

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

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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 1 α (PGC-1 α). Elevation of PGC-1 α 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-1 α knockout animals, as is the ability of porphyrogenic drugs to dysregulate heme biosynthesis. These data show that PGC-1 α links nutritional status to heme biosynthesis and acute hepatic porphyria.

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 (Figure 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 aminolevulinate synthase (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 nonerythroid-synthesized heme is produced in the liver for various heme proteins, in particular

microsomal cytochromes P450. Because either a deficiency or an 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 δ -aminolevulinic acid (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 coproporphyrin, 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 and/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.

Peroxisome proliferator-activated receptor γ coactivator 1 α (PGC-1 α) is a coactivator of nuclear receptors and other transcription factors (Puigserver and Spiegelman, 2003). PGC-1 α 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-1 α is induced during fasting, when the liver ceases using glucose as an energy supply and changes to the β -oxidation of fatty

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³This paper is dedicated to the memory of our dear colleague and friend, Stanley J. Korsmeyer.

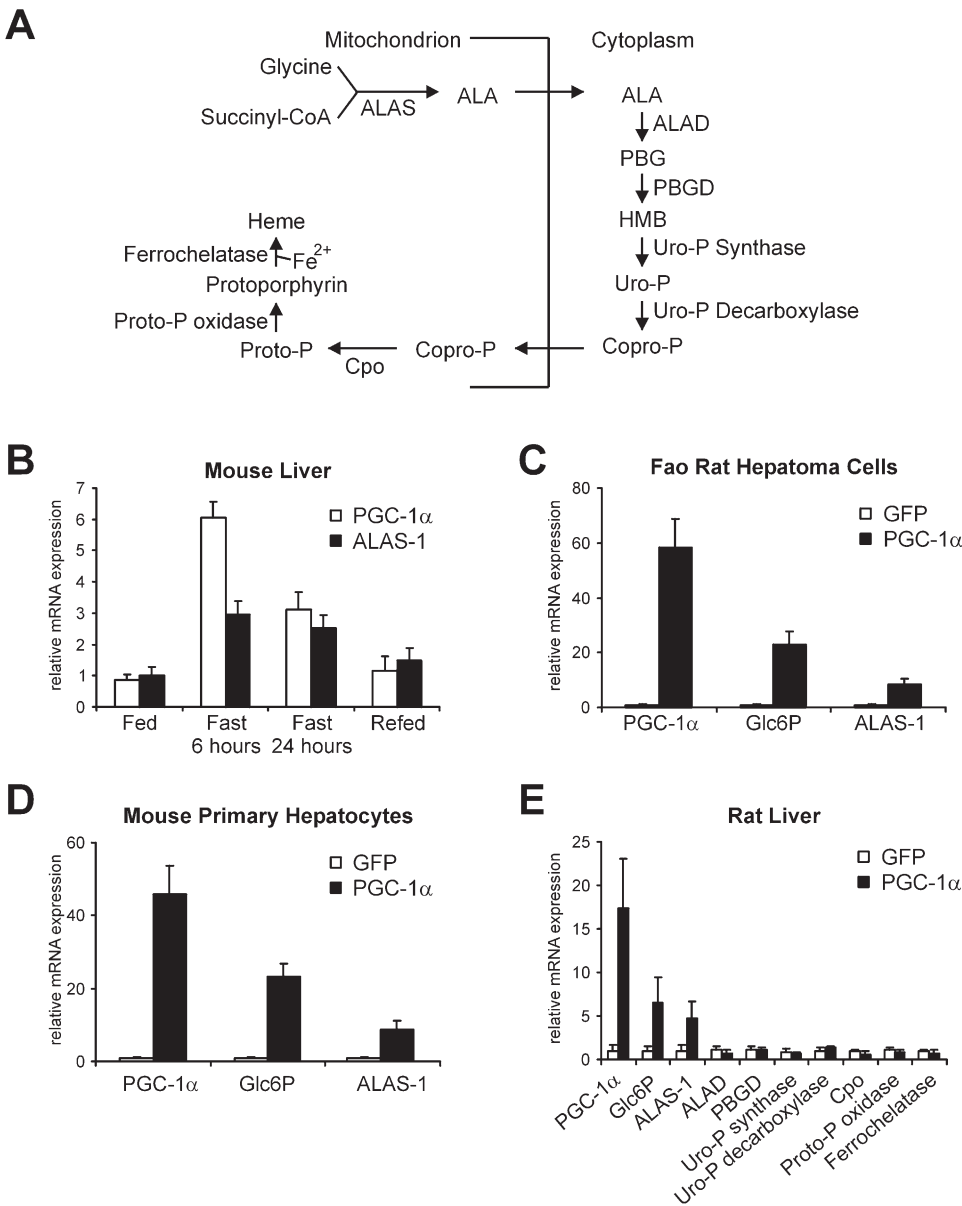


Figure 1. ALAS-1 Expression Is Activated by PGC-1 α in Hepatocytes and Liver In Vivo

(A) Heme biosynthesis pathway. ALA, 5-aminolevulinic acid; ALAD, ALA dehydratase; PBG, porphobilinogen; PBGD, PBG deaminase; HMB, hydroxymethylbilane; Uro-P, uroporphyrinogen; Copro-P, coproporphyrinogen; Cpo, coproporphyrinogen oxidase; Proto-P, protoporphyrinogen.

(B) ALAS-1 and PGC-1 α mRNAs are coinduced in fasting. Mice were fasted for 6 hr and 24 hr, respectively, and hepatic levels of PGC-1 α and ALAS-1 were compared to those of fed and refed animals by semiquantitative PCR.

(C and D) Adenoviral PGC-1 α 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-1 α , respectively, and relative mRNA levels of PGC-1 α , glucose-6-phosphatase (Glc6P), and ALAS-1 were determined by semiquantitative PCR 24 hr after infection.

(E) PGC-1 α induces ALAS-1 transcript levels in vivo. Male Wistar rats were tail-vein injected with adenovirus encoding for GFP and PGC-1 α , respectively. Five days postinjection, animals were sacrificed, and hepatic mRNAs were analyzed for changes in expression using semiquantitative PCR. Data in (B)–(E) are represented as mean \pm standard deviation.

acids. This increase in fatty-acid β -oxidation and elevation of hepatic gluconeogenesis are both under control of PGC-1 α (Herzig et al., 2001; Yoon et al., 2001).

Thus, because of the key role of PGC-1 α in liver energy homeostasis and the finding that many PGC-1 α

targets are heme proteins, we investigated the role of PGC-1 α in the regulation of hepatic heme biosynthesis by nutrition. We found that PGC-1 α is an important factor controlling the expression of ALAS-1 in the fasted and fed liver. Moreover, we showed that hepatic PGC-

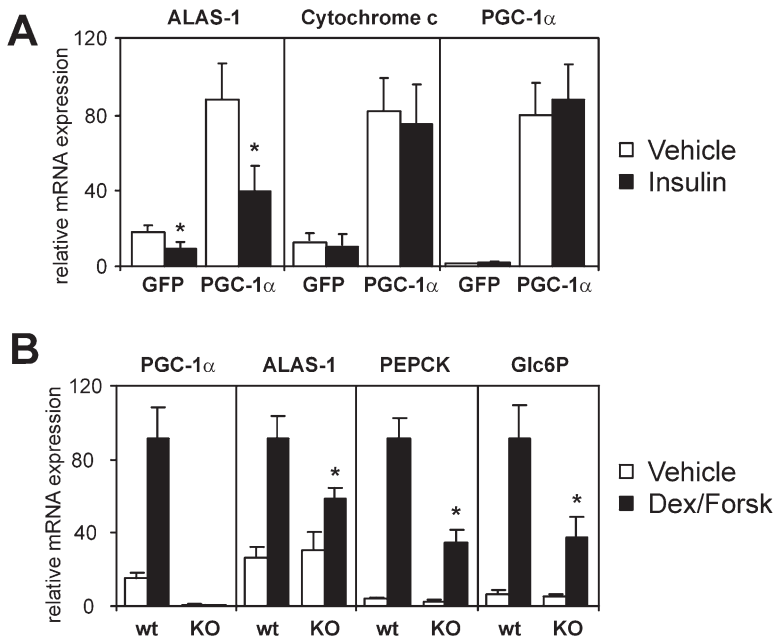


Figure 2. *ALAS-1* Expression Is Regulated by PGC-1 α and Insulin

(A) Insulin represses PGC-1 α -mediated induction of *ALAS-1*. H2.35 SV-40-transformed hepatocyte cells were infected with adenoviral GFP and PGC-1 α for 24 hr and subsequently treated with vehicle (PBS) or 10 nM insulin for 12 hr before relative *ALAS-1*, cytochrome c, and PGC-1 α 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-1 α . Mouse primary hepatocyte cultures were established from wild-type and PGC-1 α total knockout animals. These cells were subsequently treated with dexamethasone (Dex, 1 μ M) and forskolin (Forsk, 0.2 μ M) for 3 hr, and relative mRNA levels were determined by semiquantitative PCR. Data in (A) and (B) are represented as mean \pm standard deviation. * $p < 0.05$ between wild-type and knockout cells in Student's *t* test.

1 α is a major determinant of the severity of acute porphyric attacks in mouse models of chemical porphyria.

Results

Hepatic PGC-1 α and ALAS-1 Are Coregulated 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-1 α 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-1 α are coregulated in fasted mice, with increased mRNA levels (Figure 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-1 α . In all of these systems, ectopic expression of PGC-1 α increased *ALAS-1* transcript levels in a manner similar to that of glucose-6-phosphatase (*Glc6P*), a PGC-1 α target gene involved in gluconeogenesis (Yoon et al., 2001) (Figures 1C–1E). In contrast to *ALAS-1*, none of the other seven genes of the heme biosynthetic pathway were induced by PGC-1 α in rat liver (Figure 1E).

Insulin and Glucagon Regulation of ALAS-1 Involves PGC-1 α

Regulation of *ALAS-1* in fasting and feeding is mediated by the counterregulatory 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 (Figure 2A). Furthermore,

PGC-1 α -induced *ALAS-1* transcript levels are reduced by insulin, suggesting that PGC-1 α is in the pathway of the insulin regulation of *ALAS-1* (Figure 2A). Primary hepatocytes from wild-type and PGC-1 α knockout mice (Lin et al., 2004) were used to elucidate the function of PGC-1 α 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-1 α knockout hepatocytes as compared to wild-type cells (Figure 2B). Similarly, the response of the gluconeogenic genes phosphoenolpyruvate carboxykinase (*PEPCK*) and *Glc6P* to these hormones was blunted. These findings imply that PGC-1 α is involved in the fasting/feeding regulation of all three genes.

Insulin Repression of ALAS-1 Is Mediated by FOXO1 and PGC-1 α

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 coactivated by PGC-1 α (Wu et al., 1999). Thus, NRF-1 is a potential binding partner by which PGC-1 α controls *ALAS-1* expression. In addition to the NRF-1 site, an insulin-responsive element (IRE) has been defined in the *ALAS-1* promoter (Scassa et al., 2001, 2004), but the identity of transcription factors binding to this element has remained elusive (Figure 3A).

Chromatin immunoprecipitation assays in mouse hepatoma cells illustrate that PGC-1 α is recruited to both the NRF-1 and the IRE regions (Figure 3B). Moreover, PGC-1 α recruitment to the IRE region is sensitive

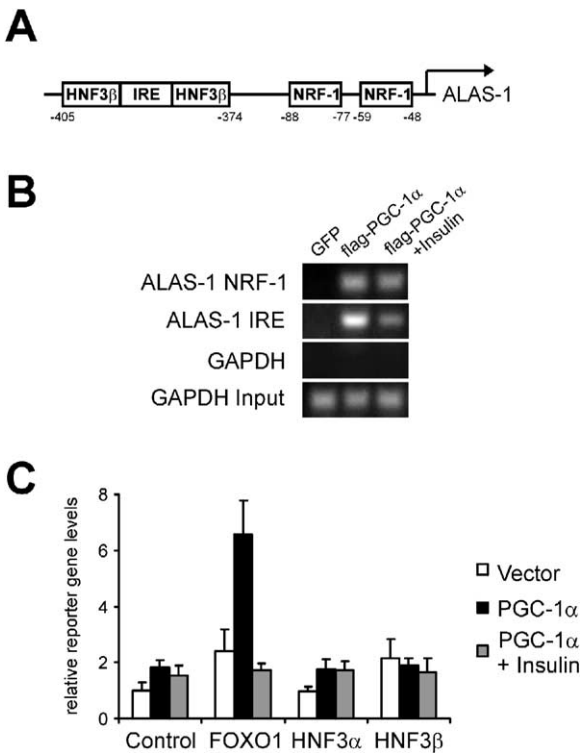


Figure 3. PGC-1 α Regulates *ALAS-1* Expression via NRF-1 and FOXO1

(A) Structure of the *ALAS-1* promoter.
 (B) PGC-1 α 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-1 α . Cells were treated with 10 nM insulin for 12 hr before cells were harvested and chromatin immunoprecipitation was performed using an anti-FLAG antibody.
 (C) PGC-1 α coactivates FOXO1 on the *ALAS-1* promoter. H.35 cells were transfected with *ALAS-1*-promoter construct and expression plasmids for FOXO1, HNF3 α (FOXA1), HNF3 β (FOXA2), and PGC-1 α . After transfection, cells were treated with 10 nM insulin for 12 hr before reporter-gene levels were determined. Data are represented as mean \pm standard deviation.

to insulin, in contrast to PGC-1 α binding to the NRF-1 site (Figure 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-1 α binds to FOXO1 at these elements. After insulin exposure, FOXO1 is phosphorylated, its binding to PGC-1 α 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 coworkers described the hepatocyte nuclear factor 3 β (HNF3 β , alternatively called FOXA2) to bind to sites adjacent to the *ALAS-1* IRE (Scassa et al., 2004) (Figure 3A). They found that integrity of the IRE and not of the HNF3 β sites is obligatory for insulin regulation of *ALAS-1* and that the regulation of *ALAS-1* by HNF3 β cannot account for the repression of *ALAS-1* by insulin (Scassa et al., 2004). We thus

tested the ability of PGC-1 α to coactivate the different Forkhead box family members FOXO1, hepatocyte nuclear factor 3 α (HNF3 α , FOXA1), and HNF3 β on the *ALAS-1* promoter. As described by Scassa et al., HNF3 β increased reporter-gene levels controlled by the *ALAS-1* promoter, whereas HNF3 α had no effect (Figure 3C). However, of the three transcription factors, PGC-1 α only coactivated FOXO1 in this context. In addition, merely the FOXO1-PGC-1 α -mediated induction of the *ALAS-1* promoter was repressed by insulin (Figure 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 (Figure 4A, lanes 4–7) and NRF-1 with the NRF-1 site (Figure 4A, lanes 1–3) in electrophoretic mobility shift assays. Site-directed mutagenesis of the FOXO1 site abolished binding of FOXO1 to this element (Figure 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 (Figure 4A, lane 6). Functionally, PGC-1 α coactivates both NRF-1 and FOXO1 in reporter-gene assays using the *ALAS-1* promoter in mouse H2.35 SV-40-transformed hepatocytes (Figure 4B). Mutagenesis of the NRF-1 or the IRE sites reduced the ability of PGC-1 α 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-1 α , strongly suggesting that NRF-1 and FOXO1 are the major binding partners of PGC-1 α in the *ALAS-1* promoter.

As shown in Figure 4C, insulin represses induction of *ALAS-1*-promoter-driven reporter-gene expression by either FOXO1 alone or in combination with PGC-1 α . In contrast, a nonphosphorylatable 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-1 α coactivation of FOXO1 3A. This suggests that the insulin repression of the *ALAS-1* promoter is controlled by the FOXO1-PGC-1 α interaction.

Liver-Specific PGC-1 α Knockout Animals Have a Blunted Induction of *ALAS-1* in Fasting

To investigate whether PGC-1 α 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-1 α (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-1 α , 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 (Figure 5A). It is unclear whether the same compensatory mechanisms (elevation of C/EBP β) 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-1 α knockout animals by crossing mice with a floxed PGC-1 α allele to transgenic

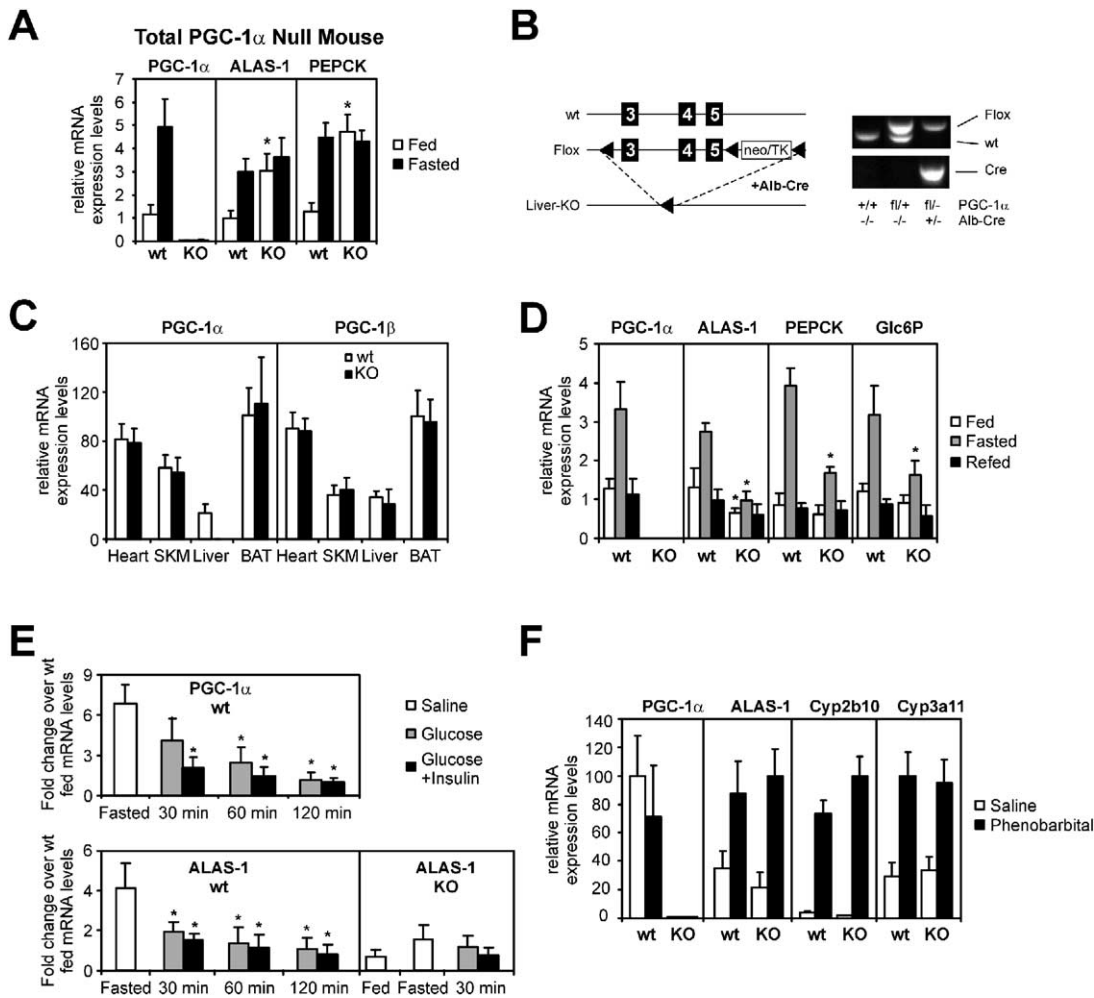


Figure 5. Fasting-Mediated Induction of *ALAS-1* Is Drastically Reduced in a Liver-Specific *PGC-1α* Knockout Model

(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 hr before relative mRNA levels for *PGC-1α*, *ALAS-1*, and phosphoenolpyruvate carboxykinase (*PEPCK*) were determined.

(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 hr, and relative transcript levels for *PGC-1α*, *ALAS-1*, *PEPCK*, and glucose-6-phosphatase (*Glc6P*) were determined by semiquantitative PCR.

(E) Glucose reverses fasting-mediated induction of *PGC-1α* and *ALAS-1*. Wild-type and liver-specific *PGC-1α* knockout animals were fasted for 6 hr and subsequently injected with vehicle, glucose, or glucose and insulin. After 30, 60, or 120 min, respectively, mice were sacrificed, livers were harvested, and relative *PGC-1α* and *ALAS-1* expression levels were determined.

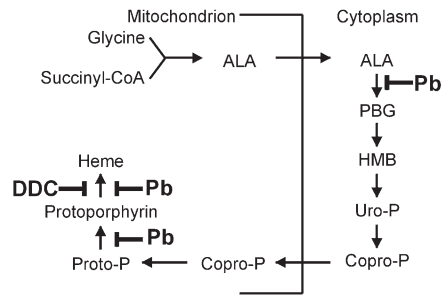
(F) Phenobarbital induces *ALAS-1* independent of *PGC-1α*. Wild-type and liver-specific *PGC-1α* knockout animals were injected i.p. with vehicle (saline) or phenobarbital (100 mg/kg). Sixteen hours after injection, livers were harvested, and relative mRNA levels of *PGC-1α*, *ALAS-1*, and cytochrome P450 2b10 and 3a11 (*Cyp2b10* and *Cyp3a11*, respectively) were determined by semiquantitative PCR. Data in (A) and (C)–(F) are represented as mean ± standard deviation. **p* < 0.05 between wild-type and knockout animals in Student's *t* test.

ficial 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 in the knockout mice, and, thus, no effect of glucose and/or insulin on *ALAS-1* transcript levels could be observed.

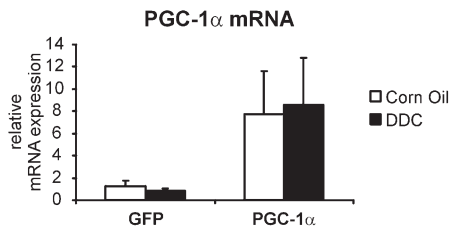
Taken together, these data strongly suggest that *PGC-1α* may be involved in fasting-induced acute porphyric attacks. Apart from fasting, certain drugs

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,

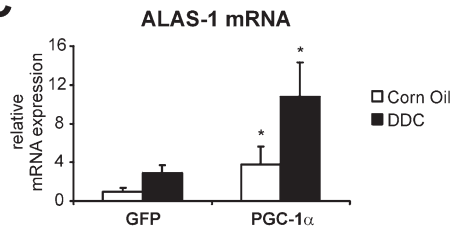
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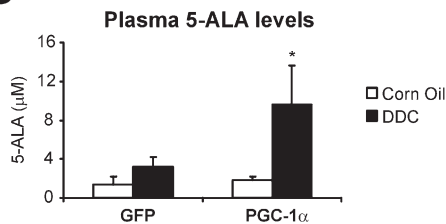
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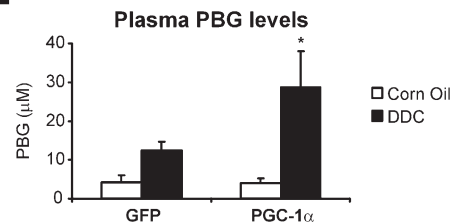


Figure 6. Ectopic Expression of PGC-1 α Elicits an Acute Porphyric Attack

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

we chose the barbiturate phenobarbital (PB), a classical drug that precipitates porphyric attacks in patients. As shown in Figure 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-1 α knockout animals. These data indicate that the role of PGC-1 α in ALAS-1 regulation does not extend universally to all other mechanisms, such as the induction by barbiturate drugs (Fraser et al., 2002, 2003; Podvinec et al., 2004).

Elevated Expression of PGC-1 α Causes Acute Attacks in Chemical Porphyria

The consequence of ALAS-1 regulation by PGC-1 α in fasting and feeding for acute porphyric attacks was

subsequently tested using proporphrogenic 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 (DDC) (Figure 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, DDC, 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-1 α . These animals were subsequently fasted and treated with vehicle (corn oil) or DDC for 24 hr. DDC did not change adenoviral ex-

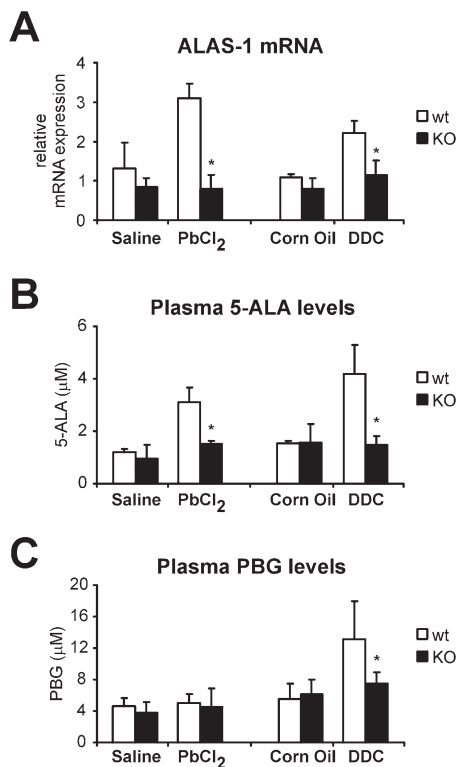


Figure 7. Liver-Specific *PGC-1 α* Knockout Animals Are Protected from Chemical Porphyrria

Lack of increase in *ALAS-1* mRNA and heme biosynthesis intermediates in liver-specific *PGC-1 α* knockout animals. Fasted wild-type and knockout animals were i.p. injected with saline and lead chloride (PbCl_2 , 20 mg/kg) or corn oil and DDC (10 mg/kg) for 24 hr. 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 \pm standard deviation. * $p < 0.05$ between wild-type and knockout animals in Student's *t* test.

pression of *PGC-1 α* mRNA (Figure 6B). In contrast, *ALAS-1* transcript levels were elevated 10-fold in animals that received both *PGC-1 α* adenovirus and DDC (Figure 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 (Figures 6D and 6E), 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-1 α* 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., 1996, 1999).

Liver-Specific *PGC-1 α* Knockout Animals Are Protected from Chemical Porphyrria

The requirement for *PGC-1 α* in fasting-induced porphyria was tested in the liver-specific *PGC-1 α* knockout animals. In fasted wild-type animals treated with lead chloride and DDC for 24 hr, increased *ALAS-1* mRNA levels were observed as compared to treatment with their vehicles saline and corn oil, respectively (Figure 7A). In contrast, neither lead chloride nor DDC changed

endogenous *PGC-1 α* levels (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 DDC treatment (Figure 7B). Since lead and DDC inhibit heme biosynthesis at different steps (Figure 6A), only DDC elevates PBG levels (Figure 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 Figure 6D and 6E. Strikingly, lead and DDC completely fail to induce either *ALAS-1* mRNA (Figure 7A) or plasma 5-ALA (Figure 7B) or PBG levels (Figure 7C) in the liver-specific *PGC-1 α* knockout mouse. These data indicate that *PGC-1 α* 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 coactivator *PGC-1 α* 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-1 α* 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-1 α* promoter (Herzig et al., 2001). In addition, CREB can also directly activate the *ALAS-1* promoter (Varone et al., 1999).

PGC-1 α activates the *ALAS-1* promoter by coactivating NRF-1 and FOXO1, both of which directly bind to the *ALAS-1* promoter (Figure 6). The ability of *PGC-1 α* to positively regulate the *ALAS-1* gene and the requirement for *PGC-1 α* 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-1 α* in mice with chemical inhibition of enzymes 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-1 α* 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-1 α . Glucagon is important in PGC-1 α 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-1 α and its export from the nucleus (Puigserver et al., 2003), thus inhibiting PGC-1 α action. Increased blood glucose levels would therefore be expected to alter the PGC-1 α 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-1 α levels after tail-vein injection of adenoviral vectors (see Figure S1 in the Supplemental Data available with this article online). Thus, despite high PGC-1 α 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-1 α -induced *ALAS-1* mRNA levels (Figure S2). 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 that elevate hepatic PGC-1 α levels are therefore potentially dangerous for patients with hepatic porphyrias. Accordingly, drugs and foods that induce PGC-1 α 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 μ g 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 $\Delta\Delta$ Ct method after amplification using a iCycler iQ real-time PCR detection system (Bio-Rad) and SYBRGreen (Bio-Rad). Data are represented as mean \pm 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. Ani-

mals were fed standard rodent chow and housed in a controlled environment with 12 hr 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; PbCl₂, 20 mg/kg; DDC, 10 mg/kg) were injected *i.p.*, and livers and blood were harvested after 16 or 24 hr. All groups consisted of at least three to six mice. Glucose (1 g/kg) and insulin (1.0 U/kg) were injected *i.p.* into mice that were fasted for 6 hr. Data are represented as mean \pm standard deviation. Significance is defined as $p < 0.05$ in Student's *t* test.

Generation of Liver-Specific PGC-1 α Knockout Animals

Generation of animals with floxed PGC-1 α 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 Laboratory, strain B6.Cg-Tg(Alb-cre) 21MGn/J) to obtain liver-specific PGC-1 α knockout mice.

Adenoviral Infection

Cultured cells were infected with adenovirus as published (Yoon et al., 2001). Twenty-four to forty-eight 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 were harvested. Data are represented as mean \pm 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 μ M 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 hr 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 \pm 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 radiolabeled 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-1 α for 24 hr and treated with vehicle or 10 nM insulin for 12 hr before cells were harvested, DNA-protein complexes crosslinked, 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 were 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 were subsequently analyzed by sequential ion-exchange chromatography using columns from the ALA/PBG by Column Test Kit (Bio-Rad) 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 \pm standard deviation. Significance is defined as $p < 0.05$ in Student's *t* test.

Supplemental Data

Supplemental Data include two figures and can be found with this article online at <http://www.cell.com/cgi/content/full/122/4/505/DC1/>.

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