The homeobox protein Prox1 is a negative modulator of ERRα/PGC-1α bioenergetic functions

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Estrogen-related receptor α (ERRα) and proliferator-activated receptor γ coactivator-1α (PGC-1α) play central roles in the transcriptional control of energy homeostasis, but little is known about factors regulating their activity. Here we identified the homeobox protein prospero-related homeobox 1 (Prox1) as one such factor. Prox1 interacts with ERRα and PGC-1α, occupies promoters of metabolic genes on a genome-wide scale, and inhibits the activity of the ERRα/PGC-1α complex. DNA motif analysis suggests that Prox1 interacts with the genome through tethering to ERRα and other factors. Importantly, ablation of Prox1 and ERRα have opposite effects on the respiratory capacity of liver cells, revealing an unexpected role for Prox1 in the control of energy homeostasis.

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Regulation of energy homeostasis involves elaborate biochemical pathways that have evolved to react to the metabolic needs of the organism in response to specific physiological states. While homeostatic regulation is generally under hormonal control and achieved through allosteric control and post-translational modifications of metabolic enzymes for immediate needs, organ-specific requirements and lasting adaptation require regulation of metabolic genes at the transcriptional level via the action of diverse classes of transcription factors and coregulatory proteins (Desvergne et al. 2006; Feige and Auwerx 2007). Among those factors, the orphan nuclear receptor estrogen-related receptor α (ERRα, NR3B1) and the coregulator peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) have been shown to play a predominant role in controlling several aspects of energy metabolism, most notably mitochondrial biogenesis and oxidative phosphorylation (Oxphos) (Lin et al. 2005; Giguère 2008).

Prospero-related homeobox 1 (Prox1) is a transcription factor essential for the development of numerous tissues, including the liver (Sosa-Pineda et al. 2000; Burke and Oliver 2002; Dudas et al. 2006). In particular, Prox1 plays a critical role in determining the fate of lymphatic endothelial cells and, consequently, *Prox1*-null embryos are devoid of lymphatic vasculature and die in utero at approximately embryonic day 14.5 (Wigle and Oliver 1999; Johnson et al. 2008). *Prox1* haploinsufficient mice also display lymphatic vascular defects that have been proposed to lead to adult-onset obesity via the promotion of adipogenesis and increased fat storage in lymphatic-rich regions (Harvey et al. 2005). Prox1 is also known to regulate the activity of a specific subset of nuclear receptors (Qin et al. 2004; Song et al. 2006; Lee et al. 2009; Yamazaki et al. 2009). Of particular interest, Prox1 was shown to regulate the activity of hepatocyte nuclear factor 4α (HNF4α, NR2A1) and liver receptor homolog-1 (LRH-1, NR5A2) on the *CYP7A1* and *PCK1* promoters, suggesting a possible role for Prox1 in the regulation of bile acid synthesis and gluconeogenesis in the liver (Qin et al. 2004; Song et al. 2006). Whether Prox1 plays a more comprehensive role in the regulation of energy metabolism is currently unknown.

Results and Discussion

Prox1 interacts with and modulates the activity of the ERRα/PGC-1α complex

An automated yeast two-hybrid interaction screen previously identified fragments of Prox1 as interactors of ERRα (Albers et al. 2005). We first sought to validate the physiological significance of this interaction by performing coimmunoprecipitation experiments with endogenous proteins present in the mouse liver. As observed in Figure 1A, ERRα could be detected in extract immunoprecipitated with a Prox1 antibody, while Prox1 could be detected in liver lysate immunoprecipitated with an ERRα antibody. As expected, but not shown previously, a potent in vivo interaction was observed in the mouse liver between ERRα and PGC-1α (Fig. 1A). Prox1 can be also found in a complex with PGC-1α. Direct interactions were detected between Prox1 and both ERRα and PGC-1α via in vitro GST pull-down experiments (Fig. 1B). Only the N terminus of Prox1 binds to PGC-1α, while both the N terminus and C terminus of Prox1 interact with ERRα. Prox1 interacts with ERRα solely through its DNA-binding domain (DBD) (Fig. 1C). Indeed, an altered Prox1 protein containing inactivation mutations for the two putative LxxLL interaction motifs (NR1/2mut) known to be required for the interaction with LRH1 and HNF4α (Qin et al. 2004; Song et al. 2006) was able to interact physically with ERRα. Finally, Prox1 was found to interact

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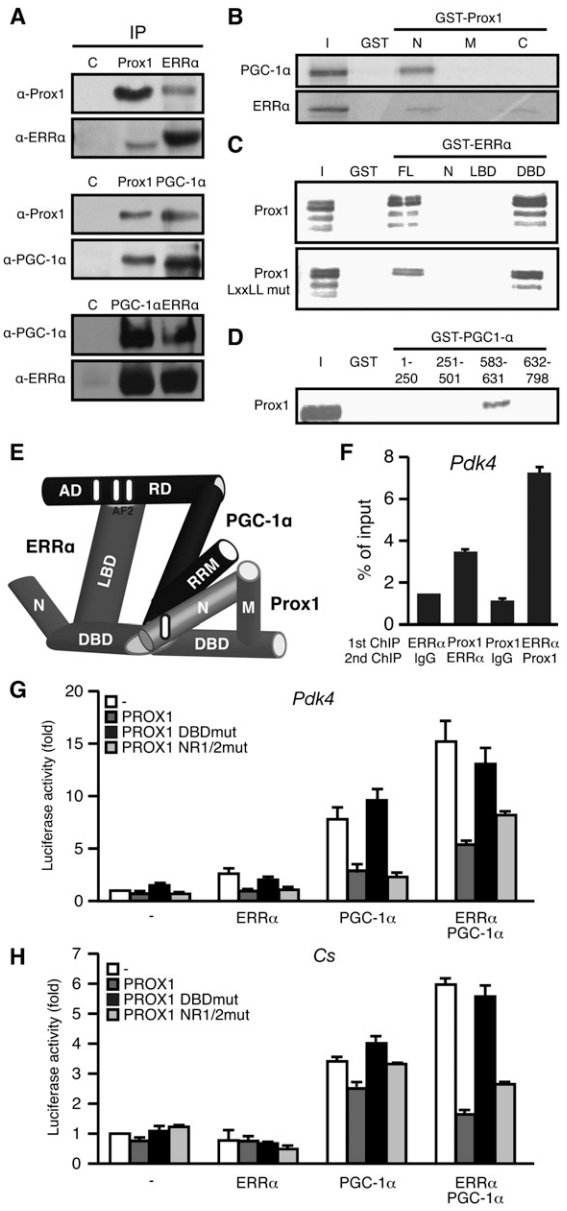

Figure 1. Prox1 interacts with and influences the transcriptional activity of ERRα and PGC-1α. (*A*) Prox1, ERRα, and PGC-1α interact in vivo. Lysates from mouse liver were subjected to immunoprecipitation and immunoblot analyses with the indicated antibodies. (*B*) Direct interactions between Prox1, PGC-1α, and ERRα. In vitro translated ERRα and PGC-1α were subjected to pull-down analysis with GST-Prox1 fragments. (N) N terminus; (M) middle; (C) C terminus. (*C*) Prox1 interacts with the DNA-binding domain (DBD) of ERRα. In vitro translated Prox1 and a LxxLL 1/2 mutant were subjected to pull-down analysis with GST-ERRα fragments. (FL) Full-length; (N) N terminus; (LBD) ligand-binding domain. (*D*) Prox1 interacts with a new functional domain of PGC-1α. In vitro translated Prox1 with GST-PGC-1α fragments. (*E*) Schematic representation of a potential trimeric interaction between Prox1, ERRα, and PGC-1α. (AD) Activation domain; (RD) repression domain; (RRM) RNA recognition motif; (AF-2) activation function 2; (white bars) LxxLL motifs. (*F*) Re-ChIP experiments performed in the mouse liver on the *Pdk4* promoter using either anti-ERRα or anti-Prox1 antibodies in a serial manner. (*G*) Effects of wild-type and mutant Prox1 proteins on the transcriptional activity of ERRα and PGC-1α. The *Pdk4* promoter–luciferase reporter gene was cotransfected in HepG2 cells with empty vector (−), ERRα, PGC-1α, or a combination of both expression vectors in the presence or absence of wild-type or mutant Prox1. (*H*) Same assay as in *G* using the *Cs* promoter as the reporter gene.

with PGC-1α via a domain comprised of residues 483–631, a domain without a previously assigned function (Fig. 1D). A schematic representation of the potential ERRα/Prox1/PGC-1α trimeric complex is shown in Figure 1E.

We next tested whether ERRα and Prox1 could form a complex on chromatin by performing a serial chromatin immunoprecipitation (ChIP) experiment in the liver at the *Pdk4* promoter. As shown in Figure 1F, re-ChIP for ERRα generated further enrichment following an initial ChIP for Prox1, while the converse re-ChIP experiment generated even more enrichment for Prox1 at the *Pdk4* promoter. Next, the *Pdk4* promoter was fused to the luciferase reporter gene, and the construct was cotransfected in HepG2 cells together with expression vectors for ERRα and PGC-1α. As shown in Figure 1G, introduction of Prox1 decreased both the basal and ERRα-induced and/or PGC-1α-induced luciferase activity. In agreement with the physical interaction data, the altered Prox1 protein containing inactivation mutations of the two putative LxxLL interaction motifs (NR1/2mut) still retains a repressive effect, while a Prox1 DBD mutant is no longer functional for ERRα target gene repression. Similar results were obtained when using reporter constructs linked to the *Cs*, *Cycs*, and *ApoC3/Apoa4* promoters (Fig. 1H; Supplemental Fig. S1).

ChIP-on-chip analyses identify a genomic relationship between ERRα and Prox1

We next performed genome-wide location analyses to assess the extent of the functional interaction between Prox1 and ERRα. To be able to directly relate the binding events to a specific target gene, we performed two distinct ChIP-on-chip experiments using tiled genomic DNA arrays covering the extended promoter regions (−5.5 to +2.5 kb from transcriptional start sites) of ~17,000 mouse genes and antibodies specific to ERRα and Prox1. Analysis of the ChIP-on-chip data sets identified 2479 and 2266 high-confidence binding sites mapping to the promoters of 2373 and 2069 genes in the mouse liver for ERRα and Prox1, respectively (Fig. 2A; Supplemental Tables S1, S2). Comparison of the data sets from both factors revealed that a total of 937 ERRα target genes are also targets of Prox1 (39.5% of all ERRα targets) (Fig. 2A). Standard ChIP validation and examination of the bound segments revealed four different classes of promoter regions targeted by the two factors (Fig. 2B,C). Of the 937 promoters shared by ERRα and Prox1, 527 contain a common segment bound by both factors (22% of all ERRα targets) (Fig. 2B). Considering that PGC-1α can interact with both Prox1 and ERRα (Fig. 1), we next tested whether PGC-1α associates with chromatin at sites bound specifically by Prox1 or ERRα, or only when both factors are present. Standard ChIP analysis revealed that PGC-1α is recruited at DNA segments recognized by Prox1 or ERRα (Fig. 2B), consistent with the observation that PGC-1α interacts directly with both partners (Fig. 1B).

The results of the ChIP-on-chip experiments were then analyzed using motif-finding algorithms. First, we searched for known transcription factor-binding motifs that were enriched in bound segments, and found that, in both ERRα-specific and ERRα/Prox1 shared segments, the most enriched motifs were ERREs (Supplemental Table S3). In agreement with our previous analysis (Dufour et al. 2007), CREB-binding motifs were also enriched in ERRα-specific segments, suggesting that the functional interaction between ERRα and CREB observed

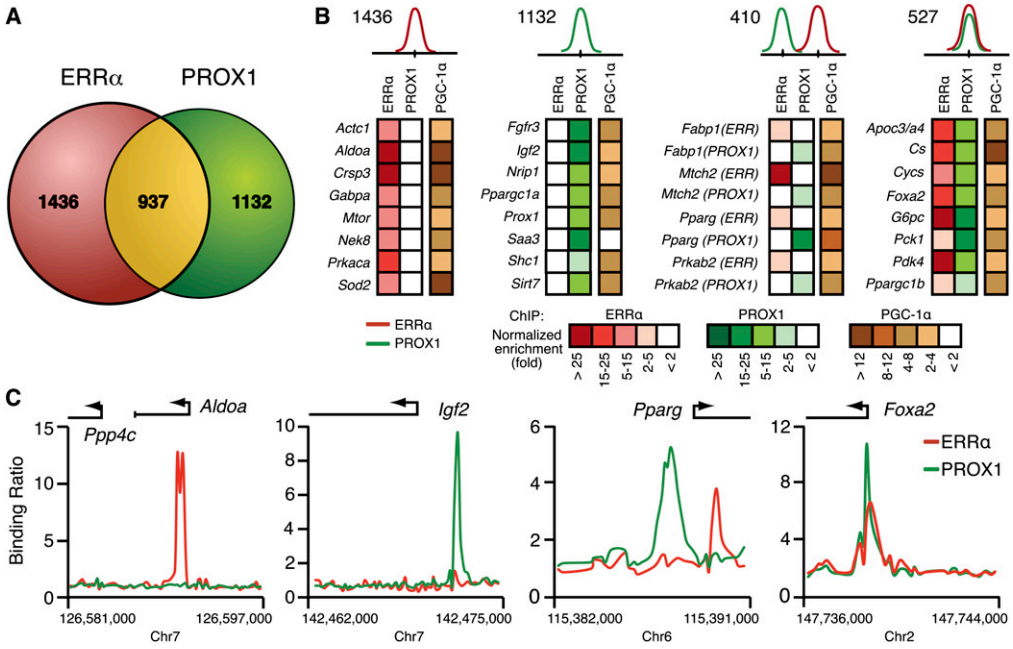

Figure 2. Genome-wide promoter occupancy of ERRα and Prox1 in mouse liver. (*A*) Venn diagrams illustrating the overlap in ERRα (red) and Prox1 (green) direct target genes from ChIP-on-chip analyses in the mouse liver. (*B*) Standard ChIP validation of a subset of ERRα-enriched (red) and Prox1-enriched (green) segments. Occupancy of PGC-1α on these selected DNA segments bound by ERRα, Prox1, or both factors as assayed by standard ChIP is also shown. (*C*) Representative binding profiles of ERRα (red line) and Prox1 (green line) on specific or common target extended promoters containing either distinct or overlapping binding sites.

in the heart is also operational in the liver. On the other hand, analysis of Prox1-specific segments revealed enrichment of HNF4α- and C/EBPβ-binding sites (Supplemental Table S3). Next, we tested a dictionary of 741 motifs identified by conservation across four mammals (Xie et al. 2005) for enrichment in bound segments using motifADE (Mootha et al. 2004), and again found that the most significantly enriched motifs in both ERRα-specific and ERRα/Prox1 shared segments were ERREs (Table 1). However, while several motifs were enriched in Prox1-specific segments, we were not able to identify with high confidence any homeobox-like motifs.

The ERRα regulon

We next evaluated the biological processes associated with genes with promoter regions that are enriched specifically for ERRα, Prox1 or both factors. As expected from previous work (Mootha et al. 2004; Schreiber et al. 2004; Dufour et al. 2007; Sonoda et al. 2007; Deblois et al. 2009), analysis showed ERRα target genes highly enriched for processes linked to metabolism (Fig. 3A; Supplemental Fig. S2). ERRα/Prox1 shared genes were also significantly enriched for the tricarboxylic acid (TCA) cycle as well as pyruvate metabolism. Similarly, Prox1-specific genes were significantly enriched for glycolysis/gluconeogenesis and pyruvate metabolism, and also for bile acid, histidine, and purine metabolism, but were virtually absent for Oxphos and the TCA cycle (Supplemental Fig. S3). Remarkably, precise assignment of ERRα and Prox1 target genes to pathways involved in energy production revealed that ERRα binds to the extended promoter regions of genes encoding enzymes at every step in the glycolytic pathway, pyruvate metabolism, and TCA cycle (Fig. 3B). This cluster of functionally linked genes is subsequently referred to as the ERRα bioenergetic regulon. ERRα-bound segments can also be found in the promoter regions of a large number of genes encoding proteins that constitute the five complexes of the Oxphos pathway (Fig. 3B). The ERRα bioenergetic regulon also includes a significant number of genes whose extended promoter regions are bound by Prox1, most notably genes encoding enzymes at key entry points in energy production pathways such as *G6pc*, *Ldhb*, *Pdk4*, *Pcx*, *Pck1*, *Cs*, and *Fh1*.

Divergent regulation of bioenergetic functions by ERRα and Prox1

We then examined the role of ERRα and Prox1 in the regulation of bioenergetic functions in HepG2 cells. We first demonstrated that ERRα, Prox1, and PGC-1α are indeed present and can interact with each other in HepG2 cells (Supplemental Fig. S4). We also showed, using siRNAs to silence ERRα and Prox1 expression, that both factors can regulate a subset of metabolic genes indentified as ERRα target genes in the mouse liver (Supplemental Fig. S5). The HepG2 expression data set indicates that ERRα and Prox1 have, in general, contrasting effects on the expression of genes involved in bioenergetic pathways. We next measured in vivo cellular respiration and glycolytic rates in HepG2 cells in the presence or absence of two specific sets of siRNAs against either ERRα or Prox1 (Fig. 4A; Supplemental Fig. S6). HepG2 cells treated with control siRNA displayed an expected cellular respiration rate (OCR) profile that was first inhibited by addition of the ATP synthase (Complex V) inhibitor oligomycin, then enhanced with the uncoupling agent p-trifluoromethoxy carbonyl cyanide phenyl hydrazone (FCCP), and repressed again with the Complex I

Table 1. *De novo enriched motifs in ERRα, ERRα/Prox1, or Prox1 target promoters at* $P < 0.01$

Motif	Annotation	ERRα	ERRα/Prox1	Prox1
TGACCTY	NR	5.75×10^{-144}	1.58×10^{-27}	NS
TGACCTTG	SF1	7.10×10^{-132}	1.06×10^{-13}	NS
TGACCT	NR	1.64×10^{-113}	3.75×10^{-24}	NS
GTGACCY	NR	5.08×10^{-47}	1.08×10^{-3}	NS
GTGWMCTT	SF1	8.04×10^{-40}	NS	NS
GTGNCMTTG	SF1	3.98×10^{-38}	1.50×10^{-4}	NS
YSACCWTGG	SF1	3.71×10^{-30}	9.00×10^{-3}	NS
CTGWCCTTNR	NR	2.02×10^{-25}	NS	NS
GAAGGTMR	NR	4.96×10^{-25}	1.82×10^{-3}	NS
GGTNACNTTG	CREB	1.09×10^{-21}	NS	NS
GAAKKTCA	—	3.48×10^{-17}	NS	NS
RGGTGACNY	CREB	8.78×10^{-16}	NS	NS
GGTGACNT	CREB	4.34×10^{-15}	NS	NS
YTTGAMCTT	NR	8.81×10^{-14}	NS	NS
GTGRNYTTGG	SF1	2.25×10^{-12}	NS	NS
YYTTGACCY	NR	1.80×10^{-11}	2.91×10^{-4}	NS
YGTCCTTGT	—	4.19×10^{-5}	NS	NS
TGAMCTTT	NR	5.98×10^{-4}	5.64×10^{-4}	8.09×10^{-6}
AGGTGA	MYOD	1.89×10^{-3}	NS	NS
RAGTGACNY	CREB	2.47×10^{-3}	NS	NS
TGCCAAR	NF1	NS	5.52×10^{-4}	3.46×10^{-10}
RATCRATA	CDP	NS	NS	3.31×10^{-6}
GGACTTY	NFKB, TEF1	NS	6.02×10^{-3}	5.55×10^{-5}
TAANMAAG	NKX61, LHX3	NS	NS	5.93×10^{-4}
AAAYATT	FOXJ2, TBP	NS	NS	7.63×10^{-4}
TTCYNRGAA	IK1, STAT	NS	NS	2.03×10^{-3}
$TTGRN_6TCCAR$	—	NS	NS	4.88×10^{-3}
GCCARGAA	ETS2, ELK1	NS	NS	5.09×10^{-3}
TTTNAAC	POU3F2, OCT	NS	NS	5.91×10^{-3}

Nuclear receptor (NR) includes ERRα (NR3B1), estrogen receptor α (NR3A1), GNCF (NR6A1), thyroid hormone receptor α (NR1A1), RORA (NR1F1), and COUP-TF (NR2F1). (NS) Not significant.

inhibitor rotenone (Fig. 4B; Supplemental Fig. S6). HepG2 cells lacking ERRα and Prox1 were found to have a decreased and increased response to FCCP relative to control cells, respectively. The FCCP-stimulated OCRs show that HepG2 cells lacking ERRα display impaired mitochondrial function, and that cells lacking Prox1 have a greater cellular respiratory capacity. Our experiment also revealed that cells treated with ERRα siRNA were found to have significantly lower extracellular acidification rates following oligomycin and FCCP addition (Fig. 4C; Supplemental Fig. S6), indicating that the presence of ERRα is indispensable for the ability of HepG2 cells to switch from oxidative to glycolytic metabolism.

The work presented herein not only extends the repertoire of nuclear receptors with which Prox1 physically interacts to include ERRα, but broadens these functional interactions to a coactivator protein, PGC-1α. Furthermore, the identification of ERRα and Prox1 target genes in the mouse liver establishes a unique relationship between the two factors at both a genomic and functional level. Our study also demonstrates the efficacy of the ChIP-on-chip on promoter array approach to define the regulon of a eukaryotic transcription factor. Indeed, we show that ERRα binds to the extended promoter regions of genes encoding virtually all enzymes involved in glycolysis, pyruvate metabolism, and the TCA cycle. The relevance of the metabolic role of ERRα and Prox1 was further probed by monitoring in vivo cellular respiration and glycolytic rates in HepG2 liver cells upon ERRα and Prox1 knockdown. The results indicate that ERRα and Prox1 have opposite effects on the respiratory capacity of liver cells, and that the presence of ERRα is essential for the switch to glycolysis when mitochondrial Oxphos is unable to meet the energy demands of the cell.

One of the unexpected elements of this study is the identification of the ERRα bioenergetic regulon. In prokaryotes and lower eukaryotes, regulons represent a widespread mechanism to coordinate the concurrent expression of a group of genes by a common transcription factor. In higher eukaryotes, the complexity of gene regulation is often linked to a multitude of extracellular signals, and this may preclude the use of a common factor to regulate all genetic components of an integrated biochemical pathway. Remarkably, ERRα occupies the extended promoter regions of practically all genes encoding enzymes of three well-defined biochemical pathways involved in the generation of energy from glucose. The potential to regulate linked biochemical pathways involved in energy metabolism likely evolved to ensure a coordinated increase in energy output in response to physiological stressors that are known to up-regulate the expression of the ERRα protein ligands PGC-1α and/or PGC-1β.

Prox1 is a homeobox protein, and, as expected, most studies probing its functions focused on embryonic development (Wigle and Oliver 1999; Sosa-Pineda et al. 2000; Kamiya et al. 2008). The results of our study demonstrate a novel and comprehensive role for Prox1

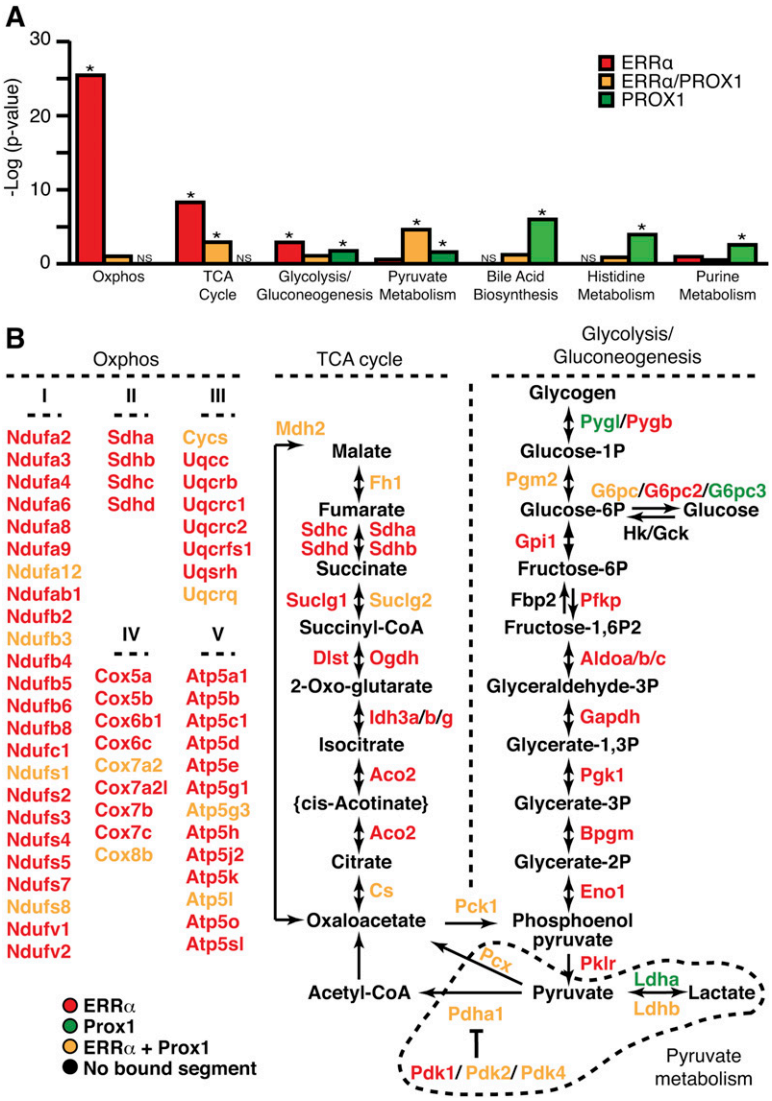

Figure 3. The ERRα bioenergetic regulon. (*A*) Enrichment of canonical metabolic pathways in the ChIP-on-chip target genes determined to be common (yellow) or specific to either ERRα (red) or Prox1 (green). (NS) Not significant; (*) $P < 0.05$. (*B*) ChIP-on-chip direct target genes specific to ERRα (red) or Prox1 (green) or shared by both factors (yellow) involved in metabolic pathways are shown. All genes involved in glycolysis, pyruvate metabolism, and the TCA cycle are targets of ERRα, a cluster of genes defining the ERRα bioenergetic regulon. Genes labeled in black were not identified as being enriched by either ERRα or Prox1.

in the direct control of energy homeostasis. Prox1 thus joins HNF1β as a rare example of a homeobox-containing factor involved directly in the transcriptional regulation of metabolism (Desvergne et al. 2006). However, our computational analysis of sequence-specific DNA recognition suggests that Prox1's sequence-specific binding to the genome in the adult liver is likely through interaction with other factors, most prominently ERRα, C/EBPβ, and HNF4α. Our results thus suggest that Prox1's main mode of action in this context is that of a corepressor. While the exact molecular mechanism by which Prox1 exerts its repressor effect remains to be determined, preliminary analysis indicates that immunoprecipitated Prox1 is not associated with histone deacetylase (HDAC) activity (Supplemental Fig. S7).

In conclusion, we demonstrated that Prox1 acts as a negative modulator of the ERRα/PGC-1α axis, and, as such, regulates a broad transcriptional program implicated in the control of energy homeostasis in the liver that includes a newly defined bioenergetic regulon controlled by ERRα. Our results also reinforce the concept that the ERR isoforms are essential factors controlling the transition from carbohydrate-based to lipid-based oxidative metabolism (Alaynick et al. 2007; Dufour et al. 2007). The physiological significance of these findings is further highlighted by the recent identification of Prox1 as a genetic locus implicated in fasting glucose homeostasis and increased risk for type II diabetes (Dupuis et al. 2010). Thus, the identification of Prox1 as an important regulator of the ERRα/PGC-1α axis suggests that novel strategies for managing diseases involving long-term energy imbalance can be envisaged.

Materials and methods

Animals

Adult male C57BL/6J mice were housed and fed standard chow in the animal facility at the McGill University Health Center. In all experiments involving mouse livers, mice were sacrificed during the day at ZT (Zeitgeber time) 4.

Cell culture and reporter assays

Luciferase constructs and reporter assays in HepG2 cells are described in the Supplemental Material.

Coimmunoprecipitation, immunoblotting, and GST pull-down assays

Coimmunoprecipitation, Western blot, and GST pull-down assays involving ERRα, PGC-1α, and Prox1 are described in the Supplemental Material.

ChIP, re-ChIP, ChIP-on-chip, ChIP-qPCR, and functional analysis of target genes

Mouse liver ERRα, Prox1, and PGC-1α ChIP, serial ChIP, and genome-wide location analyses performed using Agilent extended promoter arrays are described in the Supplemental Material. Bed files are available on request. Primer sequences used for ChIP-qPCR are shown in Supplemental Table S4. Ingenuity Pathway Analysis software (Ingenuity Systems, http://www.ingenuity.com) was used for functional analysis of target genes (see the Supplemental Material).

Computational motif discovery

Enriched motifs within ERRα and Prox1 ChIP-on-chip targets were identified using MOTIFCLASS (Smith et al. 2006) and motifADE (Mootha et al. 2004) as described in the Supplemental Material.

siRNA, qRT–PCR, and extracellular flux (XF) analysis

ERRα and Prox1 knockdown experiments in HepG2 cells with subsequent qRT–PCR and bioenergetic analysis using a Seahorse Extracellular Flux (XF24) Analyzer are described in the Supplemental Material. Primers used for qRT–PCR are shown in Supplemental Table S5.

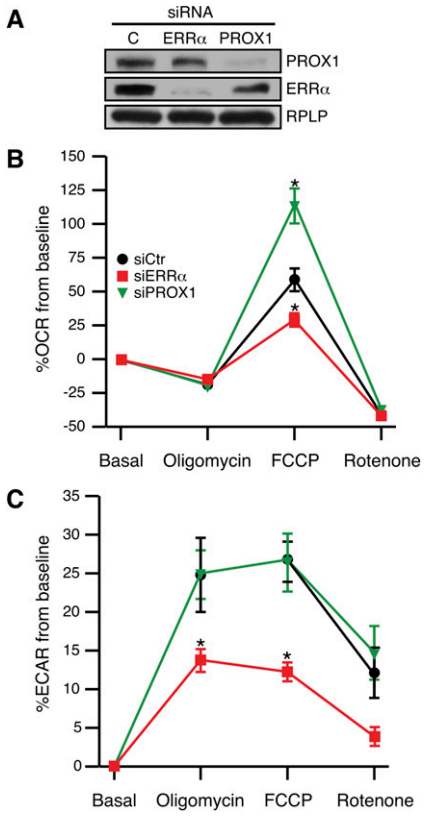

Figure 4. Divergent regulation of mitochondrial functions by ERRα and Prox1. (*A*) Western blot analysis on lysates prepared from the HepG2 knockdown samples is shown with the respective antibodies as indicated. Detection of RPLP was used a control. (*B*,*C*) Cellular oxygen consumption (*B*) and extracellular acidification rates (*C*) were measured in intact HepG2 cells treated with either control siRNA or an siRNA against ERRα or Prox1. Rates determined following sequential addition of oligomycin, FCCP, and rotenone were taken from an average of two measurements and are expressed as a percentage of the baseline rates. (*) $P < 0.05$.

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