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# A Single-Base Mutation in the Peroxisome Proliferator-Activated Receptor $\gamma$ 4 Promoter Associated with Altered *in Vitro* Expression and Partial Lipodystrophy

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Familial partial lipodystrophy (FPLD) results from coding sequence mutations either in *LMNA*, encoding nuclear lamin A/C, or in *PPARG*, encoding peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ). The *LMNA* form is called FPLD2 (MIM 151660), and the *PPARG* form is called FPLD3 (MIM 604367). We now report a 21-yr-old female with FPLD and no coding sequence mutations in either *LMNA* or *PPARG*. She was heterozygous for a novel A>G mutation at position –14 of intron B upstream of *PPARG* exon 1 within the promoter of the PPAR $\gamma$ 4 isoform. Her less severely affected father, who had features of the metabolic syndrome and a paucity of limb and

gluteal fat, was also heterozygous for –14A>G. This mutation was absent among 600 alleles from normal Caucasians. A minimal promoter sequence bearing the mutation had significantly reduced promoter activity when used to drive reporter expression in *in vitro* expression in two cell lines, compared with the wild-type sequence. This is the first report of a human mutation in the promoter of a PPAR $\gamma$  isoform. Because the mutation affects PPAR $\gamma$ 4 expression and is associated with FPLD, this implies that PPAR $\gamma$ 4 might be important for fat depot distribution and metabolism *in vivo*. (*J Clin Endocrinol Metab* 89: 5655–5660, 2004)

DUNNIGAN-TYPE FAMILIAL PARTIAL lipodystrophy (FPLD; MIM 151660) is considered to be a monogenic model of the common syndrome of insulin resistance, or metabolic syndrome (1). FPLD is a genetically heterogeneous autosomal dominant phenotype characterized by repartitioning of adipose tissue and is associated with multiple metabolic disturbances. FPLD results from coding sequence mutations either in *LMNA*, encoding nuclear lamin A/C, or in *PPARG*, encoding peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ) (1). The *LMNA* form is called FPLD2 (MIM 151660), and the *PPARG* form is called FPLD3 (MIM 604367). The presence of lipodystrophy in subjects with dysfunctional *PPARG* missense mutations, such as R425C, F388L, V290M, and P467L (2–5), and in PPAR $\gamma$ -deficient murine models (6, 7) has confirmed the central role of PPAR $\gamma$  in adipogenesis.

The tissue expression of PPAR $\gamma$  mRNA is complex: four *PPARG* mRNA isoforms, called PPAR $\gamma$ 1,  $\gamma$ 2,  $\gamma$ 3, and  $\gamma$ 4 (8–10), result from different promoter usage and alternative splicing. A map of the mRNA isoforms and promoters of the PPAR $\gamma$  isoforms is shown in Fig. 1. PPAR $\gamma$ 1 and PPAR $\gamma$ 3 mRNAs are relatively widely expressed (8, 11, 12), whereas PPAR $\gamma$ 2 mRNA is expressed exclusively in adipose tissue (8, 13), suggesting that it is functionally important for that tis-

sue. Primer extension studies have confirmed that PPAR $\gamma$ 4 mRNA is also present in adipose tissue (9). However, little is known of PPAR $\gamma$ 4's potential role in adipocyte biology or metabolism. We now present human genetic evidence suggesting that the PPAR $\gamma$ 4 isoform has a role in adipocyte biology. Specifically, in a subject with FPLD3, we found a rare *PPARG* mutation within the PPAR $\gamma$ 4 promoter that was associated with decreased promoter activity in two cell lines.

## Subjects and Methods

### Study subjects

*FPLD proband.* The proband was a 21-yr-old Dutch female. Menarche occurred at age 11, and she has had regular menstrual cycles since then. At about age 13, she was noted to have grossly abnormal fat distribution consistent with FPLD. At age 20, she was diagnosed with type 2 diabetes, and was treated with metformin (1500 mg daily). On examination, she was obese: weight, 109 kg; height, 178 cm; and body mass index (BMI), 34.4 kg/m<sup>2</sup>. Waist and hip circumferences were 130 and 114.5 cm, respectively, with a ratio of waist to hip circumference of 1.14. Her resting blood pressure was 140/65 mm Hg. Clinically, she had excess sc fat on the face, neck, trunk, and abdomen, with relative lack of sc fat on the gluteal region, arms, and legs. This was confirmed with magnetic resonance imaging (Fig. 2), which showed excessive and relatively symmetrical deposition of sc fat on the face, neck, and upper trunk, with disproportionate depletion of sc fat in the lower body, especially dorsally in the gluteal region and thigh. She had no acanthosis nigricans or hirsutism. Measurements in fasting plasma included: glucose, 8.5 mmol/liter (153 mg/dl); insulin, 19 mU/liter (normal, <11 mU/liter); C-peptide, 1.22 nmol/liter (normal, <0.9 nmol/liter); total cholesterol, 4.1 mmol/liter (159 mg/dl); triglycerides, 0.94 mmol/liter (85 mg/dl); high-density lipoprotein cholesterol, 0.88 mmol/liter (34 mg/dl); and low-density lipoprotein cholesterol, 2.83 mmol/liter (110 mg/dl). Based on the 2001 National Cholesterol Education Panel (NCEP) criteria (14), she had the metabolic syndrome. DNA sequence of the exons, intron-

Abbreviations: B-Gal,  $\beta$ -Galactosidase; FPLD, familial partial lipodystrophy; PPAR, peroxisome proliferator-activated receptor; RLU, reporter luciferase unit; RORE, receptor-related orphan receptor response element.

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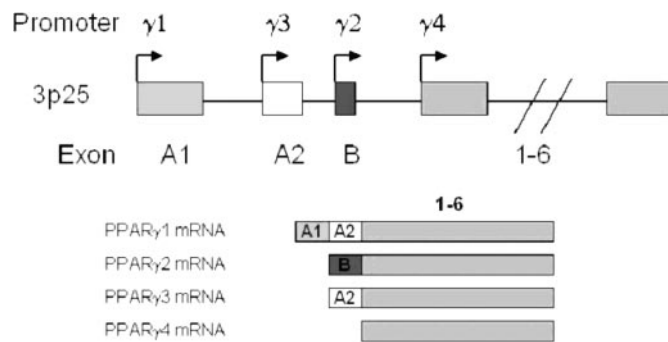


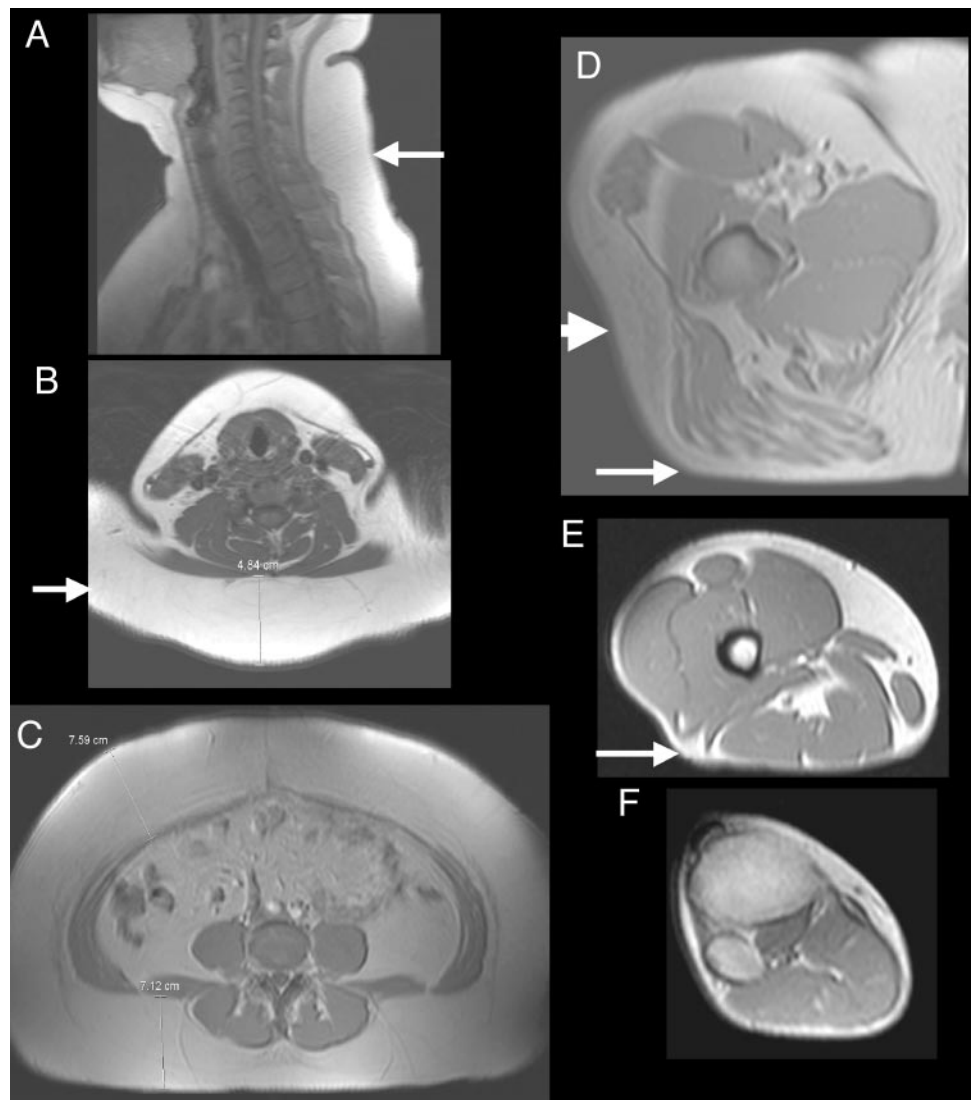
FIG. 1. Organizational structure of the *PPARG* gene on chromosome 3p25 and the four PPAR $\gamma$  mRNA isoforms. Arrows, Start site of transcription for the specific mRNA isoform. Exons are indicated as boxes on the genomic map. The schematic structure of the four mRNA isoforms is shown under the genomic map. Exons A1 and A2 are untranslated, and exon B is translated. PPAR $\gamma$ 1,  $\gamma$ 3, and  $\gamma$ 4 mRNAs translate into a 477-amino-acid protein. PPAR $\gamma$ 2 mRNA translates into a 505-amino-acid protein with 28 extra amino acids at the N-terminal end.

exon boundaries, and flanking regions of her *LMNA* gene, using a described procedure (15), was normal.

**Proband's father.** Her father was 56 yr old and apparently healthy, although he reported a sister and paternal female first cousin with similar physical appearance to the proband. On examination, he was overweight: weight, 94 kg; height, 186 cm; and BMI, 27.2 kg/m<sup>2</sup>. The circumferences of his waist and hip were 110 and 103 cm, respectively, with a ratio of waist to hip circumference of 1.07. His resting blood pressure was 132/78 mm Hg. Like his daughter, he had relative depletion of sc fat from the arms, legs, and gluteal area, with limited truncal fat, although the phenotype was much less severe than that of his daughter. Fasting plasma determinations included: glucose, 4.8 mmol/liter (86 mg/dl); insulin, 8 mU/liter; C-peptide, 0.74 nmol/liter; total cholesterol, 5.8 mmol/liter (224 mg/dl); triglycerides, 1.7 mmol/liter (153 mg/dl); high-density lipoprotein cholesterol, 1.0 mmol/liter (38 mg/dl); and low-density lipoprotein cholesterol, 4.07 mmol/liter (158 mg/dl). Based on the 2001 NCEP criteria (14), he had the metabolic syndrome. DNA sequence of the exons, intron-exon boundaries, and flanking regions of his *LMNA* gene, using a described procedure (15), was normal.

**Other subjects.** The proband's 47-yr-old mother and 18-yr-old brother were each clinically and biochemically normal, with normal fat distribution. Neither had clinical or biochemical criteria for diagnosis of the metabolic syndrome. Three hundred additional control DNA samples of

FIG. 2. Magnetic resonance imaging (MRI) tissue scans of the proband at various anatomical sites. A, Sagittal scan of the neck, with the arrow showing a prominent dorsal suprascapular sc fat pad; B, cross-section at the level of the upper thorax, with the arrow showing a layer of dorsal sc fat measuring 4.84 cm at the thickest point; C, cross-section at the abdomen, showing a symmetrical circumferential layer of sc fat measuring 7.59 cm at the thickest point; D, cross-section at the right gluteal region, with the large arrow showing concavity of dorsolateral sc fat pad and the small arrow showing paucity of dorsal sc fat, measuring 0.5 cm at its thickest point; E, cross-section at the right midthigh, with the arrow showing paucity of dorsal sc fat, measuring 0.3 cm at its thickest point; F, cross-section of the right leg, showing a thin layer of sc fat. The scans are consistent with excess sc fat deposition centrally in the upper body, with relative lack of sc fat on extremities and gluteal region and preferential loss of fat dorsally in the lower body.



normal subjects of European descent without diabetes or the metabolic syndrome were studied to determine mutation frequency.

### DNA analysis

Participants from The Netherlands provided informed consent, and all genetic analyses were performed with approval from the University of Western Ontario Ethics Review Board. *PPARG* was amplified from the proband's genomic DNA. Oligonucleotides were designed using genomic DNA sequences of human *PPARG* obtained from GenBank accession numbers AB005520–AB005526, AF548352, and AY043357 and from published sequences (8, 9, 16). *PPARG* primers spanned all translated exons, namely exon B and exons 1–6, with more than 50 nucleotides at each intron-exon boundary, 400 bp of the PPAR $\gamma$ 2 promoter, 660 bp that spanned the PPAR $\gamma$ 3 promoter and exon A2, and the 433-bp PPAR $\gamma$ 4 promoter. Amplified fragments were purified on a 2% agarose gel (QIAquick Gel Extraction Kit; Qiagen, Mississauga, Ontario, Canada). Direct DNA sequencing was carried out using the dideoxynucleotide chain termination method with the designed primers, with electrophoresis on a Prism 377 Automated DNA Sequencer and analysis using Sequence Navigator software (both from Applied Biosystems, Mississauga, Ontario, Canada).

To detect rapidly the PPAR $\gamma$ 4 promoter mutation in three first-degree relatives of the proband and in 300 control DNA samples, an allele-specific method called SNaPshot (Applied Biosystems) was used (17). The sequence of the common amplification primer was 5'-CTG GGA TAA CAG GTG TGA GCC A. The sequence of the bracketing primer for the wild-type promoter was 5'-TTT CTG AAA GGA AAA ATA GAC TAG CTG TG. The sequence of the bracketing primer for the mutant promoter was 5'-TTT CTG AAA GGA AAA ACA GAC TAG CTG TG (mutated nucleotide shown in *bold* and *underlined*). The SNaPshot primer sequence was 5'-GGA CTT AAC TTC ACA GCT AGT CT. The procedure was performed as described, with fragment analysis using GeneScan Software (Applied Biosystems).

### In vitro expression studies

**Expression vector constructs.** The PPAR $\gamma$ 4 promoter was amplified from subjects with and without the  $-14A>G$  mutation using primers. All sequences for promoter studies were derived from GenBank number AY043357. Purified 436-bp products representing wild-type and mutated PPAR $\gamma$ 4 promoter were ligated into the pCR2.1 vector using the TA Cloning Kit protocol (Invitrogen, Carlsbad, CA). Plasmid DNA was prepared using the Qiagen kit (Qiagen), and sequencing identified a clone with a perfect forward (5'→3') wild-type promoter sequence, a clone with a perfect reverse (3'→5') wild-type promoter sequence (antisense), and a clone with a perfect forward (5'→3') mutation promoter sequence. After digestion with endonucleases *SacI* and *XhoI*, the inserts were directionally subcloned in the luciferase reporter PGL3 basic vector (Promega, Madison, WI). After transfection and purification, sequencing was used to confirm orientation and fidelity of purified plasmid DNA samples for three types of constructs: 5'→3' wild-type, 3'→5' wild-type, and 5'→3' mutation.

**Cell lines and culture.** Murine cell lines NIH/3T3 (CRL-1658) and 3T3-L1 (CL-173) were obtained from American Type Culture Collection (Manassas, VA). The overall strategy for *in vitro* expression studies was described previously (18). Cells were maintained in a DMEM (Invitrogen) containing 4.5 g/liter D-glucose, 1.5 g/liter NaHCO<sub>3</sub>, 25 mM HEPES, 4 mM L-glutamine, and 110 mg/liter sodium pyruvate in a humidified atmosphere of 95% air-5% CO<sub>2</sub> at 37 C. Medium was supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin, and 10% heat-inactivated fetal bovine serum (Invitrogen). Each cell line was grown on a 100-mm culture dish (Nunc; Nalge Nunc, Mississauga, Ontario, Canada). Cells were seeded on six-well plates to achieve 60% confluence and transfected using calcium phosphate precipitation with one of the three PPAR $\gamma$ /Luc reporter constructs. For each transfection, 2  $\mu$ g pSV- $\beta$ -galactosidase vector was cotransfected according to manufacturer's instructions (ProFection Mammalian Transfection Systems, Promega). Cells were harvested 48 h post transfection. Transfection experiments were repeated in triplicate, three times on separate days for each cell line. Luciferase activity was assayed according to the manufacturer's instructions (Promega), and luminescence was determined

using a Lumat LB9507 luminometer (Berthold Systems, Pittsburgh, PA). Luciferase activities were normalized for transfection efficiencies using reporter luciferase units (RLU) per unit of activity of  $\beta$ -galactosidase (B-Gal). The  $\beta$ -galactosidase enzyme assay system was used according to manufacturer's directions (Promega).

### Statistical analysis

SAS version 6.12 (SAS Institute, Cary, NC) was used for statistical analysis. A two-way ANOVA was performed using the general linear model computing procedure of SAS, with independent variables being single-nucleotide polymorphism type (wild-type or mutation) and experiment number (first, second, or third experiment). The dependent variable was RLU/B-Gal. Because transfection was done in triplicates in each experiment, each ANOVA cell had three data points.

## Results

### Identification of mutation in promoter of PPAR $\gamma$ 4 isoform

In the genome of the proband, we found a heterozygous nucleotide substitution in *PPARG* intron B,  $-14A>G$  upstream of exon 1 within the PPAR $\gamma$ 4 promoter (9). All other regions were free of DNA sequence changes. By genotyping, we found that this mutation was also present in the heterozygous father but was absent from the genomes of her unaffected mother and brother and also from 300 normal Caucasian controls. Furthermore, our DNA sequence analysis revealed that the PPAR $\gamma$ 4 promoter consists of 433 bp rather than 432 bp, as previously published (9), with the difference in size attributable to an extra A nucleotide at position  $-383$  of intron B. This 433-bp sequence length was confirmed in an additional 10 control DNA samples.

### PPAR $\gamma$ 4 promoter activity

Adjusted (least squares) means of normalized luciferase activities from triplicate experiments performed on three different days are shown in Fig. 3. Compared with wild-type promoter, the mutant promoter had 60% decreased normalized luciferase activity in 3T3 cells ( $P < 0.05$ ) and 70% decreased activity in 3T3-L1 cells ( $P < 0.0001$ ).

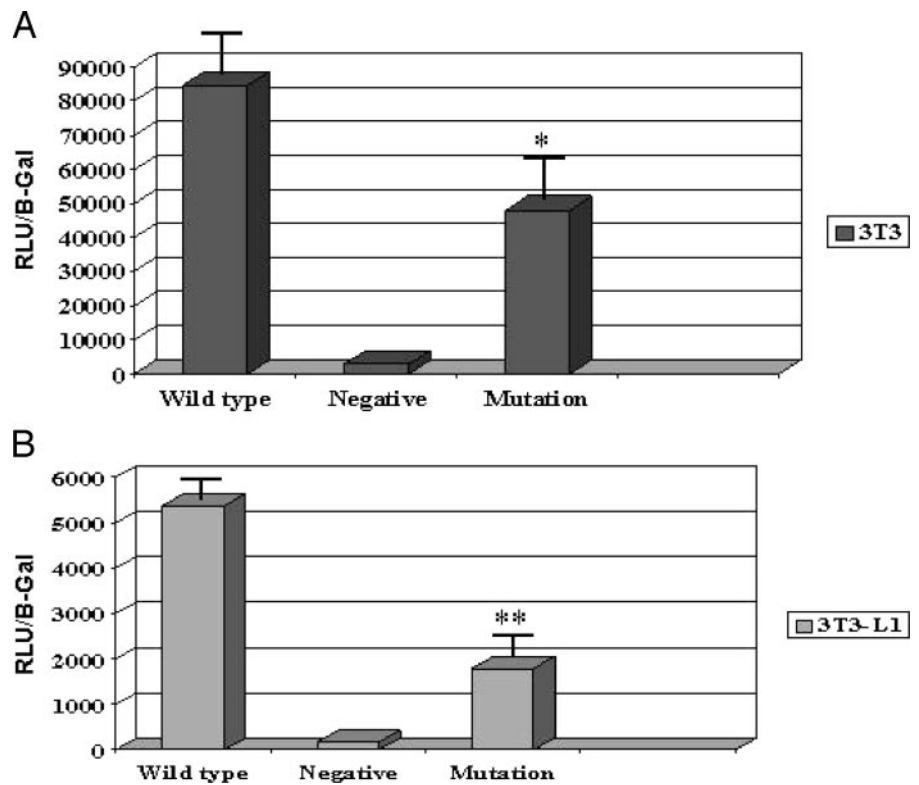
## Discussion

In the *PPARG* gene of a proband with the FPLD phenotype and type 2 diabetes and marked fat repartitioning, we found the  $-14A>G$  heterozygous mutation in the promoter of PPAR $\gamma$ 4. The clinical phenotype of the subject was notable for a relative excess of sc fat centrally in the upper body and a relative paucity of fat in the gluteal region and extremities, especially dorsally (Fig. 2). This mutation was also found in the genome of her less severely affected father but neither in her clinically normal mother nor brother nor among the genomes of 300 normal Caucasian control subjects. *In vitro* expression studies showed that this mutation resulted in a significant reduction in promoter activity in both 3T3 and 3T3-L1 cell lines, the latter of which is an adipogenic cell line derived from mouse Swiss 3T3 fibroblasts (19, 20). The findings suggest that noncoding mutations in *PPARG* are associated with partial lipodystrophy and implicate the PPAR $\gamma$ 4 isoform as being potentially important in adipocyte biology. Because the mutation is in *PPARG*, this patient and father have FPLD3.

Differential splicing of *PPARG* gives rise to four distinct



FIG. 3. *In vitro* expression studies of wild-type and mutant PPAR $\gamma$ 4 promoter. Normalized RLU/B-Gal are shown for three sets of triplicate experiments for two cell lines: 3T3 (A) and 3T3-L1 (B). Adjusted (least squares) means  $\pm$  SE values are shown for luciferase reporter constructs containing wild-type PPAR $\gamma$ 4 promoter, a negative control construct made by cloning of wild-type promoter in the reverse orientation, and a construct containing the  $-14A>G$  mutation. Asterisks, Significant difference between mean normalized expression from wild-type and mutant constructions (\*,  $P < 0.05$ ; \*\*,  $P < 0.0001$ ).



mRNA isoforms, called PPAR $\gamma$ 1,  $\gamma$ 2,  $\gamma$ 3, and  $\gamma$ 4, respectively (Fig. 1). PPAR $\gamma$ 1,  $\gamma$ 3, and  $\gamma$ 4 mRNA species give rise to the identical 477-amino-acid protein, whereas PPAR $\gamma$ 2 mRNA gives rise to a 505-amino-acid protein with 28 extra N-terminal amino acids (8–10). Although the expressed protein is the same for each mRNA isoform, the differences in the promoters and noncoding exons result in differential tissue expression. For instance, the well-studied PPAR $\gamma$ 2 isoform is primarily expressed in fat and is considered to be the key isoform related to adipose metabolism. In contrast, the recently characterized PPAR $\gamma$ 4 isoform, though also expressed in fat, has largely unknown metabolic attributes. Expression studies to identify tissue distribution and relative quantity of the PPAR $\gamma$ 4 mRNA have not been possible because the PPAR $\gamma$ 4 nucleotide sequence is common to all four PPAR $\gamma$  mRNA isoforms. At minimum, it is known that PPAR $\gamma$ 4 mRNA is present in human adipose tissue, because it was detected there by primer extension analysis of total RNA (9). The current findings indicate that deficiency of the PPAR $\gamma$ 4 isoform resulting from diminished expression in carriers of the  $-14A>G$  mutation is associated with partial lipodystrophy.

The PPAR $\gamma$ 4 promoter contains a TATA-like sequence at nt  $-170$  to  $-166$ , an AP-1 site at position nt  $-187$  to  $-184$ , and a retinoic acid receptor-related orphan receptor response element (RORE) at position nt  $-303$  to  $-298$ , all relative to the start site of transcription (9). The latter element has been proven to be regulatory, because overexpression of ROR $\alpha$ 1, which specifically binds to RORE, resulted in a 40-fold increase in promoter activity in transient transfection assays in several cell lines (9). However, the  $-14A>G$  mutation is not located within RORE or in any potential regulatory element. Instead, the mutation is very close to the transcription ini-

tiation site and, as such, may affect the transcription initiation complex machinery. Alternatively, an unknown transcription factor might fail to bind and transactivate transcription in the presence of the  $-14A>G$  mutation. Detection of such a DNA binding protein would require EMSA, which is beyond the scope of this report. However, our findings highlight the importance of more careful characterization of the PPAR $\gamma$ 4 promoter.

The proband's phenotype was consistent with that seen in other female heterozygotes for coding sequence mutations in *PPARG*. To date, four missense mutations in *PPARG* resulting in PPAR $\gamma$  deficiency have been shown to cause partial lipodystrophy, namely R425C (2), F388L (3), and V290M and P467L (4, 5). Clinical features of these patients and the two subjects with the  $-14A>G$  mutation are shown in Table 1. Careful examination of subphenotypes (also called phenomic examination) has shown that patients with *PPARG* mutations (FPLD3) have a distinctive phenotype including a specific distribution of adipose tissue loss, relatively severe biochemical and clinical signs of insulin resistance, and relatively early onset of type 2 diabetes. There have been suggestions that the biochemical phenotype in PPAR $\gamma$  deficiency is out of proportion to the extent of adipose loss, compared with *LMNA*-associated partial lipodystrophy (1, 3). This would suggest that, in addition to adipose tissue redistribution, *PPARG* mutations may have additional independent effects on metabolism.

Furthermore, there appeared to be a difference in phenotypic severity in the PPAR $\gamma$ 4  $-14A>G$  proband and her father. Specifically, the proband's father had a relatively mild clinical and biochemical phenotype, which contrasted with the proband's more striking phenotype. This appears to be

**TABLE 1.** Clinical and biochemical features in FPLD3 subjects with *PPARG* mutations

	<i>PPARG</i> F388L	<i>PPARG</i> R425C	<i>PPARG</i> V290M	<i>PPARG</i> P467L	<i>PPARG</i> $\gamma$ 4 promoter - 14A > G
No.	4	1	1	2	2
Age (yr)	44.3 $\pm$ 6.1	64	21	32 and 56	21 and 56
% Female	2 of 4 subjects	1 subject	1 subject	1 of 2 subjects	1 of 2 subjects
Ethnicity	N. European	Hispanic	N. European	N. European	N. European
Limb fat	Absent, mainly distal	Absent, mainly distal	Decreased	Absent, mainly distal	Absent, mainly distal
Facial fat	Present	Absent	Decreased	Decreased	Present
Gluteal fat	Present	Present	Decreased	Decreased	Decreased
Truncal fat	Increased	Increased	Increased	Increased	Present
Lipodystrophy onset (yr)	Females: ~15; males: unknown	Mid-adult	Early adult	Mid-adult	Proband: ~13; father: unknown
Diabetes onset (yr)	45.5 $\pm$ 6.7	32	17	30.0 $\pm$ 4.0	20
Hypertension	Present	Present	Severe	Severe	Absent
Acanthosis nigricans	2/4	Absent	Present	Present	Probably absent
Hirsutism	Absent	Present	Present	Present	Absent
Hepatic steatosis	Absent	Present	Present	Present	Absent
Polycystic ovaries	1 of 2 women	Absent	Dysmenorrhea	Dysmenorrhea	Absent
Body mass index	1.0-fold	0.9-fold	1.1-fold	1.0-fold	1.0-fold
Insulin	2.7-fold	1.6-fold	6.4-fold	4.8-fold	1.5-fold
MetS criteria met	Yes, all four	Yes	Yes	Yes, both	Yes, both

MetS, Patient meets criteria for metabolic syndrome as proposed by the National Cholesterol Education Program (14); values for continuous traits are mean  $\pm$  SEM. Explanation of fold changes for quantitative variables: 1) for *PPARG* F388L mutation, group means for mutation carriers were compared with group means for family controls from Ref. 3. For other *PPARG* mutations, individual values or mean for two observations were compared with the upper limit value for normal reference ranges.

consistent with observations in the small numbers of PPAR $\gamma$ -deficient subjects studied so far (2–5). For instance, female carriers of the *PPARG* F388L mutation appeared to be more severely affected, with respect to anthropometry, hypertension, and dyslipidemia, than male carriers (3). Similar, between-sex differences in phenotype severity have been noted in *LMNA*-related partial lipodystrophy (1, 21). Although interesting, the anecdotal nature of the between-sex differences in phenotypic severity in PPAR $\gamma$  deficiency and in partial lipodystrophy precludes speculation regarding potential mechanisms. Ascertainment of additional subjects and families and specific physiological studies are required.

Finally, we cannot exclude the possibility that there is a defect at another genetic locus that interacts with the PPAR $\gamma$ 4 –14A>G mutation. For instance, Savage and colleagues (22) described heterozygosity for a loss-of-function premature stop mutation in *PPARG* in a large Caucasian European kindred. However, in that family, the *PPARG* defect alone was not associated with an abnormal metabolic phenotype. Instead, subjects who were doubly heterozygous for the *PPARG* defect together with a defect in a separate unrelated gene had severe insulin resistance (22). In an analogous manner, therefore, it is possible that the haploinsufficiency resulting from the PPAR $\gamma$ 4 –14A>G mutation requires a second genetic defect for the affected subjects to express their disease. In our sample, the search for such a second defect is limited by the small family size.

In summary, we have found a rare loss-of-function mutation in the promoter of PPAR $\gamma$ 4 in a proband with partial lipodystrophy. This extends the range of *PPARG* mutations (23) and indicates that noncoding mutations in *PPARG* are associated with the FPLD3 subtype of partial lipodystrophy. Furthermore, the findings provide additional confirmation of the concept that PPAR $\gamma$  deficiency is a cause of partial lipodystrophy with associated metabolic disturbances. Fi-

nally, the findings implicate the PPAR $\gamma$ 4 isoform as being potentially important in adipocyte biology.

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