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Nutritional Regulation of Hepatic Heme Biosynthesis and Porphyria through PGC-1α

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Summary

Inducible hepatic porphyrias are inherited genetic disorders of enzymes of heme biosynthesis. The main clinical manifestations are acute attacks of neuropsychiatric symptoms frequently precipitated by drugs, hormones or fasting, associated with increased urinary excretion of δ-aminolevulinic acid (ALA). Acute attacks are treated by heme infusion and glucose administration but the mechanisms underlying the precipitating effects of fasting and the beneficial effects of glucose are unknown. We show that the rate-limiting enzyme in hepatic heme biosynthesis, 5-aminolevulinate synthase (ALAS-1), is regulated by the peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α). Elevation of PGC-1α in mice via adenoviral vectors increases the levels of heme precursors *in vivo* as observed in acute attacks. The induction of ALAS-1 by fasting is lost in liver-specific PGC-1α knockout animals, as is the ability of porphyrogenic drugs to dysregulate heme biosynthesis. These data show that PGC-1α links nutritional status to heme biosynthesis and acute hepatic porphyria.

Running title: Regulation of heme biosynthesis by PGC-1α

Introduction

The heme biosynthetic pathway in eukaryotic cells is comprised of eight enzymatic steps; the first and the last three enzymes are located in the mitochondria, while the rest are in the cytoplasm (Fig. 1A). Eighty to ninety percent of total heme in mammals is synthesized in erythroid cells for incorporation into hemoglobin. Regulation of heme biosynthesis in these cells involves the erythroid-specific ALAS gene ALAS-2. In contrast, the housekeeping ALAS gene ALAS-1 also called ALAS-N or ALAS-H, is ubiquitously expressed, given that all nucleated cells must synthesize heme for respiratory cytochromes. The bulk of the non-erythroid synthesized heme is produced in the liver for various heme proteins, in particular microsomal cytochromes P450. Because either a deficiency or excess of heme is toxic to the cell, hepatic heme production has to be tightly controlled, mostly via its rate-limiting step ALAS-1. Accordingly, hepatic ALAS-1 is highly regulated in different contexts to ensure adequate levels of intracellular heme (May et al., 1995). Inherited mutations in all genes encoding for heme biosynthetic enzymes have been described except for ALAS-1 and the resulting diseases are referred to as porphyrias (Elder, 1998). Depending on the specific enzymatic defect, different patterns of overproduction, accumulation and excretion of intermediates of heme synthesis are observed.

The main clinical manifestations of porphyrias are intermittent attacks of neuropsychiatric dysfunction, and/or sensitivity of the skin to sunlight. The

neuropsychiatric syndrome occurs only in those porphyrias in which there is intermittent induction of hepatic ALAS-1 and consequent increased urinary excretion of ALA. Attacks are characteristically precipitated by drugs such as barbiturates, fasting and hormones and result in abdominal pain, tachycardia, peripheral motor neuropathies, psychosis and other mental disturbances (Elder, 1998; Thadani et al., 2000; Thunell, 2000). Inducible hepatic porphyrias are caused by rare defects in δ-aminolevulinic acid dehydratase (ALAD), porphobilinogen deaminase (PBGD, also known as hydroxymethylbilane synthase), coproporphyrinogen oxidase and protoporphyrinogen oxidase. The classical names for the corresponding diseases are ALAD-deficiency, acute intermittent porphyria, hereditary coproporphyria and variegate porphyria.

Although not definitively proven, historic personalities thought to have suffered from porphyria include King George III (Macalpine and Hunter, 1966), Friedrich Wilhelm I of Prussia (Macalpine et al., 1968; Pierach and Jennewein, 1999) and Vincent van Gogh (Bonkovsky et al., 1992; Loftus and Arnold, 1991). Thus, the psychoses arising from their disease potentially influenced the course of the American War for Independence or the creative genius of van Gogh. Acute attacks of inducible hepatic porphyria are treated by discontinuing exposure to the precipitating agents, heme infusions and high carbohydrate load. The carbohydrates are typically given as concentrated glucose infusion. Heme directly represses its own biosynthesis in a negative feedback loop (May et al., 1995). In contrast, the underlying mechanisms of the beneficial effects of carbohydrates are not understood.

PGC-1α is a co-activator of nuclear receptors and other transcription factors (Puigserver and Spiegelman, 2003). PGC-1α controls mitochondrial biogenesis and oxidative metabolism in many tissues, including brown adipose tissue, skeletal muscle, heart and liver (Lehman et al., 2000; Puigserver et al., 1998; Wu et al., 1999; Yoon et al., 2001). In the liver, PGC-1α is induced during fasting, when the liver ceases using glucose as an energy supply and changes to the β-oxidation of fatty acids. This increase in fatty acid β-oxidation and elevation of hepatic gluconeogenesis are both under control of PGC-1α (Herzig et al., 2001; Yoon et al., 2001).

Thus, because of the key role of PGC-1α in liver energy homeostasis and the finding that many PGC-1α targets are heme proteins, we investigated the role of PGC-1α in the regulation of hepatic heme biosynthesis by nutrition. We found that PGC-1α is an important factor controlling the expression of ALAS-1 in the fasted and fed liver. Moreover, we could show that hepatic PGC-1α is a major determinant of the severity of acute porphyric attacks in mouse models of chemical porphyria.

Results

Hepatic PGC-1α and ALAS-1 are co-regulated in fasting and feeding

Since fasting can be a powerful stimulus to induce an acute porphyric attack and the liver is central to the fasting response in mammals, the metabolic status of the liver should be crucial for the regulation of heme biosynthesis. The transcriptional coactivator PGC-1α has been described as a key factor in the control of hepatic gluconeogenesis in the fasted liver (Herzig et al., 2001; Yoon et al., 2001). Interestingly, ALAS-1 and PGC-1α are co-regulated in fasted mice, with increased mRNA levels (Fig. 1B). To test the relationship between the regulation of these two genes, Fao rat hepatoma cells, mouse primary hepatocytes and rat liver *in vivo* were infected with adenovirus expressing PGC-1α. In all of these systems, ectopic expression of PGC-1α increased ALAS-1 transcript levels in a manner similar to that of glucose-6-phosphatase (Glc6P), a PGC-1α target gene involved in gluconeogenesis (Yoon et al., 2001) (Fig. 1C-E). In contrast to ALAS-1, none of the other seven genes of the heme biosynthetic pathway were induced by PGC-1α in rat liver (Fig. 1E).

Insulin and glucagon regulation of ALAS-1 involves PGC-1α

Regulation of ALAS-1 in fasting and feeding is mediated by the counter-regulatory hormones insulin and glucagon (Scassa et al., 1998; Varone et al., 1999). Insulin treatment of primary mouse hepatocytes reduces basal levels of

ALAS-1 mRNA (Fig. 2A). Furthermore, PGC-1α-induced ALAS-1 transcript levels are reduced by insulin, suggesting that PGC-1α is in the pathway of the insulin regulation of ALAS-1 (Fig. 2A). Primary hepatocytes from wild-type and PGC-1α knockout mice (Lin et al., 2004) were used to elucidate the function of PGC-1α in the ALAS-1 induction in fasting with dexamethasone and forskolin representing the effects of glucocorticoids and glucagon that are elevated when blood glucose levels are low. Induction of ALAS-1 mRNA by these agents was reduced in the PGC-1α knockout hepatocytes as compared to wild-type cells (Fig. 2B). Similarly, the response of the gluconeogenic genes phosphoenolpyruvate carboxykinase (PEPCK) and Glc6P to these hormones was blunted. These findings imply that PGC-1α is involved in the fasting-feeding regulation of all three genes.

Insulin-repression of ALAS-1 is mediated by FOXO1 and PGC-1α

In the ALAS-1 promoter, two binding sites for the nuclear respiratory factor-1 (NRF-1) have been identified (Braidotti et al., 1993). NRF-1 is a transcription factor that increases expression of nuclear-encoded mitochondrial genes (Virbasius and Scarpulla, 1994) and is known to be potently co-activated by PGC-1α (Wu et al., 1999). Thus, NRF-1 is a potential binding partner by which PGC-1α controls ALAS-1 expression. In addition to the NRF-1 site, an insulin-responsive region (IRE) has been defined in the ALAS-1 promoter (Scassa et al., 2004; Scassa et al., 2001) but the identity of transcription factors binding to this element has remained elusive (Fig. 3A).

Chromatin immunoprecipitation assays in mouse hepatoma cells illustrate that PGC-1α is recruited to both the NRF-1 and the IRE regions (Fig. 3B). Moreover, PGC-1α recruitment to the IRE region is sensitive to insulin, in contrast to PGC-1α binding to the NRF-1 site (Fig. 3B). Sequence comparison of the ALAS-1 IRE to those of the gluconeogenic genes PEPCK and Glc6P revealed high sequence conservation between these sites. In the flanking regions of the gluconeogenic genes, PGC-1α binds to FOXO1 at these elements. After insulin exposure, FOXO1 is phosphorylated, its binding to PGC-1α disrupted and subsequently, FOXO1 is exported from the nucleus (Puigserver et al., 2003). Thus, FOXO1 is a plausible candidate to bind to the ALAS-1 promoter.

Recently, Scassa and co-workers described the hepatocyte nuclear factor 3β (HNF3β, alternatively called FOXA2) to bind to sites adjacent to the ALAS-1 IRE (Scassa et al., 2004) (Fig. 3A). They found that integrity of the IRE and not of the HNF3β sites is obligatory for insulin regulation of ALAS-1 and that the regulation of ALAS-1 by HNF3β cannot account for the repression of ALAS-1 by insulin (Scassa et al., 2004). We thus tested the ability of PGC-1α to co-activate the different Forkhead box family members FOXO1, hepatocyte nuclear factor 3α (HNF3α, FOXA1) and HNF3β on the ALAS-1 promoter. As described by Scassa *et al.*, HNF3β increased reporter gene levels controlled by the ALAS-1 promoter whereas HNF3α had no effect (Fig. 3C). However, of the three transcription factors, PGC-1α only co-activated FOXO1 in this context. In

addition, merely the FOXO1-PGC-1α-mediated induction of the ALAS-1 promoter was repressed by insulin (Fig. 3C).

To further characterize the role for FOXO1 in the regulation of the ALAS-1 promoter, we showed direct physical interaction of FOXO1 with the DNA probes containing the ALAS-1 IRE (Fig. 4A, lanes 4-7) and NRF-1 with the NRF-1 site (Fig. 4A, lanes 1-3) in electrophoretic mobility shift assays. Site-directed mutagenesis of the FOXO1 site abolished binding of FOXO1 to this element (Fig. 4A, lanes 3 and 7, respectively). The specificity of the FOXO1-IRE complex was confirmed by using an anti-FOXO1 antibody that resulted in a supershift (Fig. 4A, lane 6). Functionally, PGC-1α co-activates both NRF-1 and FOXO1 in reporter gene assays using the ALAS-1 promoter in mouse H2.35 SV-40 transformed hepatocytes (Fig. 4B). Mutagenesis of the NRF-1 or the IRE sites reduced the ability of PGC-1α to augment the activity from the ALAS-1 promoter stimulated by NRF-1 and FOXO1, respectively. Moreover, an ALAS-1 promoter allele with a combined mutation of both the NRF-1 and IRE sites is completely insensitive to PGC-1α, strongly suggesting that NRF-1 and FOXO1 are the major binding partners of PGC-1α in the ALAS-1 promoter.

As shown in Fig. 4C, insulin represses induction of ALAS-1 promoter-driven reporter gene expression by either FOXO1 alone or in combination with PGC-1α. In contrast, an non-phosphorylatable mutant of FOXO1 with three alanines in place of the serine/threonine residues targeted by Akt kinase (termed FOXO1 3A) prevented repression by insulin. Similarly, insulin is unable to inhibit PGC-

1α co-activation of FOXO1 3A. This suggests that the insulin repression of the ALAS-1 promoter is controlled by the FOXO1-PGC-1α interaction.

Liver-specific PGC-1α knockout animals have a blunted induction of ALAS-1 in fasting

To investigate whether PGC-1α is a key mediator of the metabolic regulation of ALAS-1 in an *in vivo* setting, we first examined animals with a total knockout of PGC-1α (Lin et al., 2004) under fasting and feeding conditions. Unfortunately, regulation of ALAS-1 is masked by systemic effects of the whole-body knockout of PGC-1α: similar to what has previously been described for the gluconeogenic genes (Lin et al., 2004): ALAS-1 mRNA is constitutively induced to fasted levels in the total knockout animals, even in the fed state (Fig. 5A). It is unclear whether the same compensatory mechanisms (elevation of C/EBPβ) account for the constitutive expression of the gluconeogenic genes and ALAS-1 (Lin et al., 2004). Thus, in order to dissect systemic effects of the total knockout from the liver phenotype, we had to generate liver-specific PGC-1α knockout animals by crossing mice with a floxed PGC-1α allele to transgenic animals expressing cre recombinase under the control of the albumin promoter (Fig. 5B). Tissue-specific ablation of PGC-1α in the liver was verified by mRNA analysis (Fig. 5C). PGC-1α levels in the heart, skeletal muscle (SKM) and brown adipose tissue (BAT) were unaltered. Moreover, liver-specific knockout of PGC-1α did

not change the expression of the closely related family member PGC-1β in liver, heart, skeletal muscle or brown adipose tissue.

In these mice, basal levels of ALAS-1, PEPCK and Glc6P were not elevated, as was seen in the total knockout (Fig. 5D). Indeed, ALAS-1 basal expression was significantly lower in the liver-specific PGC-1α knockout as compared to wild-type animals. Moreover, the induction of ALAS-1 and the gluconeogenic genes after 16 hours of fasting was severely blunted in the liver-specific knockout animals compared to wild-type mice (Fig. 5D). These findings indicate a requirement for PGC-1α in the fasting/feeding regulation of the gluconeogenic genes and, furthermore, imply a key role for PGC-1α in the dietary control of ALAS-1 transcription. Patients suffering from porphyric attacks are given a high carbohydrate load. Thus, in addition to re-feeding, fasted mice were *i.p.* injected with a bolus of glucose (1 g/kg) or glucose (1g/kg) and insulin (1.0 U/kg) 30 min., 60 min. or 120 min. before mice were sacrificed and hepatic PGC-1α and ALAS-1 mRNA levels determined (Fig. 5E). Glucose loading reduces ALAS-1 transcript levels 30 min. after injection. The combination of glucose and insulin is even more potent in inhibiting fasting-mediated induction of PGC-1α and ALAS-1 supporting the hypothesis that at least part of the beneficial effect of glucose in acute porphyric attacks is mediated by the glucose-triggered increase of plasma insulin. ALAS-1 mRNA is not regulated by fasting and thus, no effect of glucose and/or insulin on ALAS-1 transcript levels could be observed.

Taken together, these data strongly suggest that PGC-1α may be involved in fasting-induced acute porphyric attacks. Apart from fasting, certain drugs strongly regulate ALAS-1 levels and therefore are able to precipitate porphyric attacks (Elder et al., 1997; Thadani et al., 2000). There are two different classes of chemicals that perturb heme homeostasis: first, drugs that increase heme biosynthesis by inducing ALAS-1 and second, compounds that block different steps of heme biosynthesis and thus generate various heme intermediates. Representative for the first class of drugs, we chose the barbiturate phenobarbital (PB), a classical drug that precipitates porphyric attacks in patients. As shown in Fig. 5F, no significant difference in PB-induction of ALAS-1 and the prototypical PB-target genes cytochromes P450 Cyp2b10 and Cyp3a11, two microsomal cytochromes P450 with a heme moiety as prosthetic group, was observed between wild-type and liver-specific PGC-1α knockout animals. These data indicate that the role of PGC-1α in ALAS-1 regulation does not extend universally to all other mechanisms, such as the induction by barbiturate drugs (Fraser et al., 2002; Fraser et al., 2003; Podvinec et al., 2004).

Elevated expression of PGC-1α causes acute attacks in chemical porphyria

The consequence of ALAS-1 regulation by PGC-1α in fasting and feeding for acute porphyric attacks was subsequently tested using pro-porphyrogenic drugs that are known to function by disruption of the pathway of hepatic heme

biosynthesis. Two members of this class of chemicals are lead (Pb) and 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DCC) (Fig. 6A). Lead intoxication produces symptoms resembling those of acute hepatic porphyria (May et al., 1995). Because of its ability to replace other ions such as zinc and to block thiol groups, lead inhibits several enzymes in heme biosynthesis, most importantly ALAD. Another drug widely used to induce porphyria in systems that lack the genetic predisposition for this disorder, DCC, causes accumulation of N-methyl protoporphyrin, a potent inhibitor of ferrochelatase (De Matteis et al., 1973). In gain-of-function experiments, mice were injected *i.v.* with adenoviral GFP and PGC-1α. These animals were subsequently fasted and treated with vehicle (corn oil) or DDC for 24 hours. DDC did not change adenoviral expression of PGC-1α mRNA (Fig. 6B). In contrast, ALAS-1 transcript levels were elevated 10 fold in animals that received both PGC-1α adenovirus and DDC (Fig. 6C). This high induction of ALAS-1 was reflected in the dramatically increased levels of the heme precursors 5-ALA and PBG in their plasma (Fig. 6D and E), to 9 μM and 27 μM, respectively. Importantly, these are levels that are seen in acute attacks in mouse models of porphyria. Thus, in wild-type animals with chemical porphyria, elevation of PGC-1α expression in the liver results in accumulation of heme precursors comparable to that classically observed in drug-precipitated acute attacks in genetic mouse models of porphyria (Lindberg et al., 1999; Lindberg et al., 1996).

Liver-specific PGC-1α knockout animals are protected from chemical porphyria

The requirement for PGC-1α in fasting-induced porphyria was tested in the liver-specific PGC-1α knockout animals. In fasted wild-type animals treated with lead chloride and DDC for 24 hours, increased ALAS-1 mRNA levels were observed as compared to treatment with their vehicles saline and corn oil, respectively (Fig. 7A). In contrast, neither lead chloride nor DDC changed endogenous PGC-1α levels, respectively (data not shown). As a consequence of the ALAS-1 induction and the chemical block in the biosynthetic pathway, 5-ALA accumulates in plasma after lead and DCC treatment (Fig. 7B). Since lead and DCC inhibit heme biosynthesis at different steps (Fig. 6A), only DCC elevates PBG levels (Fig. 7C). Thus, as reported, blocking of heme biosynthesis with porphyrogenic drugs results in a state of latent porphyria in wild-type mice, comparable to the status of patients between attacks, which is characterized by moderately elevated levels of 5-ALA and PBG (De Matteis, 1973). In an actual acute attack, 5-ALA and PBG levels rise to those observed in the gain-of-function experiment shown in Fig. 6D and 6E. Strikingly, lead and DCC completely fail to induce either ALAS-1 mRNA (Fig. 7A), plasma 5-ALA (Fig. 7B) or PBG levels (Fig. 7C), respectively, in the liver-specific PGC-1α knockout mouse. These data indicate that PGC-1α is absolutely required for animals treated with porphyrogenic drugs to enter a state of latent porphyria.

Discussion

While the biochemical consequences of mutations in the heme biosynthetic pathway are well known, the molecular mechanisms underlying the nutritional regulation of hepatic porphyrias have been poorly understood. Specifically, questions regarding how fasting can precipitate porphyric attacks and why glucose infusions provide therapeutic benefit have remained unanswered. The results presented here provide a clear-cut mechanism, deduced from biochemical and genetic evidence: the transcriptional co-activator PGC-1α is induced in the liver in fasting, and potently turns on expression of the ALAS-1 gene in hepatocytes and in liver *in vivo*. The induction of PGC-1α in fasting has previously been shown to be a consequence of glucagon action and the transcription factor cAMP element binding protein (CREB), which binds directly to the PGC-1α promoter (Herzig et al., 2001). In addition, CREB can also directly activate the ALAS-1 promoter (Varone et al., 1999).

PGC-1α activates the ALAS-1 promoter by co-activating NRF-1 and FOXO1, both of which directly bind to the ALAS-1 promoter (Fig. 6). The ability of PGC-1α to positively regulate the ALAS-1 gene, and the requirement for PGC-1α in the fasting induction of ALAS-1 together provide a direct explanation for how fasting can provoke an acute attack in an individual with a mutation in the pathway of heme biosynthesis that results in hepatic porphyria. Indeed, adenoviral expression of PGC-1α in mice with chemical inhibition of enzmyes of heme biosynthesis results in significantly elevated porphyrin precursor levels

reminiscent of acute porphyric attacks. In contrast, the excess production of heme intermediates by porphyrogenic drugs is lost in the liver-specific PGC-1α knockout.

The therapeutic effect of glucose on acute hepatic porphyria is well documented (Robert et al., 1994). Moreover, it has been established that ALAS-1 transcription is inhibited by the insulin pathway involving Akt (Kappas et al., 1995; Scassa et al., 2001). Our data for the first time illustrate likely mechanisms by which glucose, and the subsequent elevation of insulin, which occurs *in vivo* in response to glucose, can ameliorate an acute porphyric attack. First, increased levels of insulin will certainly blunt the expression of PGC-1α. Glucagon is important in PGC-1α expression and rising blood glucose dampens glucagon secretion (Herzig et al., 2001; Yoon et al., 2001). Second, insulin has been shown to activate the protein kinase Akt in the liver and Akt in turn phosphorylates FOXO1 (Brunet et al., 1999; Nakae et al., 2001). Phosphorylation of FOXO1 results in disruption of its binding to PGC-1α and its export from the nucleus (Puigserver et al., 2003), thus inhibiting PGC-1α action. Increased blood glucose levels would therefore be expected to alter the PGC-1α modulation of ALAS-1 gene expression by two related but independent mechanisms. This hypothesis is supported by the reduced ALAS-1 mRNA levels in fed mice that have constitutively elevated PGC-1α levels after tail-vein injection of adenoviral vectors (Supplemental data 1). Thus, despite high PGC-1α levels, ALAS-1 mRNA transcription can be reduced by insulin-triggered nuclear exclusion of FOXO1. Moreover, like insulin, glucose has been shown to

have an inhibitory effect on ALAS-1 transcription in cell culture (Canepa et al., 1984; Giger and Meyer, 1981). Under our experimental conditions, glucose alone did not significantly repress basal or PGC-1α-induced ALAS-1 mRNA levels (Supplemental data 2). However, insulin and glucose together were more efficient than insulin in reducing ALAS-1 expression. Thus, in addition to its effect on insulin secretion, glucose could directly affect ALAS-1 transcription. Candidate pathways include the AMP-activated protein kinase (AMPK), protein phosphatase 2A (PP2A) and carbohydrate-response element binding protein (ChREBP) (Kawaguchi et al., 2002; Yamashita et al., 2001), although an effect of this signaling cascade on ALAS-1 remains to be shown.

Agents which elevate hepatic PGC-1α levels are therefore potentially dangerous for patients with hepatic porphyrias. Accordingly, drugs and foods that induce PGC-1α in the liver should be avoided. Unfortunately, because of the therapeutic high carbohydrate intake, patients with hepatic porphyrias are prone to weight gain. Losing excess weight is very difficult for some of these patients because of fasting-induced acute attacks. Hopefully, our findings described here might lead to the development of more specific treatments for these patients.

Experimental procedures

RNA isolation and analysis. Total RNA was isolated from liver or cultured cells using the Trizol reagent (Invitrogen) according to the manufacturer's protocol. For semiquantitative real-time PCR analysis, 1 μg of total RNA was treated with RNase-free DNase and subsequently reverse transcribed with random hexamer primers (Roche Applied Science). Relative mRNA abundance normalized to 18S rRNA levels was determined with the ΔΔCt method after amplification using a iCycler iQ real-time PCR detection system (Biorad) and SYBRGreen (Biorad). Data are represented as mean +/- standard deviation. Significance is defined as $p<0.05$ in Student's t-test.

Animal experiments. All animal experiments were performed according to procedures approved by the Institutional Animal Care and Use Committee. Animals were fed standard rodent chow and housed in a controlled environment with 12-hours light and dark cycles. For fasting experiments, mice were deprived of food for the indicated amount of time before animals were sacrificed. Drugs (phenobarbital, 100 mg/kg; $PbCl_2$, 20 mg/kg; DDC, 10 mg/kg) were injected *i.p.* and livers and blood harvested after 16 or 24 hours. All groups consisted of at least 3-6 mice. Glucose (1 g/kg) and insulin (1.0 U/kg) were injected *i.p.* into mice that were fasted for 6 hours. Data are represented as mean +/- standard deviation. Significance is defined as $p<0.05$ in Student's t-test.

Generation of liver-specific PGC-1α knockout animals. Generation of animals with floxed PGC-1α alleles has been described (Lin et al., 2004). These mice were crossed with mice that transgenically express cre recombinase under the control of the rat albumin promoter (Jackson Laboratories, strain B6.Cg-Tg(Alb-cre)21MGn/J) to obtain liver-specific PGC-1α knockout mice.

Adenoviral infection. Cultured cells were infected with adenovirus as published (Yoon et al., 2001). 24 to 48 hours after infection, cells were harvested. Male Wistar rats were transduced with purified adenovirus via tail-vein injection as described (Yoon et al., 2001). Mice were tail-vein injected with 0.2 OD of cesium-chloride-gradient purified adenovirus. Five days later, mice were sacrificed and livers and plasma harvested. Data are represented as mean +/- standard deviation. Significance is defined as $p<0.05$ in Student's t-test.

Cell culture (Fao, H2.35, primary hepatocytes), transfection and reporter gene assays. Fao rat hepatoma cells were cultured in RPMI medium with 10% fetal calf serum. H2.35 mouse SV-40 transformed hepatocyte cells were kept in DMEM supplemented with 4% fetal calf serum and 0.2 μM dexamethasone. Primary mouse hepatocytes were isolated and cultured as described (Lin et al., 2004). Cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. For reporter gene assays, cells were harvested 48 hours after transfection. Luciferase levels were determined and normalized to β-galactosidase expression as published (Iniguez-Lluhi et al., 1997). For

treatment with glucose (33 mM), cells were cultured in glucose-free DMEM (Mediatech). Data are represented as mean +/- standard deviation. Significance is defined as $p<0.05$ in Student's t-test.

Cloning of promoter constructs and site-directed mutagenesis. The rat ALAS-1 promoter (Braidotti et al., 1993) was amplified by PCR and cloned into the pGL3 basic luciferase reporter gene vector (Promega). Site-directed mutagenesis was performed using overlapping primers. All constructs were verified by sequencing.

Electrophoretic mobility shift assays. Electrophoretic mobility shift assays were performed as described (Handschin et al., 2003). Briefly, wild-type and mutant ALAS-1 promoter fragments were radiolabelled and used as probes together with in vitro transcribed/translated proteins. Protein-DNA complexes were subsequently separated by polyacrylamide gel electrophoresis. FOXO1 antibody was purchased from Santa Cruz Biotechnology.

Chromatin immunoprecipitation. Experiments were performed using the Chromatin Immunoprecipitation (ChIP) Assay Kit (Upstate) following the manufacturer's protocol. H2.35 cells were infected with adenoviral GFP and flag-tagged PGC-1α for 24 hours and treated with vehicle or 10 nM insulin for 12 hours before cells were harvested, DNA-protein complexes cross-linked, and immunoprecipitation reactions performed using anti-flag beads (Sigma). After

reverse crosslinking, DNA was purified by phenol/chloroform extraction and ethanol precipitation and relative levels subsequently analyzed by PCR.

Determination of ALA and PBG plasma levels. When animals were sacrificed, blood was harvested by cardiac puncture. Blood plasma was purified by centrifugation in heparin tubes (Becton Dickinson) and treated as described (Mendez et al., 1999). ALA and PBG levels in the plasma was subsequently analyzed by sequential ion exchange chromatography using columns from the ALA/PBG by Column Test Kit (Biorad) using a modified protocol (Davis and Andelman, 1967). PBG and ALA were absorbed on anion and cation exchange resins, respectively. Following elution and conversion to pyrrole in the case of ALA, Ehrlich's reagent was added and PBG and ALA levels were determined colorimetrically. Data are represented as mean +/- standard deviation. Significance is defined as $p<0.05$ in Student's t-test.

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Figure Legends

Figure 1. ALAS-1 expression is activated by PGC-1α in hepatocytes and liver *in vivo*. A, Heme biosynthesis pathway. ALA, 5-aminolevulinic acid; ALAD, ALA dehydratase; PBG, porphobilinogen; PBGD, PBG deaminase; HMB, hydroxymethylbiline; Uro-P, uroporphyrinogen; Copro-P, Coproporphyrinogen; Cpo, Coproporphyrinogen oxidase; Proto-P, protoporphyrinogen. **B,** ALAS-1 and PGC-1α mRNAs are co-induced in fasting. Mice were fasted for 6 hours and 24 hours, respectively, and hepatic levels of PGC-1α and ALAS-1 were compared to those of fed and re-fed animals by semiquantitative PCR. **C, D,** Adenoviral PGC-1α increases ALAS-1 gene expression in cell culture. Fao rat hepatoma cells (C) and mouse primary hepatocytes (D) were infected with adenovirus encoding GFP or PGC-1α, respectively, and relative mRNA levels of PGC-1α, glucose-6-phosphatase (Glc6P) and ALAS-1 were determined by semiquantitative PCR 24 hours after infection. **E,** PGC-1α induces ALAS-1 transcript levels in vivo. Male Wistar rats were tail-vein injected with adenovirus encoding for GFP and PGC-1α, respectively. 5 days post injection, animals were sacrificed and hepatic mRNAs analyzed for changes in expression using semiquantitative PCR. Data are represented as mean +/- standard deviation.

Figure 2. ALAS-1 expression is regulated by PGC-1α and insulin. A, Insulin represses PGC-1α-mediated induction of ALAS-1. H2.35 SV-40 transformed hepatocyte cells were infected with adenoviral GFP and PGC-1α for 24 hours

and subsequently treated with vehicle (PBS) or 10 nM insulin for 12 hours before relative ALAS-1, cytochrome c and PGC-1α mRNA levels were determined. * $p<0.05$ between vehicle and insulin treated cells. **B,** ALAS-1 induction by dexamethasone and forskolin is partially dependent on PGC-1α. Mouse primary hepatocyte cultures were established from wild-type and PGC-1α total knockout animals. These cells were subsequently treated with dexamethasone (dex, 1 μM) and forskolin (forsk, 0.2 μM) for 3 hours and relative mRNA levels were determined by semiquantitative PCR. Data are represented as mean +/- standard deviation. * $p<0.05$ between wild-type and knockout cells in Student's t-test.

Figure 3. PGC-1α regulates ALAS-1 expression via NRF-1 and FOXO1. A, Structure of the ALAS-1 promoter. **B,** PGC-1α binds to the NRF-1 and the IRE sites on the ALAS-1 promoter. H2.35 cells were infected with adenoviral GFP or flag-tagged PGC-1α. Cells were treated with 10 nM insulin for 12 hours before cells were harvested and chromatin immunoprecipitation performed using an anti-flag antibody. **C,** PGC-1α co-activates FOXO1 on the ALAS-1 promoter. H.35 cells were transfected with ALAS-1 promoter construct and expression plasmids for FOXO1, HNF3α (FOXA1), HNF3β (FOXA2) and PGC-1α. After transfection, cells were treated with 10 nM insulin for 12 hours before reporter gene levels were determined. Data are represented as mean +/- standard deviation.

Figure 4. FOXO1-PGC-1α mediate the repression of the ALAS-1 promoter by insulin. A, FOXO1 binds to the ALAS-1 promoter. Electrophoretic mobility shift assays were performed using radiolabelled wild-type and mutated ALAS-1 promoter as probe together with *in vitro* transcribed/translated NRF-1 and FOXO1, respectively. **B,** PGC-1α induction of the ALAS-1 promoter is mediated by NRF-1 and FOXO1. H2.35 cells were transfected with wild-type and ALAS-1 promoter constructs with mutations in the NRF-1 and the IRE sites together with expression plasmids for PGC-1α, NRF-1 and FOXO1. Reporter gene levels were determined 16 hours after transfection. **C,** Insulin regulation of the ALAS-1 promoter is inhibited by a mutant FOXO1 allele. H2.35 cells were transfected with a reporter gene plasmid driven by the wild-type ALAS-1 promoter together with expression plasmids for PGC-1α, FOXO1 and FOXO1 3A, a FOXO1 allele that is not phosphorylated by insulin signaling. After transfection, cells were treated with 10 nM insulin for 12 hours before reporter gene levels were determined. Data are represented as mean +/- standard deviation. * $p<0.05$ between vehicle and insulin treated cells in Student's t-test.

Figure 5. Fasting-mediated induction of ALAS-1 is drastically reduced in a liver-specific PGC-1α knockout model. A, Hepatic ALAS-1 is constitutively increased in fasting and feeding in PGC-1α total knockout mice. PGC-1α total knockout animals were fasted for 12 hours before relative mRNA levels for PGC-1α, ALAS-1 and phosphoenolpyruvate carboxykinase (PEPCK) were determined. Data are represented as mean +/- standard deviation. * $p<0.05$ between wild-type and knockout animals in Student's t-test. **B,** Generation of

liver-specific PGC-1α knockout animals. Mice with a floxed PGC-1α allele were crossed with animals that transgenically express cre recombinase under the control of the albumin promoter. **C,** Hepatic expression of PGC-1α is absent in the liver-specific knockout animals. Different tissues were harvested from wild-type and knockout mice and relative PGC-1α and PGC-1β levels were determined by semiquantitative PCR. SKM, skeletal muscle; BAT, brown adipose tissue. **D,** Absence of PGC-1α abolishes ALAS-1 induction in the liver by fasting. Wild-type and liver-specific PGC-1α knockout mice were fasted for 12 hours and relative transcript levels for PGC-1α, ALAS-1, PEPCK and glucose-6-phosphatase (Glc6P) were determined by semiquantitative PCR. Data are represented as mean +/- standard deviation. * $p<0.05$ between wild-type and knockout animals in Student's t-test. **E,** Glucose reverses fasting-mediated induction of PGC-1a and ALAS-1. Wild-type and liver-specific PGC-1α knockout animals were fasted for 6 hours and subsequently injected with vehicle, glucose or glucose and insulin. After 30, 60 or 120 minutes, respectively, mice were sacrificed, livers harvested and relative PGC-1α and ALAS-1 expression levels determined. **F,** Phenobarbital induces ALAS-1 independent of PGC-1α. Wild-type and liver-specific PGC-1α knockout animals were injected *i.p.* with vehicle (saline) or phenobarbital (100 mg/kg). 16 hours after injection, livers were harvested and relative mRNA levels of PGC-1α, ALAS-1, cytochrome P450 2b10 and 3a11 (Cyp2b10 and Cyp3a11, respectively) were determined by semiquantitative PCR.

Figure 6. Ectopic expression of PGC-1α elicits an acute porphyric attack. **A,** Lead (Pb) and 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) inhibit heme biosynthesis at different steps. **B, C,** Ectopic PGC-1α expression increases ALAS-1 levels and heme intermediates. Adenoviral GFP or PGC-1α was injected into the tail veins of wild-type animals, which were fasted four days later and treated with corn oil and DDC (10 mg/kg) for 24 hours. Transcript levels of PGC-1α (B) and ALAS-1 (C) were determined after the mice were sacrificed 24 hours later. **D, E,** Plasma of tail-vein injected animals was collected and 5-aminolevulinic acid (ALA) levels (D) and porphobilinogen (PBG) levels (E) determined. Data are represented as mean +/- standard deviation. * $p<0.05$ between GFP and PGC-1α infected animals in Student's t-test.

Figure 7. Liver-specific PGC-1a knockout animals are protected from chemical porphyria. A, B, C, Lack of increase in ALAS-1 mRNA and heme biosynthesis intermediates in liver-specific PGC-1α knockout animals. Fasted wild-type and knockout animals were *i. p.* injected with saline and lead chloride (PbCl2, 20 mg/kg) or corn oil and DDC (10 mg/kg) for 24 hours. After animals were sacrificed, ALAS-1 mRNA levels (A), ALA plasma levels (B) and PBG plasma levels (C) were determined. Data are represented as mean +/- standard deviation. * $p<0.05$ between wild-type and knockout animals in Student's t-test.

Supplemental data 1. Feeding reduces ALAS-1 levels despite constitutively high PGC-1α expression. Adenoviral vectors for GFP and PGC-1α were tail-vein injected into animals. After four days later, mice were

fasted overnight and hepatic PGC-1α and ALAS-1 expression compared to fed control animals. Despite constant adenoviral production of PGC-1α, ALAS-1 is significantly lower in the fed vs. the fasted state. Data are represented as mean +/- standard deviation. n.s., non-significant; *p<0.05 between fasted and fed samples in Student's t-test.

Supplemental data 2. Distinct effects of glucose and insulin H2.35 mouse hepatoma cells. H2.35 cells were infected with adenoviral GFP and PGC-1α and cultured in glucose-free medium for 24 hours. Cells were subsequently treated with 33 mM glucose and 10 nM insulin for 12 hours before RNA was isolated and relative expression of ALAS-1 determined. Although glucose alone did not significantly inhibit basal or PGC-1α-induced ALAS-1 levels, the combination of insulin and glucose was more potent that insulin by itself at repressing ALAS-1 transcription. Data are represented as mean +/- standard deviation. *p<0.05 between vehicle and glucose/insulin-treated cells in Student's t-test.

Figure 1

Fig. 1
Handschin *et al.*

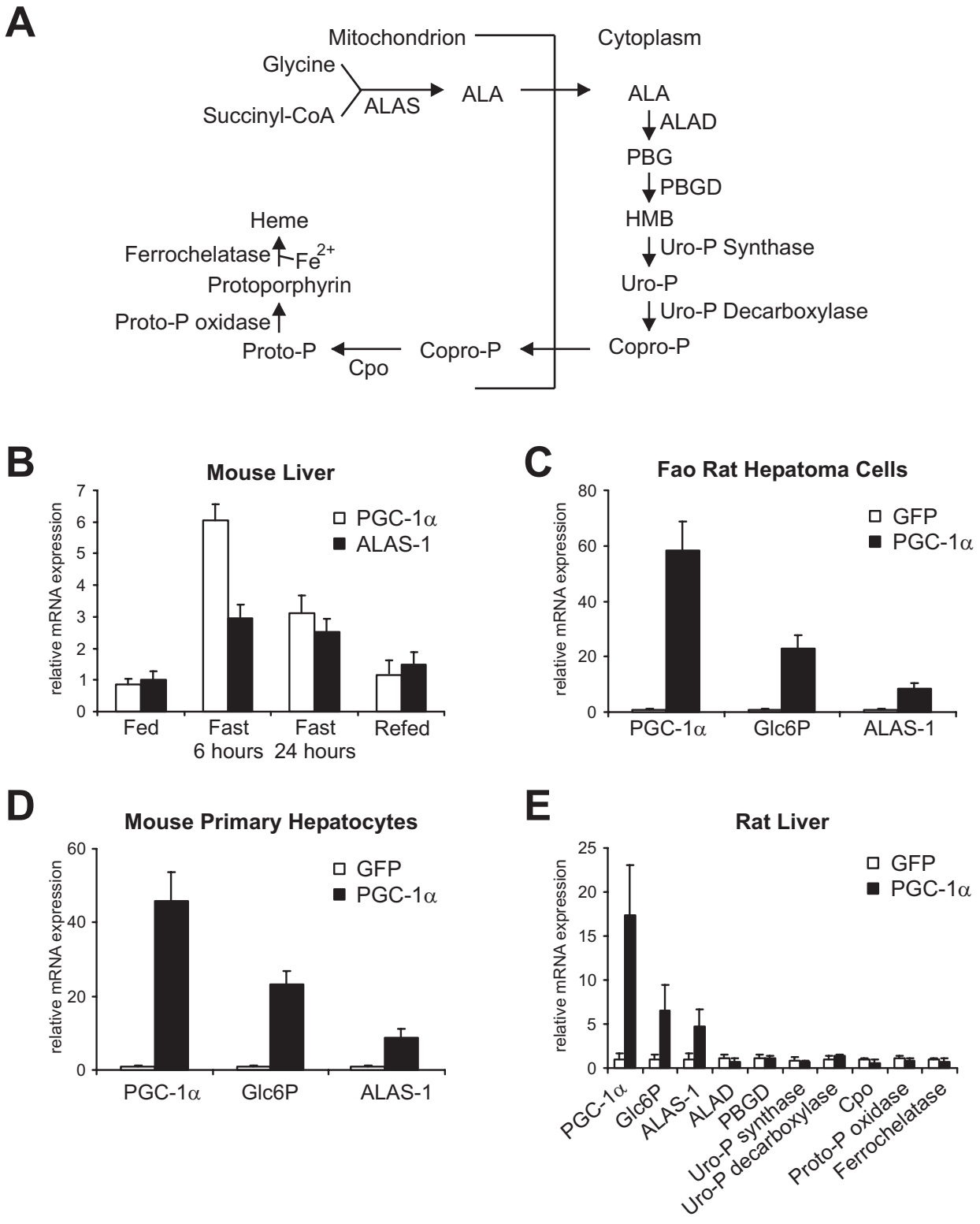

Fig. 2
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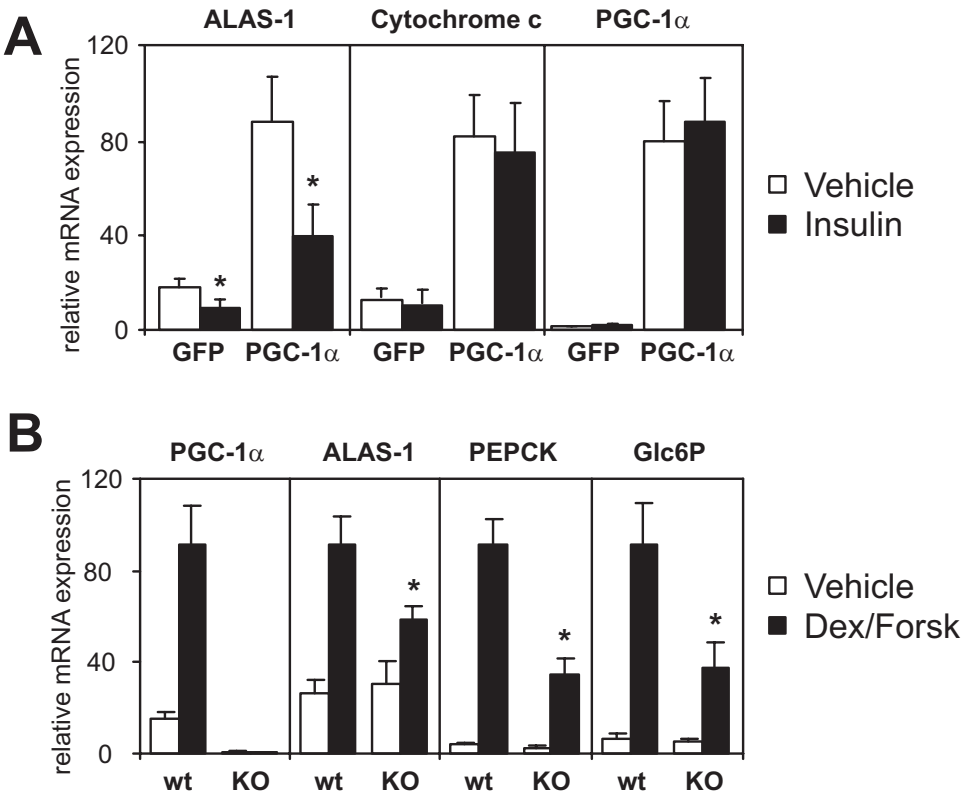

Figure 3

Fig. 3
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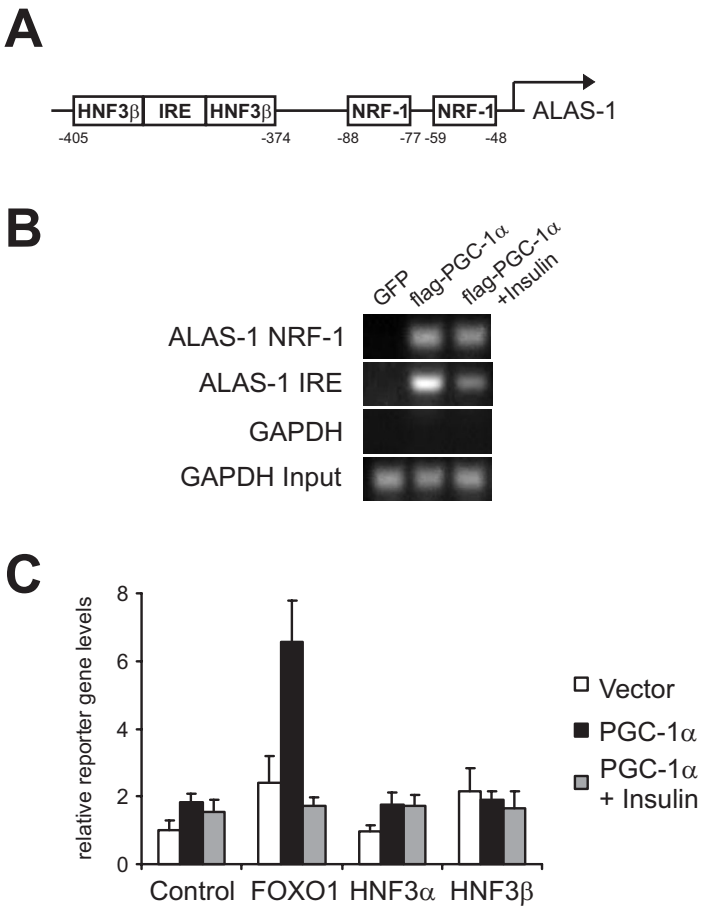

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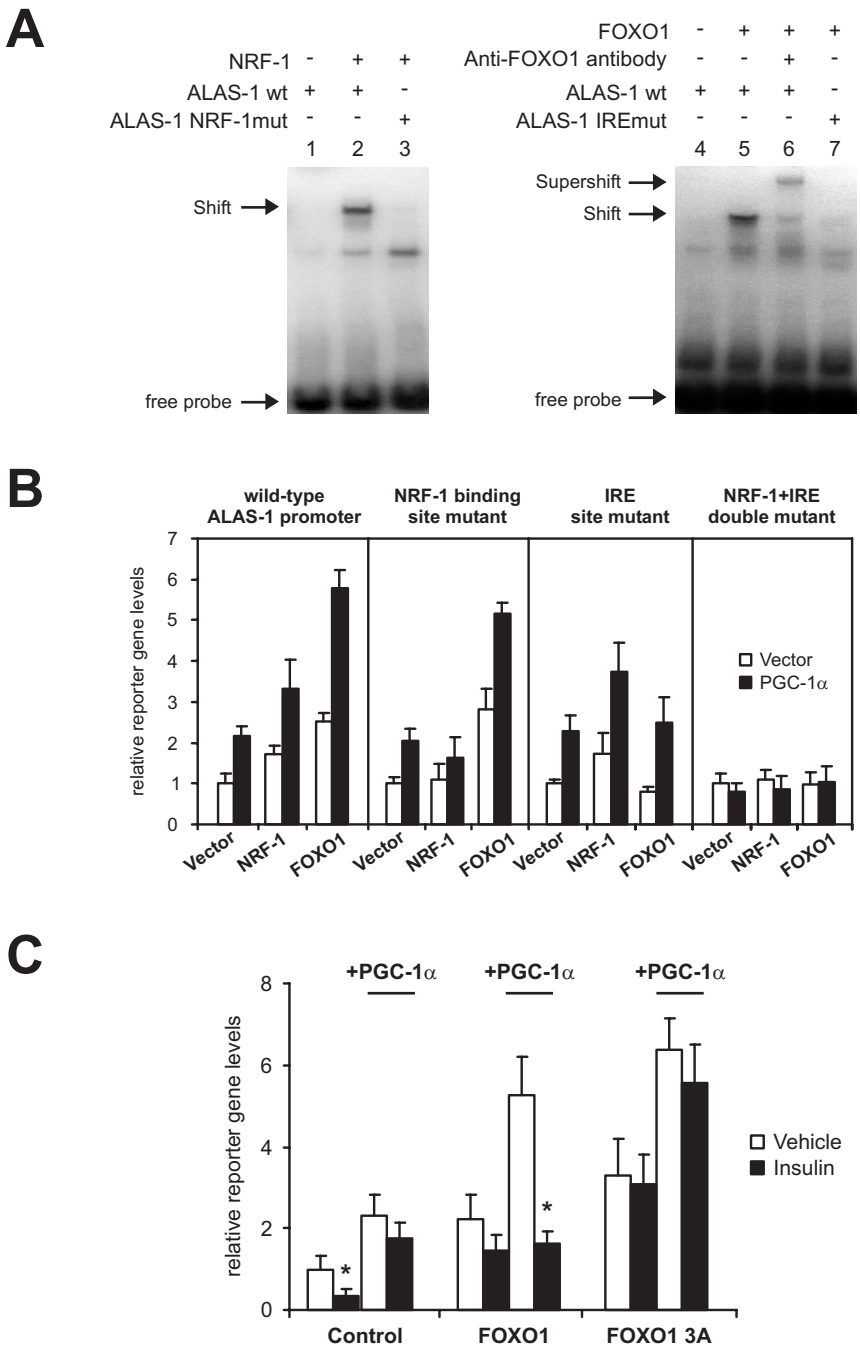

Figure 5

Fig. 5
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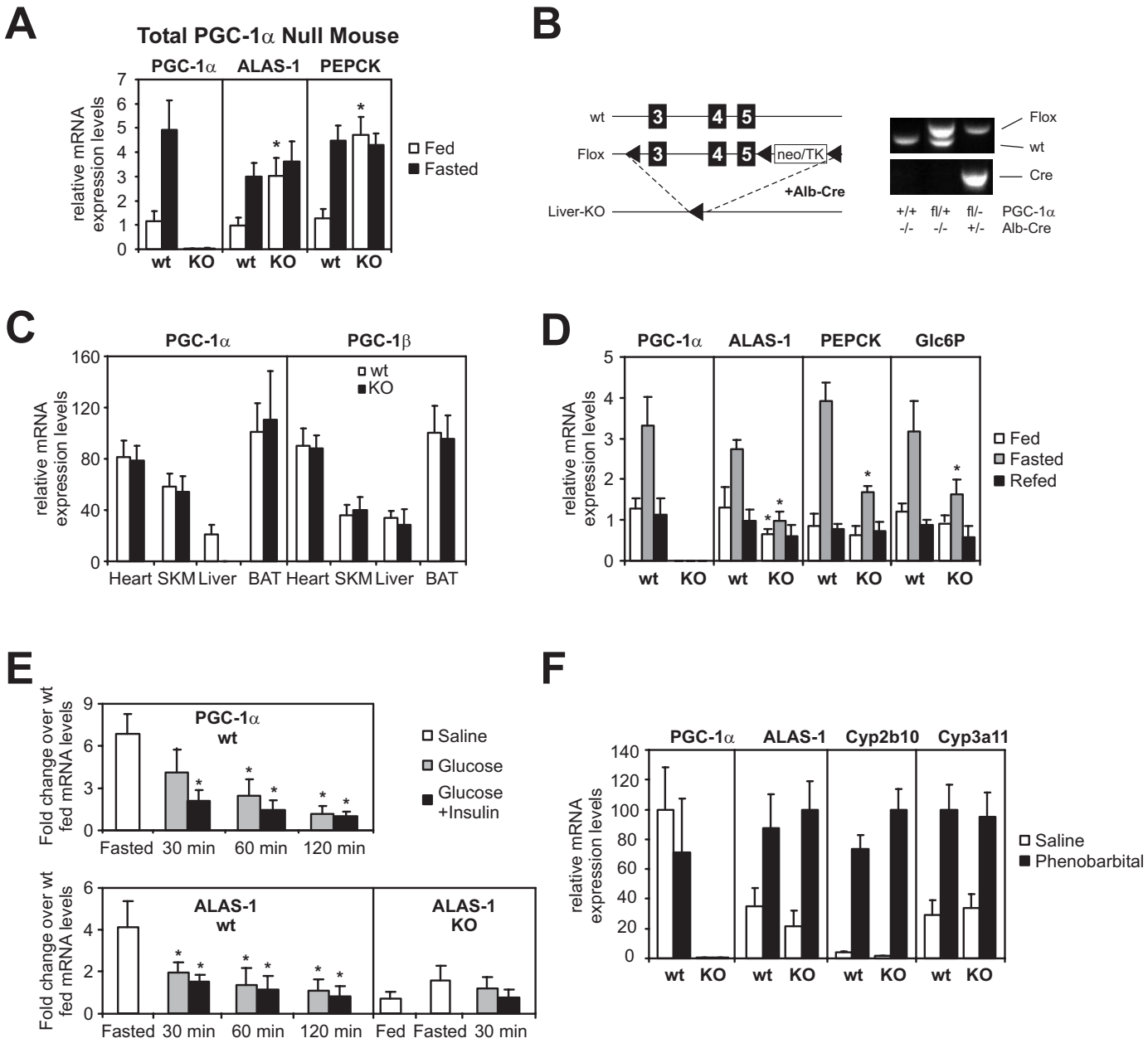

Fig. 6
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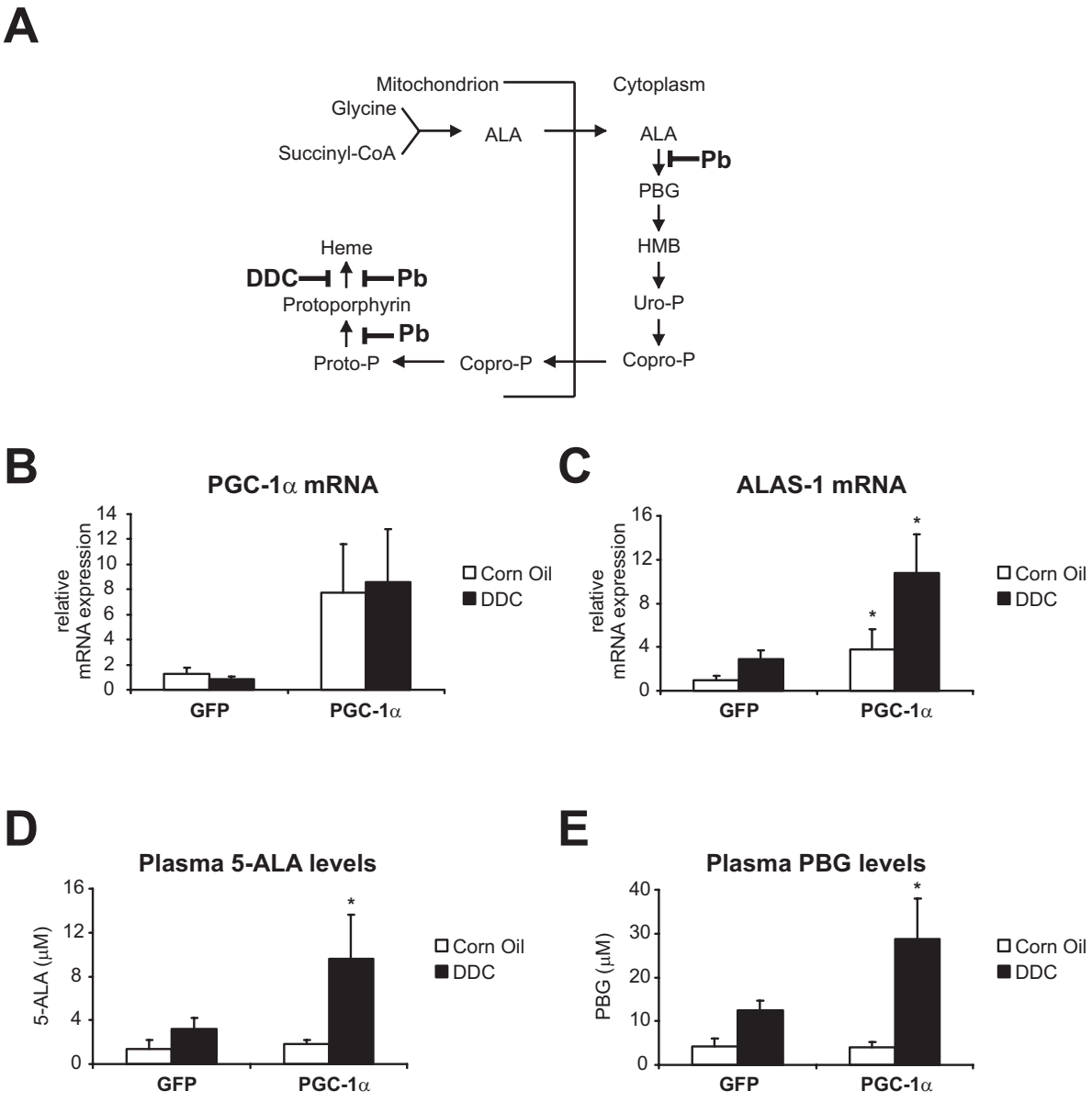
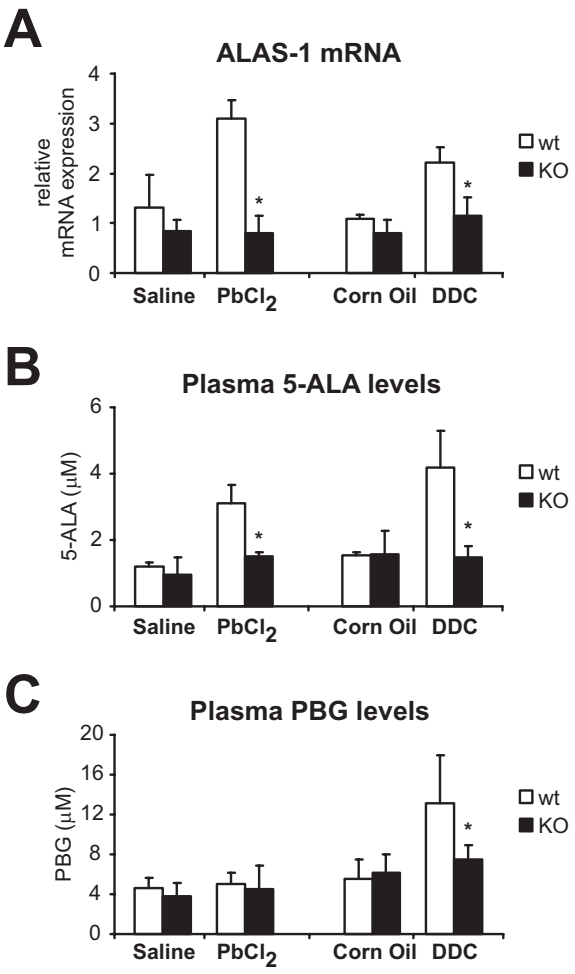


Figure 7

Fig. 7
Handschin *et al.*



Supplemental data 1
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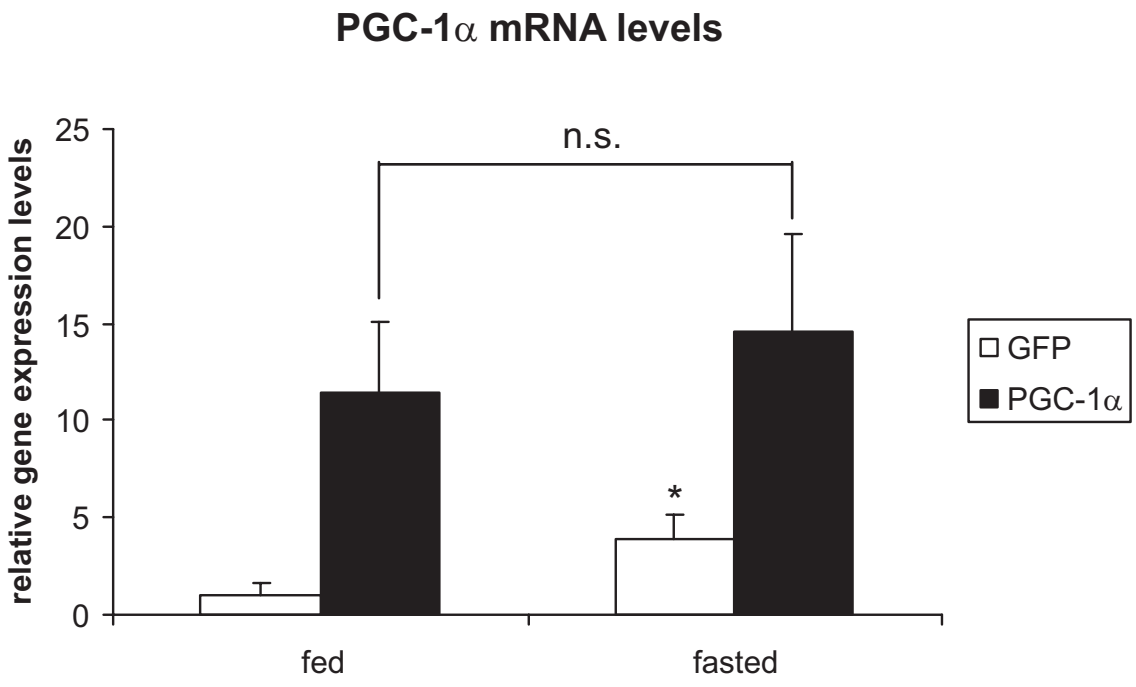

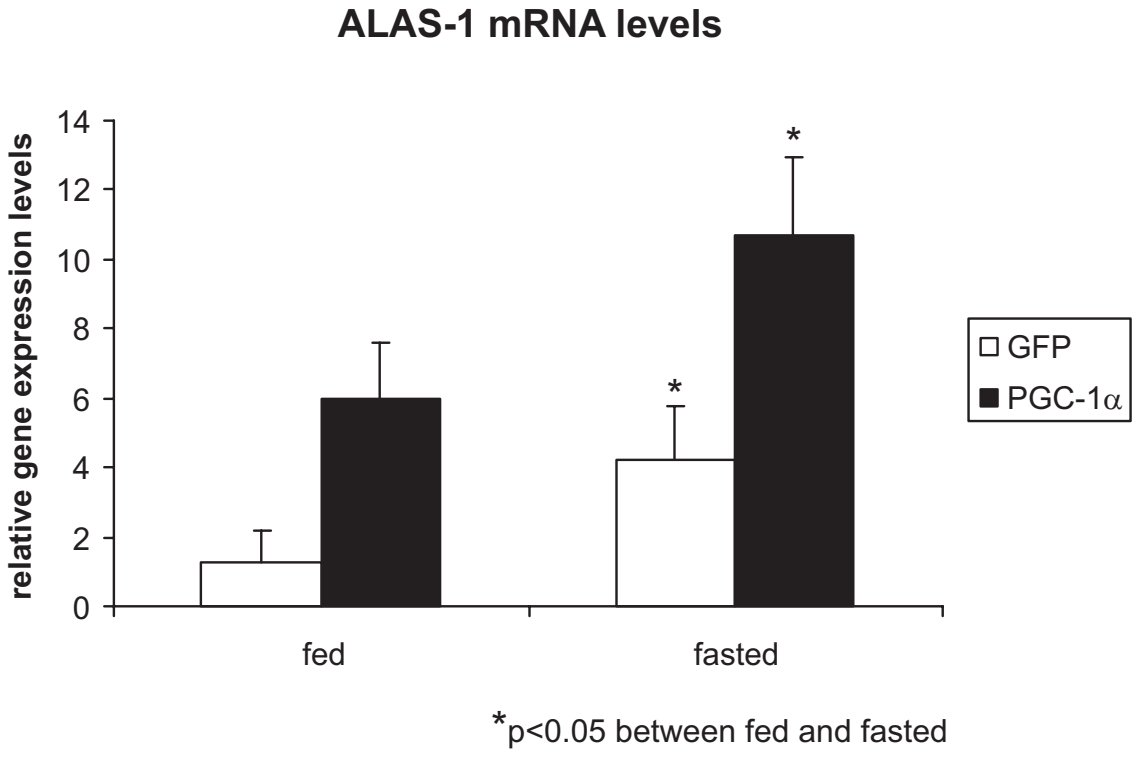

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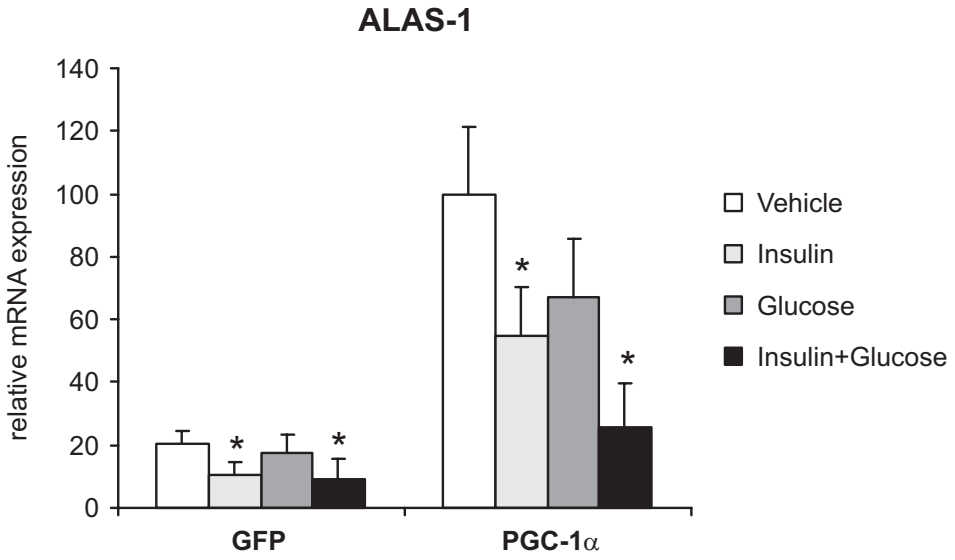

*p<0.05 between vehicle and glucose/insulin-treated cells