

## Reduced Fat Mass in Mice Lacking Orphan Nuclear Receptor Estrogen-Related Receptor $\alpha$

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**The estrogen-related receptor  $\alpha$  (ERR $\alpha$ ) is an orphan member of the superfamily of nuclear hormone receptors expressed in tissues that preferentially metabolize fatty acids. Despite the molecular characterization of ERR $\alpha$  and identification of target genes, determination of its physiological function has been hampered by the lack of a natural ligand. To further understand the *in vivo* function of ERR $\alpha$ , we generated and analyzed *Estrra*-null (ERR $\alpha^{-/-}$ ) mutant mice. Here we show that ERR $\alpha^{-/-}$  mice are viable, fertile and display no gross anatomical alterations, with the exception of reduced body weight and peripheral fat deposits. No significant changes in food consumption and energy expenditure or serum biochemistry parameters were observed in the mutant animals. However, the mutant animals are resistant to a high-fat diet-induced obesity. Importantly, DNA microarray analysis of gene expression in adipose tissue demonstrates altered regulation of several enzymes involved in lipid, eicosanoid, and steroid synthesis, suggesting that the loss of ERR $\alpha$  might interfere with other nuclear receptor signaling pathways. In addition, the microarray study shows alteration in the expression of genes regulating adipogenesis as well as energy metabolism. In agreement with these findings, metabolic studies showed reduced lipogenesis in adipose tissues. This study suggests that ERR $\alpha$  functions as a metabolic regulator and that the ERR $\alpha^{-/-}$  mice provide a novel model for the investigation of metabolic regulation by nuclear receptors.**

Nuclear receptors are ligand-regulated transcription factors that control key pathways required for normal development and maintenance of homeostasis throughout life (37). Nuclear receptors now comprise a family of 48 genes in mice and humans that encode structurally and functionally related proteins. However, the existence of fewer than 10 receptors had been predicted by classic physiological and biochemical studies (10). Since the discovery of many nuclear receptors had not been anticipated and thus is not linked to recognized natural ligands, these new gene products were referred to as orphan nuclear receptors. During the last decade, extensive study of this gene family revealed that orphan nuclear receptors control essential developmental and metabolic functions in response to natural ligands as diverse as steroid hormones, retinoic acids, leukotrienes, bile acids, cholesterol metabolites, and long-chain fatty acids (reviewed in references 5, 26, and 48). In addition, orphan nuclear receptors have been shown to react to the presence of exogenous ligands such as pesticides (15, 70), phenobarbital (53, 61), and a wide variety of xenobiotic agents and drugs (2, 24, 39, 55, 66, 68, 69). Gene deletion analyses in mice have been particularly useful to uncover biological functions of orphan nuclear receptors. Nuclear orphan receptors have been shown to participate in the development and/or maintenance of the placenta, somitogenesis, brain, heart, hypothalamus-pituitary axis, immune system, and pathways controlling steroidogenesis and cholesterol metabolism.

The estrogen-related receptor  $\alpha$  (ERR $\alpha$ ) (NR3B1) was the first orphan nuclear receptor identified more than a decade ago on the basis of its close homology to the classic estrogen receptor  $\alpha$  (ER $\alpha$ ) (NR3A1) (14). ERR $\alpha$  was also identified as a repressor of the simian virus 40 major late promoter, revealing that ERR $\alpha$  had the ability to regulate gene expression in the absence of exogenous stimuli (67). Two other orphan nuclear receptors, ERR $\beta$  (NR3B2) (6, 14) and ERR $\gamma$  (NR3B3) (9, 16, 18), also belong to the same subfamily.

ERR $\alpha$  expression begins at day 9.5 post coitum in extraembryonic tissues and is later detected in the heart, intestine, brain, spinal cord, brown fat, and bone (4, 50, 62). ERR $\alpha$  is expressed in a nearly ubiquitous fashion in adult tissues but most prominently in tissues demonstrating a high capacity for fatty acid  $\beta$ -oxidation or activation, suggesting that ERR $\alpha$  may play a role in regulating cellular energy balance (14, 21, 50). This is further supported by the observations that ERR $\alpha$  is a direct regulator of the medium-chain acyl coenzyme A dehydrogenase gene (MCAD) (50, 64) and that PGC-1, a coactivator regulating transcription in response to signals relaying metabolic needs, is a potent regulator of ERR $\alpha$  activity (19, 21, 49).

Recent experiments have also shown that ERR $\alpha$  is functionally closer to the ERs than originally anticipated (reviewed in reference 13). Like the ERs, the ERRs recognize the ERE as a homodimer, suggesting that these receptors may control overlapping regulatory pathways (32, 45, 73). In addition, ERR $\alpha$  binds to extended half-sites with the consensus sequence TCAAGGTCA referred to as an ERRE (22, 50, 64, 67, 71). ER $\alpha$  has also been shown to recognize a subset of ERREs

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and stimulate the transcriptional activity of promoters containing such sites, reinforcing the concept that the two classes of related receptors share common target genes (63). It has also been discovered that the synthetic estrogen diethylstilbestrol and the partial estrogen antagonist 4-hydroxytamoxifen can act as selective inverse agonists on the three ERR isoforms, leading to the dissociation of coactivator proteins and loss of transcriptional activation function (8, 58, 59). In addition, we have recently demonstrated that the ERR isoforms can regulate the transcriptional activity of the human breast cancer marker gene pS2 and, unexpectedly, that the full transcriptional activity of the ERs and the ERRs on the pS2 gene is dependent on the presence of a previously unidentified ERRE within its promoter (32). Taken together, these results suggest that ERR $\alpha$  may also influence classic endocrine estrogenic pathways. However, in spite of these studies, the exact physiological roles of ERR $\alpha$  remain unknown.

To explore the biological functions of ERR $\alpha$  in vivo, we used gene targeting technology to generate ERR $\alpha$  deficient mice (ERR $\alpha^{-/-}$  mice). These ERR $\alpha^{-/-}$  mice are viable and fertile and show no gross anatomical abnormalities. However, ERR $\alpha^{-/-}$  mice display reduced fat mass and are resistant to high-fat diet-induced obesity, and their adipose tissue shows alterations in the expression of several genes directly linked to lipid metabolism and adipogenesis. ERR $\alpha^{-/-}$  mice thus provide a new model to investigate nuclear receptor-regulated metabolic pathways and associated physiology and pathology.

#### MATERIALS AND METHODS

**Targeting of ERR $\alpha$  and production of chimeric mice.** Three overlapping  $\lambda$  clones containing the mouse *Estrra* locus were isolated from a 129Sv genomic library (a gift from A. Joyner, Skirball Institute, New York, N.Y.) and characterized by restriction mapping and direct sequencing of the exon boundaries. The knockout construct was created by using pNT (60) and contained 6.73 kb of genomic DNA flanking the second exon of *Estrra*. An endfilled 4.36-kb *Bam*HI/*Not*I fragment, lying upstream of the second exon, was cloned into the *Xho*I site of pNT, whereas a 2.37-kb *Hind*III fragment was cloned between the *neo*<sup>r</sup> and thymidine kinase cassettes to provide the 3' arm of the construct. Correct targeting of the *Estrra* locus replaces the second exon of the receptor, which encodes the amino-terminal region and an essential component of its DNA-binding domain, with a *neo* cassette. The linearized construct was electroporated into R1 embryonic stem (ES) cells (41), which were selected with G418 (150  $\mu$ g/ml) and ganciclovir (2  $\mu$ M). Two ES cell clones were isolated and injected into C57BL/6 blastocysts to generate chimeras, and three chimeras transmitted the mutation to their offspring. Heterozygous mice, generated by mating the chimeric animals with 129SvJ mice were mated with C57BL/6 animals to generate hybrid F<sub>1</sub> animals: physiologic studies were performed by using the F<sub>2</sub>-null mutant and wild-type offspring obtained by mating the F<sub>1</sub> hybrid heterozygotes, with the exception of the study on high-fat diet-induced obesity, which was performed with ERR $\alpha$ -null mice in the C57BL/6 background. Complete disruption of the *Estrra* allele was verified by performing Northern and Western blots with RNA and proteins obtained from kidney and intestine, respectively, as previously described (50).

**Studies of *Estrra*<sup>-/-</sup> mice.** Mice were housed in a specific-pathogen-free facility with a daily 12-h light cycle (7:00 a.m. to 7:00 p.m.) and with free access to food and water. Between two and four mice were housed in each cage. Growth curves were obtained by weighing mice of defined ages between 10:00 and 12:00 a.m. Fasting serum and biochemical studies were performed between 10:00 and 12:00 a.m. with animals that had been deprived of food for 18 h. Body composition was determined by desiccating mouse carcasses from which the intestines had been removed (29). After desiccation, the carcass was homogenized and a 1-g aliquot was saponified by using potassium hydroxide and extracted with petroleum ether. After complete evaporation of the ether, the residue was weighed to determine the fat content. Statistical comparisons of the body composition data was performed by using the Mann-Whitney U test. Baseline biochemical studies were performed with serum samples obtained from tail bleeds

of restrained animals at between 20 and 28 weeks of age. Enzymatic assays were used to determine serum triglycerides (GPO-PAP; Boehringer Mannheim) and glycerol (TC Glycerin; Boehringer Mannheim), glucose (Glucose Oxidase-Trinder; Sigma), free fatty acids (GPO-PAP Half Micro Test; Boehringer Mannheim), and  $\beta$ -hydroxybutyrate (TC  $\beta$ -hydroxybutyrate; Boehringer Mannheim). Energy balance and body composition measurements were performed as previously described (46). For high-fat feeding, a diet containing 45% (wt/wt) fat content (D12451; Research Diets, Inc., New Brunswick, N.J.) was used.

**DNA microarrays and quantitative PCR.** Microarray analysis was performed with pooled adipose tissue obtained from two wild-type and two ERR $\alpha^{-/-}$  adult male mice. The animals were fasted overnight and euthanized by using Avertin. Total RNA was prepared from homogenized adipose tissue by using Trizol reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, Calif.). Probes for microarray analysis were prepared by using 10  $\mu$ g of total RNA and hybridized to Affymetrix (Santa Clara, Calif.) Mu74Av2 GeneChips. Detailed protocols for the probe synthesis and hybridization reactions, as well as for the posthybridization washing and staining, have been previously described (43). The hybridized arrays were scanned and raw data extracted by using Microarray Analysis Suite 5.0 (Affymetrix). Expression profiles obtained from both knockout animals were compared individually to those obtained from each wild-type mouse by using Microarray Analysis Suite 5.0, which examines the hybridization intensities of individual probe pairs in order to estimate the significance of observed changes in gene expression: in the present study, the default range for the change *P* value (lower cutoff, 0.0025; upper cutoff, 0.9975) was used to identify genes whose expression changed in each comparison. Genes whose expression levels differed in three of four comparisons were considered to show significantly different levels of expression between the wild-type and knockout animals. For quantitative PCR, total RNA was extracted from white and brown fat tissues of wild-type and knockout animals by using Trizol reagent. cDNA was prepared by reverse transcription of 1  $\mu$ g of total RNA with Superscript II enzyme and oligo(dT) primer. The resulting cDNAs were amplified by using the LightCycler FastStart DNA Master SYBR Green I kit and a LightCycler instrument according to the manufacturer's instructions (Roche Diagnostics, Indianapolis, Ind.). All mRNA expression data was normalized to hypoxanthine phosphoribosyltransferase expression in the corresponding sample.

**Lipogenesis rate.** Mice were studied at 10:00 a.m. after free access to food overnight. The animals were conditioned by sham intraperitoneal injections of water. On the day of the experiment, the animals were injected intraperitoneally with <sup>3</sup>H<sub>2</sub>O (0.5 mCi per 100 g of body weight) and sacrificed by cervical dislocation 30 min later. Adipose tissue samples were harvested and stored at -80°C. The tissues were homogenized and heated in ethanolic KOH. The resulting tissue extracts, which contained saponified lipids, were acidified with concentrated sulfuric acid and extracted by using petroleum ether. The extracts were dried by evaporation, and <sup>3</sup>H incorporation in the lipid fraction was determined by scintillation counting.

#### RESULTS

**Targeting of the murine *Estrra* gene.** The gene targeting strategy was designed to produce mice null for *Estrra* by removing the exon encoding the amino-terminal domain and the first zinc finger (DNA-binding domain) and replacing it with a *neo*<sup>r</sup> cassette (Fig. 1A). Southern blot analysis of genomic DNA with an external probe and a *neo* probe from two ERR $\alpha^{+/-}$  ES cell lines (Fig. 1B) confirmed that the locus had been targeted as expected.

**ERR $\alpha^{-/-}$  mice are viable and fertile.** Heterozygotes for the disrupted ERR $\alpha$  allele were apparently normal and were crossed to generate homozygous mutant mice. Figure 1C shows a representative Southern blot of 4-week-old offspring from crosses of heterozygous mice. Homozygous ERR $\alpha^{-/-}$  mice were obtained at the expected Mendelian frequency and did not differ from their wild-type littermates in overall health, fertility (including interbreeding between ERR $\alpha^{-/-}$  mice), and longevity. Histological examinations detected no obvious abnormalities in the brain, spinal cord, pituitary, heart, kidneys, adrenal, testis, ovary, intestine, and liver (data not shown). Northern blot analysis with total mRNA extracted

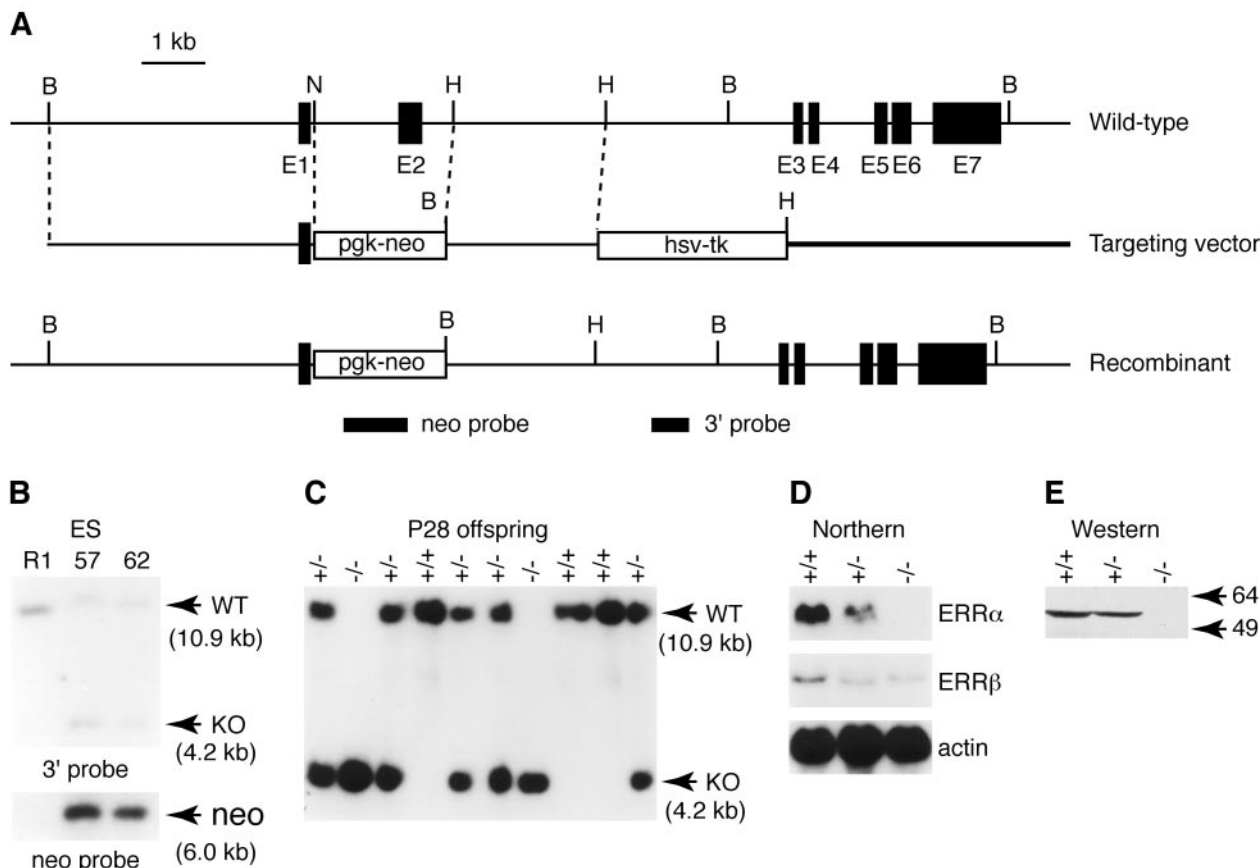


FIG. 1. Targeted disruption of the murine *Estrra* gene in ES cells and mice. (A) Schematic representation of the *Estrra* locus and targeting vector. Black boxes in the wild-type allele schematics represent exons of the *Estrra* gene. The open boxes in the targeting vector schematics correspond to the *pgk-neo* and *hsv-tk* selectable marker genes. The position of the 3' flanking and neomycin probes used in Southern blot analysis are indicated by filled boxes. (B) Homologous recombination of the targeting vector in ES cells was verified by Southern blot analysis, digesting genomic DNA with *Bam*HI, and hybridization with a 3'-flanking probe (upper panel) and a neomycin probe (lower panel). The wild-type allele generated a 10.9-kb band, while the mutant allele produced a 4.2-kb band due to the introduction of a *Bam*HI site in the targeting vector. The neomycin probe recognized a 6.0-kb band in the targeted locus. (C) Southern blot analysis of 4-week-old offspring from heterozygote intercrosses with the 3'-flanking probe. (D) Northern blot analysis of ERR $\alpha$  expression. Total RNA isolated from adult wild-type, heterozygous, and homozygous kidneys. Twenty micrograms of total RNA were used in each lane. ERR $\alpha$  and ERR $\beta$  transcripts were detected by using mouse cDNA probes (51). A mouse  $\beta$ -actin cDNA probe was used as a loading control (lower panel). (E) Western analysis of ERR $\alpha$  knockout mice. Intestine lysates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The blot was probed with antibodies against mouse ERR $\alpha$ , which were made by using the amino-terminal domain of the ERR $\alpha$  protein.

from kidneys failed to detect ERR $\alpha$  transcripts in ERR $\alpha$ <sup>-/-</sup> mice, whereas approximately half the amount of transcripts was observed in ERR $\alpha$ <sup>+/-</sup> mice (Fig. 1D). Interestingly, the levels of the ERR $\beta$  transcript were found to be downregulated in both heterozygous and ERR $\alpha$  knockout animals, suggesting that ERR $\alpha$  may regulate ERR $\beta$  expression in the kidneys. Finally, no ERR $\alpha$  protein could be detected by Western blot analysis of proteins extracted from the intestine of knockout animals (Fig. 1E).

**ERR $\alpha$ <sup>-/-</sup> mice have reduced fat mass.** To verify the general well-being of ERR $\alpha$ <sup>-/-</sup> mice and explore the possibility that ERR $\alpha$  may play a role in maintaining homeostasis in the adult animal, their growth rates relative to those of wild-type littermate controls were measured in terms of total body weight (Fig. 2A and B). During the first postnatal week, the mean body weight of ERR $\alpha$ <sup>-/-</sup> mice was slightly decreased, although it was not statistically different from wild-type litter-

mates. However, beginning at postnatal week 2, ERR $\alpha$ <sup>-/-</sup> mice displayed a significant decrease in body weight. At postnatal week 10, for example, both male and female ERR $\alpha$ <sup>-/-</sup> mice weighed ca. 15% less than their wild-type littermates ( $P < 0.01$ ). No significant reduction in body weight was observed in heterozygous ERR $\alpha$ <sup>+/-</sup> mice (Table 1 and data not shown). The body length (nose to anus) of 10-week-old male and female mice showed that linear growth was not impaired in ERR $\alpha$ <sup>-/-</sup> mice (Fig. 2C). In addition, the weight of the heart did not differ in ERR $\alpha$ <sup>-/-</sup> mice (Fig. 2D). However, ERR $\alpha$ <sup>-/-</sup> had a 50 to 60% reduction in the weight of the inguinal, epididymal and peritoneal fat pads (Fig. 2E to G). Biochemical characterization of carcass composition showed that the ERR $\alpha$ -null mice had, in comparison with control littermates, 80.6% fat, 105% lean mass, and 106.4% water (Table 1). The histology of fat tissue shows an apparent normal number of adipocytes with decreased adipocyte size (Fig. 3).

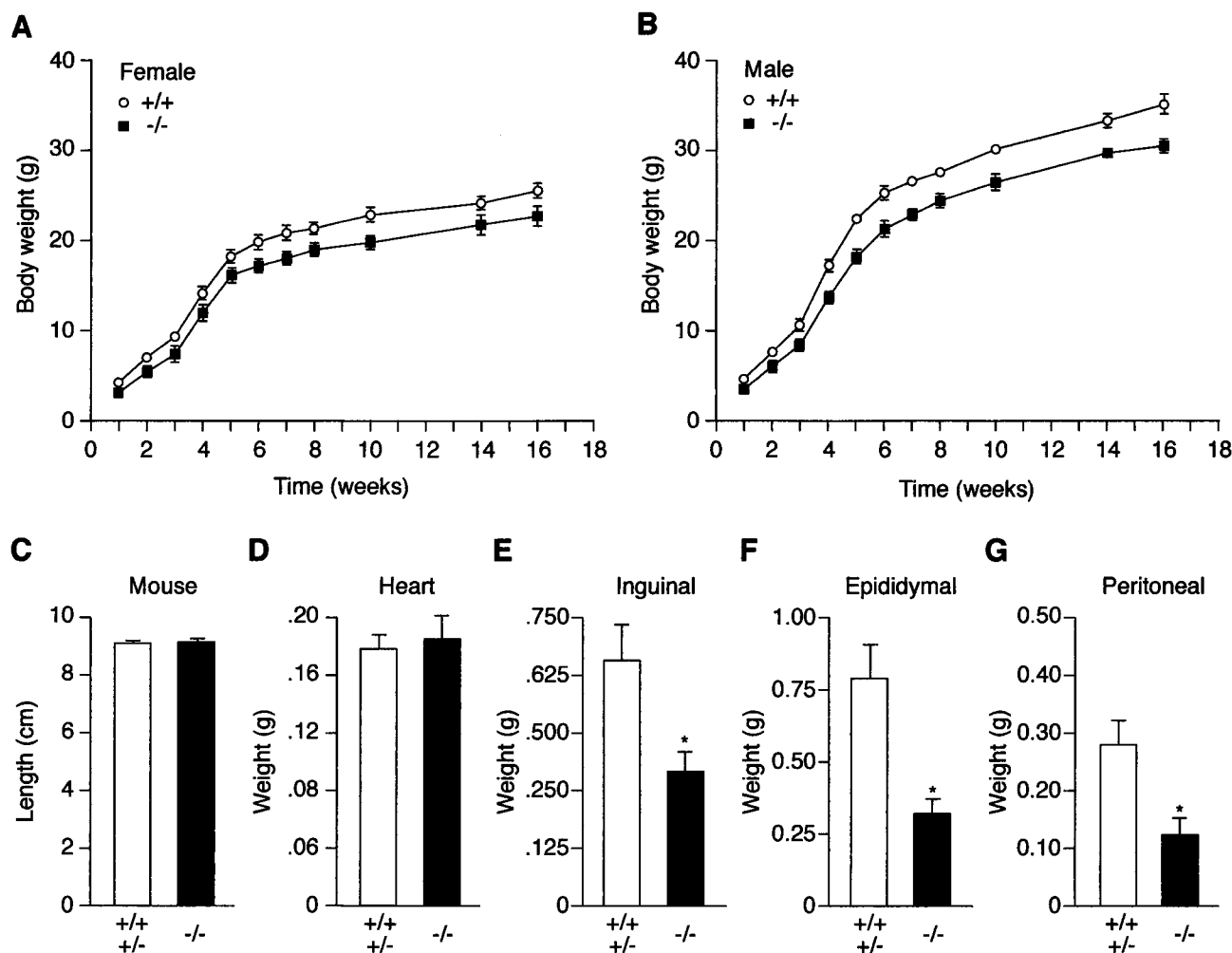


FIG. 2. Comparison of total weight, length, and fat pad weight of wild-type and  $ERR\alpha$ -null mice. (A) Growth of female  $ERR\alpha$ -null mice.  $ERR\alpha^{-/-}$  F<sub>2</sub> mice (■;  $n = 15$ ) and littermate wild-type controls (○;  $n = 16$ ) were weighed, and the data were binned by using 1- to 4-week intervals. The data are means  $\pm$  the standard error of the mean (SEM). (B) Growth of male  $ERR\alpha$ -null mice.  $ERR\alpha^{-/-}$  F<sub>2</sub> mice (■;  $n = 13$ ) and littermate wild-type controls (○;  $n = 10$ ) were weighed, and the data were binned by using 1- to 4-week intervals. The data are mean  $\pm$  the SEM. (C to F) Anatomical characteristics of wild-type plus heterozygote ( $n = 9$ ) and  $ERR\alpha$ -null ( $n = 7$ ) mice at 26 weeks of age. The  $P$  values (\*) were as follows: inguinal, 0.008; epididymal, 0.002; and peritoneal, 0.005.

**Serum biochemistry of  $ERR\alpha^{-/-}$  mice.** To further investigate the biological basis of this phenotype, a series of physiological tests were performed on the  $ERR\alpha^{-/-}$  mice. Fasting glucose levels, as well as serum free fatty acids and triglyceride levels, were not significantly affected by the absence of  $ERR\alpha$  (data not shown).

**Food consumption and energy expenditure.** To examine whether the reduction in fat mass observed in  $ERR\alpha^{-/-}$  mice was caused by decreased food intake or increased energy expenditure, both parameters were measured over a 4-day period. We observed no statistically significant change in either parameter (Fig. 4 and data not shown).

**$ERR\alpha^{-/-}$  mice are resistant to high-fat diet-induced obesity.** We next challenged 10-week-old mice with a high-fat diet containing 45% (wt/wt) fat for 5 weeks to stimulate weight gain. As shown in Fig. 5A, weight gain was significantly reduced in the  $ERR\alpha^{-/-}$  mice relative to the control littermates.

A statistical difference between the two groups was observed in the third week of feeding. By the end of the experiment, the control littermates had become mildly obese (Fig. 5B), whereas the  $ERR\alpha^{-/-}$  mice weighed only slightly more than wild-type mice fed a normal diet.

**DNA microarrays.** Microarray studies were performed to determine whether  $ERR\alpha$  ablation causes changes in gene expression in adipose tissue (Table 2). Pooled adipose tissue was harvested from fasted adult male mice and studied by using Affymetrix GeneChips. The expression studies show downregulation of  $ERR\alpha$  and changes in the expression of two known  $ERR\alpha$  target genes: lactotransferrin and MCAD. The expression studies also demonstrate altered regulation of several enzymes involved in lipid metabolism (fatty acid synthase [gene *Fasn*], stearoyl-coenzyme A desaturase 2 [*Scd2*], fatty acid coenzyme A ligase long-chain 5, [*Facl5*], acetyl-coenzyme A dehydrogenase medium chain [*Acadm*], and acetyl-coen-



TABLE 1. Body composition of ERR $\alpha$  knockout mice and control littermates<sup>a</sup>

Parameter	Mean value $\pm$ SEM for mouse genotype:			Ratio (-/- vs +/+) (%)	P	
	+/+	+/-	-/-		-/- vs +/+	-/- vs +/+ and +/-
Wt (g)	32.9 $\pm$ 1.3	32.5 $\pm$ 1.8	27.3 $\pm$ 1.0	83.0	0.0014	0.00034
Fat mass (g)	6.9 $\pm$ 1.0	6.9 $\pm$ 1.2	4.6 $\pm$ 0.5	67.4	0.039	0.014
Lean mass (g)	6.9 $\pm$ 0.2	6.8 $\pm$ 0.3	6.0 $\pm$ 0.2	87.4	0.0669	0.0016
Water content (g)	14.9 $\pm$ 0.3	14.6 $\pm$ 0.4	13.1 $\pm$ 0.4	88.0	0.0054	0.0011
Fat mass (%)	23.5 $\pm$ 2.2	23.6 $\pm$ 2.7	19.0 $\pm$ 1.6	80.7	0.053	0.029
Lean mass (%)	24.2 $\pm$ 0.6	24.2 $\pm$ 0.8	25.4 $\pm$ 0.4	105.0	0.061	0.036
Water content (%)	52.2 $\pm$ 1.8	52.2 $\pm$ 2.0	55.6 $\pm$ 1.3	106.4	0.12	0.071

<sup>a</sup> The results are shown for wild-type ( $n = 7$ ), heterozygous ( $n = 6$ ), and knockout mice ( $n = 15$ ). The mean body weight of the ERR $\alpha$  knockout mice was significantly decreased and was accompanied by decreased total fat mass and compensatory increases in lean mass and water content.

zyme A dehydrogenase long chain [*Acadl*] and energy metabolism (cytochrome *c* somatic [*Cycs*], carnitine acetyltransferase [*Crat*], creatine kinase mitochondrial 2 [*Ckmt2*], and uncoupling protein 1 [*Ucp1*]). These changes create an imbalance between enzymes involved in fat synthesis and fat catabolism that may result in decreased levels of de novo triglyceride synthesis in the knockout animals. The alterations in transcript expression observed in the microarray studies may include genes that are direct ERR $\alpha$  targets, as well as genes that are regulated by other transcription factors or cell signaling pathways. In particular, the microarray studies demonstrate alterations in sterol regulatory element binding factor 1 (*Srebf1*)

and CCAAT/enhancer binding protein gamma (*Cebpg*) expression, transcription factors that have been implicated in the regulation of adipogenesis (reviewed in reference 11). In addition, decreased expression of prostaglandin D2 synthase (*Ptgds*) and hydroxysteroid dehydrogenase-4  $\delta(5)$ -3- $\beta$  (*Hsd3b4*) and hydroxysteroid dehydrogenase-6  $\delta(5)$ -3- $\beta$  (*Hsd3b6*) isoforms may alter peroxisome proliferator-activated receptor (PPAR) activity by regulating levels of their putative endogenous ligands, which in turn may explain prostaglandin-endoperoxide synthase 2 (*Ptgs2*) upregulation (25, 72). Finally, adipose tissue from ERR $\alpha$  knockout animals also expresses increased levels of adrenergic receptor  $\beta_3$ , a known regulator of murine adiposity (54). The quality of the array data was further validated by quantitative PCR analysis of 15 selected genes indicated in bold in Table 1. Of these genes, changes in the expression of 11 of 15 (74%) were validated by quantitative PCR, including *Esrra*, whose expression could not be detected as expected. The levels of expression for two genes, *Ckmt2l* and *Hdac6*, in white adipose tissue (WAT) were also too low to obtain accurate measurements. For two other genes, *Ptgds* and *Ucp1*, we observed upregulation (~30- and 2.7-fold, respectively) by quantitative PCR, while the microarray data indi-

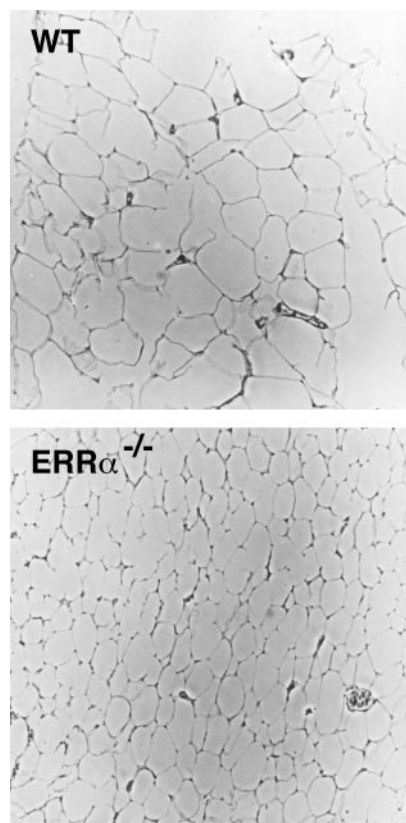


FIG. 3. Histology of adipose tissue. Hematoxylin-and-eosin-stained wild-type (WT) and ERR $\alpha$ <sup>-/-</sup> epididymal WATs are shown. Magnification,  $\times 150$ .

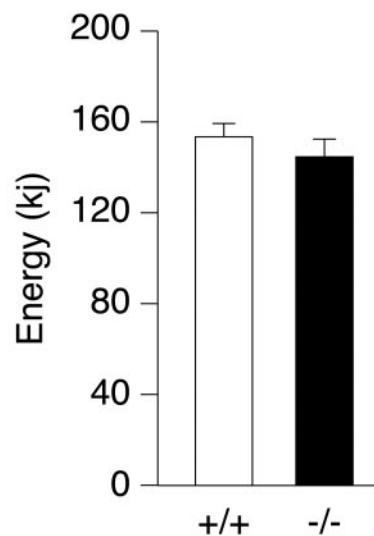


FIG. 4. Energy consumption in wild-type ( $n = 4$ ) and ERR $\alpha$ <sup>-/-</sup> ( $n = 8$ ) male mice over a 4-day period.

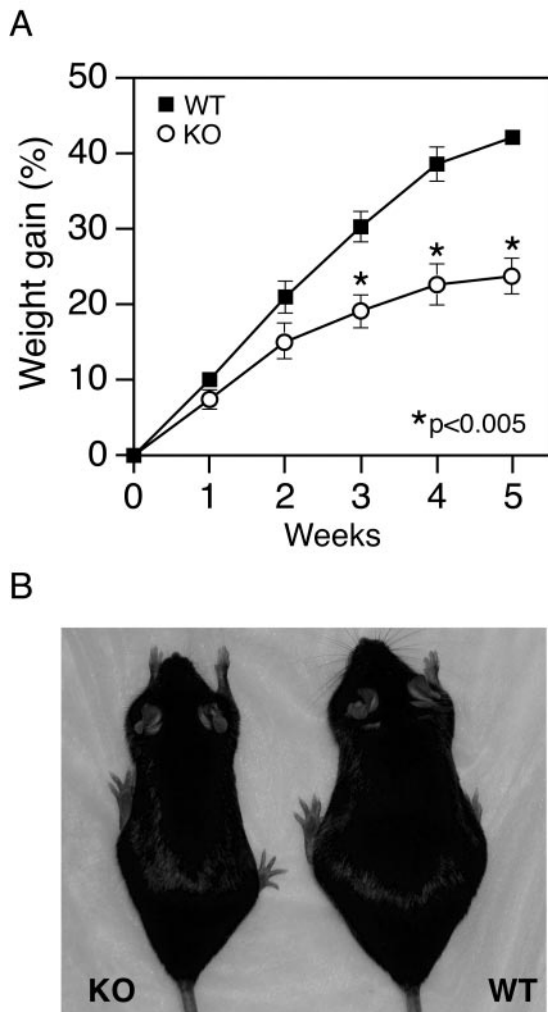


FIG. 5.  $ERR\alpha^{-/-}$  mice are resistant to high-fat diet-induced obesity. (A) Ten-week-old male mice ( $n = 7$  for each group) were fed a high-fat diet. The increases in body weights were calculated based on the initial body weight at day 0 of high-fat feeding. The average initial body weight was 10% smaller ( $P < 0.05$ ) for the  $ERR\alpha^{-/-}$  mice compared to the control littermates. (B) Representative male  $ERR\alpha^{-/-}$  mouse and control littermate after 5 weeks of feeding with a high fat diet.

cated that the expression of these genes was downregulated. Thus, we are confident that, for more than 75% of genes identified as differentially expressed, the results of our microarray gene screening method is accurate. The changes in the expression of the validated genes in the WAT of  $ERR\alpha^{-/-}$  mice compared to wild-type mice, as obtained by quantitative PCR, are shown in Fig. 6A. Of note, true fold changes are generally greater than the averaged microarray data, leading us to include genes whose level of expression was as low as 1.2-fold (Table 2). Since brown fat expressed high levels of  $ERR\alpha$ , we also measured changes in expression levels of the selected genes in this tissue (Fig. 6B). In most cases, changes in gene expression observed in brown fat parallel those seen in white fat, with the exception of *Elovl3* and *Srebf1*.

**Lipogenesis.** Given the observation of altered expression of key enzymes involved in lipid metabolism, we next investigated

whether de novo synthesis of triglyceride is affected in the knockout animals. Lipogenesis was assessed by treating  $ERR\alpha^{-/-}$  mice with  $^3H_2O$ : the amount of radioactive label incorporated into triacylglycerol can be measured by saponification and ether extraction of adipose tissues and other organs (12).  $ERR\alpha^{-/-}$  mice demonstrated significantly decreased lipogenesis in comparison to littermate controls: knockout animals show a 30 to 55% decrease in  $^3H$  incorporation into adipose tissue lipids (Fig. 7). This finding demonstrates that adipose tissues of knockout mice possess a defect in triglyceride synthesis, which may result from decreased adipocyte glycolytic activity, decreased fatty acid synthesis, or decreased esterification.

## DISCUSSION

We have generated  $ERR\alpha$ -deficient mice through homologous recombination in ES cells. No  $ERR\alpha$  expression was detectable in these mice by Northern and Western blot analyses and quantitative PCR. The mice were viable and fertile and have a normal life span. Although  $ERR\alpha$ -deficient mice weighed less compared to their wild-type littermates, histological analysis of adult tissues failed to show morphological abnormalities. Therefore, we conclude that there is no absolute requirement for  $ERR\alpha$  function in development, sexual maturation, and homeostasis. This finding is in sharp contrast with the phenotype observed with the  $ERR\beta$ -null mice who die in utero due to the complete absence of the diploid trophoblast layers of the placenta (36). This finding is also surprising in view of the very high level of  $ERR\alpha$  expression in brown adipose tissue (50) and the proposed role for  $ERR\alpha$  in bone formation (3, 4).

Although  $ERR\alpha$  deficiency by itself may be largely asymptomatic, if present with other genetic defects such as null mutations in other members of the ERR or ER families, it could conceivably give rise to an overtly abnormal developmental, metabolic, or endocrine phenotype. In fact, deletion of genes encoding nuclear receptors does not always produce an apparent phenotype. Perhaps the most dramatic example is that of the genes encoding the three retinoic acid receptors ( $RAR\alpha$ ,  $\beta$ , and  $\gamma$ ). Mice devoid of either  $RAR\alpha$  (30, 34),  $RAR\beta$  (35, 40), or  $RAR\gamma$  (31) display only subtle developmental phenotypes. An extensive functional redundancy between RARs accounts for this observation as severe developmental defects results from the lack any combinations of two RAR genes (reviewed in reference 38). Disruption of other nuclear receptor genes produce a phenotype only when animals are physiologically or pharmacologically challenged, such as with a high cholesterol diet in  $LXR\alpha$  mutant mice (44) or xenobiotic agents in  $PXR/SXR$ -null mice (52, 68, 69). Therefore, a more evident phenotype may be observed only in ERR double mutants or in response to a specific physiological stress.

Although the  $ERR\alpha^{-/-}$  mice appear normal, they display reduced body weight and diminished fat mass (Fig. 2). We were unable to detect statistically significant changes in eating behavior or energy expenditure between the wild-type and  $ERR\alpha$ -null littermates. One possible explanation for these results is that the putative increase in energy expenditure is small and significant only over a long period of time. How the absence of  $ERR\alpha$  leads to this phenotype at the molecular level

TABLE 2. Differentially expressed genes in the adipose tissue of ERR $\alpha$  knockout mice<sup>a</sup>

Function	Probe set	Gene	Fold change	Unigene no.	LocusLink no.	Cytoband no. (centimorgans)	Description
Fat metabolism	92536_at	<i>Adrb3</i>	1.7	Mm.4369	11556	8 (10.0)	Adrenergic receptor, beta 3
	92581_at	<i>Acadm</i>	1.5	Mm.10530	11364	3 (73.6)	Acetyl coenzyme A dehydrogenase, medium chain
	160921_at	<i>Acas2</i>	1.5	Mm.7044	68738		Acetyl coenzyme A synthetase 2
	95425_at	<i>Acad1</i>	1.4	Mm.198049	98243	1	Acetyl coenzyme A dehydrogenase, long chain
	93754_at	<i>Ech1</i>	1.3	Mm.2112	51798		Enoyl coenzyme A hydratase 1, peroxisomal
	103646_at	<i>Crat</i>	1.3	Mm.20396	12908	2 (18.0)	Carnitine acetyltransferase
	97456_at	<i>Fac15</i>	-1.3	Mm.30263	71879		Fatty acid coenzyme A ligase, long chain 5
	95758_at	<i>Scd2</i>	-1.5	Mm.193096	20250	19 (43.0)	Stearoyl coenzyme A desaturase 2
	98575_at	<i>Fasn</i>	-1.7	Mm.3760	14104	11 (72.0)	Fatty acid synthase
	103469_at	<i>Elov13</i>	-7.6	Mm.21806	12686	19 (47.0)	Elongation of very long chain fatty acids (FEN1/Elo2, SUR4/Elo3, yeast)-like 3
Eicosanoid metabolism	104647_at	<i>Ptgs2</i>	1.5	Mm.3137	19225	1 (76.2)	Prostaglandin-endoperoxide synthase 2
	92545_f_at	<i>Ptgsd</i>	-3.6	Mm.1008	19215	2 (12.9)	Prostaglandin D2 synthase (21 kDa, brain)
Sterol or steroid metabolism	92869_at	<i>Hsd3b4</i>	4.8	Mm.14309	15495	3 (49.1)	Hydroxysteroid dehydrogenase-4,delta <sup>5</sup> -3-beta
	102729_f_at	<i>Hsd3b6</i>	2.6	Mm.14435	15497		Hydroxysteroid dehydrogenase-6,delta <sup>5</sup> -3-beta
	104509_at	<i>Ch25h</i>	-2.7	Mm.30824	12642		Cholesterol 25-hydroxylase
Electron transport	98132_at	<i>Cytc</i>	-1.4	Mm.35389	13063	6 (23.0)	Cytochrome c, somatic
	99507_at	<i>Ucp1</i>	-2.6	Mm.4177	22227	8 (38.0)	Uncoupling protein 1, mitochondrial
Other metabolism	102049_at	<i>Pdk4</i>	1.8	Mm.10283	27273	6 (0.63)	Pyruvate dehydrogenase kinase 4
	97950_at	<i>Xdh</i>	1.3	Mm.11223	22436	17 (45.3)	Xanthine dehydrogenase
	94966_at	<i>G6pdx</i>	-1.3	Mm.27210	14381	X (30.02)	Glucose-6-phosphate dehydrogenase X linked
	101044_at	<i>Alad</i>	-1.4	Mm.6988	17025	4 (30.6)	Aminolevulinate, delta-, dehydratase
	104172_at	<i>Folr2</i>	-1.5	Mm.2724	14276	7	Folate receptor 2 (fetal)
	101408_at	<i>Gamt</i>	-1.5	Mm.7329	14431	10 (43.0)	Guanidinoacetate methyltransferase
	100046_at	<i>Mthfd2</i>	-1.7	Mm.443	17768	6 (35.15)	Methylenetetrahydrofolate dehydrogenase (NAD <sup>+</sup> dependent)
	162015_f_at	<i>Ckmt2</i>	-1.9	Mm.20240	76722		Creatine kinase, mitochondrial 2
	97089_at	<i>Folh1</i>	-1.9	Mm.7522	53320	7 (D1-D2)	Folate hydrolase
	Gene transcription	160901_at	<i>Fos</i>	2.3	Mm.5043	14281	12 (40.0)
99532_at		<i>Tob1</i>	1.5	Mm.4218	22057		Transducer of ErbB-2.1
160526_s_at		<i>Crem</i>	1.3	Mm.5244	12916	18 A	Cyclic cAMP responsive element modulator
92195_at		<i>Cebpg</i>	1.3	Mm.3402	12611		CCAAT/enhancer binding protein (C/EBP), gamma
94812_at		<i>Gtf2h1</i>	1.3	Mm.22700	14884		General transcription factor IIIH, polypeptide 1 (62-kDa subunit)
100984_at		<i>Atf1</i>	1.2	Mm.676	11908		Activating transcription factor 1
100094_at		<i>Supt5h</i>	1.2	Mm.460	20924	(710.0)	Suppressor of Ty5 homolog ( <i>Saccharomyces cerevisiae</i> )
96633_s_at		<i>Mrgx-pendi</i>	-1.2	Mm.27218	56397		MORF-related gene X
94288_at		<i>H1f2</i>	-1.4	Mm.193539	50708		H1 histone family, member 2
103634_at		<i>Isgf3g</i>	-1.4	Mm.2032	16391	14 (21.5)	Interferon-dependent positive acting transcription factor 3 gamma
93264_at		<i>Srebfl</i>	-1.5	Mm.214958	20787	8 (33.0)	Sterol regulatory element binding factor 1
92502_at		<i>Zac1</i>	-1.6	Mm.42200	22634	10 (15.0)	Zinc finger protein regulator of apoptosis and cell cycle arrest
99059_at		<i>Elf3</i>	-1.6	Mm.3963	13710		E74-like factor 3
104471_at		<i>Hdac6</i>	-1.9	Mm.29854	15185	X (2.0)	Histone deacetylase 6
101913_at		<i>Hcy1</i>	-2.0	Mm.29581	15213	3 (2.4)	Hairy/enhancer-of-split related with YRPW motif 1
103964_at		<i>Esrra</i>	-2.0	Mm.2563	26379	19 (3.0)	ERR $\alpha$
93915_at		<i>Pou2af1</i>	-2.1	Mm.897	18985		POU domain, class 2, associating factor 1

<sup>a</sup> The results of microarray analysis performed on the WAT of male ERR $\alpha$  knockout mice are shown for replicated studies performed with two wild-type and two ERR $\alpha^{-/-}$  mice. The fold change represents an average of the values obtained by a pairwise comparison of the animals. A total of 305 probe sets were differentially regulated in the ERR $\alpha^{-/-}$  mice: those implicated in lipid metabolism or its regulation, as well as transcriptional regulation, are shown here. Genes in boldface were tested by quantitative PCR analysis (Fig. 6 and data not shown).

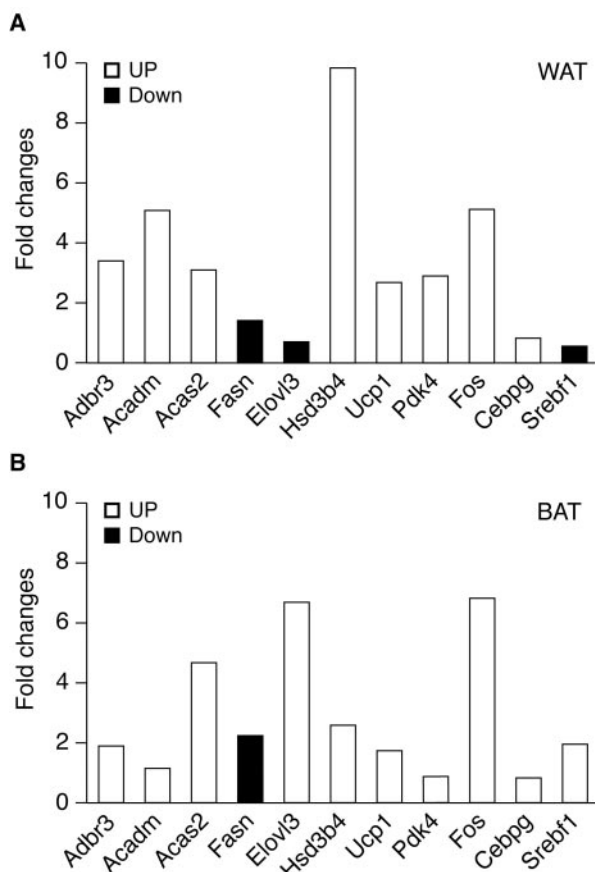


FIG. 6. The absence of  $ERR\alpha^{-/-}$  influences the expression of genes involved in lipid metabolism in both white (A) and brown (B) adipose tissues. Quantitative PCR data shows the difference in expression of both upregulated ( $\square$ ) and downregulated ( $\blacksquare$ ) genes in these tissues. The data displayed represent the means of at least two experiments.

is currently unknown. However, gene expression profiling experiments in adipose tissue of wild-type versus knockout mice are providing interesting avenues of investigation. First, the expression studies confirmed a role of  $ERR\alpha$  in MCAD (*Acadm*) gene regulation, predominantly as a repressor, since MCAD expression is upregulated in the adipose tissue of the knockout mice. This result indicates that  $ERR\alpha$  can indeed act as a direct regulator of metabolic genes. Second, the loss of  $ERR\alpha$  function leads to alterations in the expression of a wide variety of genes encoding transcriptional regulators, two of them (*Srebf1* and *Cebpg*) directly implicated in the regulation of adipogenesis. The absence of  $ERR\alpha$  might therefore induce significant changes in a regulatory cascade that normally controls the expression of genes involved in fat and sterol metabolism. We have also noted significant alterations in the expression of genes involved in sterol, steroid, and prostaglandin synthesis, suggesting that the loss of  $ERR\alpha$  might indirectly influence the activity of other nuclear receptor-based signaling pathways, many of which are known to regulate lipid metabolism and adipogenesis (1, 11, 33, 47). Third, the gene microarray analysis detected changes in the expression of metabolic enzymes directly involved in lipid synthesis, an observation cor-

related with a marked decrease in lipogenesis rate in white fat tissues. Finally, the observed upregulation of the gene encoding the  $\beta_3$  adrenergic receptor suggests that lipolysis in white adipocytes could also be affected (7), although more specific studies will be required to validate this hypothesis. It is, however, interesting that the *Adb3* promoter has been shown to encode a putative binding site for  $ERR\alpha$  (28). Although the physiological significance of the alteration in expression of each specific gene remains to be fully investigated, this ensemble is in agreement with the observed reduced fat mass in the  $ERR\alpha^{-/-}$  mice.

As introduced above,  $ERR\alpha$  has been shown to possess the ability to interfere with estrogen signaling (reviewed in reference 13). Estrogen is known to play an important role in WAT regulation: in rodents, ovariectomy increases the amount of WAT, whereas estrogen replacement decreases the amount of WAT (65). Similar observations were made in postmenopausal women subjected to hormone replacement therapy (56). More recently, increased adipose tissue in male and female  $ER\alpha$ -null mice was observed, demonstrating conclusively a role for estrogen and  $ER\alpha$  in WAT functions (17). The  $ERR\alpha$ -null mice displays the opposite phenotype, an observation consistent with the fact that  $ERR\alpha$  can act as a potent transcriptional repressor in specific biological settings and compete with the ERs for binding sites and coactivators (27, 50, 57, 67, 73). The upregulation of *Fos*, a direct target of  $ER\alpha$  signaling (20), in the white and brown adipose tissues of the  $ERR\alpha$ -null mice is in agreement with the proposed cross talk between the two nuclear receptor-based regulatory pathways. In addition, the inhibition of estrogen production in aromatase-deficient mice results in increased adipose tissue mass and hepatic steatosis (23), which is associated with reduced rates of hepatic  $\beta$ -oxidation and decreased expression of AOX, VLACS, and MCAD (42). It will be interesting to test whether the combined loss of  $ER\alpha$  and  $ERR\alpha$  in mice "resets" their metabolism toward a more wild-type-like state.

In light of the well-known caveat that mouse development

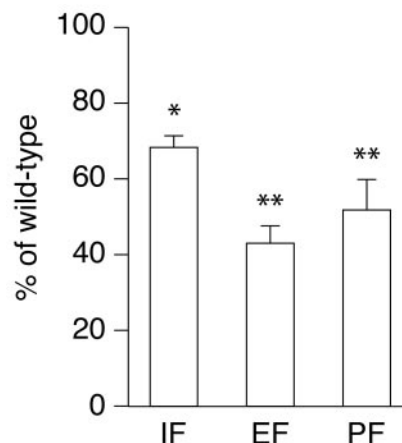


FIG. 7.  $ERR\alpha^{-/-}$  mice demonstrate decreased lipogenesis in comparison to littermate controls. After intraperitoneal injection of with  $^3H_2O$ ,  $ERR\alpha^{-/-}$  mice incorporate 30 to 55% less  $^3H$  into adipose tissue lipids. IF, inguinal fat; EF, epididymal fat; PF, perirenal fat. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ .



and life under controlled laboratory conditions do not reproduce the environment that mice find themselves in the wild, it is now evident that ERR $\alpha$  is not absolutely required for normal mouse development and growth. However, it is also evident that the ERR $\alpha$ -null mice display metabolic abnormalities that warrant further investigation. The development of ERR $\alpha$  specific ligands and the current availability of high-throughput gene expression profiling technology coupled with future ERR $\alpha$ <sup>-/-</sup>-based mouse models will provide further insight into the role played by ERR $\alpha$  in metabolic control and other biological processes.

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