We recently identified mutations in the lipin gene, Lipin1, as the cause of lipodystrophy in the fatty liver dystrophy (fld) mouse. Here we identify impaired adipocyte differentiation as the basis for lipodystrophy in lipin-deficient mice and demonstrate that lipin is required for normal induction of the adipogenic gene transcription program. We found that the reduced adiposity in chow fed fld mice and resistance to obesity in fld mice fed a high-fat diet is associated with reduced adipogenic gene expression. Using primary mouse embryonic fibroblasts isolated from fld mice, we confirmed that lipin deficiency prevents normal lipid accumulation and induction of key adipogenic genes, including peroxisome proliferator-activated receptor (PPAR)γ and CCAAT enhancer-binding protein (C/EBPα). However, our previous studies of daily gene expression at 10-h intervals during 3T3-L1 cell differentiation indicated that lipin expression is undetectable until about day 3 of differentiation, at a point after PPARγ and C/EBPα gene expression is established. This paradox was resolved by examining gene expression at 10-h intervals during 3T3-L1 cell differentiation, leading to detection of transient lipin expression at 10 h into the differentiation program, prior to the induction of PPARγ and C/EBPα. Consistent with a requirement for lipin expression upstream of PPARγ, differentiation of lipin-deficient mouse embryonic fibroblasts could be rescued by ectopic expression of PPARγ. Thus, we conclude that lipin expression is required prior to PPARγ during adipocyte differentiation.

The increasing prevalence of obesity and type II diabetes in our society has focused attention on the development and function of adipose tissue. It is well established that adipose tissue performs a variety of metabolic functions, including energy partitioning and production of endocrine hormones. Studies in human and mouse models of obesity have revealed that excess adipose tissue confers increased risk for diabetes, dyslipidemia, and coronary heart disease (1–3). Studies of lipodystrophy reveal that insufficient adipose tissue can predispose to these same conditions, indicating that normal adipose tissue development and function is critical for metabolic homeostasis (4–7).

The gene defects and cellular mechanisms underlying congenital lipodystrophies in humans have become a focus of intensive investigation in recent years (reviewed in Refs. 4, 8–11). Heterogeneity exists in both the genetic basis and in the potential cellular mechanisms for different forms of lipodystrophy in humans. Thus far, mutations in four genes have been identified in human congenital lipodystrophies. Mutations in LMNA, encoding the nuclear envelope proteins lamin A and C, cause familial partial lipodystrophy (Dunnigan variety) (12, 13). A distinct form of partial lipodystrophy has been associated with heterozygous missense mutations in PPARγ1 nuclear transcription factor, which plays a key role in adipogenesis (14, 15). Mutations in BSCL2, a novel gene of unknown function with prominent expression in the brain, cause Bardinelli-Seip complete lipodystrophy (16). Mutations in AGPAT2 (acylglycerol-3-phosphate acyltransferase), encoding an enzyme required for triglyceride synthesis, also cause congenital generalized lipodystrophy (17). Although defined mechanisms exist by which PPARγ and AGPAT2 mutations might confer adipose tissue deficiency, the role of LMNA and BSCL2 mutations are not well established. It has been proposed that LMNA mutations impair adipocyte differentiation by affecting the activity of sterol regulatory element binding protein-1, a transcription factor for lipogenic gene expression during adipocyte differentiation (18), but further work is required to fully establish such a mechanism.

Several mouse models of lipodystrophy and obesity resistance have been generated through gene knockout and transgene technologies (reviewed in Refs. 7, 19–21). Analysis of the physiological basis for fat depletion in these models has revealed that alterations in multiple processes can lead to reduced adipose tissue stores. These alterations include impaired fat cell precursor proliferation (null mutations in the high mobility group protein 1c, Ref. 22), impaired fatty acid delivery to adipose tissue (very low density lipoprotein receptor-null mutants, Ref. 23), altered lipogenesis (glycerol 3-phosphate dehydrogenase transgenic mice, Ref. 24), altered balance between fat storage and lipolysis (acylation-stimulating protein-null mice, Ref. 25), increased fatty acid oxidation or energy expenditure (acetyl CoA carboxylase 2-null mice, Ref. 26), peroxisome proliferator activated receptor δ transgenic mice, Ref. 27), and altered activity of adipogenic transcription factors (C/EBPα, β, or δ-null mutants, Refs. 28 and 29; A-ZIP/F transgenics, Ref. 30; aP2-mSREBP-1c transgenics, Ref. 31).

The fatty liver dystrophy (fld) mouse is unique among the lipodystrophic mouse models mentioned above in that it is the...
result of a spontaneous mutation in an endogenous gene. The fld mouse resembles human patients with generalized lipodystrophy, having dramatically reduced adipose tissue mass throughout the body and acquired insulin resistance and increased susceptibility to atherosclerosis (32). Using a positional cloning approach, we identified the causative mutation in the Lipin1 gene, which encodes a novel protein, lipin (33). Consistent with the lipo dystrophic phenotype of fld mice, lipin is prominently expressed in white and brown adipose tissue and is induced during differentiation of 3T3-L1 preadipocytes. Although the molecular function of lipin remains unknown, recent studies of the lipin homolog in Schizosaccharomyces pombe revealed that yeast lipin interacts with three proteins having roles in nuclear envelope structure and nucleocytoplasmic transport (34). This finding is consistent with our observation that lipin localizes to the nucleus (33).

Here, we investigate the cellular mechanisms underlying lipodystrophy in lipin-deficient fld mice. We found that the lack of adipose tissue in these animals is a direct consequence of impaired adipocyte differentiation due to a critical role for lipin in this process. Specifically, we establish that lipin is required during the initial stages of adipogenesis for the induction of key adipogenic factors, including PPARγ and CCAAT enhancer-binding protein (C/EBPα), such that lipin deficiency prevents maturation of adipocytes in vitro and causes lipodystrophy in vivo.

EXPERIMENTAL PROCEDURES

Mice and Diet—BALB/cByJ® mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and bred to produce lipin-deficient (fld/fld) and wild-type (+/+ ) mice for studies. Mice were fed a standard laboratory chow diet (Purina 5001) or high-fat diet containing 35% fat and 33% carbohydrate (Diet F3282, Bio-Serve, Frenchtown, NJ). All animal studies were performed under approved institutional protocols and according to guidelines established in the "Guide for the Care and Use of Laboratory Animals."

Adipose Tissue Measurements and Feed Conversion Efficiency—Fat pads (inguinal subcutaneous, gonadal, and retroperitoneal) were dissected and weighed after conclusion of the high-fat diet, or at 5 months of age in chow fed mice. Cell numbers in adipose tissue depots were determined by fluorometric DNA quantitation using Hoechst dye 33258 from pre-weighed inguinal adipose tissue depots (36). Food intake was determined over 10 days of ad libitum feeding. Feed conversion efficiency was calculated as the weight gain per effective food intake (food consumption normalized for fecal lipid output). Fecal lipid content was determined by quantitation of triglycerides and fatty acids in lipid extracts prepared from dried feces (37).

RNA Quantitation—Total RNA was isolated from adipose tissue or cultured cells with TRIzol (Invitrogen), and treated with RNase-free DNase (Ambion, Austin, TX) to remove any contaminating genomic DNA. First-strand cDNA synthesis was performed using oligo dT primers (Invitrogen). Real-time PCR were performed on the iCycler IQ real-time detection system (Bio-Rad) using SYBR Green PCR Quanti-Tect reagent kit (Qiagen, Valencia, CA). Each assay included (in triplicate): a standard curve of four serial dilution points of control cDNA (ranging from 100 ng to 100 pg), a no-template control, and 25–50 ng of each sample CDNA. The relative concentrations of the endogenous controls, TATA box binding protein (TBP) and hypoxan thine phosphoribosyl transferase, and genes of interest were determined by plotting the threshold cycle (Ct) versus the log of the serial dilution points, and the ratio of expression of the target gene was determined after normalizing to endogenous controls. Primers used for real-time PCR are as follows:

PPARγ (caagagcatggctgcttgatc; caagaacatgattgactcaactc)

CEBPα (caacagccaagctcagaatgc; cagctgcaactgctgatcagc)

C/EBPβ (caacagccaagctcagaatgc; cagctgcaactgctgatcagc)

DGAT (ctctgctgctgctgctgctg; ctctgctgctgctgctgctg)

Pref-1 (ctgctgctgctgctgctgctg; ctgctgctgctgctgctgctg)

TBP (acctactcactcactcactc; actactcactcactcactc)

hypoxanthine phosphoribosyltransferase (caagagcatggctgcttgatc; caagaacatgattgactcaactc).

Cell Culture—The 3T3-L1 cell line was maintained in Dulbecco’s modified Eagle’s medium containing 10% bovine serum. Mouse embryonic fibroblasts (MEFs) were derived from 18 day wild-type (WT) and fld embryos. Early passage cells (passage 3 or earlier) were used for differentiation and retroviral infection of primary cells. Spontaneously immortalized cell lines were developed as described (38) and used after passage 15. Adipocyte differentiation was initiated after 2 days in confluence with the addition of differentiation medium as described (39). For differentiation of MEFs and retrovirus-infected cells, adipocyte differentiation medium was supplemented with the PPARγ ligand, rosiglitazone (BRL 49653, a generous gift from Dr. Todd Leff) as described (40). After 2 days, differentiation mixture was removed and culture was continued in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum with insulin and rosiglitazone. At day 6, cells were lysed and homogenized for RNA isolation and triglyceride determinations, or fixed in 4% paraformaldehyde and stained with Oil Red O (41). All experiments were performed in triplicate.

The pBabe-PPARγ2 retroviral expression vector was obtained from Dr. Bruce Spiegelman. To produce the viral vector, lipid cDNA (GenBank accession no. AF180471) was subcloned into pBabe. Retroviral infection of cells was performed essentially as described (42).

Statistical Analysis—The number of mice or cell culture replicates for each study is indicated in the figure legends. Values are presented as means ± S.E. A two-tailed Student’s t test was used to calculate p values.

RESULTS

Lipin Deficiency Prevents Diet-induced Obesity and Impairs Adipose Tissue Gene Expression—The phenotype of the fld mouse revealed that lipin deficiency prevents adipose tissue accumulation under normal dietary conditions. However, it was unclear whether the effect of lipin deficiency on adipose tissue could be overcome by dietary manipulation to enhance fat accumulation. Therefore, we investigated whether fld mice could be made to gain fat by feeding a high-fat diet. After 15 weeks on the high-fat diet, WT mice increased their body weight by 27% (8.3 ± 1.8 g), whereas fld mice gained virtually no weight (0.4 ± 1.1 g) (Fig. 1a). The fld mice maintained 15-fold lower subcutaneous and retroperitoneal fat pad mass compared with WT animals (Fig. 1, b and c), with no increase over the fat mass of these mice on a chow diet (32). To determine whether reduced adipose tissue mass could result from impaired preadipocyte proliferation, we determined fat pad cellularity in mice fed chow and high-fat diets. Wild-type and fld mice had comparable cellularity on the chow diet, and both exhibited a trend toward slightly increased cellularity on the high-fat diet, indicating that lipin deficiency does not result in reduced fat pad cellularity (Fig. 1d). Nor could reduced fat in fld mice be attributed to reduced food intake or absorption. Wild-type and fld mice had identical food intake (Fig. 1e), but fld mice exhibited a 6-fold reduction in feed conversion efficiency (weight gain per food intake, normalized to food absorption; Fig. 1f).

We investigated whether lipin deficiency impairs adipose tissue accumulation through altered expression of adipogenic genes. On the chow diet, fld mice exhibited ~2-fold reductions in expression of the adipogenic transcriptional regulators, PPARγ and C/EBPα, and in the adipocyte fatty acid binding protein (aP2), a marker of adipocyte maturation and PPARγ target gene (Fig. 2). The expression of diacylglycerol acyltransferase (DGAT), a rate-limiting enzyme of triglyceride synthesis, was reduced 3-fold in fld tissue, thus likely contributing to reduced triglyceride accumulation. Wild-type mice exhibited modest (PPARγ, C/EBPα) to substantial increases (aP2, DGAT) in gene expression levels in response to the high-fat diet. In contrast, fld expression levels were not increased by the high-fat diet. These data indicate that lipin deficiency alters basal expression of adipogenic genes in adipose tissue and impairs the response of these genes to changes typically observed with
dietary manipulation, reflecting the failure offld mice to
develop increased adipose tissue mass on the high-fat diet.

Lipin Is Required for Adipocyte Differentiation in Vitro—The
results described above implicate impaired adipocyte differen-
tiation as the mechanism for diminished adiposity in lipin-
deficient mice. To verify that this depends upon the expression
of lipin within the adipocyte, we examined the adipocyte dif-
ferentiation capacity offld MEFs. Mouse embryonic fibroblasts
were induced to differentiate into adipocytes by incubation in
adipocyte differentiation mixture supplemented with the

Fig. 1. fld mice are resistant to weight gain on a high-fat diet. a, body weights of 7-month-old male wild-type and fld mice during 15 weeks
of the high-fat diet (n = 5 for each genotype). *, p < 0.01 versus wild-type. b and c, weights of subcutaneous and retroperitoneal fat pads normalized
by body weight taken fromfld and WT mice after 15 weeks of the high-fat diet (n = 5 of each genotype). *, p < 0.01. d, cell number in inguinal
adipose depot of female mice fed a chow diet (n = 4 for each genotype) or the high-fat diet for 15 weeks (n = 5 for each genotype). e and f, daily
food intake and feed conversion efficiency in mice from panel a, calculated from the weight gain per effective food intake (see "Experimental
Procedures") after 6 weeks on the high-fat diet. *, p < 0.01.
PPARγ ligand, rosiglitazone (40). After 6 days of treatment, ~20% of WT MEFs assumed a mature adipocyte morphology and accumulated large lipid droplets, as detected by Oil Red O staining (Fig. 3a). In contrast, less than 1% of the fld cells accumulated lipid droplets, which were sparse and small in size. Concordant with the reduced lipid staining, intracellular triglyceride content in fld MEFs was only 3.5% of WT levels (Fig. 3b). Real-time RT-PCR quantitation of mRNA for PPARγ, C/EBPα, and aP2 showed the expected induction by day 3 of differentiation in WT MEFs, but virtually no induction in fld cells (Fig. 3, c–e). Pref-1, a negative regulator of adipogenesis, which is normally expressed at high levels in preadipocytes and decreases during adipocyte differentiation (43), remained elevated in fld compared with WT MEFs (Fig. 3f).

To confirm that the impaired adipogenesis observed in fld MEFs was a direct result of lipin deficiency, we complemented fld MEFs by infection with a retroviral vector expressing lipin, and then induced adipocyte differentiation. Ectopic expression of lipin, but not vector alone, rescued the adipogenic defects in fld MEFs, leading to the development of cells that morphologically resembled adipocytes (Fig. 4a). Expression of PPARγ, C/EBPα, and aP2 was dramatically increased by lipin reconstitution (Fig. 4b), reaching levels similar to those observed in differentiated WT MEFs (compare with Fig. 3, c–e). These data demonstrate that reduced triglyceride accumulation and failure to induce key adipogenic genes in fld cells is a direct consequence of lipin deficiency.

**Lipin Acts Upstream of PPARγ during Adipogenesis**—The observation that PPARγ gene expression is compromised in lipin-deficient adipocytes and adipose tissue suggested that lipin func-
The adipogenic defect in \textit{fld} MEFs is rescued by ectopic expression of lipin. \textit{fld} MEFs were infected with either a control viral vector or one expressing lipin and induced to differentiate. a, Oil Red O staining illustrates restoration of lipid accumulation in lipin reconstituted \textit{fld} cells. b, real-time RT-PCR quantitation of adipogenic gene expression after vector only and lipin infection. *, \( p < 0.01 \).

**DISCUSSION**

The findings presented here establish impaired adipocyte differentiation as the basis for lipodystrophy and resistance to diet-induced obesity in \textit{fld} mice. Our evidence for the role of lipin in adipocyte differentiation derives from a combination of \textit{in vivo} and \textit{in vitro} studies. \textit{In vivo}, lipin deficiency prevents normal adipose tissue development, with dramatically reduced tissue mass and aberrant adipocyte gene expression. The requirement for lipin in adipogenesis also explains why \textit{fld} mice fail to increase adipose tissue mass on a high-fat diet, even
lipin and rosiglitazone treatment. Lipin Expression Is Critical for Adipogenesis

when supplied with excess substrates for lipid storage. Evidence that the differentiation defect is intrinsic to preadipocytes includes the demonstration that MEFs isolated from fld mice proliferate normally in culture but fail to differentiate, mirroring the reduced triglyceride accumulation and aberrant gene expression observed in adipose tissue from fld mice. We further determined that enhancing PPARγ expression or activity in fld MEFs overcomes the block in differentiation, indicating a requirement for lipin in attaining optimal PPARγ function during early stages of adipogenesis.

The induction of lipin expression at 10 h of 3T3-L1 adipocyte differentiation is consistent with a role for lipin in determining subsequent events in adipogenesis, such as down-regulation of the adipogenic inhibitor, Pref-1, or induction of adipogenesis-promoting factors such as PPARγ or C/EBPα. Previous studies (28, 42, 43) indicated that failure of any of these events is sufficient to prevent normal adipocyte differentiation. The mechanism by which lipin-deficiency attenuates adipogenic gene expression is not known, but recent studies of the fission yeast lipin homolog reveal interactions with three proteins having roles in nuclear envelope structure or nucleocytoplasmic transport (34). This is consistent with the nuclear localization of lipin (33), and suggests that, unlike other known adipogenic factors, lipin may have a novel molecular function.

The expression profile of lipin during the early stages of adipocyte differentiation is nearly identical to that of C/EBPβ and C/EBPδ, which are also induced at high levels at 10 h after stimulation with differentiation mixture followed by a sharp decline before the onset of PPARγ and C/EBPα mRNA accumulation (48). The similarity in expression dynamics between lipin and these two C/EBP family members suggests that the three genes may be induced by common factors, or even be co-regulated, during early adipogenesis. The expression of C/EBPβ and C/EBPδ genes is known to be induced directly by adipogenic stimuli, with C/EBPδ responding to dexamethasone through the glucocorticoid signaling pathway, and C/EBPδ responding to methylisobutylxanthine induction of the cAMP signaling pathway (48). It has also recently been shown (49) that C/EBPβ and C/EBPδ are target genes of the sterol regulatory element binding protein-1c transcription factor, raising the possibility that the lipin gene may share some of these same regulatory mechanisms.

It is notable that the adipogenic gene expression defect was more pronounced in isolated fld than in adipose tissue from fld mice. A similar situation has been observed in the comparison of gene expression in cells versus adipose tissue from double-knockout mice carrying null alleles for both C/EBPβ and C/EBPδ. As with lipin-deficient mice, the C/EBPβ-δ double-knockout mice exhibit profoundly reduced adipose tissue mass in vivo and failure of embryonic fibroblasts to differentiate in vitro, but adipogenic gene expression was much more strongly impaired in vitro (29). It was proposed that this may reflect a compensatory response in vivo that does not occur in vitro. Additional support for an in vivo compensatory response in fld mice is provided by our previous observation (32) that adipose tissue from 2-week-old fld mice exhibits pronounced PPARγ expression, perhaps corresponding to a developmental period in which lipin-independent mechanisms are active. Nevertheless, the failure to develop significant adipose tissue or mature adipocytes in fld mice indicates that compensatory mechanisms are not adequate to overcome the requirement for lipin in adipose tissue development.

Our findings that lipin expression is biphasic, with a transient induction during early stages of preadipocyte differentiation, as well as high levels in mature adipocytes (33), suggests that lipin may have dual roles in adipocyte biology. Because lipin-deficient mice never develop mature adipocytes, additional models will be required to evaluate the role of lipin in mature adipose tissue. We have recently developed a transgenic mouse model with enhanced lipin expression driven by regulatory elements expressed specifically in mature adipoh
cytes. Preliminary studies with this model indicate that increased lipin expression in mature adipose tissue is associated with increased adiposity, lending support to the proposal that lipin functions in mature as well as differentiating adipocytes.

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Lipin Expression Preceding Peroxisome Proliferator-activated Receptor-γ Is Critical for Adipogenesis in Vivo and in Vitro
Jack Phan, Miklós Péterfy and Karen Reue

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