Lean Phenotype and Resistance to Diet-Induced Obesity in Vitamin D Receptor Knockout Mice Correlates with Induction of Uncoupling Protein-1 in White Adipose Tissue

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Increased adiposity is a feature of aging in both mice and humans, but the molecular mechanisms underlying age-related changes in adipose tissue stores remain unclear. In previous studies, we noted that 18-month-old normocalcemic vitamin D receptor (VDR) knockout (VDRKO) mice exhibited atrophy of the mammary adipose compartment relative to wild-type (WT) littermates, suggesting a role for VDR in adiposity. Here we monitored body fat depots, food intake, metabolic factors, and gene expression in WT and VDRKO mice on the C57BL6 and CD1 genetic backgrounds. Regardless of genetic background, both sc and visceral white adipose tissue depots were smaller in VDRKO mice than WT mice. The lean phenotype of VDRKO mice was associated with reduced serum leptin and compensatory increased food intake. Similar effects on adipose tissue, leptin and food intake were observed in mice lacking Cyp27b1, the 1α-hydroxylase enzyme that generates 1,25-dihydroxyvitamin D_3, the VDR ligand. Although VDR ablation did not reduce expression of peroxisome proliferator-activated receptor-γ or fatty acid synthase, PCR array screening identified several differentially expressed genes in white adipose tissue from WT and VDRKO mice. Uncoupling protein-1, which mediates dissociation of cellular respiration from energy production, was greater than 25-fold elevated in VDRKO white adipose tissue. Consistent with elevation in uncoupling protein-1, VDRKO mice were resistant to high-fat diet-induced weight gain. Collectively, these studies identify a novel role for 1,25-dihydroxyvitamin D_3 and the VDR in the control of adipocyte metabolism and lipid storage *in vivo*. **(*Endocrinology* 150: 651–661, 2009)**

Whereas it is clear that the peroxisome proliferator-activated receptor (PPAR)-γ is an essential regulator of adipogenesis, emerging studies have implicated additional nuclear receptors in the maintenance of adiposity. For example, mice with adipose-specific ablation of retinoid X receptor (RXR)-α, a heterodimer partner for PPARγ, display impaired adipogenesis and resistance to high fat diet-induced obesity (1). Studies in mutant mice also demonstrated critical roles for other RXR interacting partners, such as the liver X receptor and the thyroid hormone receptor, in adipocyte biology (2, 3). The vitamin D receptor (VDR), another nuclear receptor that dimerizes with RXRα, is expressed in adipose tissue *in vivo* and is dynamically up-regulated during adipogenic differentiation *in vitro* (4–6). However, the impact of the VDR and its ligand 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] on adipocyte biology remains unclear. Both stimulation and inhibition of adipogenesis by 1,25$(OH)_2D_3$ have been reported in cell culture models, including 3T3-L1 cells (7–11). These discrepancies likely reflect the distinct model systems used as well as different ligand concentrations because dose-response studies indicated that 1,25$(OH)_2D_3$ stimulated adipogenesis at low concentrations but inhibited differentiation and induced apoptosis at high concentrations (8, 12). In human adipocytes, low concentrations of 1,25$(OH)_2D_3$ inhibited, and VDR knockdown enhanced, the expression of uncoupling pro-

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Abbreviations: FAS, Fatty acid synthase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; H&E, hematoxylin and eosin; 1,25$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; iBAT, intrascapular brown adipose tissue; PPAR, peroxisome proliferator-activated receptor; RXR, retinoid X receptor; ucp, uncoupling protein; VDR, vitamin D receptor; VDRKO, VDR knockout; WAT, white adipose tissue; WT, wild type.

tein (ucp)-2, a member of the uncoupling protein family involved in thermogenesis, energy metabolism and obesity (12). In *ex vivo* cultures of bone marrow mesenchymal cells, $1,25(OH)_2D_3$ enhanced the recruitment of bipotential progenitors capable of differentiation along the adipogenic and osteogenic lineages (13), suggesting that vitamin D signaling may coordinately enhance both osteogenesis and adipogenesis. A stimulatory effect of vitamin D signaling on adipocyte differentiation was supported by the demonstration that VDR knockdown in 3T3-L1 cells inhibited adipogenesis (11).

Consistent with the *in vitro* data supporting enhancement of adipogenesis by $1,25(OH)_2D_3$, VDR knockout (VDRKO) mice displayed atrophy of adipose tissue surrounding the prostate and mammary glands (14, 15). In our previous studies, the atrophy of the mammary adipose tissue in VDRKO mice developed despite normalization of calcium homeostasis with a high-calcium rescue diet and was significantly aggravated with age (15). Because bone marrow stromal cells from perinatal VDR null mice did not exhibit defective adipogenesis *in vitro* (16), we hypothesized that vitamin D signaling may become important in the control of adipogenesis during the aging process. Indeed, several phenotypes that have been described in VDR null mice (including alopecia, reduction in osteoblast progenitor formation, and defects in keratinocyte stem cells) develop as a function of age (17–19).

The goal of the studies presented here was to use genetic mouse models to clarify the effects of vitamin D signaling on adipose tissue *in vivo*. Specifically we assessed age-related changes in adipose tissue mass, morphology, and gene expression in mice with ablation of either VDR or Cyp27b1, the enzyme that generates $1,25(OH)_2D_3$. Our data indicate that deficiency of VDR signaling results in age-related adipose atrophy, hypoleptinemia, hyperphagia, and altered gene expression in adipose tissue. Furthermore, adiposity is not increased when VDRKO mice are challenged with a high-fat diet. These data identify the vitamin D signaling pathway as a novel modulator of adipose tissue homeostasis with age.

Materials and Methods

Animals and diets

Initial studies were conducted with female wild-type (WT), VDRKO (17), and Cyp27b1 KO (20) mice, which were created and maintained on the C57BL6 background. Eight to 10 mice per genotype per time point, from at least two different litters, were studied. Dietary modification experiments were conducted with VDRKO mice backcrossed onto the CD1 outbred background (achieved by mating VDRKO mice over eight generations to stock CD1 mice from Charles River Laboratories, Wilmington, MA). In all studies, both VDRKO mice and WT controls were maintained from weaning on a high-calcium, high-lactose rescue diet (TD96348; Teklad, Madison, WI) that prevents hypocalcemia secondary to VDR/$1,25(OH)_2D_3$ deficiency (21, 22). Diets were provided *ad libitum*. In some studies, the fat content of the rescue diet was increased from 5 to 15% by the addition of corn oil. Before the animals were killed, four to six mice per genotype for each age group (2, 4, and 6 months) were adapted to individual cages for measurement of food intake over a 10-d period. At the time the animals were killed, serum was collected and analyzed for leptin (mouse leptin kit; Linco Research, St. Charles, MO), insulin (mouse insulin ELISA; ALPCO Diagnostics, Salem, NH), glucose (GO assay kit; Sigma, St. Louis, MO), and triglycerides (GPO assay kit; Pointe Scientific, Canton, MI) according to the manufacturer's directions. The abdominal and inguinal white adipose tissue (WAT) depots, intrascapular brown adipose tissue (iBAT), kidney, uterus/ovaries, and liver were dissected, weighed, and partitioned for histology (formalin fixation), mRNA isolation (Trizol), and protein expression (flash frozen) analyses. In some cases, sample size was limiting and not all assays could be conducted for all animals. All studies were conducted under protocols approved by the University of Notre Dame Institutional Animal Care and Use Committee.

Histological analyses

For morphological assessment, formalin-fixed, paraffin-embedded samples (WAT, iBAT, liver) were sectioned at 5 μm and stained with hematoxylin and eosin (H&E) according to standard protocols. Slides were viewed using bright-field microscopy with an AX70 microscope and Spot RT digital camera (Olympus, Tokyo, Japan). The number of adipocytes within a microscopic field was determined by quantitation of three to four individual mice of each genotype using AxioVision software (Carl Zeiss Vision, Munich, Germany).

Immunohistochemistry of ucp1 was conducted with formalin-fixed sections of WAT from 6-month-old CD1 WT and VDRKO mice fed the rescue diet containing 5% fat. Sections were incubated with 5 μg/ml ucp1 antibody (affinity purified rabbit polyclonal; α Diagnostic International, San Antonio, TX) and developed with the diaminobenzidine procedure. Controls included sections of iBAT from WT mice incubated with or without primary ucp1 antibody. All slides were counterstained with hematoxylin and photographed with a Spot RT digital camera.

Gene expression analyses

For real-time PCR, total RNA was extracted from WAT and iBAT with Trizol, purified, and used for cDNA synthesis. Three independent cDNA stocks were prepared from each sample, using random hexamer primers (Applied Biosystems, Foster City, CA), and each cDNA stock was independently analyzed in duplicate. VDR, fatty acid synthase (FAS), and leptin were detected with TaqMan primer and probe sets; PPARγ and ucp-1 were analyzed by SYBR green technology and mouse-specific primers. Data were calculated by the comparative cycle threshold method, normalized against 18S, and expressed relative to values from WT mice, which were set at 1. To identify additional regulators of adiposity that might be altered in VDRKO mice, a PCR screening array for 84 genes in the mouse insulin signaling pathway (catalog no. PAMM-030; Superarray, Frederick, MD) was conducted with WAT from 6-month-old WT ($n = 4$) and VDRKO ($n = 3$) mice fed the rescue diet with 5% fat. PCR array data were calculated by the comparative cycle threshold method, normalized against multiple housekeeping genes, and expressed as mean fold change in VDRKO samples relative to WT control samples.

Western blotting

Lysates from adipose tissues were separated on SDS-PAGE, transferred to nitrocellulose, and blotted with primary antibodies directed against ucp-1 (affinity purified rabbit polyclonal; α Diagnostic International) or PPARγ (rabbit polyclonal 81B8; Cell Signaling Technology, Danvers, MA), followed by appropriate secondary antibodies and chemiluminescent detection. After detection of the primary antigen of interest, blots were stripped and reprobed with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (mouse monoclonal; AbD Serotec, Raleigh, NC) as loading control.

Statistical analysis

Data are expressed as mean $\pm$ SE, and statistical evaluation was by Student's *t* test or one-way ANOVA (as appropriate) using GraphPad Instat software (Intuitive Software for Science, San Diego, CA). For ANOVA, Bonferroni's *post hoc* test was used if the F test indicated significant differences between data sets. For real-time PCR analyses, mean values of relative gene expression were analyzed using the Student-

Newman-Keuls multiple comparisons test for significant differences. In all cases, $P < 0.05$ was considered to be significant.

Results

Effect of VDR ablation on body and tissue weights and food intake

Our initial indication that VDR might contribute to maintenance of adipose stores was obtained in experiments focused on the effect of VDR ablation on mammary tumorigenesis (15). As shown in Fig. 1A, 18-month-old VDRKO mice from this previous study exhibited complete absence of abdominal adipose tissue, whereas age-matched WT mice had abundant adipose stores. This difference in adipose tissue was not secondary to disturbed extracellular calcium homeostasis because VDRKO mice and their WT counterparts were maintained on a high-calcium rescue diet, which normalizes extracellular calcium and bone metabolism. To follow up this finding, we assessed growth and tissue homeostasis in younger WT and VDRKO mice on the C57BL6 background fed the rescue diet. As demonstrated in Fig. 1B, body weights increased in both WT and VDRKO mice from 2 to 6 months of age, but mice lacking VDR weighed significantly less than age-matched WT mice at all time points.

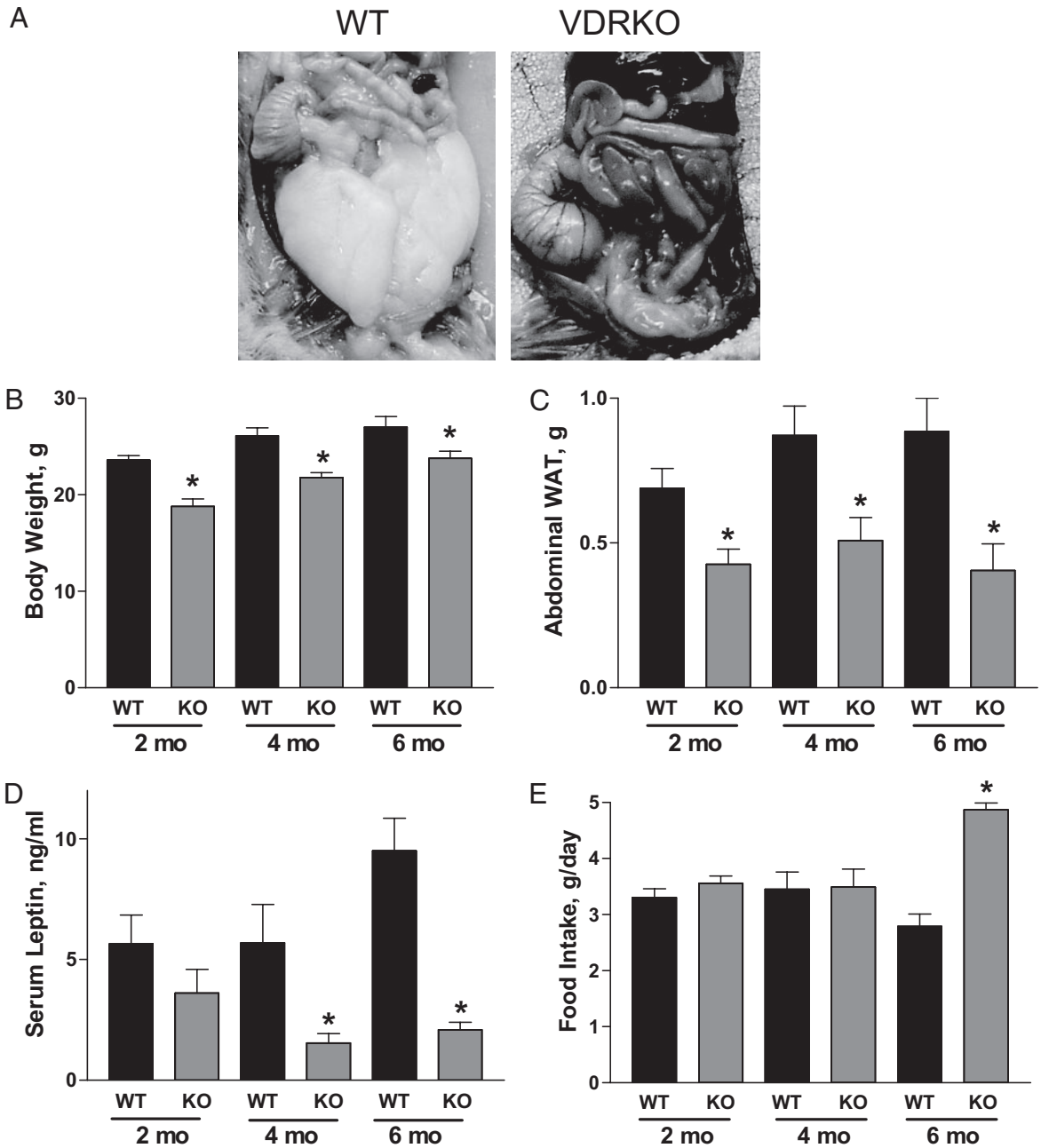


FIG. 1. Adipose stores and food intake in WT and VDRKO mice. A, Representative images of visceral adipose stores in 18-month WT (*left panel*) and VDRKO (*right panel*) mice fed the high-calcium rescue diet from weaning. Body weights (B) and abdominal WAT (C) were measured in normocalcemic WT and VDRKO mice on the C57BL6 background at 2, 4, and 6 months of age. D, Serum leptin was measured by ELISA. E, Individual food intake was measured over the 10-d period preceding the time the animals were killed. All data are mean ± SE. *, $P < 0.05$, WT *vs.* VDRKO.

To determine whether changes in body weights reflected changes in adipose tissue mass, the sc (inguinal) and visceral (abdominal) fat depots were dissected and weighed at each time point. Data for the abdominal (Fig. 1C) and inguinal fat pads (not shown) indicated that VDRKO mice had significantly reduced adipose tissue mass by 2 months of age. At 6 months, the abdominal adipose depots in VDRKO mice averaged 50% of age-matched WT mice. Consistent with this evidence of reduced adiposity, serum leptin, an adipokine that reflects total body fat stores, was significantly reduced in VDRKO mice at both 4 and 6 months of age (Fig. 1D). Surprisingly, the reduced fat stores in VDRKO mice were not secondary to inadequate food intake, and 6-month-old VDRKO mice actually consumed more food than their WT counterparts (Fig. 1E). Similar deficits in body weight were observed in VDRKO male mice over the same age period (data not shown).

To determine whether VDR ablation had similar effects on brown adipose tissue mass, the intrascapular depots were removed, extensively cleaned of white adipose tissue and muscle and weighed. No differences in absolute iBAT weights were detected between WT and VDRKO mice at any age (supple-

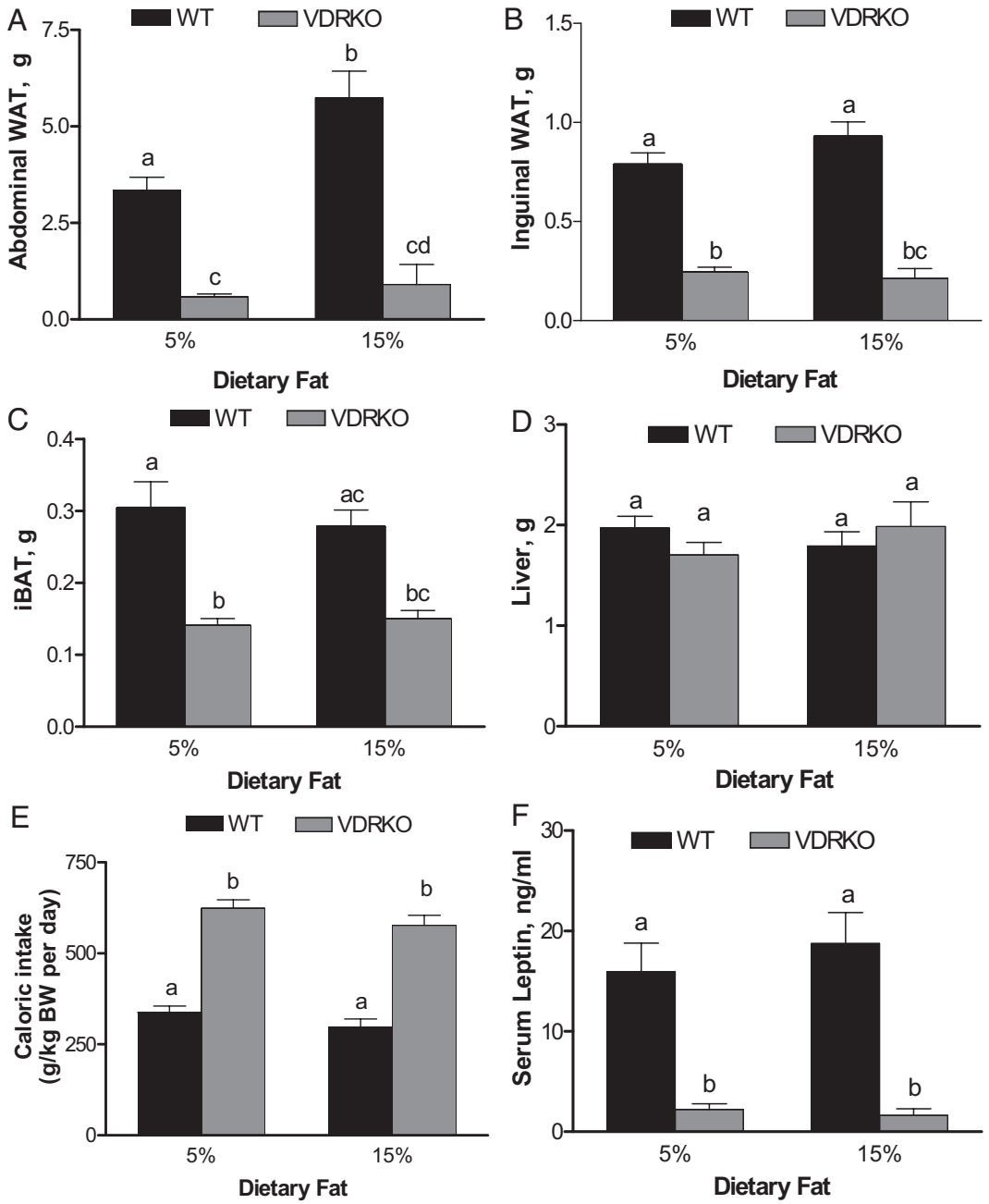


FIG. 2. Effects of high-fat diet on tissue weights, food intake, and serum leptin in WT and VDRKO mice on the CD1 genetic background. WT and VDRKO mice on the CD1 background were weaned onto the high-calcium rescue diet containing either 5 or 15% fat from corn oil. Diets were provided *ad libitum* and mice were killed at 6 months of age. A, Abdominal WAT. B, inguinal WAT. C, iBAT. D, Liver. E, Caloric intake was measured in individually caged mice for the 10 d preceding the time the animals were killed. F, Serum leptin was assessed by ELISA. All data are mean ± SE; *bars with different letters* (a–d) are significantly different by ANOVA ($P < 0.05$).

mental Table 1, published as supplemental data on The Endocrine Society's Journals Online web site at http://endo.endojournals.org). Furthermore, there were no age-related reductions in the absolute weight of the kidney or the female reproductive tract (uterus and ovaries) in VDRKO mice (supplemental Table 1).

Effect of genetic background and dietary fat on weight gain and adiposity of VDRKO mice

Because the C57BL6 strain of mice can exhibit considerable heterogeneity in body weight and susceptibility to obesity, we were concerned that the lean phenotype of our VDRKO mice may have been selected for during breeding of our colony. To exclude this possibility, additional studies were conducted with VDRKO and WT mice that were backcrossed to CD1 mice, a strain that is prone to obesity and impaired glucose tolerance (23). In these experiments we also investigated the effect of increasing dietary fat content of the rescue diet from 5 to 15% through the addition of corn oil. Mice (n = 4–6/group) were assigned to the diets at weaning and studied at the age of 6 months.

Compared with their counterparts on the C57BL6 background, WT and VDRKO mice on the CD1 background were heavier and displayed more lipid accumulation. When fed the standard rescue diet (which contains 5% fat), the mean body

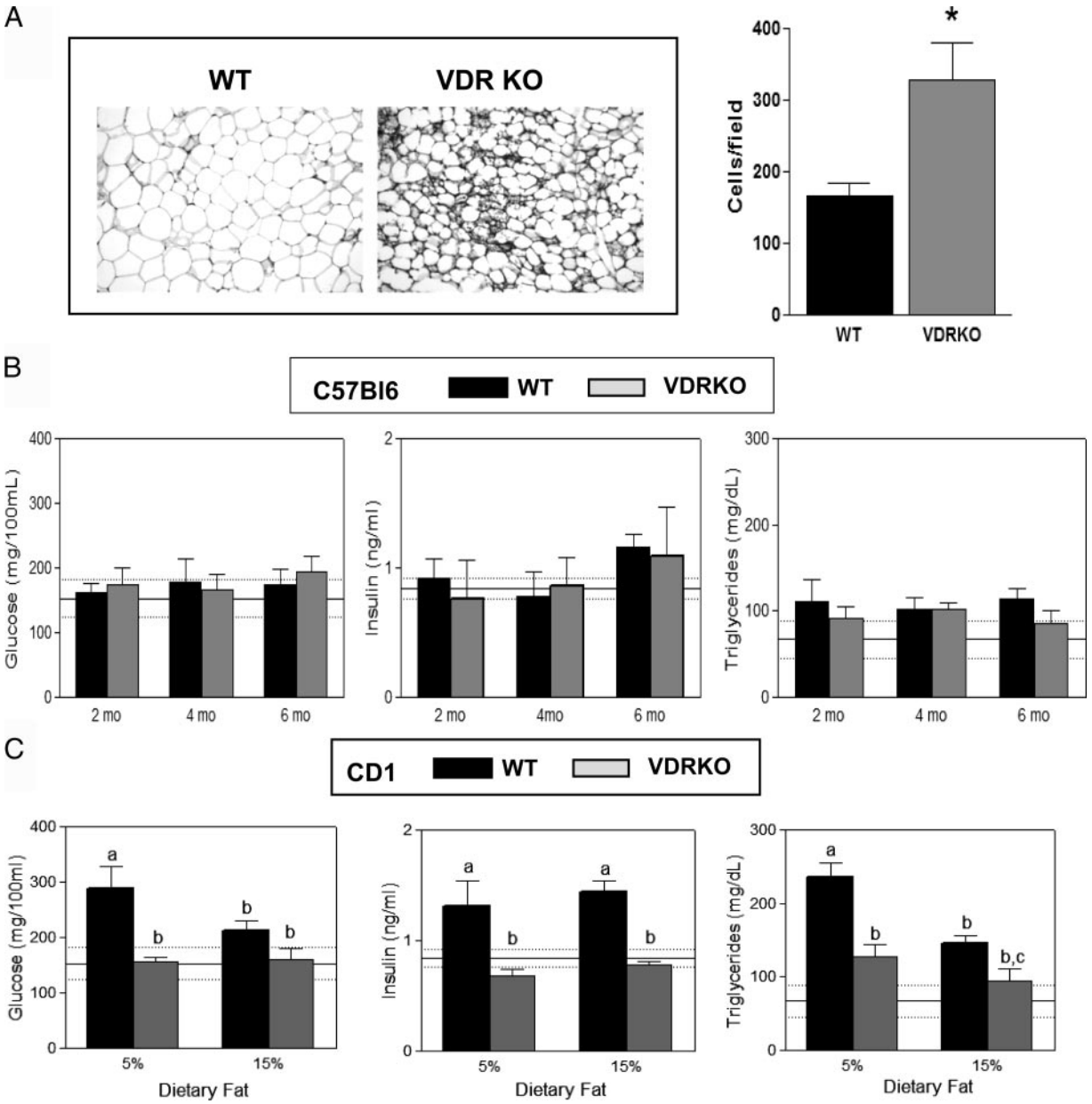
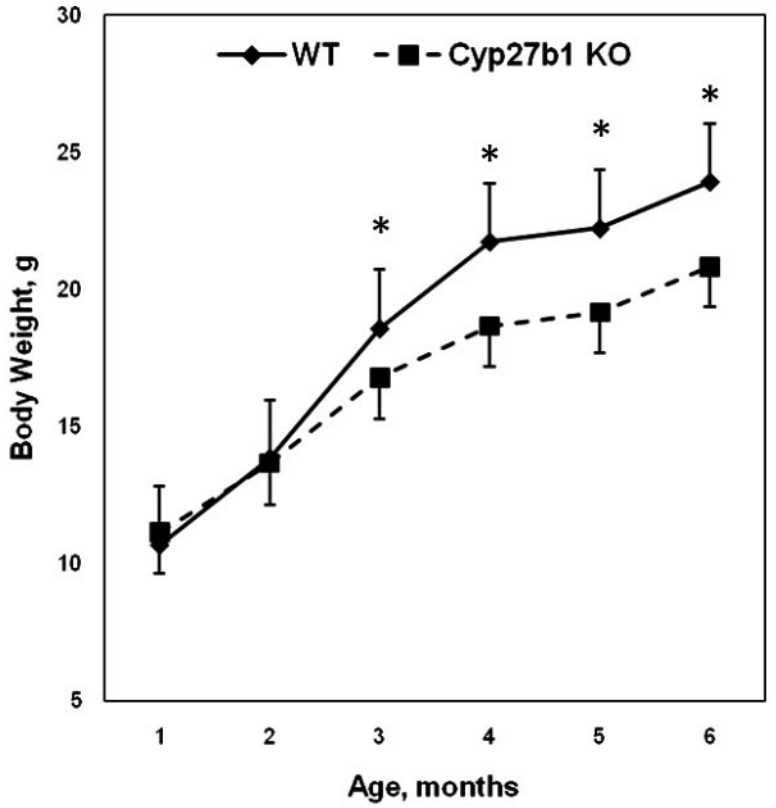


FIG. 3. WAT histology and metabolic parameters in WT and VDRKO mice. A. *Left panel*, Representative H&E-stained sections of abdominal WAT from 6-month-old WT and VDRKO mice on the C57BL6 background. Fat pads were collected, fixed, and subjected to H&E staining. A representative image is shown for each fat pad/genotype. *Right panel*, The number of cells per field was counted for three to four mice per genotype on the CD1 background; *bars* represent mean ± SE. *, $P < 0.05$ by Student's *t* test. Serum was collected from C57BL6 (B) and CD1 (C) mice for analysis of glucose, insulin, and triglycerides as described in *Materials and Methods*. *Background lines* represent the reference values (mean ± SE) for C57BL6 mice (obtained from the Mouse Phenome Database at www.jax.org) for each parameter. *Data bars* are mean ± SE; *bars with different letters* (a–c) are significantly different by ANOVA ($P < 0.05$).

weight of CD1 WT mice (42.0 ± 1.5 g) was significantly higher than that of CD1 VDRKO mice (33.1 ± 1.7 g), and both abdominal and sc adipose depots were smaller in VDRKO mice (Fig. 2, A and B). In response to high-fat feeding, body weights remained higher in WT mice (53.5 ± 2.0 g) than VDRKO mice (41.8 ± 0.5 g). Furthermore, in contrast to the increase in adiposity observed in WT mice reared on the 15% fat rescue diet, abdominal adipose mass of VDRKO mice did not increase in response to higher fat intake (Fig. 2, A and B). iBAT mass was not affected by dietary fat in either genotype, but in contrast to the C57BL6 mice, iBAT mass was significantly smaller in CD1 VDRKO mice compared with CD1 WT mice on both diets (Fig. 2C). There was no significant effect of VDR ablation on liver mass (Fig. 2D). On both low- and high-fat diets, CD1 VDRKO mice consumed significantly more calories than WT mice (Fig. 2E). Consistent with the reduced adipose stores, serum leptin was markedly reduced in the CD1 VDRKO mice, even on the high-fat diet (Fig. 2F).

Effect of VDR ablation and dietary fat on tissue histology and metabolic parameters

Histology of WAT, iBAT, and liver of 6-month-old mice was assessed by H&E staining. In WT mice on both genetic backgrounds, WAT was composed of characteristic large, unilocular triglyceride-filled adipocytes (Fig. 3A, *left panel*). In contrast, WAT from VDRKO mice exhibited smaller unilocular adipocytes and frequent areas of multilocular cell clusters reminiscent of BAT. The mean number of cells per field (Fig. 3A, *right panel*), quantitated from the H&E images, was significantly higher in VDRKO tissue, confirming that the reduced tissue mass reflects a reduction in cell size. Similar morphology was observed for both genetic backgrounds. No obvious differences in iBAT or liver morphology were observed in 6-month-old WT and VDRKO animals on either the 5% fat diet (supplemental Fig. 1) or the 15% fat diet (not shown). In particular, there was no evidence of fatty liver on H&E stained sections in either genotype even on the high-fat diet.



C57Bl6	Abdominal WAT, g/kg BW	iBAT, g/kg BW	Liver, g/kg BW	Serum Leptin, ng/ml	Food Intake, g/day
WT	22.7 ± 1.9	2.9 ± 0.1	41.5 ± 1.6	3.76 ± 1.0	2.99 ± 0.17
Cyp27b1 KO	14.7 ± 0.7*	2.8 ± 0.2	43.8 ± 2.0	1.15 ± 0.17*	4.11 ± 0.31*

FIG. 4. Analysis of WT and Cyp27b1 knockout (KO) mice. *Top panel*, Female WT and Cyp27b1 KO mice on the C57BL6 background were fed a high-calcium rescue diet containing 15% fat from weaning and body weights (BW) were monitored through 6 months of age. Data are mean values of four to seven mice. *, $P < 0.05$, WT *vs.* Cyp27b1 knockout. *Bottom panel*, Tissue weights, serum leptin, and food intake of WT and Cyp27b1 knockout mice on the standard rescue diet were measured at 9 months of age as described in *Materials and Methods*. Data represent mean ± SE. *, $P < 0.05$, WT *vs.* Cyp27b1 knockout.

The effects of diet and genotype on metabolic parameters were assessed in WT and VDRKO mice on both genetic backgrounds. Serum glucose, insulin, and triglyceride levels in WT and VDRKO C57BL6 mice on the 5% fat rescue diet were within the normal ranges for this strain and were not significantly different at any age (Fig. 3B). On the CD1 genetic background (a strain prone to impaired glucose tolerance), WT mice fed 5% fat exhibited higher serum glucose, insulin, and triglyceride levels than age- and diet-matched WT mice on the C57BL6 background (Fig. 3C). In contrast, serum glucose and triglyceride levels were similar in VDRKO mice on both genetic backgrounds. Thus, on the CD1 background, VDRKO mice were protected against the elevations in serum triglycerides, insulin, and glucose that are characteristic of this strain, even on the high-fat diet.

Mice lacking 1,25-dihydroxyvitamin D, the VDR ligand, also exhibit lean phenotype

The VDR is a transcription factor that can be activated by binding to $1,25(OH)_2D_3$, a steroid ligand that is generated from circulating 25-hydroxyvitamin D_3 by the enzyme Cyp27b1. However, the VDR also exerts $1,25(OH)_2D_3$-independent effects in some tissues (notably skin and hair), as demonstrated by distinct phenotypes in humans and mice lacking VDR compared with those lacking $1,25(OH)_2D_3$ (17, 18, 24). These precedents of $1,25(OH)_2D_3$-independent actions of VDR *in vivo* prompted us to investigate whether the effects of VDR ablation on adiposity would be phenocopied in mice lacking Cyp27b1. Cyp27b1 KO mice, which are on the C57BL6 genetic background, have undetectable levels of circulating $1,25(OH)_2D_3$ and are routinely maintained on the identical 5% fat rescue diet that is used for maintenance of VDRKO mice. On this diet, young male Cyp27b1 KO mice maintain normal serum calcium and appropriate bone mineralization but exhibit 20% lower body weights than WT mice (25). To assess whether body weight of Cyp27b1 KO mice would be enhanced by increasing dietary fat, female WT and Cyp27b1 KO mice were fed the rescue diet containing 15% fat from weaning. Beginning at 3 months of age, body weights were significantly lower in Cyp27b1 KO mice than their WT counterparts (Fig. 4, *top panel*). We followed up these data by assessing tissue weights and food intake in older Cyp27b1 KO mice fed the standard rescue diet (Fig. 4, *bottom panel*). Although the mean body weight of 9-month-old Cyp27b1 KO mice (21.8 g) fed the 5% fat rescue diet was only marginally reduced compared with age-matched WT mice (23.2 g), the abdominal adipose tissue was significantly smaller in Cyp27b1 KO mice than WT mice. In addition, Cyp27b1 KO mice were hypoleptinemic and consumed significantly more food than their WT counterparts. Cyp27b1 ablation did not affect weight of either iBAT or liver. Thus, these data on Cyp27b1 KO mice on the C57BL6 background are similar to those obtained in the C57BL6 VDRKO mice, suggesting that both VDR and its ligand impact on adipose tissue maintenance.

VDR ablation alters gene expression in WAT and iBAT

To assess whether VDR might exert direct effects on adipose tissue, we used real-time PCR to measure VDR gene expression in WAT and iBAT of WT mice, with VDRKO tissue used as negative control. Figure 5A demonstrates that VDR gene expression was detected in both WAT and iBAT of WT mice but was below detection limits in tissue from VDRKO mice. In WAT, VDR was decreased approximately 50% on high-fat feeding, but in iBAT, VDR was minimally affected by high-fat feeding.

To determine whether VDR ablation altered expression of known genes involved in adipogenesis in WAT or iBAT, we measured expression of leptin, PPARγ and FAS genes, with the expectation that all three of these genes would be reduced in VDRKO mouse adipose tissue. Indeed, the pattern of leptin mRNA expression (Fig. 5B) in WAT was, as expected, significantly higher in WAT of WT mice in response to increased fat intake and stores and lower in VDRKO mice, regardless of diet. Leptin was also significantly reduced in iBAT of VDRKO mice. These data are consistent with the serum leptin analyses presented in Figs. 1 and 2.

Because FAS catalyzes the synthesis of long chain fatty acids for incorporation into triglycerides, we expected that FAS gene expression patterns in WAT would be similar to that of leptin, *i.e.* lower in VDRKO mice with reduced adiposity. Contrary to this expectation, FAS was significantly higher in WAT from VDRKO mice compared with WT mice on the 5% fat diet and tended to be higher in VDRKO mice on the 15% fat diet (Fig. 5C). FAS expression in iBAT was not significantly affected by genotype or diet.

We also considered whether the lean phenotype in VDRKO mice might be secondary to decreased expression of PPARγ, a nuclear receptor that is highly expressed in adipose tissue and is essential for adipogenesis. Surprisingly, no significant differences in PPARγ gene (Fig. 5D) or protein (Fig. 5E) expression were detected in WAT of VDRKO and WT mice. PPARγ gene expression tended to be lower in iBAT of VDRKO mice, but this was not statistically significant.

Because the expression data for the key lipogenic genes PPARγ and FAS did not clarify the underlying basis for the lean phenotype of VDRKO mice, we used a PCR screening array to simultaneously profile the expression of 84 genes involved in insulin signaling in WAT of 6-month-old CD1 WT and VDRKO mice fed the 5% fat rescue diet. Table 1 summarizes the top 12 genes that were identified through this screen. Classes of differentially expressed genes included those involved in catabolism (ucp-1, gpd1, hk2, acox1), lipid uptake and synthesis (ldlr1, acaca), insulin signaling (akt1, akt2, dok1), and lipid regulatory transcription factors (steroid regulatory element binding protein-1 and CCAAT/enhancer-binding protein-β).

Of the genes implicated by the screening approach, we chose to follow up ucp-1, which was 25-fold elevated in WAT of VDRKO mice compared with WT mice. Ucp-1 is of particular interest because it functions as an uncoupler of mitochondrial ATP production, and thus, its up-regulation in WAT could potentially explain the failure of VDRKO mice to maintain adipose stores. Furthermore, ucp-1 is a highly specific marker for brown adipocytes, yet our screen detected induction of ucp-1 in WAT of VDRKO mice. Figure 6A demonstrates the normalized expression of ucp-1 from the screening array data. Ucp-1 RNA expression was undetectable in WAT of WT mice but was strongly induced in all three samples tested from VDRKO mice. Up-reg-

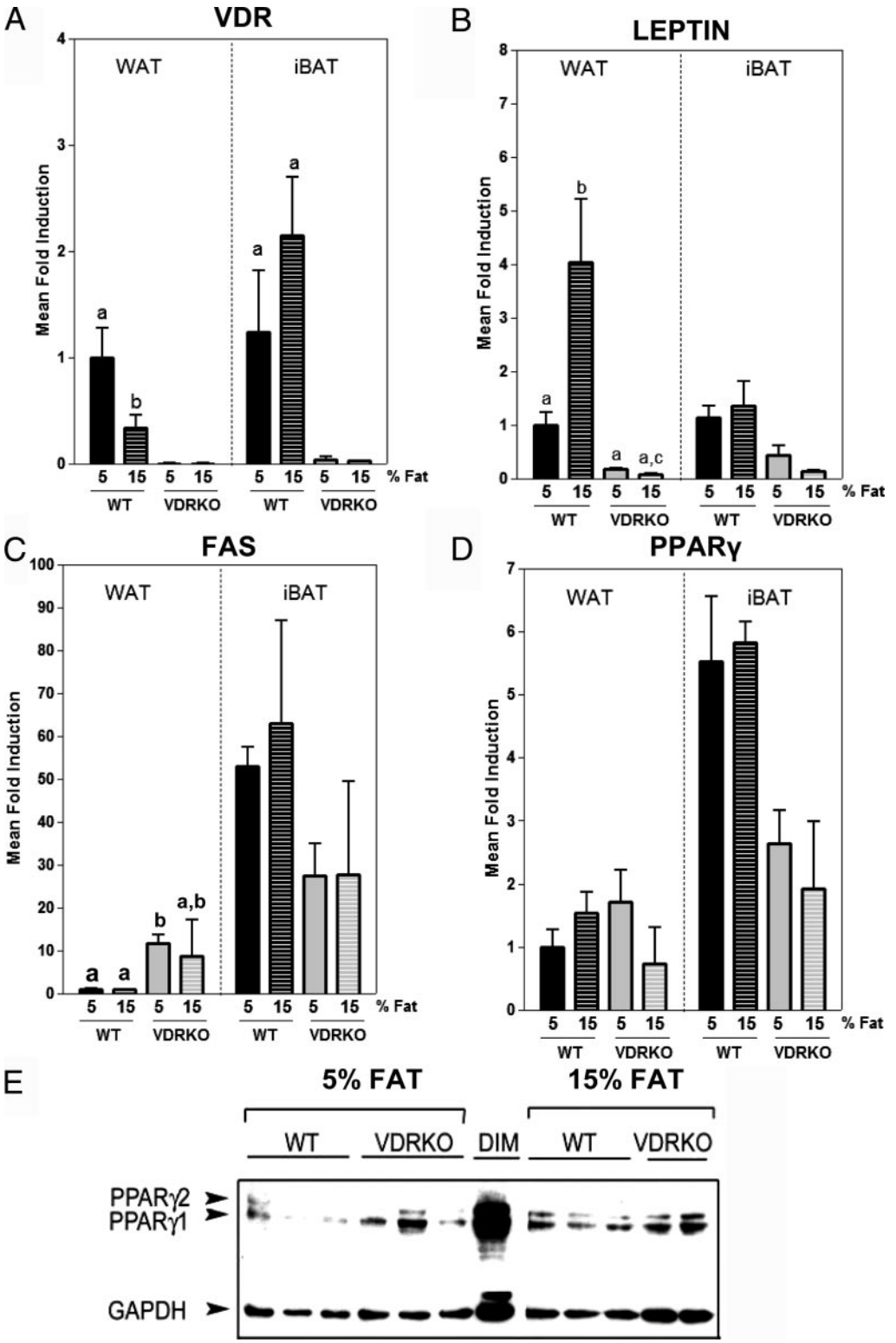


FIG. 5. Effect of VDR genotype on gene and protein expression in white and brown adipose tissue. Gene expression was determined by real-time PCR in WAT and iBAT from normocalcemic 6-month-old WT mice on the CD1 background fed two levels of dietary fat (5 *vs.* 15%) from weaning. Data were calculated by the comparative cycle threshold method, normalized against 18S and expressed relative to values from WAT of WT mice fed 5% fat, which was set at 1. A, VDR. B, Leptin. C, FAS. D, PPARγ. For A–D, *bars* represent mean ± SE. *, Significantly different ($P < 0.05$) compared with WT mice fed 5% fat. *Bars with different letters* are significantly different by ANOVA ($P < 0.05$). E, Western blot of PPARγ protein expression in WAT from two to three individual mice per group. Both PPARγ1 and PPARγ2 isoforms were detected in WAT, but no consistent differences were observed by diet or genotype. DIM, Cells treated with adipogenic cocktail used as positive control.

ulation of ucp-1 mRNA was independently confirmed with additional samples from the same study by real-time PCR analysis (Fig. 6B). Western blotting of WAT lysates from these mice was then conducted to determine whether the PCR results translated to differential expression of ucp-1 protein. As demonstrated in Fig. 6C, ucp-1 was strongly detected in lanes 5 and 6, corre-

TABLE 1. Genes differentially expressed in WAT tissue of 6-month-old CD1 WT and VDRKO mice identified by PCR-focused array

Gene name	Fold change VDRKO/WT	Pathway
Ucp-1	26.7	Thermogenesis
Acetyl-coenzyme A carboxylase-α (acaca)	26.2	FAS
Glycerol-3-phosphate dehydrogenase (gpd1)	25.2	Glycerol degradation
Hexokinase (hk2)	6.4	Glycolysis/pentose shunt
LDL receptor (ldlr)	5.7	Lipid uptake
Akt2	5.5	Insulin receptor signaling
Acyl-coA oxidase (acox1)	4.2	Fatty acid oxidation
Akt1	3.5	Insulin receptor signaling
CCAAT/enhancer-binding protein-β	3.4	Transcription factor
Steroid regulatory element-binding protein (srebp1)	2.7	Transcription factor
Protein tyrosine phosphatase receptor type F (ptprf)	−3.6	Intracellular signaling
Docking protein 1 (dok1, p62)	−4.5	Insulin receptor signaling

sponding to samples from VDRKO mice, but not in lanes 1–3, corresponding to samples from WT mice. These lanes each contained 50 μg total WAT protein, and comparable GAPDH expression confirmed equal loading. Due to the small amount of WAT in VDRKO mice, sample volume was often limiting, as demonstrated in lane 4, which contained insufficient protein (evidenced by minimal GAPDH staining) to detect ucp1 protein. Further confirmation of ucp-1 protein expression in white adipocytes was obtained by immunohistochemistry, which detected large patches of ucp-1-positive cells in WAT of VDRKO mice but negligible staining in tissue from WT mice (Fig. 6D). Collectively, these approaches confirmed the lack of ucp-1 expression in WAT of WT mice and its induction in WAT of VDRKO mice.

Discussion

Our data clearly show that loss of VDR function negatively impacts on total adipose tissue stores *in vivo*. This conclusion is based on the phenotype of reduced body and adipose tissue weights, decreased adipocyte size, and hypoleptinemia in VDRKO mice which begins in young adulthood. By 6 months of age, leptin gene expression was low and adipocytes were smaller in WAT P10 of VDRKO mice. VDRKO mice were also highly resistant to high fat diet-induced weight gain despite hyperphagia relative to WT mice. Importantly, mice lacking Cyp27b1, the 25-hydroxyvitamin D_3 1α-hydoxylase enzyme that generates $1,25(OH)_2D_3$, the VDR ligand, display a similar lean phenotype with hypoleptinemia and hyperphagia. Although a more detailed comparison of the phenotypes of the VDRKO and Cyp27b1 KO mice (with age and dietary manipulation) is needed, these studies suggest that both VDR and its ligand contribute to the maintenance of adipose tissue. This suggestion is consistent with previous incidental descriptions of adipose tissue atrophy in the prostate and mammary glands of VDRKO mice (14, 15) and smaller body weights of Cyp27b1 KO mice (25). The observation that the adipose tissue phenotype of VDRKO mice is more pronounced with age is consistent with other data demonstrating age-related impairments in hair follicle signaling, osteoblast progenitor formation, and keratinocyte stem cell renewal in these mice (17–19). Furthermore, new insights into the mechanisms of action of klotho, a putative aging suppressor gene, hint at complex interactions between fibroblast growth factors, $1,25(OH)_2D_3$, and the VDR in the aging process (26). Control of metabolism and adiposity may therefore be another age-related process under control by this regulatory network.

Although $1,25(OH)_2D_3$ and the VDR are critical for maintenance of calcium homeostasis, the observed effects on adiposity are unlikely to be related to regulation of extracellular calcium because both VDRKO and Cyp27b1 KO mice received a high-calcium diet that normalizes serum calcium (15, 21, 22). However, more detailed assessment of calcemic regulatory hormones such as PTH is necessary to completely exclude this possibility. Our data also indicate that VDR ablation did not alter growth or histology of other VDR target organs such as liver or kidney, excluding global growth disturbances and generalized tissue atrophy.

In these studies, we have confirmed the expression of VDR in WAT, suggesting that age-related changes in $1,25(OH)_2D_3$-VDR signaling in adipocytes could contribute to the lean phenotype of Cyp27b1 knockout and VDRKO mice. VDR is dynamically up-regulated in cell culture models of adipogenesis and VDR knockdown in 3T3-L1 cells inhibits adipogenic differentiation (4–6, 11). If the lean phenotype of VDRKO mice was secondary to impaired adipocyte differentiation, we would have expected changes in key regulators such as PPARγ and FAS, but these were not reduced in WAT of VDRKO mice. Furthermore, adipogenic differentiation was not impaired in bone marrow stromal cells derived from perinatal VDR null mice (16). Collectively, these data indicate that $1,25(OH)_2D_3$ signaling in cell culture models of preadipocyte differentiation differs substantially from that in mature adipocytes *in vivo*. Creation of an adipose-specific VDR null mouse model would be an important step toward determining whether VDR signaling plays a direct role in adipocyte metabolism *in vivo*.

Using a PCR screening approach, we identified several candidate genes, including ucp-1, that are differentially expressed in WAT of 6-month-old WT and VDRKO mice. Interestingly, the majority of the differentially expressed genes were up-regulated in WAT of VDRKO mice, suggesting that if these are direct target genes, VDR might act primarily as a ligand-dependent transcriptional repressor in the context of adipose tissue function. We confirmed that ucp-1 protein, which is normally restricted to brown adipocytes, is induced in WAT of VDRKO mice. It is unclear whether this induction reflects transdifferentiation of white adipocytes into brown adipocytes in VDRKO tissue or the absence of VDR mediated repression of ucp-1 in white adipo-

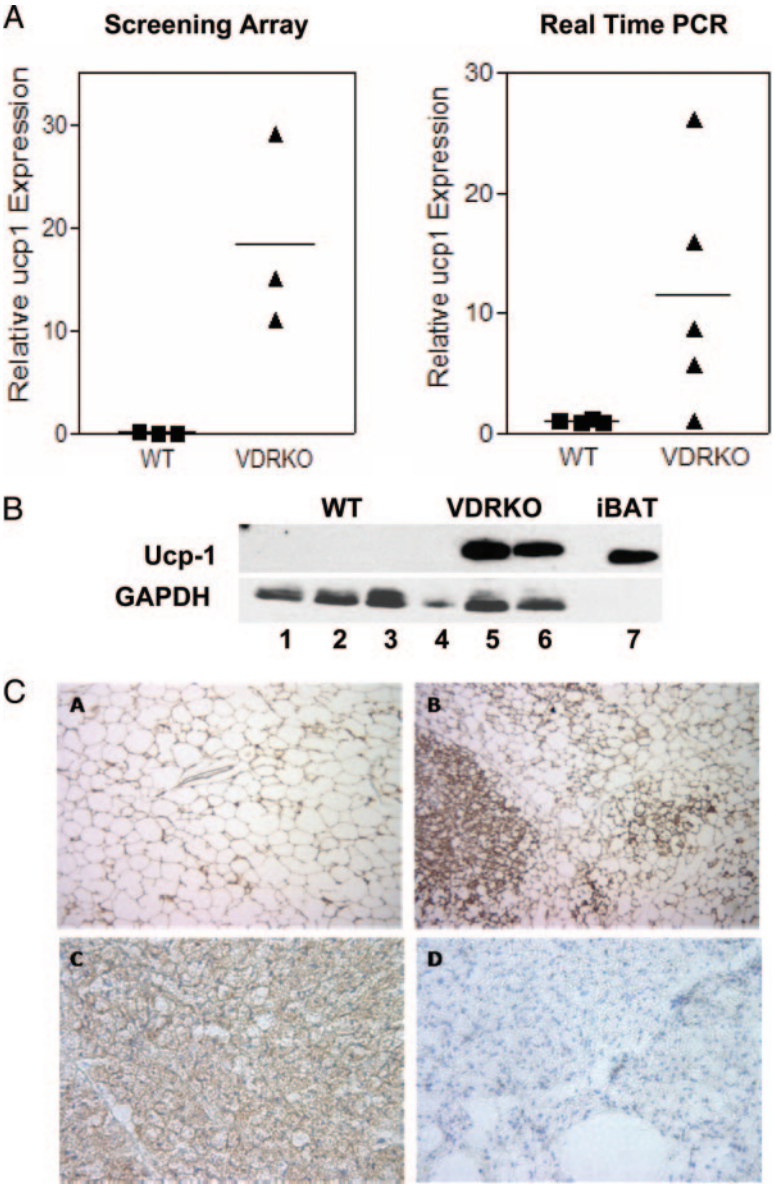


FIG. 6. Effect of VDR ablation on ucp-1 expression in WAT. A, Relative ucp-1 gene expression normalized against hypoxanthine-guanine phosphoribosyltransferase (calculated from screening array data) in abdominal WAT of 6-month-old CD1 WT and VDRKO mice fed the high-calcium rescue diet containing 5% fat. Each *symbol* represents tissue from an individual mouse, and *horizontal bar* represents group mean. B, Confirmation of ucp-1 up-regulation in WAT from 6-month-old VDRKO mice by real-time PCR. Each *symbol* represents tissue from an individual WT or VDRKO CD1 mouse fed the high-calcium rescue diet containing 5% fat; *horizontal bar* represents group mean. C, Western blot of ucp-1 protein expression in WAT (50 μg/lane) from three individual mice per group (6 month old WT or VDRKO mice fed 5% fat). Lanes 1–3, WT; lanes 4–6, VDRKO; lane 7, iBAT lysate (2 μg/lane) was used as positive control for ucp-1. Blots were stripped and reprobed with anti-GAPDH as loading control. D, Immunohistochemistry of ucp-1 in formalin-fixed sections of WAT from 6-month-old CD1 WT (A) and VDRKO (B) mice fed 5% fat. Sections were incubated with antibody against ucp-1 (5 μg/ml) and developed with the diaminobenzidine procedure. Brown adipose tissue was used as positive control (C) and no antibody negative control (D). Slides were counterstained with hematoxylin.

cytes. Ucp-1 and related proteins ucp-2 and ucp-3 display tissue-specific expression, but all function as proton carriers in the mitochondrial membrane and mediate dissociation of oxidative phosphorylation from ATP synthesis. Consistent with a role for vitamin D signaling in control of mitochondrial respiration, Shi *et al.* (12) reported that physiological concentrations of $1,25(OH)_2D_3$ inhibited ucp-2 in human white adipocytes, an effect that was prevented by VDR knockdown. Further studies will be necessary to determine whether the $1,25(OH)_2D_3$-VDR complex directly represses ucp-1 transcription in murine white adipocytes. Regardless of mechanisms, because ucp-1 functions in mitochondria to uncouple respiration and oxidative phosphorylation, up-regulation of this protein in WAT could contribute to the limited capacity of VDRKO mice to increase adipose stores. In support of this concept, resistance to diet-induced obesity in liver X receptor null mice has been linked to ectopic expression of ucp-1 in WAT and skeletal muscle, resulting in uncoupled oxidative phosphorylation and abnormal energy dissipation (2). If a similar disturbance occurs in the absence of VDR, we would predict that VDRKO mice would exhibit age-related increases in energy expenditure that would be enhanced in response to high-fat feeding.

In addition to potential direct effects of VDR on adipocytes, $1,25(OH)_2D_3$/VDR deficiency may exert other, more global effects that impact on overall energy balance. Although our data do not specifically rule out effects of VDR ablation on the central nervous system, the observation that VDRKO and Cyp27b1 mice consumed more calories than WT mice suggests that chronic hypoleptinemia in these animals stimulated appetite and argues against an effect at the level of the hypothalamus. However, studies to assess the sensitivity of VDRKO mice to exogenous leptin administration would be necessary to confirm this suggestion. Previous behavioral studies have also demonstrated that general activity patterns are not increased in VDRKO mice (28), although as noted above, increased metabolism has not been ruled out.

Vitamin D and the VDR have long been implicated in control of insulin secretion (29, 30), and insulin secretion in response to glucose challenge was impaired in a distinct strain of VDR mutant mice (31). In our studies, VDRKO mice on the C57BL6 genetic background had normal circulating insulin and glucose, but VDRKO mice did not exhibit the characteristic hyperglycemia and hyperinsulinemia when bred onto the obesity-prone CD1 background. Therefore, characterization of pancreatic insulin secretory capacity and peripheral insulin sensitivity in VDRKO mice on these two genetic backgrounds as a function of age and diet will be an important future direction.

In summary, in addition to its well-known role in osteogenesis, the VDR and its ligand impact on lipid accumulation and gene expression in adipose tissue. The VDR is expressed in both white and brown adipocytes, and vitamin D and its metabolites are stored in adipose tissue (32). Furthermore, obesity is associated with abnormal vitamin D hydroxylation (33) and leptin secreted from adipocytes modulates bone turnover (34). Other recent studies demonstrated that the $1,25(OH)_2D_3$ regu-

lated, secreted bone protein osteocalcin mediates effects on adipose tissue, β-cells, and energy metabolism (35). Thus, emerging data suggest that $1,25(OH)_2D_3$ and the VDR likely function in a complex network that coordinately regulates bone formation, adipose stores, and energy metabolism in response to physiological and nutritional cues. Indeed, many nuclear receptors that also heterodimerize with RXR (notably liver X receptor and PPARs) sense environmental and dietary signals and function as global regulators of metabolism. Dissecting the interactions of these receptors and their ligands in regulation of adiposity and energy metabolism *in vivo* represents a challenge for future studies.

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