since microarrays may not accurately provide data at the single gene level, qPCR validation was performed with respect to genes of further interest.

1.4.3 Quantitative polymerase chain reaction (qPCR)

In all three papers, qPCR was used to measure target gene expression in terms mRNA level. Briefly, total RNA was isolated from sample cells and reverse-transcribed into cDNA prior to real-time qPCR analysis. Primers were designed *in silico* to amplify complementary regions of the target gene mRNA. We also validated primer efficiencies and melting temperature curves. RT-qPCR was run on a Roche

LightCycler 480 using SYBR I green dye that binds specifically to dsDNA, emitting fluorescence when excited by light after each qPCR cycle. In all experiments, the mRNA level of the target gene of interest was normalized to that of an internal reference gene. Depending on which gene exhibited the most stable mRNA level in response to experimental conditions, we employed glyceraldehyde 3-phosphate dehydrogenase (GAPDH), TATA-binding protein (TBP) or ribosomal protein L4 (RPL4) as reference genes. Notably, we observed that the glycolytic enzyme GAPDH was downregulated at the transcriptional level by metformin treatment, and consequently RPL4 was used as reference gene instead. The relative mRNA level of the gene of interest compared to reference gene was calculated using the delta-delta Ct method, assuming parallel primer efficiencies of 2.0 per PCR cycle. This calculation subtracts the mean [Ct (target gene)] – [Ct (reference gene)] for the control treatment and relates the resulting difference to that of equivalently calculated samples where cells were subjected to treatment. Thus if the normalizer sample exhibits Ct target gene = 25 and Ct reference gene = 25 ($\Delta Ct = 0$), and a treatment sample exhibits Ct target gene = 26 and Ct reference gene = 25 ($\Delta Ct = 1$), this returns a $\Delta\Delta Ct = 1$. Since the number of qPCR cycles (n) is exponentially inversely related to the original amplicon amount and amplifies amplicons at an projected efficiency of 2.0, the relative amount of mRNA is can be expressed as 2^{-n}. In the above example, $2^{-1} = 0.5$, suggesting that expression of the target gene was reduced by 50 % relative to the control treatment. Ct values exceeding 35 PCR cycles were considered invalid.

1.4.4 Chromatin immunoprecipitation (ChIP)

ChIP allows for quantitative detection of transcription factors and associated coregulators on genomic promoters of interest [126]. Functional ChIP assays were performed to assay changes in recruitment (enrichment) resulting from distinctive experimental conditions. For this purpose, we used the Magna EZ-ChIP kit in accordance with the manufacturer's instructions. Briefly, cells were treated as indicated and fixed with formaldehyde prior to lysis and sonication of lysate. Sonication was optimized to shear genomic DNA into 200-1000 base pair fragments, as verified by electrophoretic agarose gel. In conjunction with magnetic protein A/G

beads, sonicated lysate samples were incubated overnight with positive control (anti-RNA polymerase II), background noise negative control (normal IgG) or target (anti-SRC-2) antibodies. Eluted DNA was analyzed by qPCR using primers to amplify specific genomic promoter regions. The recent ChIP-sequencing technology is based on these same principles, but retrieves far more data with the combined sequencing of all eluted DNA fragments. Notably, good results from ChIP assays greatly depend on the specific interaction between antibody and target antigen. In paper III, failure to immunoprecipitate the rat SRC-2 antigen in FaO cells with three separate ChIP-grade antibodies prompted us to employ human HepG2 cells for this purpose instead. The resolution of ChIP assay for "footprinting" of a target transcription factor is limited by the chromatin fragment sizes that are normally distributed around 500 base pairs.

1.4.5 Electrophoretic mobility shift assay (EMSA)

Affinity and binding of transcription factors to cognate DNA recognition sequences can be verified *in vitro* using EMSA. Affinity of the nuclear receptor RORα to its consensus RORα response element (RORE) recognition sequence is well established [127, 128]. In paper II, we demonstrate binding of RORα to an atypical RORE in the proximal mouse G6Pase promoter has not been previously shown by EMSA. Briefly, purified V5-tagged RORα protein was incubated with hot, radioactively labelled positive control probe (consensus RORE) or probe of interest (G6Pase RORE). Protein-DNA complexes were separated by gel electrophoresis and visualized by autoradiography. Parallel addition of excess cold probes and antibody-mediated supershift of RORα was performed in order to validate the specificity of the interaction.

1.4.6 siRNA

All papers included in this thesis employed small interfering RNA (siRNA) to knock down genes of interest in cell cultures. It is of crucial importance to optimize the knock-down protocol and verify that the siRNA significantly and specifically downregulates the gene of interest. Optimization of our protocol suggested that knock-down at both mRNA and protein level peaked when cells were incubated with

siRNA for 72 hours prior to analysis. This relatively large window of time allows for prolonged silencing of the gene of interest while the existing target protein component is depleted during several half-life cycles. In all experiments, transfection with non-targeting (NT) siRNA was included as control. Although siRNA is inferior to knock-down and CRISPR genomic approaches, it represents a cost-efficient way to assess the functional implications of the gene of interest.

2. Aims

The aims of this thesis were to elucidate novel mechanisms by which transcriptional and post-translational regulation of SRC-2 determines hepatocellular metabolism.

The specific aims of this thesis were:

Paper I

To elucidate the molecular events and components that execute the previously identified PKA-mediated degradation of SRC-2 protein.

Paper II

To determine whether the cAMP/PKA pathway regulates gluconeogenic G6Pase expression via RORα and its coactivator SRC-2.

Paper III

To determine whether metformin transcriptionally inhibits hepatocellular glucose, lipid and cholesterol biosynthetic pathways via SRC-2.

3. Summary of papers

Paper I

In this study we demonstrated that the inhibitory effect of PKA on SRC-2 protein level is mediated by the transcription factor CREB. Overexpression of CREB reduced SRC-2 protein level, intrinsic transactivation activity and ability to coactivate ERα. Degradation of SRC-2 occurred regardless of CREB Ser-133 phosphorylation status, and was abrogated in the presence of the proteasome inhibitor MG132. The ability of CREB to target SRC-2 for degradation was mediated by a direct protein-protein interaction between the CREB bZIP domain and two functionally independent protein domains of SRC-2 (amino acids 347-758 and 1121-1462). Interestingly, PKA-stimulated degradation of SRC-2 was accompanied by changes in gene expression of several ERα target genes in MCF-7 breast cancer cells.

Paper II

In this study we explored the function of SRC-2 in regulating expression of the gluconeogenic enzyme G6Pase. SRC-2 stimulated G6Pase expression by coactivating RORα at an atypical RORE located on the proximal G6Pase promoter. We observed that the activity of this RORα/SRC-2 complex was markedly inhibited by PKA activity due to proteasomal degradation of SRC-2. Binding of RORα to the atypical G6Pase RORE was not affected by PKA activity, as confirmed by EMSA. Potent activation of PKA reduced recruitment of SRC-2 and RNA polymerase II to the G6Pase promoter. Using the synthetic RORα ligand SR1001 to disrupt coactivator recruitment, we found that SRC-2 is required for the transactivational effect of PGC-1α on the G6Pase promoter. This observation was confirmed by siRNA and transactivation assays using G6Pase promoter constructs with mutated nuclear receptor binding sites. PGC-1α is a coactivator of HNF4α, which is recruited to a binding site adjacent to the G6Pase RORE. Our findings may imply that SRC-2 is necessary for PGC-1α to coactivate HNF4α on the G6Pase promoter.

Paper III

In this study we demonstrated that the anti-diabetic drug metformin represses expression of SRC-2 in a dose-dependent manner. Microarray analysis of FaO hepatoma cells revealed that rate-limiting enzymes involved in gluconeogenesis (*G6pc*) and biosynthesis of lipids (*Fasn*) and cholesterol (*Hmgcr, Hmgcs1*) were downregulated by metformin. These genes were validated as SRC-2 target genes and several of the corresponding gene promoters were characterized by sterol regulatory elements. Transactivation experiments confirmed that SRC-2 acts as a coactivator for SREBP-1, but not SREBP-2. We also found that recruitment of SRC-2 and RNA polymerase II to target gene promoters was markedly reduced in presence of metformin. Treatment of cells with metformin or knock-down of SRC-2 and SREBP-1 significantly reduced hepatocellular lipogenesis and fat content.

4. General discussion

Nuclear receptors recruit transcriptional coregulators that enhance or repress expression of hormonally regulated target genes. The stimulatory and inhibitory properties of coactivators and corepressors, respectively, provide an additional layer of transcriptional regulation and fine-tuning of cellular metabolism [129]. While nuclear receptors have become a paradigm for therapeutic targeting, the potential for pharmacological modulation of coregulators remains largely unexplored [130]. Pertaining to the p160 steroid receptor coactivator family, SRC-2 is widely recognized as a coactivator of several nuclear receptors. Compared to wild type littermates, SRC-2 knock-out mice are phenotypically characterized by (i) fasting hypoglycemia and glycogen immobilization due to reduced expression of hepatic G6Pase [19], (ii) reduced expression of hepatic enzymes pertaining to biosynthesis of lipids and cholesterol [28] and (iii) protection from both obesity and insulin resistance when fed a high-fat diet [22, 26]. These clear phenotypic traits point to SRC-2 as a mediator of anabolic pathways (i.e. energy storage) in both liver and adipose tissue, with implications for whole-body physiology. In humans, single nucleotide polymorphisms of the SRC-2 may be linked to obesity [27]. The above studies and observations warrant additional knowledge of how SRC-2 is regulated to affect cell metabolism. Importantly, identification of relevant cell signaling pathways and novel DNA-binding molecular interaction partners is a key to elucidating the mechanisms by which SRC-2 regulates metabolism in a dynamic and tissue-specific manner.

4.1 Transcriptional regulation of SRC-2

Transcriptional regulation can be defined as the net rate by which an array of transcription factors and coregulators facilitate the ability of RNA polymerase to transcribe mRNA from a target gene promoter. In paper III we demonstrated that the anti-diabetic drug metformin dose-dependently represses hepatocellular expression of SRC-2, whereas SRC-1 and SRC-3 were not affected. In light of the known physiological functions of SRC-2, this finding is innately reconcilable with the ability

of metformin to inhibit hepatic gluconeogenesis and lipogenesis. Since SRC-2 binds to and stimulates transcription of its own promoter [131], our findings may imply that metformin breaks this autonomous self-stimulating loop. We also observed that treatment of cells with metformin drastically downregulated the inflammatory transcription factor EGR1, which previously has been identified as an SRC-2 target gene [18]. The inhibitory effect of metformin on EGR1 expression in monocytes has been established previously [132]. A study conducted by Zhang *et al*. demonstrated that EGR1 knock-out mice were protected from diet-induced obesity, fatty liver and insulin resistance [133]. Interestingly, these phenotypic traits are remarkably overlapping with that observed in SRC-2 knock-out animals. Bioinformatic analysis of the SRC-2 promoter revealed the presence of an EGR1 binding site. It may thus be possible that EGR1 stimulates expression of SRC-2, and vice versa, in a feed-forward loop. Furthermore, SRC-2 by promoting lipogenesis and EGR1 by unknown mechanisms are both unequivocally implicated in facilitating survival of prostate cancer cells in an androgen-depleted environment [30, 134, 135]. Interestingly, administration of metformin reduced the mortality after diagnosis in prostate cancer patients [136]. Generating data to support the hypothesis concerning the possible mutual transcriptional association of SRC-2 and EGR1 was unfortunately outside the scope of paper III, and will consequently be subject to a possible future investigation. In contrast to the inhibitory effect of metformin on SRC-2 expression described in paper III, activation of the cAMP/PKA pathway in paper II did not affect SRC-2 mRNA levels.

4.2 Post-translational regulation of SRC-2

The SRCs are subject to regulation by intracellular signaling, thus modulating the ability of associated nuclear receptors to promote transcription of target genes [137]. Although several PTMs have been characterized to influence the function and activity of SRC-3, much less is known about PTMs affecting parameters of SRC-2 function. In paper I, we described a previously undefined mechanism by which prolonged activation of the cAMP/PKA pathway stimulates proteasomal degradation of ubiquitylated SRC-2. Physiologically, this work relates to hormonal activation of the

cAMP/PKA pathway but with no particular hormone in mind. Others have shown that the incretin hormone GLP-1 negatively affects the growth of breast cancer cells by activating the cAMP/PKA pathway [138]. Previously, it was shown that PKA inhibited SRC-2 coactivator function by targeting SRC-2 for destruction via the ubiquitin-proteasome pathway [46]. The missing components of this mechanism were addressed in paper I and include: characterization of CREB as an executing factor of the PKA-mediated degradation of SRC-2; confirmation of a molecular interaction between CREB and SRC-2 and the corresponding mapping of involved protein domains of CREB and SRC-2, respectively. Importantly, we were also able to demonstrate that the mRNA levels of SRC-2 target genes were downregulated as a result of the described CREB-induced degradation of SRC-2. Although this finding from paper I was primarily thought to be relevant in terms of breast cancer and ERα coactivation, we decided to investigate whether the same basic mechanism, by which the cAMP/PKA pathway inhibits expression of SRC-2 target genes, may also be relevant with regard to physiological events in the liver. The implication of PKA-mediated degradation of SRC-2 in context of hepatocellular regulation of gluconeogenesis was therefore investigated in paper II. Here, we demonstrated that potent PKA activity is accompanied by proteasomal degradation of SRC-2 and inability of SRC-2 to coactivate its partner nuclear receptor RORα on the proximal G6Pase promoter. Furthermore, the master gluconeogenic coactivator PGC-1α was unable to transactivate the G6Pase promoter in the absence of RORα and/or SRC-2. This observation may be explained by the observation that PGC-1α is known to coactivate HNF4α at an adjacent binding site, 20 base pairs upstream of the relevant RORE, on the G6Pase promoter. In paper II we demonstrate direct binding of RORα to this RORE by EMSA. Although this particular RORE has been previously reported to greatly affect the ability PGC-1α to transactivate the G6Pase promoter, its atypical nucleotide sequence occluded its pertinence to RORα [29]. We observed that binding of RORα to its consensus RORE was markedly stronger than to that of the atypical G6Pase RORE. However, EMSA and transactivation assays unequivocally demonstrated the affinity of RORα to both binding sites.

Dipeptidyl peptidase-IV (DPP-IV) inhibitors attenuate the degradation of incretin hormones including GLP-1, and these promising drugs have recently been introduced as treatment for hyperglycemia associated with type 2 diabetes [139]. By inhibiting the degradation of GLP-1, this class of drugs increases pancreatic β-cell insulin secretion and inhibit α-cell glucagon secretion to indirectly reduce hepatic glucose production [140]. Several studies have also shown that DPP-IV inhibitors reverse the progression of NAFLD by directly affecting hepatocyte metabolism [141, 142]. The mechanisms by which DPP-IV inhibitors directly inhibit hepatic gluconeogenesis and lipogenesis remain unknown. Interestingly, it has been shown that hepatocytes express the GLP-1 receptor, and that PKA is activated in response to stimulation of hepatocytes with GLP-1 [143, 144]. It is thus likely that DPP-IV inhibitors prolong the postprandial surges of GLP-1-mediated cAMP/PKA pathway activity in hepatocytes. This may be related to our findings that prolonged PKA activity triggers degradation of hepatocellular SRC-2 and accordingly reduce expression of SRC-2 metabolic target genes, including G6Pase.

4.3 Physiological relevance of SRC-2 target genes

To our knowledge, no previous studies have focused on the effect of metformin on the steroid receptor coactivator family. Interestingly, a paper published by Chopra *et al.* in 2011 reported that SRC-2 is phosphorylated by AMPK *in vitro*, and that incubation of HepG2 cells with the AMP-analogue AICAR increased the ability of SRC-2 to coactivate FXR [22]. Notably, the association of FXR/SRC-2 was only found to be relevant with regard to transactivation of the bile acid export pump (BSEP/ABCB11) promoter. These findings were tied to the observed defective bile acid synthesis observed in SRC-2 knock-out mice and provide an explanation for the increased energy expenditure of SRC-2 knock-out animals compared to wild type littermates. Others have reported that metformin-induced AMPK activation is associated with perturbed bile acid homeostasis due to inhibition of FXR [145]. Physiologically, it is plausible that the AMPK pathway would both inhibit cholesterol and bile acid synthesis due to the energy-demanding nature of these anabolic pathways. It is widely established that metformin transcriptionally inhibits SREBP-1

[93], and that hepatic SREBP-1 expression levels are also decreased in response to inhibition of HMGCR and depletion of oxysterols [146]. Bile acids and oxysterols are derivatives of cholesterol and represent the endogenous ligands for FXR activation, which is known to stimulate the expression of the nuclear receptor small heterodimer partner (SHP/NR0B2) [147]. SHP consists only of a ligand-binding domain and no DNA binding domain and acts as a negative regulator of several nuclear receptors, and a transcriptional repressor of the CYP7A1 gene, the rate-limiting enzyme in bile acid synthesis [148, 149]. Thus, SHP functions as a negative feedback mediator of bile acid synthesis. Interestingly, SHP has been identified as a transcriptional target gene of SRC-2 [131] although the implications of this remains unknown. Incidentally, it has also been shown that metformin upregulates SHP [150]. Further studies are required to solve the role of SRC-2 in the complex regulatory networks of cholesterol and bile acid synthesis.

SRC-2 liver knock-out mice are phenotypically characterized by fasting hypoglycemia and glycogen immobilization due to reduced G6Pase expression [19]. In line with these results, we demonstrated in paper II that PKA-mediated proteasomal degradation of SRC-2 resulted in a complete loss of ability to coactivate RORα and transactivate the G6Pase promoter. We also observed that PGC-1α activity on the same promoter requires the presence of SRC-2. These findings point to SRC-2 as a critical mediator of gluconeogenesis by promoting expression of G6Pase. Furthermore, since SRC-2 activity is abolished in response to potent stimulation of the cAMP/PKA pathway, our findings may explain the downregulation of hepatic gluconeogenesis during long-term starvation, which is prevailingly characterized by abundant glucagon signaling.

We show in paper III that metformin transcriptionally represses SRC-2 and inhibits recruitment of SRC-2 to RORα at the proximal G6Pase promoter. This may partly explain the ability of metformin to inhibit gluconeogenesis at the transcriptional level. Our results are in line with the established importance of coactivators in the regulation of gluconeogenesis. The ability of metformin to activate the AMPK pathway and inhibit CRTC2/TORC2-mediated expression of PGC-1α is well established [74, 151]. Interestingly, it has been reported that metformin retains

its inhibitory effect on gluconeogenic gene expression in absence of both LKB1 and the AMPK enzyme [152]. The AMPK-independent effects of metformin are poorly characterized and the exact mechanisms by which this anti-diabetic drug works remain elusive. Rather than pursuing the effect of AICAR on SRC-2 function, we chose to focus on metformin instead due to its clinical relevance. Metformin hydrochloride was diluted in water and added to cell media to final concentrations in the millimolar range, which is a widely established dosage in terms of cell experiments. Metformin enters cells primarily via the organic cation transporter 1 (OCT1) membrane channel, and it has been shown that genetic variation of the OCT1 impact metformin uptake *in vivo* [153]. We confirmed expression of OCT1 in both FaO and HepG2 cell lines.

At the core of de novo lipogenesis is the rate-limiting enzyme fatty acid synthase (FASN/FAS), a multi-subunit enzyme that generates palmitate from sequential addition of two carbons to malonyl-CoA. As expected, hepatic FASN expression levels have been found to be increased in NAFLD [154]. A new paradigm to have emerged is the FASN-driven "lipogenic state" which points to FASN as a pathologic driver of insulin resistance, fat accretion and cancer [155]. Originally characterized as a LXR target gene, it is now known that hepatic FASN is regulated at the transcriptional level by also SREBP-1 and ChREBP [84]. In paper III, we demonstrated that SRC-2 acts as a coactivator of SREBP-1 on the FASN promoter. This finding is in line with the findings of a recent publication by Dasgupta *et al.,* where it was shown that SRC-2 exerts 'metabolic programming' to promote lipogenesis and survival of prostate cancer cells [30]. It was also demonstrated that the presence of glutamine and mTORC1 activity was required to enable SRC-2 coactivation of SREBP-1. Our cell experiments for paper III were performed with the required amount of glutamine to ensure stable mTORC1 activity.

5. Future perspectives

A comprehensible analysis of hepatic SRC-2 target gene networks is required for to elucidate its multifaceted roles in regulating liver metabolism. For paper III, we planned to perform a ChIP-sequencing of SRC-2 to complement the microarray data in FaO cells treated with or without metformin. However, no tested antibodies were compatible with immunoprecipitation of the rat antigen SRC-2, and no data was retrieved. If pursued for future studies, this analysis should be performed in conjunction with microarray using hepatic tissue from wild type and SRC-2 knock-out mice treated with or without metformin. Data from ChIP-sequencing may also elucidate novel nuclear receptors that are coactivated by SRC-2 on mutual target promoters. Identifying novel interaction partners of SRC-2 should be useful for elucidating mechanisms that may be relevant to human diseases including diabetes and metabolic complications associated with obesity. It would also be interesting to ascertain whether the metformin-reduced downregulation of SRC-2 is AMPK-dependent and involves EGR1. Moreover, a possible connection between the known inhibitory effect of metformin on the mTORC1 pathway with regard to mTORC1-dependent activation of SRC-2 should be explored. This was unfortunately outside the scope of paper III.

It would also be interesting to investigate whether SRC-2 in skeletal muscle and adipose tissue is relevant to the insulin sensitizing mechanisms by which metformin increases glucose uptake.

6. Conclusions

In this thesis it was demonstrated that SRC-2 is subject to both transcriptional and post-translational regulation. Furthermore, the available amount of cellular SRC-2 protein determines the rate of transcription of SRC-2 target genes. In the liver, SRC-2 regulates gluconeogenesis and biosynthesis of lipids and cholesterol at the transcriptional level.

The following conclusions can be drawn from this study:

- Prolonged activity of the cAMP/PKA signaling pathway stimulates the targeted degradation of SRC-2 protein. CREB directly interacts with SRC-2, and is an integral factor in the PKA-mediated degradation of SRC-2.

- The nuclear receptor RORα and its coactivator SRC-2 stimulate transactivation of the G6Pase promoter. In the absence of RORα/SRC-2, the ability of PGC-1α to transactivate the G6Pase promoter is diminished. By regulating the amount of SRC-2 protein available to coactivate RORα, prolonged cAMP/PKA signaling may attenuate the rate of G6Pase expression and gluconeogenesis during long-term starvation.

- The anti-diabetic drug metformin dose-dependently represses transcription of both SRC-2 and its target genes. Metformin reduces recruitment of SRC-2 to RORα on the G6Pase promoter and markedly downregulates G6Pase expression. Metformin also inhibits transcription of several SRC-2 target genes involved in biosynthesis of lipids and cholesterol where SRC-2 acts as a coactivator of SREBP-1.

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Appendix

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The cAMP-dependent protein kinase downregulates glucose-6-phosphatase expression through RORα and SRC-2 coactivator transcriptional activity

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ABSTRACT

Fasting hormones activate the cAMP/PKA signaling pathway and stimulate expression of hepatic gluconeogenic enzymes including glucose-6-phosphatase (G6Pase). Previously it was shown that steroid receptor coactivator 2 (SRC-2) knock-out mice exhibit fasting hypoglycemia and that SRC-2 coactivates RAR-related orphan receptor alpha (RORα) at the proximal G6Pase promoter. We have investigated the upstream regulation and functional implications of this RORα/SRC-2 complex on G6Pase expression. In HepG2 cells, overexpression of the catalytic PKA subunit (PKA-Cα) reduced the SRC-2 protein level, recruitment to the G6Pase promoter, and its ability to coactivate RORα. Knock-down and transactivation experiments employing G6Pase promoter constructs demonstrated that RORα and SRC-2 are required for PGC-1α to stimulate G6Pase expression. These results suggest that PKA inhibits SRC-2 coactivation of RORα and in turn reduces PGC-1α dependent regulation of G6Pase. This indirect feedback mechanism may underlie the suppression of gluconeogenesis throughout long-term starvation.

1. Introduction

During short-term fasting the continued demand for circulating glucose in mammals is supplied by mobilization of hepatic glycogen into free glucose through the process of glycogenolysis (Nordlie, 1974, 1979; van Schaftingen and Gerin, 2002). In this phase, stable plasma glucose levels rely on a finite hepatic glycogen storage that rapidly depletes. Following glycogen depletion and overnight fasting, new glucose is generated by the process of gluconeogenesis to avoid hypoglycemia (Unger et al., 1963; Cahill, 2006).

Common to both glycogenolysis and gluconeogenesis is the rate-limiting enzymatic dephosphorylation of glucose-6-phosphate into free glucose that can be secreted into plasma and this reaction is catalyzed by the catalytic subunit of glucose-6-phosphatase (G6Pase) (Nordlie, 1974, 1979; van Schaftingen and Gerin, 2002; Nordlie et al., 1999; Chopra et al., 2008). This enzyme is expressed mainly in the liver and kidneys where it is translated into the membrane of endoplasmic reticulum (Nordlie, 1979; Arion et al., 1976). The rate-limiting enzymes phosphoenolpyruvate carboxykinase (PEPCK) and G6Pase control the output of gluconeogenesis and are regulated at the transcriptional level by key metabolic hormones including glucagon, glucocorticoids, and insulin (Sever and Glass, 2013). A key downstream mediator of glucagon signaling is peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC-1α), which regulates several metabolic processes including gluconeogenesis and mitochondrial biogenesis (Lin et al., 2005; Finck and Kelly, 2006; Schilling et al.,

Abbreviations: G6Pase, glucose-6-phosphatase; SRC, steroid receptor coactivator; PKA, protein kinase A/cAMP-dependent protein kinase; RORα, retinoic acid related orphan receptor alpha; RORE, RORα response element; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; HNF4α, hepatocyte nuclear factor 4-alpha.

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2008). Fasting is accompanied by an increase in glucagon levels and activation of the cAMP/PKA-dependent pathway (Unger et al., 1963). Specifically, PKA-mediated phosphorylation of cAMP response element binding protein (CREB) at Ser133 facilitates the interaction with its coactivator CRTC2/TORC2 and enables transcription of PGC-1α (Lin et al., 2005; Finck and Kelly, 2006). PGC-1α in turn promotes expression of G6Pase by coactivating hepatocyte nuclear factor 4-alpha (HNF4α) (Sever and Glass, 2013). Conversely, insulin signaling represses PGC-1α expression by stimulating ubiquitin-mediated degradation of TORC2. In type 2 diabetes, the combination of insulin resistance and elevated serum glucagon exacerbates hepatic gluconeogenesis resulting in increased endogenous glucose production and fasting hyperglycemia (Sloop et al., 2005).

Steroid receptor coactivator 2 (SRC-2) is a member of the p160 steroid receptor coactivator family of three genetically distinct yet structurally and functionally similar members (Stashi et al., 2014; York and O'Malley, 2010). The SRCs associate with nuclear hormone receptors (NRs) in a ligand-dependent manner to enhance transcriptional activity (Stashi et al., 2014; Johnson and O'Malley, 2012). NRs respond to hormonal signaling by binding directly to DNA and play important roles in a variety of physiological processes including development, homeostasis, and metabolism (Picard et al., 2002; O'Malley, 2006). Phenotypic observations of global or liver-specific SRC-2 knock-out mice include hypoglycemia in fasted state and reduced expression of hepatic G6Pase in both fed and fasted state (Chopra et al., 2008). Interestingly, loss-of-function mutations of the G6Pase gene (glycogen storage disorder-1a) exhibit a similar phenotype characterized by fasting hypoglycemia due to immobilization and accumulation of hepatic glycogen (Lei et al., 1996). It has been shown that SRC-2 regulates G6Pase expression by coactivating the retinoic acid receptor-related orphan receptor alpha (RORα) at an evolutionary conserved RORα response element (RORE) sequence of the proximal G6Pase promoter (−54 to −49, relative to transcription start site (TSS) in mouse) (Chopra et al., 2008). Indeed, SRC-2 mediated coactivation of RORα greatly enhanced promoter activity and the rate of transcription of G6Pase (Chopra et al., 2008). Located adjacent to a HNF4α binding sequence (−76 to −63 relative to TSS in mouse), this particular RORE sequence has previously been identified as a nuclear receptor half-site required for PGC-1α mediated G6Pase expression (Schilling et al., 2008).

Continued fasting leads to progressively elevated levels of glucagon that corresponds to increased levels of intracellular cAMP (Unger et al., 1963). We have previously demonstrated that SRC-2 is subject to regulation by the cAMP/PKA pathway in other contexts (Hoang et al., 2004; Fenne et al., 2008). This study was designed to investigate whether stimulation of the cAMP/PKA signaling pathway regulates G6Pase expression via RORα and SRC-2.

2. Materials and methods

2.1. Cell culture and transfection assays

The human hepatoma cell line HepG2 was purchased from ATTC and cultured at 37 °C and 5% CO_2 in presence of water bath containing fungizone (Bristol-Myers Squibb, New York City, NY, USA). HepG2 cells were grown in EMEM (Lonza, Basel, Switzerland) supplemented with 10% FBS (Gibco, Waltham, MA, USA), 1% penicillin-streptomycin (Sigma, St. Louis, MO, USA) and 2 mM L-glutamine (Lonza) and seeded in multi-well plates one day prior to transfection. Plasmids were transfected using TransIT-LT1 reagent (Mirus, Madison, WI, USA) according to the manufacturer's instructions. Mutant reporters were made using the QuickChange II Site-Directed Mutagenesis Kit (Agilent, Santa Clara, CA, USA) according to manufacturer's instructions or with less overlapping primers as described (Liu and Naismith, 2008). All reporter constructs were verified by DNA sequencing. The following reporter plasmids were used: the promoter sequence between −231/+66 relative to transcriptional start site of the wild type (WT) mouse pGL3-WT-mG6Pase-luc; pGL3-ΔRORE-mG6Pase-luc; and pGL3-ΔHNF4α-mG6Pase-luc. The following expression plasmids were used: pCMV-XL5-RORα; pCMV empty vector; pCR3.1-SRC2; pCR3.1-empty vector; pcDNA4-6xHis-hRORα1-HisMax; pCMV5-Cα; pCDNA-PGC-1α and pCDNA-empty vector. For indicated experiments, cells were treated with DMSO (Sigma) as vehicle, MG132 (Sigma), RORα ligand SR1001 (Sigma), forskolin (Sigma), a combination of cAMP-elevating agents (10 μM forskolin and 50 μM IBMX (Sigma) and 500 μM N^6-monobutyryladenosine-3′,5′-cyclic monophosphate (6 MB-cAMP) (Sigma), or transfected with the PKA catalytic subunit vector pCMV5-Cα. For knock-down experiments we employed TransIT-TKO transfection reagent (Mirus) per the provided instructions. SRC-2 *(Ncoa2)*, RORα (*Rora*) or non-targeting (NT) SMARTpool ON-TARGETplus siRNA (Dharmacon, Lafayette, CO, USA) was incubated with cells at a final concentration of 100 nM for 72 h.

2.2. RNA isolation, cDNA synthesis and mRNA quantification

Cells were lysed in 350 μl RLT buffer and RNA was isolated using QIAcube and RNEasy mini kit (Qiagen, Hilden, Germany). Sample RNA concentrations were measured by NanoDrop and cDNA was synthesized from normalized amounts of total RNA with Transcriptor First Strand kit (Roche, Basel, Switzerland) according to the manufacturer's mixed primer protocol. The cDNA was diluted 1:10 prior to mRNA quantification by real-time SYBR I Green RT-qPCR of target genes relative to reference gene *RPL4*. Primer sequences are presented in the supplemental information Table 1. Relative mRNA quantification was calculated using the delta−delta Ct method.

2.3. Transactivation assays

Cells were transfected 48 h prior to lysis with buffer containing 25 mM Tris Acetate-EDTA (pH 7.8), 2 mM dithiotheitol, 1 mM EDTA, 10% glycerol and 1% Triton X-100. Sample lysate was analyzed with luciferase kit (BioThema, Handen, Sweden) on a FLUOStar Optima (BMG Labtech, Ortenberg, Germany) luminescence plate reader.

2.4. Protein quantification, SDS-PAGE and Western blot

Cells were harvested in buffer containing 50 mM Tris−HCl (pH 7.5), 200 mM NaCl, 5 mM EDTA, 1% NP-40, 1ug/ml aprotinin, 5 mM N-ethylmaleimide, 100 nM sodium orthovanate, 0.2 mM PMSF and 1× EDTA-free protease inhibitor cocktail (Roche). Sample protein concentrations were measured using a detergent compatible protein assay (Bio-Rad, Hercules, CA, USA) in accordance with manufacturer's protocol. 4× loading buffer was added to samples of equal protein amounts and boiled at 95 °C for 5min. Samples were loaded onto precast 4−20% gradient Mini-Protean TGX gels (Bio-Rad) and run at 150 V for 1 h in a mini PROTEAN tetra Powerpac system (Bio-Rad). Protein was subsequently transferred to nitrocellulose membranes using iBlot system (Invitrogen, Waltham, MA, USA). Membranes were blocked using 5% non-fat dry milk in PBS-T overnight at 4 °C, incubated with antibodies with 3% BSA in PBS-T, developed using Femto substrate (Thermo Scientific, Waltham, MA, USA) and analyzed using a ChemiDoc XRS camera (Bio-Rad) and QuantityOne software. The following antibodies were employed: anti-SRC-2 (BD Biosciences #610985); anti-beta-Actin

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(Abcam ab8227); anti-RORα (Sigma AV45607); HRP goat-anti mouse IgG (BD Biosciences #554002) and HRP goat-anti rabbit IgG (Thermo Scientific #31460).

2.5. Chromatin immunoprecipitation (ChIP)

HepG2 cells were seeded (2.0×10^6) in 92 mm plates per treatment as indicated prior to fixation in 1% (v/v) formaldehyde for 10 min and quenching with glycerol. Cells were washed in ice-cold PBS and further processed with the EZ-Magna ChIP kit (Millipore, Billerica, MA, USA). Sonication was set to 8 min (30 s on/off cycles) using a cold-water bath Bioruptor (Diagenode, Denville, NJ, USA). The following antibodies were used in conjunction with protein A magnetic beads in over-night immunoprecipitation: anti-RNA Pol II (Millipore #05-623B); normal rabbit IgG (Millipore #PP64B); anti-SRC-2 (Bethyl #300-346A) in quantities of 5 μl per sample. The primers used to direct amplification of the proximal RORE on the human promoter of Glucose-6-Phosphatase, catalytic (*G6Pc*) in end-point SYBR I Green real-time qPCR were 5′-CGTGGTTTTTGAGTCCAAAGAT-3′ (forward) and 5′-CCCCTGTTTTA-TATGCCCTGT-3′ (reverse). Primer specificity was verified *in silico* using the UCSC Genome Browser (http://genome-euro.ucsc.edu). Sample qPCR data was normalized that of respective 1% input samples for each treatment.

2.6. Electrophoretic mobility shift assay (EMSA)

The promoter sequence of Glucose-6-phosphatase, catalytic (G6pc) (NCBI gene 14377, refseq NM_008061) was retrieved from the TRED database (https://cb.utdallas.edu/cgi-bin/TRED/tred.cgi?process=home) and verified by the EPD database (http://epd.vital-it.ch). Complementary single-stranded oligonucleotides surrounding the putative proximal RORE were purchased from Sigma and 2.8 nM of each forward and reverse oligonucleotide were combined and precipitated with 1:10 volume of 3 M sodium acetate and 2 volumes of ice-cold 96% ethanol and left for minimum 1 h at −80 °C. Precipitates were centrifuged at 13000× *g* for 30 min at 4 °C prior to supernatant removal and pellet resuspension in 100 μL hybridization buffer (10 mM Tris–HCl at pH 8.0, 1 mM EDTA, 50 mM NaCl). Solution was heated to 70 °C for 10 min followed by incubation at room temperature for 90 min. Annealed double-stranded oligonucleotides were precipitated again as described above and resuspended in 37 μL TE buffer (Qiagen). The following oligonucleotides were used to create the mG6Pase-RORE probe: 5′-GGGCTGGGTTGACCT*ACAGA*CTGAATCC-3′ and 5′-GGATTCA*GT **C**TGT*AGGTCAACCCAGCCC-3′, and the consensus RORE probe mutant probe was made by combining: 5-GGGCTGGGTTGACCT *ACA**T***ACTGAATCC-3′ and 5′-GGATTCA*GT**A**TGT*AGGTCA**A**CCCAGCCC-3′. The core nuclear receptor half-site is underlined, RORα recognition site-specific A/T rich region is shown in italics and mutation site is emphasized in bold letters. 50 ng double-stranded probes were labeled using T4 polynucleotide kinase (Promega, Madison, WI, USA) and 25 μCi [γ-^{32}P]dATP (Nerliens Meszansky, Oslo, Norway) according to manufacturers' instructions. Labeled probe was diluted 1:2 in 50 mM Tris/HCl, pH 7.5 to give a probe concentration of 1 ng/μL and purified through 7K Zeba spin column (Thermo Scientific) prior to scintillation count. Typical incorporation was 300.000 cpm/ng probe. *In vitro* transcription and translation of pcDNA4-6xHis-hRORα1-HisMax, pCMV-Cα and empty vector negative control were made using cold methionine and the TNT Quick Coupled Transcription system (Promega) according to instructions. Parallel TNT expression assay using ^{35}S-methionine was performed to protein expression by SDS-PAGE and autoradiography. The following components were added to the binding reaction in subsequent order: H_2O, binding buffer (modified from Sato et al. (Sato et al., 2004) final concentrations: 10 mM Tris–HCl pH 7.5, 50 mM KCl, 1 mM DTT, 1 mM EDTA, 5% glycerol, 5 mM $MgCl_2$ and 12.5 ng/μL sonicated salmon sperm DNA), 200 ng competitive unlabeled probe (where indicated) and 2.5 μL TNT lysate. For the functional PKA assay, TNT lysate expressing RORα or empty vector was incubated with recombinant, purified PKA-Cα (0.18 μg/μL) in presence of 10 mM ATP, protease and phosphatase inhibitors (Roche) in a 1× NEB buffer (50 mM Tris–HCl pH 7.5, 10 mM MgCl, 0.1 mM EDTA, 2 mM DTT, 0.01% Igepal) at 30 °C for 30 min. After 20 min of pre-incubation on ice, 200 pg labeled probe was added and the binding reaction was performed at room temperature for 30 min. DNA-protein complexes were separated on 10% non-denaturing TBE polyacrylamide gels (Biorad) using ice-cold TBE running buffer. Visualization was performed by autoradiography.

2.7. Statistics and graphics

Two-tailed, unpaired Student's *t*-test was used to evaluate significance of results. Where explicitly indicated, One-way ANOVA with linear trend or Dunnett's multiple comparison post-test was used for statistical calculations. Significance was defined as $p \leq 0.05$ (indicated *) or $p \leq 0.01$ (indicated **) or $p \leq 0.001$ (indicated ***). Unless explicitly stated, all figures contain data from one representative out of at least three independent experiments. GraphPad Prism v5.0 (GraphPad Software Inc, La Jolla, CA, USA) and Adobe Illustrator (Adobe Systems Inc, San Jose, CA, USA) were used for statistical calculations and graphical presentation of data.

3. Results

3.1. Transcriptional activity of RORα and SRC-2 is inhibited by the overexpression of the PKA catalytic subunit

Fasting hormones stimulate expression of G6Pase by activating the cAMP/PKA signaling pathway (Sever and Glass, 2013). We have previously shown that activation of the cAMP/PKA signaling pathway can change the SRC-2 coactivator function (Fenne et al., 2013) and target promoter recruitment (Fenne et al., 2008) while stimulating SRC-2 proteasomal degradation (Hoang et al., 2004). Since SRC-2 has been shown to regulate G6Pase expression by coactivating RORα (Chopra et al., 2008), we wanted to investigate whether activation of the cAMP/PKA pathway may affect the RORα/SRC-2 dependent transcriptional regulation of G6Pase. First we investigated the cAMP/PKA effect on a reporter system containing the proximal promoter sequence of G6Pase (−231/+66 relative to transcription start site). In order to induce a moderate level of PKA activation, simulating early stages of fasting, respectively, cells were treated with forskolin (FSK) or a combination of FSK, IMBX and 6MB-cAMP. Overexpression of the catalytic PKA-Cα subunit was performed to mimic potent PKA activity corresponding to long-term starvation. Overexpression of RORα significantly stimulated the G6Pase reporter activity and we observed a synergistic effect when RORα was overexpressed together with SRC-2 (Fig. 1A). Importantly, addition of forskolin or the combination of forskolin, IBMX and 6MB-cAMP stimulated the basal level of reporter activity in presence of empty vectors proportionally to that observed upon overexpression of RORα, SRC-2 or both. This suggests that the cAMP/PKA signaling pathway does not stimulate the expression of G6Pase by enhancing activity of the RORα/SRC-2 transcriptional complex. In contrast, overexpression of PKA-Cα significantly reduced reporter activity and completely abolished the transcriptional synergy in cells overexpressing both RORα and SRC-2 (Vehicle vs. PKA-Cα, $p < 0.01$). This suggests that a strong activation of the PKA signaling pathway inhibits transcriptional activity of

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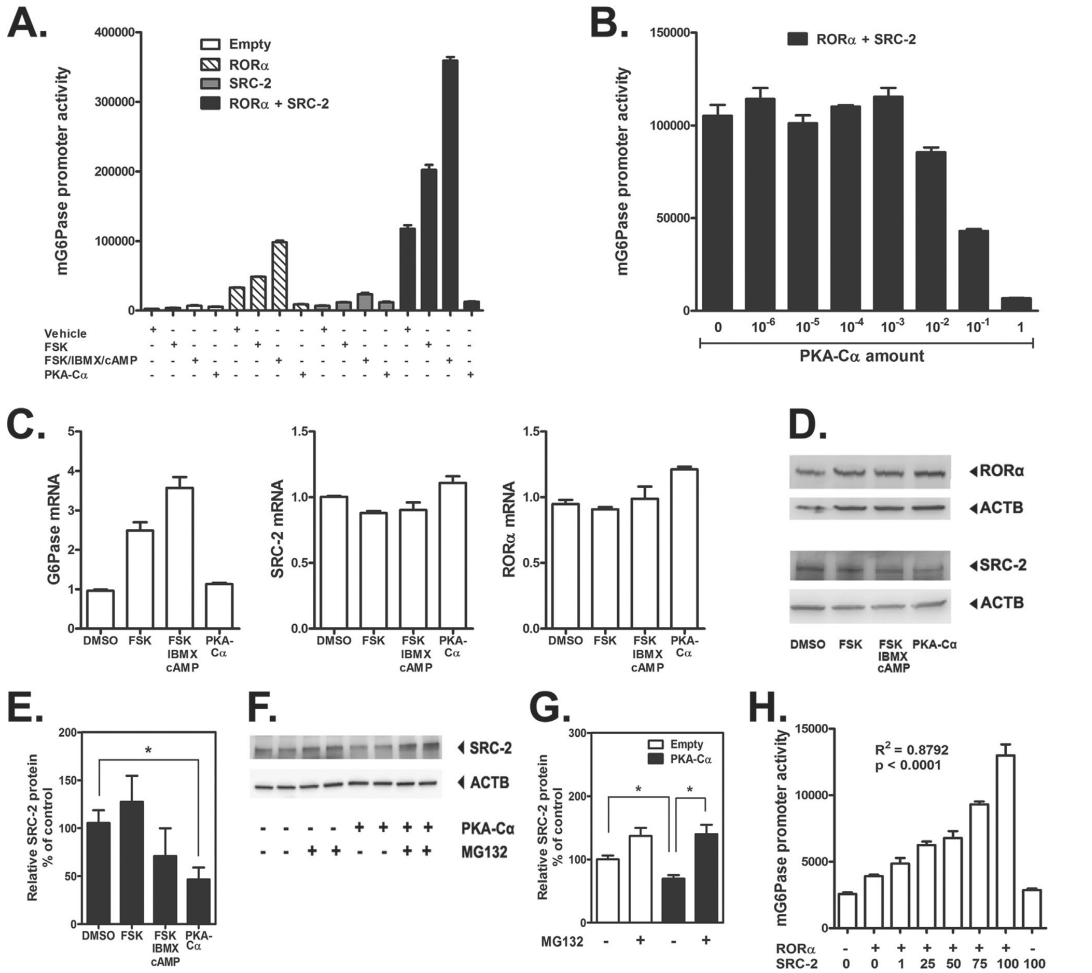

Fig. 1. Effect of the cAMP/PKA pathway on RORα/SRC-2 and G6Pase expression. A. The ability of SRC-2 to coactivate RORα and transactivate the mG6Pase reporter (−231/+66) in response to activation of the cAMP/PKA pathway was assayed by transfecting and treating HepG2 cells as indicated. Activation of the cAMP/PKA signaling pathway was accomplished by the addition of either 10 μM forskolin (FSK), or a combination of 10 μM forskolin, 50 μM IBMX and 500 μM 6 MB-cAMP (FSK/IBMX/cAMP), or overexpression of the PKA catalytic subunit (PKA-Cα). Reporter activity of sample lysate is presented as mean arbitrary luciferase units ± SEM of biological triplicates. B. HepG2 cells were transfected with mG6Pase-luc reporter, full-length RORα and SRC-2 plasmids in presence of an increasing amount of PKA-Cα. The maximal PKA-Cα plasmid amount of 1 corresponds to the default level used in all other experiments. Data is represented as mean arbitrary luciferase units ± SEM of biological quadruplicates. C. Effect of cAMP/PKA pathway activation on expression of indicated genes. HepG2 cells treated as indicated for 48 h and endogenous mRNA levels of *G6Pase*, *RORα* and *G6Pase* mRNA relative to reference gene *RPL4* were quantified by RT-qPCR. The average ± SEM of biological triplicates from a representative experiment is shown. D. Western blotting of annotated endogenous proteins with beta-Actin (ACTB) loading control from lysate of HepG2 cells treated as indicated for 48 h. A representative result of three independent experiments is shown. E. Volumetric densitometry analysis of SRC-2 protein amount relative to ACTB based on three independent experiments. F. HepG2 cells were transfected with PKA-Cα or an equivalent empty vector and incubated in the absence or presence of 1 μM MG132 proteasome inhibitor for 48 h prior to analysis of lysate by Western blot. G. SRC-2 protein level was normalized to that of ACTB in densitometry analyses of three independent experiments. H. Promoter activity of mG6Pase reporter in response to titration with increasing amounts (×100 ng transfected DNA) of SRC-2 plasmid in presence or absence of RORα. Data represent mean luciferase activity ± SEM of biological quadruplicates. 1-way analysis of variance with post test for linear trend was applied to analyze promoter activity in response to increasing amounts of SRC-2 in the presence of RORα. In figures E and G, $^{*}p < 0.05$ (Student's *t*-test).

the RORα/SRC-2 complex on the G6Pase promoter. To verify that the unchanged mG6Pase reporter activity upon treatment of cells with FSK/IMBX/6MB-cAMP corresponded to only a moderate level of PKA activation, we titrated the amount of PKA-Cα plasmid in cells overexpressing RORα and SRC-2 (Fig. 1B). We observed that small amounts of PKA-Cα did not impede the ability of RORα/SRC-2 to transactivate the mG6Pase promoter, whereas higher levels of PKA-Cα exhibited an inhibitory effect as observed before. Importantly, this observation suggests that the observed reduction of reporter activity in the transition from a moderate PKA activation (FSK/IMBX/6MB-cAMP) to a strong PKA activation (PKA-Cα overexpression) in Fig. 1A was not due to activation of different pathways. In this cell system, overexpression of the catalytic PKA-Cα subunit corresponds to a vastly stronger PKA activity than that of endogenous PKA activated by FSK or the combination of FSK/IBMX/6MB-cAMP.

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Next we subjected HepG2 cells to treatment with FSK, the combination of FSK/IMBX/6MB-cAMP, or transfection with PKA-Cα in order to determine mRNA levels of G6Pase, SRC-2 and RORα (Fig. 1C). We found that *G6Pase* mRNA was significantly upregulated in response to moderate PKA activation. Supporting the findings from the luciferase system, we observed that *G6Pase* mRNA level was significantly reduced to baseline level when PKA-Cα was overexpressed. In contrast, mRNA levels of *RORα* and *SRC-2* remained unchanged regardless of PKA activity. This implies that the ability of strong PKA activity to inhibit RORα/SRC-2 transcriptional activity is not attributable to changes in mRNA expression levels of RORα or SRC-2. Similar findings were observed in the rat hepatoma FaO cell line (data not shown). Furthermore, using HepG2 lysate from identical experiments we found that endogenous protein levels of RORα relative to load control β-actin (ACTB) were also insensitive to PKA activity (Fig. 1D). However, as previously reported (Hoang et al., 2004), a reduction of endogenous SRC-2 protein level relative to ACTB was observed when PKA-Cα was overexpressed. Densitometry analysis of Western blots from three experiments confirmed that SRC-2 protein was significantly reduced upon overexpression of PKA-Cα (Fig. 1E, $p < 0.05$). In order to verify that PKA indeed caused SRC-2 degradation, HepG2 cells were transfected with PKA-Cα or an equivalent amount of empty vector in the presence or absence of the proteasome inhibitor MG132 at a final concentration of 1 μM (Fig. 1F). Again, we observed that PKA-Cα significantly decreased the SRC-2 protein level. Densitometry analyses confirmed that addition of MG132 decreased the basal turnover and, additionally, prevented PKA-Cα mediated degradation of SRC-2 (Fig. 1G).

In light of the evident PKA-mediated degradation of SRC-2, we wanted to investigate whether this mechanism is relevant for the activity of the G6Pase promoter. Transfection assays in HepG2 cells demonstrated that SRC-2 transactivated the G6Pase promoter in a dose-dependent manner in the presence of RORα (Fig. 1H). As expected, SRC-2 was unable to stimulate promoter transactivation in absence of RORα. These data indicate that G6Pase promoter activity in part is determined by both the presence of RORα and the amount of available SRC-2. Taken together, these results demonstrate that that the ability of RORα/SRC-2 to transactivate the G6Pase promoter is not enhanced by the PKA signaling pathway. In contrast, strong PKA activity is accompanied by SRC-2 degradation, complete loss of RORα/SRC-2 activity on the G6Pase promoter and reduction of the G6Pase expression back to baseline level.

3.2. Recruitment of SRC-2 to the G6Pase promoter is determined by PKA activity

Shilling et al. (Schilling et al., 2008) observed that the ability of PGC-1α to promote transactivation of a G6Pase-luc reporter plasmid was abolished upon introduction of a mutation in a proximal RORα response element (RORE). The authors assumed that this particular NR half-site represented a HNF4α binding site. However, an enrichment of RORα at the proximal G6Pase promoter was subsequently shown by Chopra et al. (Chopra et al., 2008), demonstrated by chromatin immunoprecipitation (ChIP). The consensus RORE sequence is a classical nuclear receptor half site flanked by a 5′ A/T-rich RORα-specific recognition sequence 5′-(A/G/T)(A/T)(**A/T**)(**A/T**)N(**A/T**)**AGGTCA**-3′, where bold letters indicate residues important for RORα binding (Giguere et al., 1994). We aligned the mG6Pase-RORE reported by Chopra et al. with the consensus RORE and found no similarity (data not shown). However, we hypothesized that RORα could bind to the reverse strand and indeed, alignment of the reverse complement sequence reported by Chopra with that of the consensus RORE resulted in a near complete match. However, at the important position underlined above, the mG6Pase-RORE contains a C (on the reverse strand) as opposed to consensus and critical A or T. We therefore expected that RORα binding would be weak, yet still functional as indicated by our previous mG6Pase promoter activity assays. To verify binding of RORα to mG6Pase-RORE, we performed EMSA using a radiolabeled double-stranded probe containing the mG6Pase-RORE sequence and *in vitro* transcribed and translated V5-tagged RORα (Fig. 2A, left panel). A specific complex was indeed formed when incubating mG6Pase-RORE probe with translated RORα (lane 3), which was not observed with addition of increasing molar excess unlabeled probe (lane 4–5) or when incubated with unprogrammed reticolucyte lysate (lane 2). As a positive control, we utilized the consensus RORE probe containing a C to A gain-of-function mutation as explained above (Fig. 2A, right panel). As expected, RORα bound to the positive control consensus RORE in a strong and specific manner (lanes 9–11). To verify the specificity of the RORα-RORE complex, we performed antibody-mediated supershift-EMSA. When Anti-V5 antibody was added to the binding reaction, a supershift was clearly observed for the consensus RORE (lane 12). This supershift was less pronounced for the mG6Pase-RORE, and however, addition of the antibody was accompanied by the disappearance of the lower band corresponding to RORα bound probe (lane 6 vs lane 3). This may imply that binding of the V5-antibody to RORα has somehow disrupted interaction between RORα and the probe. This diminishing disruption effect was also observed upon supershift of RORα with respect to the consensus RORE probe compared to the corresponding intensity of basal RORα binding to the same probe in the absence of the V5-antibody (lane 12, vs lane 9). Given the already weak binding of RORα to the mG6Pase-RORE, it is likely that binding of the V5-antibody to the RORα fusion protein has caused RORα to dissociate from the probe. Having confirmed the ability of RORα to bind to the mG6Pase-RORE, we investigated whether the affinity of RORα to the probes was altered in presence of PKA-Cα *in vitro* (Fig. 2B). We observed that binding of RORα both mG6Pase-RORE and consensus RORE was not significantly affected by PKA.

We next assessed the recruitment pattern of RNA Polymerase II (RNA Pol II) and SRC-2 to the G6Pase promoter in response to stimulation of the cAMP/PKA pathway for 48 h in HepG2 cells, using ChIP analysis (Fig. 3A). Recruitment of RNA Pol II to the G6Pase promoter was in accordance with the observed *G6Pase* mRNA levels presented in Fig. 1C for respective treatment conditions. Notably, transfection with the PKA-Cα significantly decreased enrichment of RNA Pol II at the G6Pase promoter to below that of the baseline control. In presence of PKA-Cα we also observed a significant reduction of SRC-2 protein at the proximal G6Pase promoter, whereas addition of forskolin or a FSK/IBMX/6MB-cAMP had no effect compared to basal recruitment. Taken together our data suggest that strong PKA activity impedes recruitment of SRC-2 and subsequently RNA Pol II to the proximal G6Pase promoter. We performed additional ChIP experiments in order to elucidate the temporal recruitment of RNA Pol II and SRC-2 in response to treatment of HepG2 cells with FSK/IMBX/6MB-cAMP for 45 min, 24 h and 48 h (Fig. 3B). The highest observed recruitment of RNA Pol II and SRC-2 occurred at 45 min after stimulation of the cAMP/PKA pathway, and significantly declined at 24 h and 48 h. This suggests that prolonged PKA activity diminishes the presence of SRC-2 and also RNA Pol II on the proximal G6Pase promoter.

3.3. Presence of SRC-2 is required for PGC-1α dependent transactivation of the G6Pase promoter

Previous studies have demonstrated that the nuclear receptor/coactivator complexes RORα/SRC-2 (Chopra et al., 2008) and

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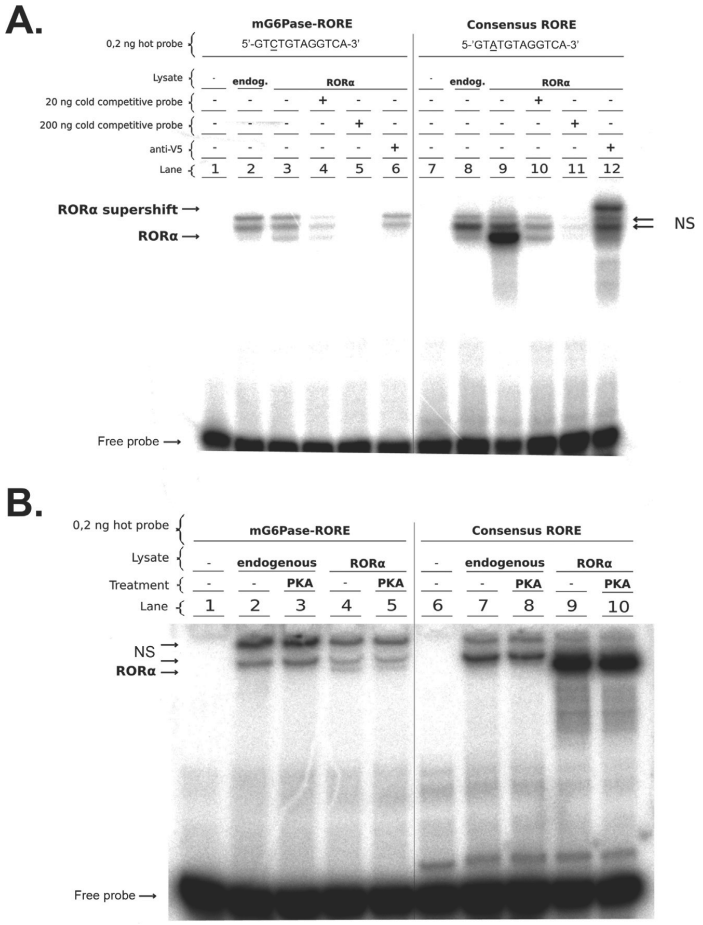

Fig. 2. Affinity of RORα to the mG6Pase-RORE and the consensus RORE is not altered by PKA. A. Binding of RORα to the mG6Pase-RORE probe shown in the left panel and consensus RORE positive control probe shown in the right panel. Probes were incubated alone (lanes 1 and 7), with endogenous, unprogrammed TNT reticulocyte lysates (lanes 2 and 8) or *in vitro* transcribed and translated V5-tagged RORα fusion protein (lanes 3–6 and 9–12). Progressive molar excess of unlabeled probes were added (lanes 4–5 and 10–11) to assess specificity of RORα binding. Antibody-mediated EMSA supershift of V5-RORα in presence of the mG6Pase-RORE (lane 6) or consensus RORE (lane 12) probes was achieved by addition of anti-V5 antibody. Apart from the upper supershift band, probes bound to RORα correspond to the lower visible band complexes on the gel, and NS denotes non-specific bands. B. Binding of RORα to the mG6Pase-RORE (lanes 1–5) and consensus RORE (lanes 6–10) probes in the presence and absence of purified catalytic PKA-Cα subunit was assessed by EMSA.

HNF4α/PGC-1α (Schilling et al., 2008; Boustead et al., 2003) are recruited to adjacent binding sites at the proximal G6Pase promoter. Shilling et al. (Schilling et al., 2008) demonstrated that the ability of PGC-1α to activate the G6Pase-luc reporter was abolished upon introduction of a mutation in this relevant RORE. Given the close proximity of these two NR-coactivator complexes, we hypothesized that RORα/SRC-2 may be important to facilitate transcriptional activity of HNF4α/PGC-1α at the G6Pase promoter.

The compound SR1001 is an inverse agonist ligand for RORα that has been shown to prevent interaction between RORα with its coactivators and trigger recruitment of corepressors. SR1001 thus effectively inhibits RORα-mediated transactivation, presumably without altering its ability to bind DNA (Solt et al., 2011). We therefore wanted to assess the implications of SR1001 on RORα-dependent transcription of G6Pase. When HepG2 cells were treated with increasing concentrations of SR1001, we observed a dose-dependent downregulation of *G6Pase* mRNA (Fig. 4A), as also observed by others (Solt et al., 2011). This emphasizes the important role of coactivation of RORα in the regulation of G6Pase expression. Even at the maximal dose, we observed no cytotoxic effect of SR1001. Next we validated that SR1001 inhibited SRC-2 mediated coactivation of RORα on the mG6Pase reporter (Fig. 4B). As expected, SR1001 at a concentration of 100 μM completely abolished the ability of SRC-2 to coactivate RORα. We also assessed whether the absence of endogenous SRC-2 would also affect the ability of overexpressed PGC-1α to transactivate the mG6Pase reporter (Fig. 4C). Intriguingly, we observed that the effect of PGC-1α was dramatically

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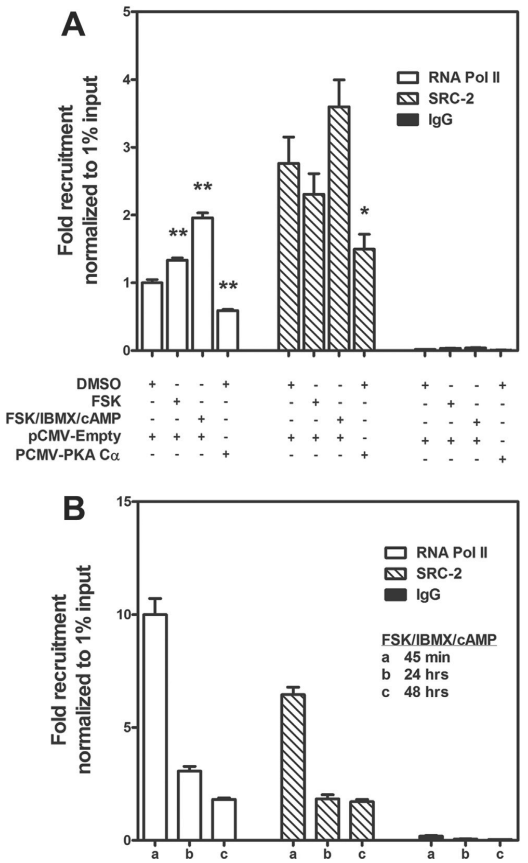

Fig. 3. [1 column] PKA-Cα reduces recruitment of SRC-2 and RNA polymerase to G6Pase promoter. A. Recruitment of endogenous RNA Pol II and SRC-2 to the genomic G6Pase promoter in HepG2 cells after 48 h treatment with forskolin (FSK) or a combination of 10 μM forskolin, 50 μM IBMX and 500 μM 6MB-cAMP (FSK/IBMX/cAMP), or overexpression of the PKA catalytic subunit (pCMV-PKA-Cα). Immunoprecipitated DNA was analyzed by qPCR using sequence specific primers to generate an amplicon flanking the proximal RORE of the genomic human G6Pase promoter. Data was normalized to respective 1% input samples and presented as mean fold recruitment relative to RNA Pol II control treatment (DMSO and Empty vector) ± SEM of technical triplicates. By convention, basal recruitment of RNA Pol II was defined as 1.0 and all other data were normalized to scale accordingly. B. Recruitment of RNA Pol II and SRC-2 at time points 45 min, 24 h and 48 h following treatment of HepG2 cells with 10 μM FSK, 50 μM IBMX and 500 μM 6MB-cAMP (FSK/IBMX/cAMP). Data was normalized to 1% input and presented as arbitrary fold recruitment relative to RNA Pol II ± SEM of technical triplicates. Negative control antibody (IgG) represents unspecific assay background. Statistical annotations $^{*}p < 0.05$ and $^{**}p < 0.01$.

reduced in the presence of 100 μM SR1001. These results indicate that G6Pase is transcriptionally repressed upon displacement of SRC-2 from RORα. Moreover, PGC-1α-dependent activation of the G6Pase promoter is reduced in the absence of SRC-2.

In order to elucidate the functional relationship between RORα and PGC-1α on the G6Pase promoter we introduced mutations in either the RORα binding site (ΔRORE) or the HNF4α binding site (ΔHNF4α) of the wild type (WT) proximal G6Pase 231/+66 promoter reporter. Luciferase assays in HepG2 cells demonstrated that the capability of PGC-1α to transactivate both ΔRORE and ΔHNF4α reporter constructs was abolished (Fig. 5A). As previously also shown by Schilling et al., this indicates that the proximal RORE is required for PGC-1α dependent activation of the reporter plasmid.

The observed synergistic effect of combining RORα and PGC-1α led us to investigate whether PGC-1α is a direct coactivator of RORα. Accordingly we overexpressed both these factors in HepG2 cells and measured promoter activity of the different mG6Pase variants. RORα clearly stimulated transactivation of the WT construct, and co-transfection with PGC-1α caused a synergistic increase in the promoter activity, suggesting that PGC-1α could potentially coactivate RORα directly (Fig. 5B). Introducing a mutation in the RORE (ΔRORE) abolished the effect of RORα, and cotransfection with PGC-1α produced only an additive effect, demonstrating the importance of the RORE in facilitating PGC-1α mediated transcription. Compared to the WT reporter, the ability of RORα alone to transactivate the ΔHNF4α reporter was not significantly impeded. However, upon introduction of a mutation in the HNF4α binding site, the contribution from PGC-1α in terms of reporter transactivation was statistically insignificant both in the presence and absence of RORα. Taken together, these results suggest that PGC-1α does not coactivate RORα directly, but rather that the presence of RORα is required to facilitate the interaction between PGC-1α and HNF4α on the proximal G6Pase promoter.

Next we used the wild type mG6Pase reporter constructs in conjunction with either wild type (WT) or dominant negative (DN, truncated and lacking DNA binding domain) RORα and simultaneous overexpression of empty vector or PGC-1α (Fig. 5C). As expected, the capability of DN RORα alone to activate the mG6Pase promoter was comparable to baseline reporter activity, and six times lower than that of WT RORα. Whereas WT RORα and PGC-1α combined to strongly induce promoter activity, the combination of DN RORα and PGC-1α exhibited near baseline level of transactivation. These results further demonstrate that presence of RORα at the G6Pase proximal RORE is required to facilitate the function of PGC-1α.

We previously demonstrated the ability of SRC-2 to coactivate RORα on the G6Pase promoter and sought to further investigate the effect of combining SRC-2 and PGC-1α in transactivation assays. In line with earlier observations, SRC-2 was convincingly able to coactivate RORα in presence of an intact RORE. Combined overexpression of SRC-2, PGC-1α and RORα resulted in an additive effect on transactivation of the WT G6Pase reporter that was severely diminished upon mutation of either the RORα (ΔRORE) or HNF4α (ΔHNF4α) binding sites (Fig. 5D). This suggests that presence of the RORα/SRC-2 facilitates the interaction between PGC-1α and HNF4α.

In order to test the hypothesis that RORα/SRC-2 facilitates the interaction between PGC-1α and HNF4α, we employed siRNA against RORα and/or SRC-2 and measured transactivation of the mG6Pase reporter with or without overexpression of PGC-1α (Fig. 6A). We observed that knock-down of RORα or SRC-2 alone and in combination dramatically decreased the effect of PGC-1α. This result strongly suggests that PCG-1α requires the presence of SRC-2 and RORα in order to stimulate G6Pase promoter activity. Based on the results presented in the current article, we suggest the following model for the cooperative transcriptional regulation of G6Pase by RORα/SRC-2 and HNF4α/PGC-1α (Fig. 6B).

4. Discussion

The conversion of glucose-6-phosphate into free glucose is catalyzed by G6Pase and marks the final and determining step in

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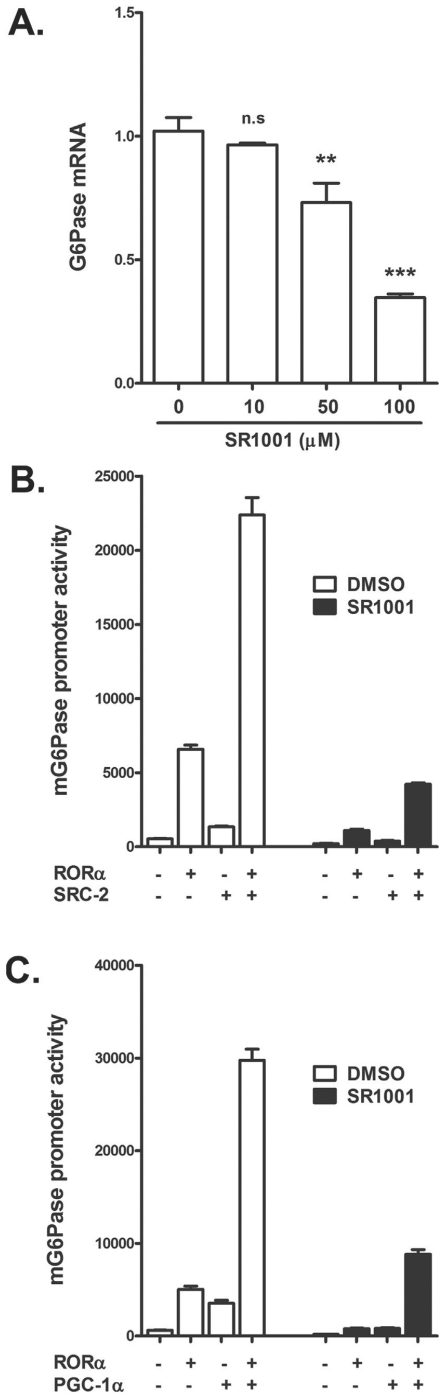

Fig. 4. The RORα ligand SR1001 inhibits transcription of G6Pase by disrupting SRC-2 and PGC-1α coactivation. A. Levels of HepG2 endogenous *G6Pase* mRNA in response to incubation for 24 h with increasing concentrations of the RORα inverse agonist SR1001 were assessed by RT-qPCR. Amount of vehicle solvent (DMSO) was kept constant for all treatments. Data represent mean G6Pase mRNA normalized to *RPL4* ± SEM of biological triplicates from one of three independent experiments. One-way ANOVA with Dunnett's multiple comparison post test was employed for statistical evaluation of this experiment. B. Promoter activity of the mG6Pase-luc reporter in the absence or presence of 100 μM SR1001 was determined by luciferase. Cells were transfected with the mG6Pase reporter in combination with RORα and SRC-2 or C. RORα and PGC-1α as indicated, 48 h prior to luminometric analysis of lysate. Data represent mean luciferase activity ± SEM of biological quadruplicates. $^{**}p < 0.01$, $^{***}p < 0.001$, n.s., not significant.

both glycogenolysis and gluconeogenesis. These two processes are physiologically related to fasting and align with the fact that G6Pase is regulated at the transcriptional level by fasting hormone signaling via the cAMP/PKA pathway. Previously, it has been shown that SRC-2 coactivates RORα at the G6Pase promoter (Chopra et al., 2008). In the current study, we demonstrate that RORα and its coactivator SRC-2 are required for PGC-1α-mediated transactivation of the G6Pase promoter. By causing protein degradation and reduced recruitment of SRC-2 to its target nuclear receptor RORα at the G6Pase promoter, PKA indirectly impedes the ability of the master gluconeogenic mediator PGC-1α to coactivate the adjacently positioned HNF4α.

It is widely established that PGC-1α, a key mediator of gluconeogenic gene expression, is induced in response to activation of the cAMP/PKA pathway. Treatment of cells with FSK or a combination of FSK/IBMX/6MB-cAMP induces moderate PKA activity and increases G6Pase expression, but not via RORα/SRC-2. Interestingly, overexpression of the PKA-Cα induced strong PKA activity and significantly reduced the ability of RORα/SRC-2 to transactivate the G6Pase promoter. These findings are partially attributable to the finding that PKA reduces both the protein level and recruitment of SRC-2 to RORα on the G6Pase proximal promoter. These results are in line with previously published data demonstrating that prolonged stimulation of the cAMP/PKA signaling pathway reduces SRC-2 coactivator activity (Fenne et al., 2013) and target promoter recruitment (Fenne et al., 2008) while stimulating SRC-2 ubiquitylation and proteasomal degradation (Hoang et al., 2013; Hoang et al., 2004).

It has been shown by Schilling et al. that introduction of a mutation shortly downstream of the HNF4α binding site at the proximal G6Pase promoter significantly prevented PGC-1α-mediated transactivation (Schilling et al., 2008). This site was later shown to be a binding site for RORα (Chopra et al., 2008). Here, we show that RORα binds this RORE located in the proximal G6Pase promoter and adjacent to an HNF4α binding site. Notably, this RORE differs from the consensus RORE in a single, but crucial residue in the RORα specification site. We found that the binding affinity of RORα to the mG6Pase-RORE was weaker than that of the consensus RORE, albeit still functional in both EMSA and transactivation assays. We also demonstrate that the coregulator-modulating RORα ligand SR1001 alone is capable of dose-dependently reducing *G6Pase* mRNA by displacing SRC-2 from RORα. This event in turn renders PGC-1α unable to activate the G6Pase promoter. These findings illustrate the importance of coactivators in the regulation of gluconeogenesis.

It is well established that PGC-1α increases expression of several metabolic enzymes including G6Pase in response to fasting and following activation of the cAMP/PKA signaling pathway. Using reporter constructs containing a mutation in either the RORα (ΔRORE) or the HNF4α (ΔHNF4α) binding sites in the proximal wild type (WT) promoter region of G6Pase, we demonstrate that the proximal RORE is necessary for PGC-1α dependent transcription of mG6Pase. Importantly, we observed that the ability of RORα to transactivate the ΔHNF4α reporter was unimpeded compared to

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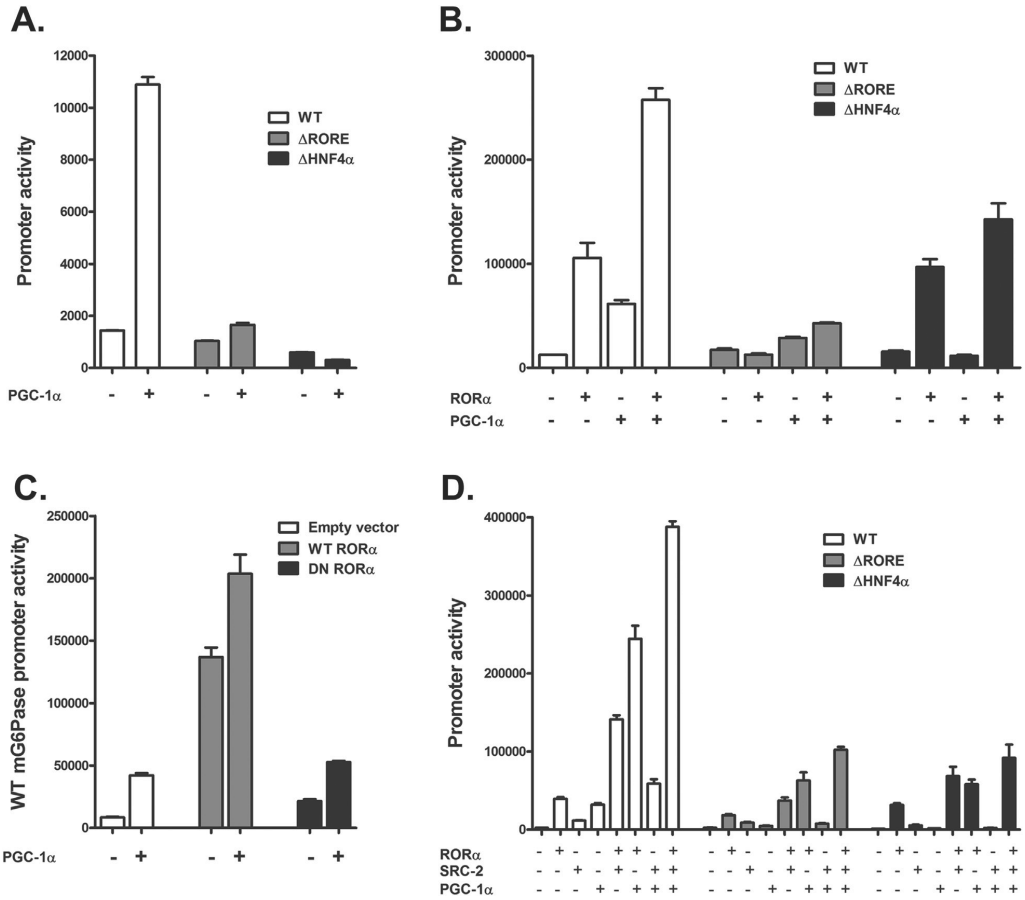

Fig. 5. RORα and SRC-2 are required for PGC-1α dependent transactivation of the G6Pase promoter. Reporters labeled ΔRORE and ΔHNF4α annotate mutations in the proximal RORα or HNF4α binding site, respectively, of the wild type (WT) mG6Pase promoter. The ability of PGC-1α to transactivate the indicated reporter constructs when overexpressed (A) alone or (B) with RORα, (C) with dominant negative (DN) or wild type (WT) RORα or (D) in combination with RORα and SRC-2 was measured by luciferase assays. Data represent mean luciferase activity units ± SEM of biological triplicates from one of three independent experiments.

the WT reporter whereas there was no significant increase in promoter activity upon addition of PGC-1α. This implies that PGC-1α coactivates HNF4α and not RORα, and that presence of adjacent RORα/SRC-2 is necessary for the full transcriptional effect of PGC-1α. By overexpressing a dominant negative RORα variant unable to bind DNA, we show that RORα is required for PGC-1α-mediated transactivation of the mG6Pase promoter. Our additional experiments employing the RORα ligand SR1001 or siRNA against RORα and/or SRC-2 verified these findings.

Overexpression of PKA-Cα caused proteasomal degradation of SRC-2 and reduced its recruitment to the G6Pase promoter, whereas binding of RORα to the mG6Pase-RORE remained unchanged. SRC-2 is subject to negative regulation by the cAMP/PKA signaling pathway. Prolonged and sustained PKA activity promotes ubiquitylation and proteasomal degradation of SRC-2 (Hoang et al., 2004; Fenne et al., 2008). By ChIP, we observed that the presence of SRC-2 at the G6Pase promoter decreased dramatically in response to both strong and prolonged PKA activity. It is plausible that the concomitantly decreased recruitment of RNA Pol II to the G6Pase promoter is a causal consequence of the elimination of SRC-2. Fasting is characterized by a substantial increase of circulating levels of hormones that stimulate the cAMP/PKA signaling pathway (Unger et al., 1963). During long-term fasting, hepatic gluconeogenesis is ultimately downregulated despite high amounts of circulating glucagon that stabilize at the postabsorptive level which is also characterized by minimal levels of insulin (Marliss et al., 1970). Accordingly, prolonged and pronounced PKA activity during long-term fasting may cause degradation of SRC-2 and, in turn, prevent PGC-1α dependent coactivation of HNF4α and thus cause downregulation of G6Pase and gluconeogenesis.

In conclusion, we have found that RORα/SRC-2 dependent transcriptional activation of G6Pase is inhibited in response to strong PKA activity. This is the result of a targeted reduction in the protein level and recruitment of SRC-2 to RORα at the proximal G6Pase promoter. Moreover, the presence of RORα and SRC-2 at the proximal promoter is required to facilitate the stimulatory effect of PGC-1α on G6Pase expression.

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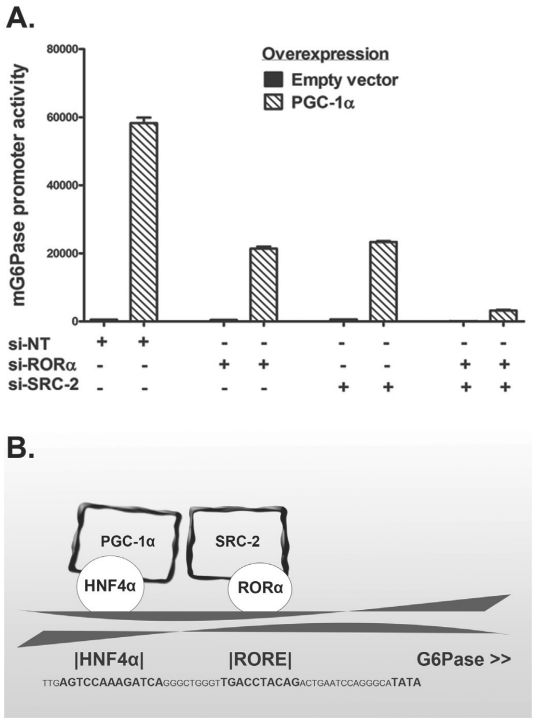

Fig. 6. Knock-down of RORα and/or SRC-2 reduces PGC-1α dependent transactivation of G6Pase reporter. A. The effect siRNA-mediated knock-down of RORα and/or SRC-2 on mG6Pase promoter transactivation. HepG2 cells were cotransfected with empty vector (black bars) or PGC-1α (striped bars) and luciferase activity was measured 72 h after transfection of siRNA at 100 nM final concentration. Data presents the mean arbitrary luciferase activity units ± SEM of biological duplicates in one of three independent experiments. B. We have demonstrated that the presence of nuclear receptor RORα and its coactivator SRC-2 at the proximal G6Pase promoter RORα response element (RORE) is required for effective transcription of G6Pase. Upon treatment of cells with the inhibitory RORα ligand SR1001 or mutation of the RORE sequence we observed that the master gluconeogenic regulator PGC-1α was unable to stimulate promoter transactivation via the adjacently positioned partner nuclear receptor HNF4α. Additionally, we have demonstrated that presence of SRC-2 is required for optimal transactivational effect of PGC-1α and that the cAMP-dependent protein kinase ultimately causes degradation and reduced recruitment of SRC-2 to the G6Pase promoter. During fasting, glucagon signaling via the cAMP/PKA pathway stimulates expression of PGC-1α and, in turn, its gluconeogenic target genes including G6Pase. We propose that RORα-bound SRC-2 facilitates the activity of PGC-1α at the G6Pase promoter and that PKA-mediated degradation of SRC-2 may represent a mechanism by which hepatocytes transition out of gluconeogenesis during long-term starvation.

Disclosure statement

The authors have nothing to disclose.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.mce.2015.10.003.

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Metformin inhibits hepatocellular glucose, lipid and cholesterol biosynthetic pathways by transcriptionally suppressing steroid receptor coactivator 2 (SRC-2)

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The ability of the anti-diabetic drug metformin to inhibit anabolic processes including gluconeogenesis and lipogenesis is partly attributable to activation of the AMP-activated protein kinase (AMPK) pathway. The p160 steroid receptor coactivator 2 (SRC-2) is a key regulator of cellular metabolism and drives expression of the gluconeogenic enzyme glucose-6-phosphatase (G6Pc). Here, we uncovered a role for SRC-2 in the metabolic reprogramming imposed by metformin. In FaO cells, metformin dose-dependently reduced mRNA expression of SRC-2. Microarray analysis of metformin-treated cells revealed an overrepresentation of downregulated genes involved in biosynthesis of lipids and cholesterol. Several metformin-regulated genes including fatty acid synthase (FASN) were validated as transcriptional targets of SRC-2 with promoters characterized by sterol regulatory element (SRE) binding protein (SREBP) recognition sequences. Transactivation assays of the FASN promoter confirmed that SRC-2 is a coactivator of SREBP-1. By suppressing SRC-2 at the transcriptional level, metformin impeded recruitment of SRC-2 and RNA polymerase II to the G6Pc promoter and to SREs of mutual SRC-2/SREBP-1 target gene promoters. Hepatocellular fat accretion was reduced by metformin or knock-down of both SRC-2 and SREBP-1. Accordingly we propose that metformin inhibits glucose and lipid biosynthesis partly by downregulating SRC-2 gene expression.

The p160 steroid receptor coactivator (SRC) family consists of the three distinct members SRC-1/NCOA1, SRC-2/NCOA2/GRIP1/TIF2 and SRC-3/NCOA3/AIB1 that aid in the function of nuclear hormone receptors and transcriptional regulation of target genes. The SRCs thus regulate metabolism and a variety of cellular processes by facilitating transcription of hormonally regulated target genes[1–3]. SRC-2 regulates physiology and metabolism in a tissue-specific manner and is accordingly subject to regulation by several cell signaling pathways and even circadian events[4–7]. Previous studies have demonstrated that SRC-2 contributes to obesity and insulin resistance[8], fat accretion[4,7], hepatic gluconeogenesis[9,10] and biosynthesis of lipids[4,9,10] and cholesterol[6,9,10] at the transcriptional level. It has previously been shown that SRC-2 liver knock-out mice exhibit fasting hypoglycemia due to reduced hepatic expression of the rate-limiting gluconeogenic enzyme glucose-6-phosphatase (*G6pc*)[10]. This observation was coupled with

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the finding that SRC-2 acts as a coactivator of the orphan nuclear receptor RAR-related orphan receptor alpha (RORα) on the proximal G6pc promoter[10]. Microarray profiling of liver from SRC-2 knock-out mice revealed reduced expression of *Srebp1* and several key metabolic enzymes involved in gluconeogenesis (*G6pc*), lipogenesis (*Fasn*, *Elovl6*) and cholesterol synthesis (*Hmgcr*, *Hmgcs1*, *Nsdhl*, *Cyp51*)[11]. Notably, the sterol regulatory element binding protein 1 (SREBP-1) represents a master regulator of biosynthesis of lipids and cholesterol[12]. It was recently demonstrated that SRC-2 is activated in response to phosphorylation by the nutrient-sensor mammalian target of rapamycin complex 1 (mTORC1) and this event enables SRC-2 coactivation of SREBP-1 to promote lipogenesis and survival of particularly lipid-reliant prostate cancer cell models[4].

Metformin, a synthetic biguanide drug, remains the preferred pharmacologic treatment for type 2 diabetes[13,14]. The ability of metformin to suppress hepatic gluconeogenesis and other anabolic pathways including lipid and cholesterol biosynthesis is partly attributed to transient inhibition of the mitochondrial respiratory chain complex I and indirect activation of the energy-sensing AMP-activated protein kinase (AMPK) pathway[15–17]. Administration of metformin is associated with activation of AMPK and transcriptional repression of hepatic gluconeogenic enzymes[18]. Furthermore, AMPK inhibits lipogenesis by reducing activity of acetyl-CoA carboxylase[14] and downregulating SREBP-1 and its target genes including fatty acid synthase (FASN)[19]. Several studies also support a role for metformin in the treatment of non-alcoholic fatty liver disease (NAFLD)[20–23]. The pleiotropic AMPK pathway regulates cellular energy balance by modulating metabolism and this is partly achieved by inhibition of mTORC1 activity[16,24]. Interestingly, metformin-induced inhibition of both mTORC1 activity and gluconeogenic gene expression is retained in the absence of the catalytic AMPK subunits[25,26]. Thus, unlike the AMP analogue 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR), the precise mechanisms by which metformin exerts its effects on hepatocyte metabolism remain elusive and several reports suggest that the central therapeutic properties of metformin in fact are mediated independently of the AMPK pathway[25,27–29].

Liver knock-out SRC-2 mice are characterized by reduced expression of rate-limiting enzymes pertaining to gluconeogenesis and biosynthesis of lipids and cholesterol[4,6,9]. This study aimed to determine whether the seemingly overlapping metabolic alterations induced by metformin may involve a downregulation of SRC-2.

Results

Metformin inhibits expression of SRC-2. The established effects of metformin on hepatocellular metabolism led us to investigate whether metformin affects the expression of members of the steroid receptor coactivator family. The effects of increasing doses of metformin on relative expression levels of the steroid receptor coactivators SRC-1, SRC-2 and SRC-3 were assessed in the rat FaO hepatoma cell line (Fig. 1a). Whereas mRNA levels of SRC-1 and SRC-3 remained essentially unchanged in response to treatment with metformin, we observed a dose-dependent downregulation of SRC-2 mRNA. This range of metformin concentration (0–5 mM) was not cytotoxic to the cells and did not significantly affect mRNA levels of the house-keeping gene *Rpl4*. Expression levels of the gluconeogenic enzyme *G6pc* was included as positive control of the pharmacological effect of metformin. Since metformin significantly reduced SRC-2 mRNA we next investigated whether this downregulation also would affect endogenous SRC-2 protein level (Fig. 1b). As confirmed by densitometry of Western blots from three independent experiments, metformin concentrations at or above 1 mM caused a significant reduction of SRC-2 protein relative to beta-Actin (ACTB) in FaO cells. The above findings were replicated in HepG2 cells (data not shown). HepG2 cells were used for transactivation assays due to more effective plasmid transfection. Transactivation of a luciferase reporter construct under transcriptional control of the endogenous SRC-2 promoter (containing bases −1500 to +1 relative to the SRC-2 transcription start site) was used to detect whether metformin downregulates SRC-2 at the transcriptional level. This reporter was dose-dependently inhibited by metformin relative to the control pGL-basic reporter (Fig. 1c). Taken together, these results demonstrate that treatment of cells with metformin decreases SRC-2 mRNA and protein levels.

Metformin inhibits transcription of SRC-2 target genes involved in biosynthesis of lipids and cholesterol. In order to elucidate how metformin affects the hepatocellular transcriptome we performed a significance analysis of microarray based on FaO cells treated with vehicle or 5 mM metformin and categorized the genes into respective biological processes using the PANTHER database (Fig. 2). As expected, mainly genes related to metabolic processes were differentially regulated by metformin and among the upregulated genes there was a significant overrepresentation of factors involved in fatty acid β-oxidation ($p < 0.01$). As for genes that were subject to transcriptional downregulation by metformin, there was a significant statistical overrepresentation of factors involved in lipid metabolic processes ($p < 0.05$). Notably, several of the genes included in this category have previously been identified as transcriptional target genes of SRC-2 (Table 1). We next performed a separate qPCR validation of the microarray and overlapping SRC-2 target genes in which FaO hepatoma cells were incubated with increasing concentrations of metformin. Combining the annotated genes from Table 1 with other known SRC-2 target genes[11], we observed that metformin dose-dependently downregulated mRNA levels of SRC-2 target genes involved in gluconeogenesis (*G6pc*), biosynthesis of lipids (*Fasn, Elovl6)* and cholesterol (*Hmgcr, Hmgcs1, Sqle, Nsdhl*, *Cyp51* and *Egr1*) (Fig. 3). In line withour microarray data, we found that the insulin receptor (*Insr*) was upregulated by metformin, and *Igfbp*, a known metformin-inducible

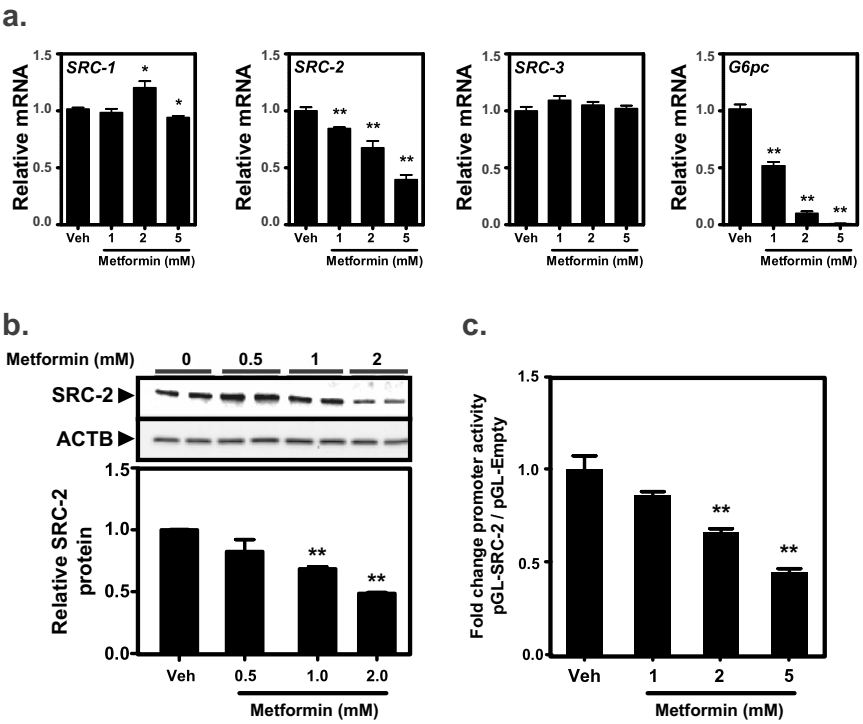

Figure 1. Metformin inhibits expression of SRC-2. (**a**) The effect of vehicle (Veh, water) or metformin treatment on the endogenous mRNA expression levels of steroid receptor coactivators SRC-1, SRC-2 and SRC-3 in FaO hepatoma cells was determined by RT-qPCR. Metformin is known to downregulate *G6pc* expression and this gene was included as a positive control. Target gene expression was normalized to reference gene *Rpl4* and represented as mean fold change relative to vehicle treatment ± SEM of biological triplicates. (**b**) FaO cells were treated as indicated and biological duplicates of 30 μg lysate were analyzed by Western blotting using antibodies against SRC-2 and ACTB load control. Volumetric densitometry band analysis of SRC-2 protein was normalized to ACTB. Data present mean relative SRC-2 protein amount ± SEM of biological duplicates in three independent experiments. **(c)** HepG2 cells were transfected with luciferase reporter constructs under transcriptional control of the bases −1500/+1 of the human SRC-2 promoter (pGL-SRC-2) or no promoter (pGL-empty). Reporter transactivation was measured by luminescence and is presented as the mean ratio between the two constructs, relative to vehicle treatment ± SEM of biological triplicates. *$p < 0.05$; **$p < 0.01$.

gene[30,31], was included as a positive control. In order to verify that these genes indeed represent SRC-2 target genes we performed transient siRNA knock-down of SRC-2 in FaO cells. Endogenous gene expression levels of all genes were measured by RT-qPCR 72 hours after siRNA transfection (Fig. 4a). We observed that knock-down of SRC-2 reduced *G6pc* expression and also significantly lowered mRNA levels of the included genes relating to lipid and cholesterol biosynthesis, with the only exception of *Egr1*. In the microarray, the *Srebp1* gene was omitted due to the unsatisfactory FDR associated with this gene. Notably, *Srebp1* but not *Srebp2* has been previously identified as a SRC-2 target gene[11] and we were only able to detect a moderate decrease of *Srebp1* mRNA in response to knock-down of SRC-2. To validate that the observed changes in gene expression were indeed caused by the absence of SRC-2 protein we performed Western blot experiments to verify that the knock-down procedure had markedly reduced the SRC-2 protein level (Fig. 4b). Taken together, these results indicate that metformin causes transcriptional repression of key metabolic genes, of which several represent SRC-2 target genes.

Metformin inhibits recruitment of SRC-2 to target gene promoters. Gene expression is preceded by binding of transcriptional activators and associated coactivators that ultimately recruit RNA polymerase II (RNAP) to target gene promoters. Bioinformatic analyses using Genomatix, UCSC and ENCODE databases revealed that sterol regulatory elements (SREs) were a common transcription factor

22957	70↑	77↓	PANTHER biological category	p-value (E-03) FC ≥ 1.5	FC ≤ -1.5
6	14	5	**Cellular component organization**	8	-
4	14	4	Cellular component morphogenesis	1	-
14	21	10	**Developmental process**	46	-
4	14	4	Anatomical structure morphogenesis	1	-
40	47	56	**Metabolic process**	-	4
1	3	6	Phosphate metabolic process	-	3
39	46	53	Primary metabolic process	-	7
17	26	25	Nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	49	-
10	16	10	Transcription	-	-
9	16	10	Transcription from RNA polymerase II promoter	-	-
7	14	6	Regulation of transcription from RNA polymerase II promoter	33	-
17	20	22	Protein metabolic process	-	-
4	1	10	Translation	-	19
0	1	3	tRNA aminoacylation for protein translation	-	10
0	3	0	Protein complex assembly	45	-
6	14	10	Protein modification process	10	-
3	9	5	Protein amino acid phosphorylation	23	-
5	10	10	Lipid metabolic process	-	34
1	3	3	Fatty acid metabolic process	-	-
0	3	0	Fatty acid beta-oxidation	9	-
29	37	29	**Cellular process**		-
8	14	9	Cell cycle	48	-
3	9	1	Mitosis	12	-
21	23	22	Cell communication	-	-
20	21	18	Signal transduction	-	-
10	7	9	Cell surface receptor linked signal transduction	-	-
6	0	5	G-protein coupled receptor protein signaling pathway	16	-
12	14	18	**Transport**	-	-
1	3	4	Lipid transport	-	48
0	1	5	Phosphate transport	-	1
3	1	9	Ion transport	-	16
3	1	8	Cation transport	-	23

Figure 2. Metformin-induced changes in the transcriptome. Based on the significance analysis of microarrays, genes that were differentially regulated by metformin were ordered into PANTHER biological categories. Only genes exhibiting a fold change (FC) equal to or greater than 1.5 (FC $\geq$ 1.5; upregulated) or equal to or less than -1.5 (FC ≤ -1.5; downregulated) and FDR $\leq$ 0.2 were included. Red and blue cells annotate significant overrepresentation of upregulated and downregulated gene categories, respectively.

Gene	Description	FC	Biosynthetic pathway
*Elovl6**	Elongation of very long chain fatty acids protein 6	−4.9	Lipid biosynthesis
*Fasn**	Fatty acid synthase	−2.4	Lipid biosynthesis
*Hmgcr**	3-hydroxy-3-methylglutaryl-CoA reductase	−1.7	Cholesterol biosynthesis
*Sqle**	Squalene monooxygenase	−2.8	Cholesterol biosynthesis
Pigm	Phosphatidylinositol glycan anchor biosynthesis class M	−4.6	Glycosylphosphatidyl-inositol biosynthesis
Hsd11b2	Corticosteroid 11-beta-dehydrogenase isozyme 2	−2.7	Cortisol metabolism
Star	Steroidogenic acute regulatory protein	−4.6	Steroid hormone biosynthesis
Gpd1	Glycerol-3-phosphate dehydrogenase	−2.1	Glycerol metabolism
Srd5a1	3-oxo-5-alpha-steroid 4-dehydrogenase 1	−2.4	Steroid hormone biosynthesis
Fabp2	Fatty acid-binding protein	−4.5	Fatty acid transport

Table 1. Transcriptional downregulation of lipid metabolic process genes by metformin. Gene entry comprising the statistically overrepresented microarray category "lipid metabolic process" ($p < 0.05$). Genes marked with *asterisks indicate previously established SRC-2 target genes[11]. Downregulation of the indicated genes in FaO hepatoma cells after treatment with metformin is annotated by the negative fold change (FC).

binding motif at the proximal promoters of the above SRC-2 target genes, including *Srebp1* itself, but with the exception of *G6pc*. On the proximal *G6pc* promoter, however, it has previously been shown that SRC-2 is a coactivator of the nuclear receptor RORα[10]. In order to determine whether metformin reduces recruitment of SRC-2 to target gene promoters we performed chromatin immunoprecipitation (ChIP) with respect to SRC-2 and RNAP protein and designed qPCR primers to amplify regions of proximal SREs of respective target gene promoters. For the *G6pc* promoter, primers were designed to amplify the relevant proximal RORα response element (RORE). Due to the inability of any tested

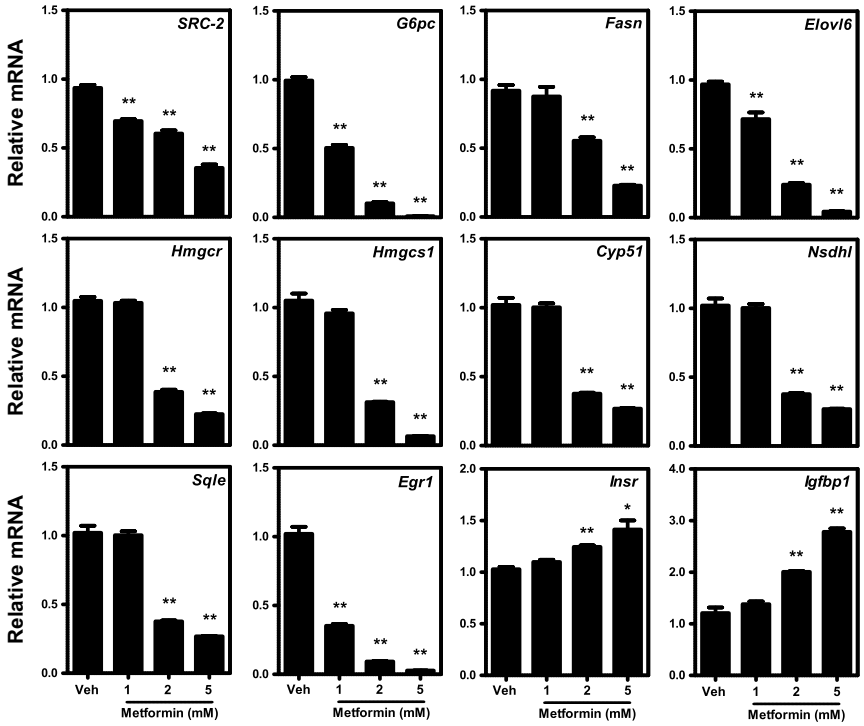

Figure 3. Downregulation of SRC-2 and its target genes by metformin. FaO cells were treated for 24 hours with increasing concentrations of metformin prior to RT-qPCR analysis of endogenous mRNA expression levels of the indicated genes. Expression levels of *Insr* and *Igfbp1* were included as positive controls of metformin-upregulated genes. Gene expression was normalized to the reference gene *Rpl4* and presented as mean fold change relative to vehicle treatment ± SEM of biological triplicates. One representative out of three independent experiments is shown. Where indicated, $^{**}p < 0.01$.

ChIP-grade antibody to immunoprecipitate the rat SRC-2 antigen, HepG2 cells were used for this assay. HepG2 cells were incubated with vehicle (water) or metformin (5 mM) for 24 hours and ChIP end-point qPCR demonstrated that recruitment of both RNAP and SRC-2 to target gene promoters (*G6PC, FASN, ELOVL6, HMGCR, HMGCS1, CYP51, NSDHL, SQLE* and *SREBP1*) was significantly reduced upon treatment with metformin (Fig. 5). These results are in agreement with the above findings, that both metformin and transient knock-down of SRC-2 reduce expression of the corresponding genes. Notably, the levels of unspecific recruitment observed with negative control IgG antibody appeared at very late qPCR cycles for all promoters. Thus, the insignificantly small background noise did not influence interpretation of results in terms of absolute quantification or by normalization according to the [Ct (Sample)] – [Ct (IgG)] subtraction method.

SRC-2 coactivates SREBP-1 at the FASN promoter. In light of the recent discovery that SRC-2 acts as a coactivator of SREBP-1 in prostate cancer cells[32] we wanted to examine whether this also occurred in a hepatocellular system. Additionally, we investigated whether SRC-2 could coactivate the related SREBP-2. A luciferase reporter construct under transcriptional control of the proximal FASN promoter, containing bases −220/+25 relative to transcription start site, was overexpressed in conjunction with full-length SREBPs and SRC-2 in HepG2 cells. The combined overexpression of SREBP-1 and SRC-2 synergistically stimulated reporter transactivation, suggesting that SRC-2 indeed coactivates SREBP-1 at the FASN promoter (Fig. 6a). In contrast, this effect was not observed with overexpression of SRC-2 and SREBP-2, suggesting that SRC-2 does not act as a coactivator for SREBP-2 in this context (Fig. 6b). It is plausible that SRC-2 coactivation of SREBP-1 is also relevant with respect to other SREBP-1 target gene promoters.

Knock-down of SRC-2 and Srebp1, and treatment with metformin reduces hepatocellular fat accumulation. In order to clarify the physiologic relevance of our above results, we next investigated

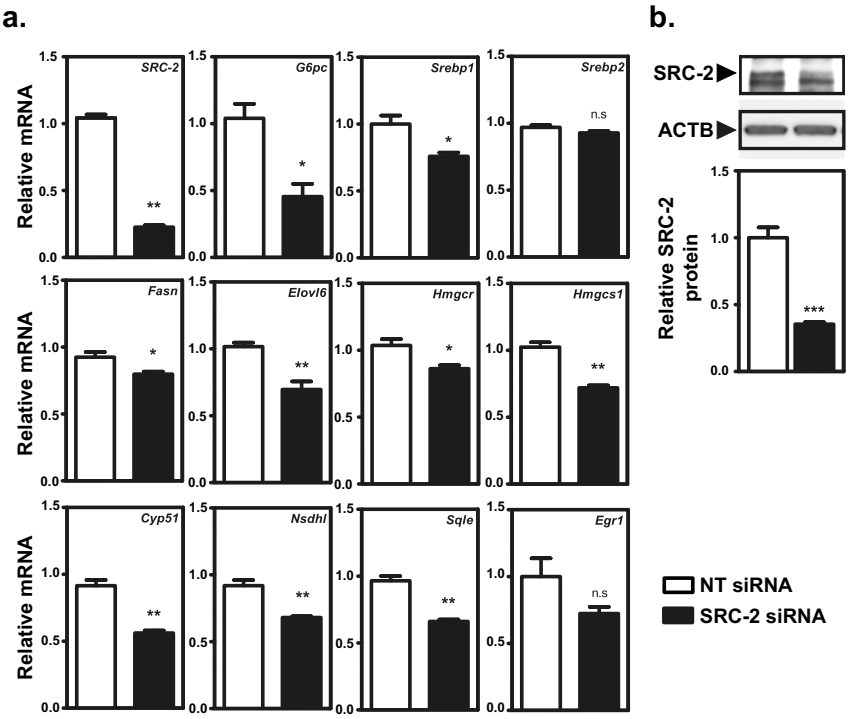

Figure 4. Knock-down of SRC-2 reduces target gene expression. (**a**) Quantification of endogenous mRNA levels of SRC-2 and its target genes in FaO cells were measured by RT-qPCR, 72 hours after transfection with non-targeting (white bars) or SRC-2 (black bars) siRNA. Gene expression was normalized to reference gene *Rpl4* and presented as mean fold change relative to transfection with NT siRNA ± SEM of biological triplicates of a representative experiment. **(b)** Protein levels of SRC-2 and load control ACTB were assessed by Western blot 72 hours after transfection of FaO cells with SRC-2 or NT siRNA. Quantification of protein bands was determined by densitometry. Data is presented as the relative SRC-2 protein amount ± SEM of nine biological replicates, representative of two independent experiments. $*p < 0.05$; $**p < 0.01$, $***p < 0.001$; n.s not significant.

whether the absence of SREBP-1 and its coactivator SRC-2 would affect actual lipogenesis and cellular lipid content. Therefore we knocked down *Srebp1* and SRC-2 in FaO hepatoma cells and starved the cells in serum-free medium for 48 hours prior to re-introduction of supplemented medium for 24 additional hours to facilitate lipogenic fat accretion. End-point Oil Red O staining demonstrated that cellular lipid content was reduced when *Srebp1* and SRC-2 were knocked down simultaneously (Fig. 7a). Optical density quantitative measurements of the total cellular Oil Red O content confirmed that lipid levels were significantly reduced when both *Srebp1* and SRC-2 were knocked down (Fig. 7b). We also starved FaO cells in serum-free medium for 48 hours prior to reintroduction of supplemented medium containing vehicle (water) or 5 mM metformin for 24 additional hours. Subsequent Oil Red O staining (Fig. 7c) and optical density quantification (Fig. 7d) demonstrated that cellular lipid contents were significantly reduced upon treatment with metformin compared to the vehicle control. These results demonstrate that Srebp1 and SRC-2 contribute to stimulate hepatocellular lipogenesis, whereas metformin has an inhibitory effect on this same process.

Discussion

Previous studies have demonstrated that hepatic SRC-2 transcriptionally promotes energy-demanding pathways including gluconeogenesis and biosynthesis of fatty acids and cholesterol. Here, we demonstrate that the pharmacological effects of metformin extend to inhibit expression of SRC-2 and, in turn, SRC-2 target genes including rate-limiting enzymes pertaining to gluconeogenesis (*G6pc*) and biosynthesis of lipids (*Fasn*) and cholesterol (*Hmgcr* and *Hmgcs1*). Previously it was shown that SRC-2 can act as a coactivator SREBP-1 to promote lipogenesis and survival of prostate cancer cells[4]. Metformin and

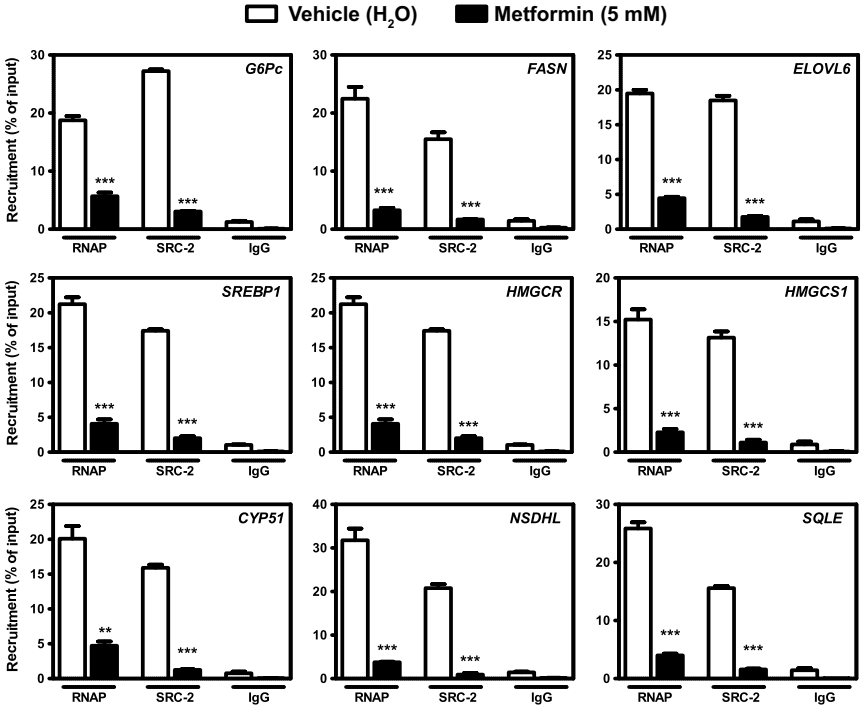

Figure 5. Recruitment of SRC-2 and RNA polymerase to target gene promoters. Chromatin immunoprecipitation of RNA polymerase II (RNAP) and SRC-2 in HepG2 cells treated with vehicle (white bars) or 5 mM metformin (black bars). IgG represents assay background noise. Eluted sample DNA was subjected to qPCR amplification of regions overlapping known SREBP-1 binding sites at the proximal promoters of indicated target genes. For G6Pc, primers were designed to amplify the genomic promoter region flanking the proximal RORE. Signal quantification was normalized to that of respective treatment 1% input samples and data is presented as mean recruitment (% of input) ± SEM of biological triplicates in one representative out of three independent experiments. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

the AMPK pathway are known to inhibit activity of both mTORC1 and SREBP-1[16,19]. However, several pharmacological properties of metformin appear to be mediated independently of the AMPK pathway. Interestingly, the ability of metformin to inhibit both gluconeogenic gene expression and mTORC1 activity is retained in absence of the AMPK enzyme[25,26].

Our initial findings show that metformin, in a concentration-dependent manner, causes transcriptional downregulation and thus reduction of the available protein level of SRC-2, and this effect was not observed with SRC-1 or SRC-3. Microarray analysis of FaO hepatoma cells treated with or without metformin revealed significant upregulation of genes involved in fatty acid β-oxidation. Conversely, there was a significant overrepresentation of downregulated genes associated with lipid biosynthesis, of which several were identified as SRC-2 target genes. Combining the latter entry with other metabolically relevant and previously established SRC-2 hepatic target genes identified by Jeong *et al.*[11], we show that metformin in a dose-dependent manner inhibits transcription of this entire panel of SRC-2 target genes. The observed difference in dose responses for these genes is likely due to metformin differentially modulating the intrinsic activity of a large array of other transcription factors and coregulators on the various promoters. In line with the microarray results and the insulin-sensitizing property of metformin, we also show that metformin stimulates expression of the insulin receptor (*Insr*). Furthermore, the stimulating effect of metformin on expression of the insulin-like growth factor binding protein 1 (*Igfbp1*) has been described previously[30,31]. We also demonstrate that transient siRNA knock-down of SRC-2 is sufficient to significantly reduce mRNA expression of indicated SRC-2 target genes, with the exception of Early growth response 1 (*Egr1*). Notably, *Egr1* has been reported to be a transcriptional target of SRC-2[11,33] and is implicated in promotion of obesity and insulin resistance[34], progression of prostate cancer[35] and transcriptional regulation of lipid and cholesterol biosynthetic genes[36]. Interestingly, *Egr1* was markedly downregulated when cells were treated with metformin, and this could potentially be related with

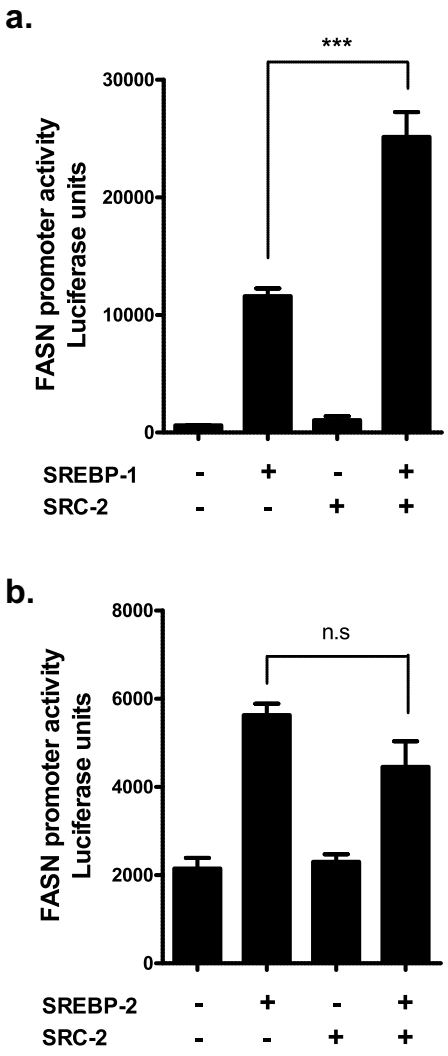

Figure 6. SRC-2 is a coactivator of SREBP-1. Transactivation of a luciferase reporter construct under transcriptional control of the endogenous −220/+25 FASN promoter in response to combinatorial overexpression of SRC-2 and (**a**) SREBP-1 or (**b**) SREBP-2. Data are presented as mean arbitrary luciferase units ± SEM of biological quadruplicates. The presence or absence of coactivation synergy is illustrated. ***$p \leq 0.001$; n.s not significant.

the recent discovery that metformin administration after prostate cancer diagnosis is associated with decreased mortality[37].

It has previously been shown that SRC-2 coactivates SREBP-1 and we therefore wanted to investigate whether the reduced SRC-2 expression and protein level in response to metformin treatment also would affect recruitment of SRC-2 to mutual target gene promoters. Sequential recruitment of the general transcription machinery and ultimately RNA polymerase II is a core property of steroid receptor coactivators, including SRC-2[6]. Using ChIP analysis we demonstrate that metformin significantly reduces recruitment of both endogenous SRC-2 and RNA polymerase II to sterol regulatory elements (SREs) located proximally at promoters of the entire panel of SRC-2 target genes. Notably, there is no SRE at the human *G6Pc* promoter. However, SRC-2 is a known coactivator of the nuclear receptor RORα on the *G6Pc* promoter[10] and we observed that recruitment of SRC-2 to the relevant proximal RORα response element (RORE)

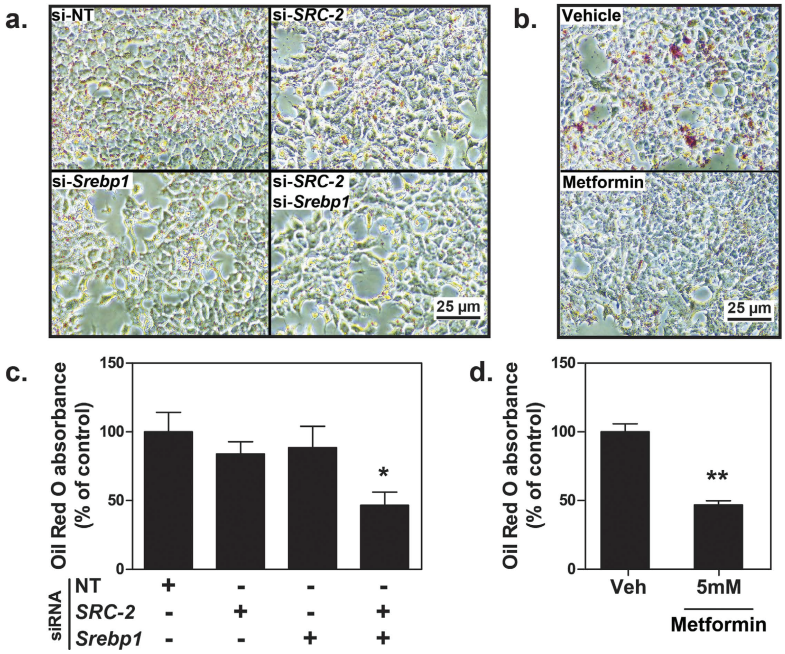

Figure 7. Inhibition of hepatocellular fat accretion. (**a**) Oil Red O staining of FaO hepatoma cells at 40x magnification after combinatorial knock-down with non-targeting (NT), SRC-2 and *Srebp1* siRNA. Cells were starved in serum-free medium for 48 hours prior to introduction of medium supplemented with 10% FBS and 2 mM glutamine for 24 additional hours. Scale bar: 25 µm. (**b**) Oil Red O staining of FaO cells at 40x magnification following starvation for 48 hours prior to introduction of medium supplemented with 10% FBS, 2 mM glutamine and vehicle (Veh, water) or increasing doses of metformin for 24 additional hours. Scale bar: 25 µm. (**c**) Quantitative analysis of Oil Red O content of cells from experiment (**a**) was done by measuring optical density of eluate at 490 nm. By convention, total Oil Red O content from cells transfected with NT siRNA was defined as 100% and all other samples were normalized accordingly. (**d**) Quantitative analysis of Oil Red O content of cells from the experiment (**b**). Data are presented as percent mean absorbance relative to cells treated with vehicle ± SEM of biological triplicates from one representative out of three or more independent experiments. $^{*}p < 0.05$; $^{**}p < 0.01$.

was significantly reduced when cells were treated with metformin. It is well established that transcriptional control of coactivators is essential to the regulation of hepatic gluconeogenesis[38]. For example, the inhibitory effect of metformin and AMPK on the CREB regulated transcription factor 2 (CRTC2/TORC2) impedes expression of the master gluconeogenic coactivator PGC-1α[39]. Our observation that metformin inhibits SRC-2 expression is thus reconcilable with the established effect of metformin.

Previous studies have shown that transcription of SREBP-1 is positively regulated by insulin, and that metformin inhibits expression of SREBP-1 *in vivo*[40]. By downregulating both SREBP-1 expression and activity, metformin effectively inhibits lipogenesis and accumulation of hepatic lipids[20,40,41]. Here, we show in a hepatocellular system that SRC-2 is able to coactivate SREBP-1, but not SREBP-2, and that also SRC-2 is subject to transcriptional suppression by metformin. Our findings complement the established inhibitory effect of metformin on SREBP-1 and provide a new layer of complexity as to how metformin suppresses the transcriptional activity of SREBP-1 and lipogenesis. In particular, a key feature of SREBP-1 is to mediate expression of FASN which is the rate-limiting enzyme in *de novo* lipogenesis. Whereas knock-down of *Srebp1* alone in our system was insufficient to functionally lower hepatocellular fat accretion, we observed a marked decline in intracellular fat content upon simultaneous knock-down of *Srebp1* and SRC-2. This implies that SRC-2 markedly contributes to hepatocellular lipogenesis by coactivating SREBP-1. Metformin alone significantly reduced hepatocellular lipogenic fat accumulation, and this may partly be due to transcriptional suppression of SRC-2.

In conclusion, we provide evidence that treatment of cells with metformin is accompanied by reduced expression of SRC-2, which in turn impedes transcription of gluconeogenic G6Pc and coactivation of SREBP-1 at key metabolic target gene promoters. We propose that the ability of metformin

to transcriptionally suppress gluconeogenesis and biosynthesis of lipids and cholesterol is in part due to transcriptional inhibition of SRC-2.

Methods

Cell culture, transfection and siRNA. Human HepG2 and rat FaO hepatoma cells were purchased from ATCC and cultured in a 5% CO_2 incubator at 37 °C. The culture media EMEM (Lonza, Basel, Switzerland) and F-12 HAM (Gibco, Waltham, MA, USA) were supplemented with 10% fetal bovine serum (FBS) (Gibco), 1% penicillin-streptomycin (Sigma, St. Louis, MO, USA) and 2 mM L-glutamine (Lonza). Cells were seeded 24 hours prior to transfection to a standardized density of 50.000 cells/24-well. For indicated experiments, cells were treated for 24 hours with metformin hydrochloride (Sigma) dissolved in sterile-filtered water. Transient plasmid transfections were performed in HepG2 cells using TransIT-LT1 reagent (Mirus, Madison, WI, USA) according to the manufacturer's protocol. All plasmids were verified by sequencing. The following reporter constructs were used: pGL-basic; pGL-SRC-2 (containing promoter bases −1500/+1) and pFASN-luc (containing promoter bases −220/+25). The following expression vectors were used: pSG5-HA-SRC-2; pSG5-empty; pcDNA3.1-2xFLAG-SREBP-1a and pcDNA3.1-2xFLAG-SREBP-2. For knock-down experiments we employed the TransIT-TKO transfection reagent (Mirus) with rat SRC-2 *(Nuclear receptor coactivator 2*) and rat non-targeting (NT) SMARTpool ON-TARGETplus siRNA (Dharmacon, Lafayette, CO, USA) at 100 nM final concentration. Cells were transfected with siRNA 72 hours prior to further analyses.

Transactivation assays. Cells were lysed with buffer containing 25 mM Tris Acetate-EDTA (pH 7.8), 2 mM dithiotheitol, 1 mM EDTA, 10% glycerol and 1% Triton X-100 and sample lysate luciferase activity was analyzed using a luciferase kit (BioThema, Handen, Sweden) and a FLUOStar Optima (BMG Labtech, Ortenberg, Germany) luminescence plate reader.

RT-qPCR analysis of gene expression. Cells were lysed in RLT buffer (Qiagen, Hilden, Germarny) and processed for RNEasy (Qiagen) RNA isolation according to manufacturer's protocol. Yield concentrations were measured by NanoDrop and 500 ng sample RNA was used for cDNA synthesis using High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Waltham, MA, USA) according to the manufacturer's instructions. Synthesized cDNA was diluted 1:10 in water prior to SYBR I Green (Roche, Basel, Switzerland) real-time RT-qPCR of target genes relative to reference gene *Rpl4*. Primer sequences are presented in the Supplementary Table S1. Relative target/*Rpl4* mRNA quantification was calculated using the delta-delta Ct method.

Microarray. FaO cells were treated with metformin (5 mM) or vehicle (water) for 24 hours prior to RNEasy (Qiagen) RNA purification. Integrity of total RNA (RIN value $\geq$ 9.0) from n = 3 biological replicates per treatment was verified by Agilent Bioanalyzer prior to sample randomization and microarray (Agilent, Santa Clara, CA, USA) at the Genomics Core Facility, University of Bergen. No sample batch effects were observed. Significance analysis of microarrays was performed with J-Express software (http://jexpress.bioinfo.no/site). Only genes exhibiting a fold-change FC ≤ -1.5 or FC ≥ 1.5 relative to vehicle and false discovery rate (FDR) ≤ 0.2 were included in subsequent analyses. Functional annotations of valid genes and statistical overrepresentation of biological categories were retrieved in accordance with instructions[42] using the PANTHER online database (pantherdb.org). The PANTHER library of 22957 annotated genes (*Rattus norvegicus*) was used as reference list for calculating statistical overrepresentation. Microarray data is included as Supplementary Dataset.

Western blotting. Sample cell lysate (30 μg) was loaded onto precast 4–20% Mini-Protean TGX gels (Bio-Rad, Hercules, CA, USA) and subjected to SDS-PAGE prior to iBlot (Invitrogen, Waltham, MA, USA) transfer to nitrocellulose membrane. The following antibodies were used: beta-Actin/ACTB (Abcam, Cambridge, UK, cat.no ab8227); SRC-2 (BD Biosciences, San Jose, CA, USA, cat. no 610985); HA-HRP (Roche, cat. no 12013819001); HRP goat-anti mouse IgG (BD Biosciences, cat. no 554002); HRP goat-anti rabbit IgG (Thermo Scientific, Waltham, MA, USA, cat. no 31460). Immunoblotted membranes were developed using Femto substrate (Thermo Scientific) and analyzed on a ChemiDoc XRS imager (Bio-Rad) equipped with QuantityOne densitometry software. For densitometry analyses, volumetric protein band intensity was normalized to that of ACTB in the same gel lane.

Chromatin immunoprecipitation (ChIP). Seeding of 2.0×10^6 HepG2 cells per 92 mm plate was performed one day prior to treatment with either 5 mM metformin or vehicle (water) for 24 hours. Cells were fixated with 1% (v/v) formaldehyde for 10 minutes and further processed using EZ-Magna ChIP kit (Millipore, Billerica, MA, USA) in accordance with the manufacturer's protocol. Sonication was optimized to 8 minutes (30 second on/off cycles) using a cold-water bath Bioruptor (Diagenode, Denville, NJ, USA). The following antibodies were used in conjunction with Protein A magnetic beads for over-night immunoprecipitation: ChIP-grade anti-RNA Pol II (Millipore #05-623B); normal IgG (Millipore #PP64B); ChIP grade anti-SRC-2 (Abcam ab9261) in quantities of 5 μl per sample. Primers for end-point SYBR Green qPCR of eluted sample DNA were designed to amplify regions flanking proximal sterol regulatory elements (SREs) at target gene promoters. Primer specificities and SRE motifs were verified *in silico* using

the UCSC Genome Browser (http://genome-euro.ucsc.edu) and Genomatix software. Primer sequences are listed in Supplementary Table S2. Quantification of samples by qPCR was normalized to parallel qPCR of 1% sonicated lysate input of respective treatments.

Oil Red O lipid staining. Cells were treated as indicated prior to fixation in 4% formaldehyde in PBS for 5 minutes followed by 1.5 hours with fresh 4% formaldehyde in PBS. Cells were washed twice with sterile water prior to incubation for 5 minutes with 60% isopropanol and being completely dried. Per well (12-well format) 1 ml of Oil Red O working solution (0.30 g Oil Red O in 100 ml 99% isopropanol, diluted 3:2 with sterile water) was added and left for 20 minutes prior to washing of each well 4 times with sterile water. Cells were depicted by Nikon TS100 light microscope and subsequent elution of cellular Oil Red O was done by gently washing of the wells with 1 ml 100% isopropanol. Optical density of eluted Oil Red O was measured at 490 nm.

Statistical analyses. Unless indicated otherwise, data values are expressed as Mean ± SEM of three or more biological replicates. All experiments were reproduced at least three times. All numerical data presented was considered to be of normal distribution and statistical differences between mean values were evaluated using two-tailed, independent Student's *t*-test. Differences of $p \leq 0.05$ (indicated*), $p \leq 0.01$ (indicated**) or $p \leq 0.001$ (indicated***) were considered significant. GraphPad Prism v5.0 (GraphPad Software Inc, La Jolla, CA, USA) was used for statistical calculations and graphical presentation of data.

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Author Contributions

A.M. performed the experiments and wrote the manuscript; O.B. assisted with experiments, data interpretation and manuscript editing; J.I.B. performed experiments; G.M. and J.V.S. critically revised the manuscript. All authors reviewed the manuscript.

Additional Information

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