

Clinical and molecular characterization of a severe form of partial lipodystrophy expanding the phenotype of PPAR γ deficiency^[S]

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Abstract Familial partial lipodystrophy (FPLD) is characterized by abnormal fat distribution and a metabolic syndrome with hypertriglyceridemia. We identified a family with a severe form of FPLD3 with never-reported clinical features and a novel mutation affecting the DNA binding domain of PPAR γ (E157D). Apart from the lipodystrophy and severe metabolic syndrome, individuals presented musculoskeletal and hematological issues. E157D heterozygotes had a muscular habitus yet displayed muscle weakness and myopathy. Also, E157D heterozygotes presented multiple cytopenias and a susceptibility to autoimmune disease. In vitro studies showed that the E157D mutation does not decrease the receptor's affinity to classical PPAR response elements or its responsiveness to a PPAR γ agonist, yet it severely reduces its target gene transcription. Microarray experiments demonstrated a decreased activation of a wide array of genes, including genes involved in the PPAR response, the immune response, hematopoiesis, and metabolism in muscle. In addition, a subset of genes with cryptic PPAR response elements was activated. In summary, we describe a large family with a novel PPAR γ mutation, which extends the clinical phenotype of FPLD3 to include muscular, immune, and hematological features. Together, our results support the role of PPAR γ in controlling homeostasis of multiple systems beyond lipid metabolism.—Campeau, P. M., O. Astapova, R. Martins, J. Bergeron, P. Couture, R. A. Hegele, T. Leff, and C. Gagné. **Clinical and molecular characterization of a severe form of partial lipodystrophy expanding the phenotype of PPAR γ deficiency.** *J. Lipid Res.* 2012. 53: 1968–1978.

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Familial partial lipodystrophy type 3 (FPLD3) is an autosomal dominant condition caused by *PPARG* mutations. Starting in adolescence, there is variable loss of adipose tissue in the face, gluteal area, and distal limbs with increased truncal adiposity and hepatic steatosis. A metabolic syndrome occurs with diabetes, hypertension, hypertriglyceridemia, and related complications (1). More than 15 FPLD3 families have been identified, and in contrast to FPLD2, which is caused by *LMNA* mutations, no FPLD3 subject has yet been reported to have any muscular, neurological, or cardiac involvement. *PPARG* encodes a nuclear receptor involved in adipocyte differentiation, whereas *LMNA* encodes lamin A/C, an essential component of the nuclear envelope. We identified a large family with a dominantly transmitted severe lipodystrophy. We identified the causative mutation, characterized its effect on PPAR γ , and studied the phenotype to uncover never-before-reported features.

MATERIALS AND METHODS

Clinical study

This clinical study was approved by the Laval University Hospital Center Institutional Review Board (Comité d'Éthique du Centre Hospitalier de l'Université Laval). Potential subjects were informed of our study by a letter distributed by affected family members

Abbreviations: EMSA, electrophoretic mobility shift assay; FPLD, familial partial lipodystrophy; PPAR γ , peroxisome proliferator-activated receptor gamma; PPRE, peroxisome proliferator response element; TK, thymidine kinase.

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[S] The online version of this article (available at <http://www.jlr.org>) contains supplementary data in the form of four tables and a dataset.

and invited to an information session. Sixty-one potential subjects attended an information session and three declined to participate because they did not want to have a blood draw. Subjects completed a questionnaire, were examined, and provided blood and urine samples after a 12 h fast. Physicians conducting the physical examinations were blinded to the genotype. Among the 58 participants, we screened eight unrelated partners and 26 relatives not shown on the Fig. 1 pedigree; 16 relatives of individual I-1 (paternal side, siblings and their descendants), and 10 relatives of individual I-2 (maternal side, siblings and their descendants). The pedigree was drawn using Cranefoot (2).

Biochemical analyses

Routine blood chemistry analyses, performed using the Hitachi 917 chemistry analyzer (Roche Diagnostics), included: glucose, electrolytes, creatinine, bilirubin, alanine aminotransferase, aspartate aminotransferase, γ glutamyl transpeptidase, alkaline phosphatase, and glycated hemoglobin. Because of the elevated triglycerides, preparative ultracentrifugation was used for accurate assessment of the different cholesterol fractions (3). Plasma C-reactive protein, apo B, and apo A-I were measured by nephelometry. Serum thyrotropin was measured by electrochemoluminescence immunoassay (Roche Diagnostics). Leptin and adiponectin were measured by ELISA (Quantikine high-sensitivity human kits, R & D Systems). Total plasma free fatty acids were measured using a colorimetric assay (Wako Diagnostics). Insulin was measured by radioimmunoassay (Linco Research, St. Charles, MO), in which 1 μ IU/ml of insulin is equivalent to 6 pmol/L. Insulin sensitivity and β -cell production were calculated using the updated Homeostasis Model Assessment model using an Excel macro (3a). C-peptide values were used to calculate β -cell secretion and insulin values were used to calculate sensitivity to insulin. One diabetic subject administered her insulin before the blood draw and her results were excluded from the analyses. Data from subjects usually on secretagogues and/or insulin were kept for the analyses because they were at a steady state at the time of the blood draw. Creatinine clearance was estimated using the Modification of Diet in Renal Disease formula (4).

DNA analysis

Mutations in the *LPL* gene encoding LPL that are known to be common in French-Canadians (G188E and P207L) as well as *LPL* polymorphism D9N, and *APOE* genotypes were assessed as described previously (5). DNA sequencing showed no mutation in *LMNA* (6). We then amplified and sequenced the six exons of *PPARG* plus at least 100 nucleotides at each intron-exon boundary and \sim 700 bp of the promoter as described (7). The mutation identified, referred to as E157D, corresponds to p.E157D [reference sequence NP_056953.2], c.A562C [reference sequence NM_015869] and g.A93633C [reference sequence NC_000003.11]. A rapid, allele-specific genotyping method was then developed (primer sequences and conditions available upon request). Genomic DNA from 244 healthy Caucasian subjects was studied, permitting with 95% power to exclude a mutation frequency \geq 1% in the healthy population (two-tailed $\alpha < 0.05$). The structure of PPAR γ for Fig. 1 is from PDB entry 3DZY and was rendered using SwissPDBviewer.

PPAR γ expression plasmids

For the PPAR γ expression vectors used in transient transfection, a 1472-bp DNA segment containing human PPAR γ 1 cDNA tagged with two flag epitopes at the 5' end was cloned into the tet-regulated expression vector pTRE (Clontech). For lentiviral expression vectors, wild-type or E157D 2 \times flag-tagged PPAR γ 2 cDNA was cloned into the polylinker region of pLenti6.3-V5

DEST (Invitrogen). The E157D mutation was introduced in PPAR γ 1 and 2 using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). All plasmids were verified by DNA sequencing.

EMSA

NIH3T3 cells were grown in a 150 mm dish in Dulbecco's Modified Eagle Medium containing 4.5 g/L glucose and 10% fetal bovine serum, and transfected using Lipofectamine-PLUS (Invitrogen) with plasmids coding for PPAR γ 1 (25 μ g), RXR (25 μ g), and pTet-Off (Clontech, 10 μ g) to induce PPAR γ 1 expression. Transfection mixture was replaced with fresh media after 4 h. Twenty-four hours posttransfection, nuclei were isolated and sonicated in lysis buffer (20 mM Hepes pH7.9, 150 mM KCl, 0.2 mM EDTA, 15% glycerol, 0.5 mM DTT). Increasing amounts of nuclear extracts from cells transfected with a PPAR γ 1 expression vector or the empty vector were incubated with biotin-labeled PPRE from and separated on a nondenaturing PAGE. PPRE sequences listed in supplementary Table II were labeled with biotin-dUTP using Klenow DNA polymerase and electrophoretic mobility shift assay (EMSA) was performed according to the manufacturer's instructions (Pierce). PPAR γ 1 expression levels were monitored by Western analysis using a mouse monoclonal PPAR γ antibody (sc-7273).

Luciferase reporter assay

PPAR γ transcription reporter plasmids were cloned by inserting PPRE sequences upstream of the TK promoter in the pGL3 luciferase reporter plasmid (Promega). NIH3T3 cells were grown in 12-well plates and transfected with plasmids encoding PPAR γ 1 (WT, E157D or the empty vector, 100 ng), mouse RXR α (100 ng), pTet-Off (50 ng), β -galactosidase (50 ng), and pcDNA3 (300 ng), as well as 200 ng of the transcription reporter plasmid, according to the manufacturer's instructions. Posttransfection, cells were treated with either 20 μ M rosiglitazone or vehicle (DMSO). Twenty-four hours later, the cells were harvested in 150 μ L tissue lysis buffer (Promega) and assayed for luciferase activity in a Berthold 96-well luminometer. PPAR γ 1 expression levels were monitored by Western analysis using a mouse monoclonal PPAR γ antibody (sc-7273). A colorimetric β -galactosidase assay was used to normalize for transfection efficiency.

Generation of stable cell lines and qRT-PCR

Wild-type or E157D PPAR γ 2 cDNA was cloned into the polylinker region of pLenti6.3-V5 DEST (Invitrogen). PPAR γ 2 was chosen over PPAR γ 1 for these experiments as being the more relevant isoform of the receptor with regard to its native transcriptional activity in adipose tissue. PPAR γ lentivirus was made according to the manufacturer's instructions and used to infect NIH3T3 cells plated in 6-well plates at 50% confluence. Infected cells were selected with 2 μ g/ml blasticidin for 2 weeks and maintained in blasticidin medium for the duration of the experiment. Total RNA for qRT-PCR was isolated after 24 h of treatment with 20 μ M rosiglitazone using TRI reagent (Sigma) according to the manufacturer's instructions, followed by cDNA synthesis from 2 μ g of RNA using a kit from Applied Biosystems. Quantitative PCR for mouse gene expression was performed in a Stratagene Mx3000P real-time PCR machine using the Cybr-Green fluorescence method (Thermo) and the primer sequences for mouse adiponectin, CD36, aP2, and perilipin can be obtained upon request.

Microarray analysis of gene expression and in silico promoter analysis

Total RNA was isolated from NP γ 2 cells treated for 24 h with Rosiglitazone (20 μ M) or vehicle (DMSO) using TRI reagent

(Sigma) and purified on RNeasy columns (Qiagen). RNA integrity was verified using Agilent Bioanalyser 2100 and ranged from 7.2 to 10. Samples were labeled and hybridized to Illumina Mouse WG-6 v2.0 cDNA arrays according to the manufacturer's protocol. The Illumina gene expression image files obtained from the Illumina iScan scanner were uploaded to GenomeStudio (version 2010.3) using the Gene Expression module (v1.8.0). Data were normalized using the Rank Invariant method, and uploaded with accession GSE37990 to NCBI's Gene Expression Omnibus. Differentially expressed probes were identified using the Illumina Custom Error Model with Benjamini and Hochberg False Discovery Rate. The genes considered to be differentially expressed were uploaded to the Genomatix Software Suite to determine over-represented Gene Ontology Biological Processes. Gene promoters were defined by the Gene2Promoter function of Genomatix and searched for PPAR γ binding sites consistent with the matrices generated by Lefterova et al. (defined in Genomatix MatInspector as V\$PPARG.02) and Nielsen et al. (V\$PPARG.03). One thousand promoters of the loss-of-function set were randomly selected for the PPRE scan. The incidences of PPREs in the promoters of gain-of-function and loss-of-function genes were compared using Fisher's exact test. Human-mouse promoter alignments were done in Rvista.

Statistical analysis

Means standard deviations are shown for continuous variables and those that were not normally distributed or did not have equal variance are expressed by their medians with interquartile ranges (Shapiro-Wilk distribution test and Levene equal variance test thresholds were set at $P < 0.05$). Comparisons between heterozygotes and the control group were performed using unpaired Student's t -tests for normally distributed data with equal variance (values presented as means \pm standard deviations); otherwise the Mann-Whitney rank sum test was used (values presented as medians with interquartile ranges). For categorical data we used a chi-square or Fisher's exact test. Nominal significance was set at a p -value < 0.05 (and a minimum power of 0.8 with $\alpha = 0.05$). The software used was Sigmaplot.

RESULTS

Identification of the kindred and genetic analyses

The index proband (II-21) was referred to Laval University's Lipid Research Centre due to severe hypertriglyceridemia and frequent episodes of pancreatitis. Evaluation and family history revealed a partial lipodystrophy phenotype segregating in an autosomal dominant manner (Fig. 1A). A clinical study was conducted to investigate relatives of the proband. Sequencing of *LMNA* revealed no mutations. However, sequencing of *PPARG* identified a novel heterozygous missense mutation in the DNA-binding domain of *PPARG* (E157D) (Fig. 1B). We identified a total of 15 individuals who were heterozygous for the *PPARG* E157D mutation and 43 individuals who were homozygous for wild-type *PPARG*. We also screened for potential modifier genes because *ApoE* $\epsilon 2/\epsilon 2$ is associated with type III hyperlipoproteinemia (8) and *LPL* has demonstrated founder mutations among French-Canadians (5) (see Table 1). Among three subjects without *PPARG* E157D who were heterozygous for *APOE* $\epsilon 2$, only one had hypertriglyceridemia. Two subjects with *PPARG* E157D were heterozygous for *LPL* D9N and both had hypertriglyceridemia, whereas one subject without

PPARG E157D but heterozygous for *LPL* D9N had normal plasma triglycerides. Thus, alleles other than *PPARG*E157D could contribute to the hypertriglyceridemia in this family but most likely in only a small minority of study participants.

Metabolic syndrome and lipodystrophy

The biochemical investigations and clinical examination revealed a severe metabolic syndrome with hypertriglyceridemia in E157D mutation carriers and a typical partial lipodystrophy phenotype (see Tables 2 and 3 and supplementary Table I). Most (6/7) E157D female heterozygotes had a readily apparent partial lipodystrophy phenotype with increased adiposity in the trunk and back with decreased adiposity in the extremities. Ten E157D heterozygotes including six males had prominent veins on their extremities (phlebectasia), which can signify lipodystrophy (9). Thus, the general phenotype was of marked lipodystrophy in the arms and legs in both men and women and increased fat in the trunk and back, especially noticeable in women, as illustrated in an excellent review by Dr. Abhimanyu Garg (10). Eight individuals were diabetic, six required insulin with three being so insulin-resistant as to require more than 100 units of insulin per day. In contrast, of 43 noncarrier relatives, only three had type 2 diabetes, all of whom were well controlled with oral hypoglycemic agents. Glycated hemoglobin, insulin, and C-peptide were higher in E157D heterozygotes, consistent with their lower insulin sensitivity and normal β cell function. Of nine E157D heterozygotes who had received abdominal ultrasound examinations, all showed hepatic steatosis. Of 15 E157D heterozygotes, four had acanthosis nigricans, and of seven E157D female heterozygotes, five had hirsutism and two had polycystic ovarian syndrome. Heterozygotes had significantly lower plasma leptin levels. Interestingly, E157D heterozygotes had significantly lower C-reactive protein and normal free fatty acid levels. Twelve E157D heterozygotes were treated for hypertriglyceridemia, and another two had triglycerides > 1.84 mmol/L. Seven E157D heterozygotes had a past history of pancreatitis, including one at age 14. Eleven of 15 E157D heterozygotes were treated for hypertension.

Muscular, neurological, and cardiac features

E157D heterozygotes were muscular despite the majority reporting no involvement in physical training or manual labor. One male with a muscular habitus had weakness on the trunk flexion test and a prior EMG showed a pattern consistent with a myopathic process affecting the deltoid and vastus medialis muscles with low amplitude polyphasic waves. A total of three heterozygotes reported unexplained muscle weakness (excluding a subject who had Guillain-Barré syndrome), and five reported unexplained myalgias (although three were taking a statin drug). Remarkably, 12/15 E157D heterozygotes had median neuropathies (carpal tunnel syndrome) of whom nine had undergone surgical decompression. The decompression relieved all symptoms of the median neuropathy. These features are reminiscent of the pseudohypertrophy,

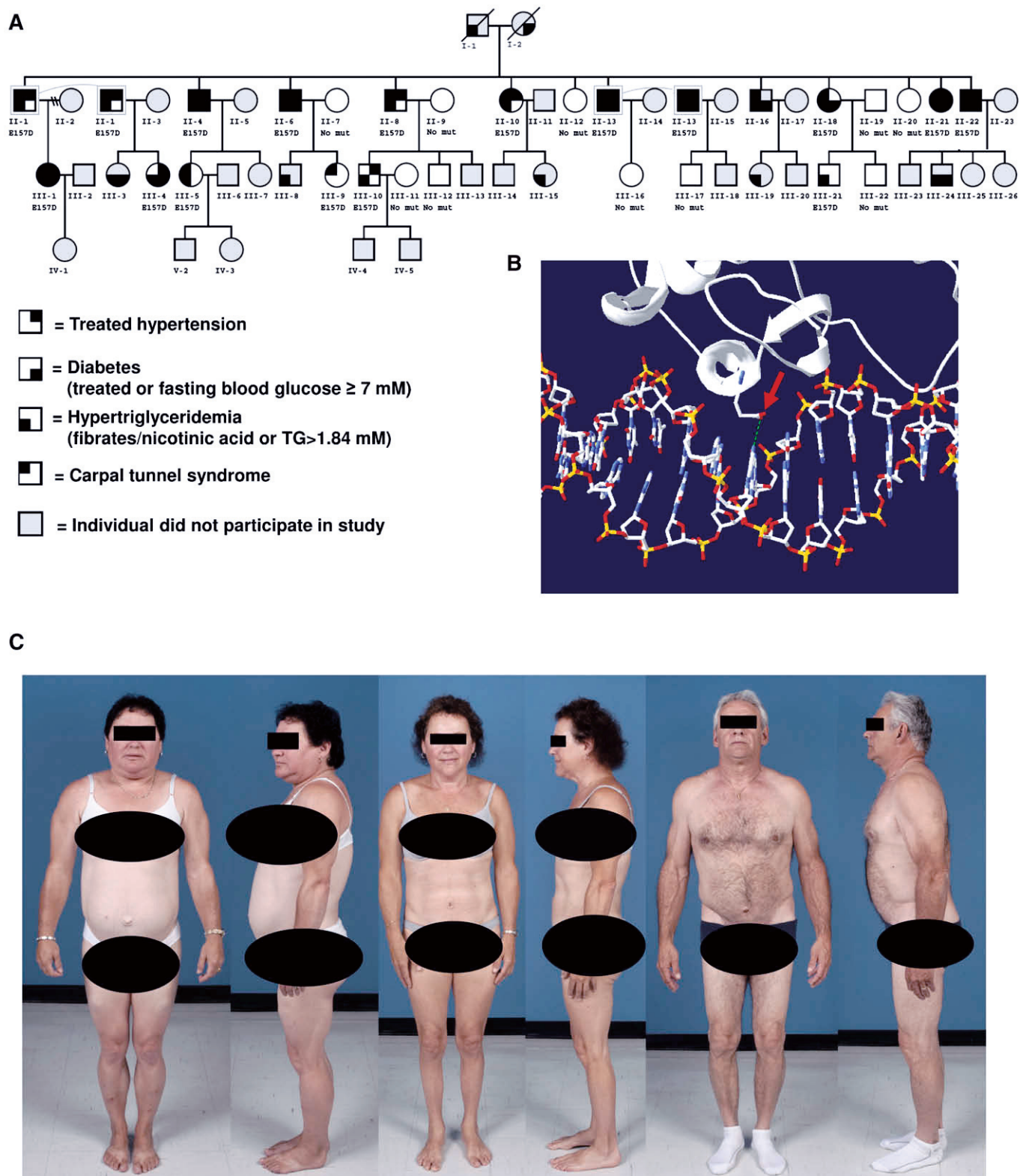


Fig. 1. A: Pedigree showing identified E157D heterozygotes. More relatives were investigated than shown in this Fig., see supplementary dataset for details. No mut = normal PPAR γ genotype. B: 3D rendering of a portion of PPAR γ bound to DNA, with an arrow pointing at the native glutamine at position 157. C: Photographs of three carriers of the PPAR γ E157D mutation (from left to right, individuals II-21, II-18, and II-1 in the pedigree). Notice the sparing of the extremities and the variable truncal adiposity. The man has phlebectasia and a good musculature despite having myogenic weakness demonstrated by EMG.

TABLE 1. Potential modifying genotypes in *APOE* and *LPL*

	PPARG E157D (N = 15)	Normal (N = 42)
<i>ApoE</i> 2/3 ^a	0	3
<i>ApoE</i> 3/3	3	20
<i>ApoE</i> 3/4	12	17
<i>ApoE</i> 4/4	1	1
<i>LPL</i> D9N allele	2	1

^aNo subject had the *ApoE* ε2/2 or ε2/4 genotype, or the *LPL* G188E or P207L alleles.

myopathy, and neuropathy reported in FPLD2 (caused by *LMNA* mutations), yet they have never been reported for FPLD3 until now (11). Four E157D heterozygotes also had a “trigger finger” tendon entrapment syndrome (stenosing flexor tenosynovitis). One heterozygote had multiple small hyperintense foci in the cerebral white matter on T2-weighted MRI, which were most likely lacunar infarcts. One heterozygote had benign ventricular ectopic activity and isolated atrial extrasystoles.

Hematological abnormalities in E157D heterozygotes

Hematological abnormalities have never been noted in patients with FPLD3. One E157D heterozygote had a non-Hodgkin lymphoma. 7/15 E157D heterozygotes had normocytic anemia, while an additional subject had a past history of anemia. The mean corpuscular volume was 86.9 fL (±4.5) in E157D heterozygotes and 88.7 fL (±3.5) in the other individuals ($P=0.11$). Ten E157D heterozygotes had leucopenia, 6 had neutropenia, 3 had lymphopenia, 1 had monocytopenia and 2 had thrombocytopenia (see **Fig. 2A** and **Table 4**). Extensive prior investigation of the anemia in four subjects showed no evidence of hemolysis, with normal levels of iron reserves, vitamin B12, folic acid, and normal serum protein electrophoresis. Two had slightly elevated reticulocyte counts. One E157D heterozygote with leucopenia had an MRI for unrelated reasons. The bone marrow was nonhomogenous with diffuse foci across the vertebral marrow hypointense in T1 and T2

weighted MRIs without hyperintensity on Short TI Inversion Recovery (STIR) (Fig. 2B). These observations are characteristic of marrow reconversion and appeared similar on another MRI performed 4 years earlier. Marrow reconversion occurs with chronically increased hematopoietic demand. Also, mild splenomegaly was observed in two individuals. The reticulocytosis, splenomegaly, and marrow reconversion, along with the multiple cytopenias, are consistent with either a decreased survival of cells or an inadequate support of hematopoiesis in its normal sites. Iliac bone marrow biopsy in the subject who had abnormal bone marrow on MRI revealed normal maturation of the three cell lineages and no proliferative lesion or metaplasia. There was, however, a sparseness of hematopoietic cells and most medullary spaces were occupied by mature adipose tissue without signs of fibrosis.

Heterozygotes appeared to have an increased risk for autoimmune diseases. For instance, among heterozygotes, we noted one case of Guillain-Barré syndrome (acute motor axonal neuropathy type) and three cases of Bell’s palsy, which is considered multifactorial with an autoimmune component (12). In contrast, the rate of autoimmune diseases in subjects with a normal *PPARG* genotype was comparable to the 5% rate reported in general population studies (2/43; Crohn’s disease and rheumatoid arthritis). The rate of autoimmune diseases in *PPARG* mutation carriers appears significantly higher than in noncarriers ($P=0.034$ by Fisher’s exact test). Also, in 11 other hypertriglyceridemic family members who did not participate in the study, there was one case each of Wegener syndrome, ulcerative colitis, and Crohn’s disease.

Binding of mutant PPARG to PPAR response elements

The proximal-box of PPARγ (CEGCKG), which contains the E157D mutation residue, is perfectly conserved from fish to mammals and is essential for distinguishing DNA sequences (13). We assessed the ability of E157D PPARγ1 to bind well-characterized PPRE DNA sequences

TABLE 2. Age and gender of study participants and some important laboratory values

	E157D	Normal	P-value
Age (years)	45 (32–55)	53 (30–61)	0.311
Gender	7 women, 8 men	26 women, 17 men	
Total Triglycerides (TG, mM/L)	5.46 (2.02–22.61)	1.23 (0.86–1.78)	<0.001
VLDL Cholesterol (mM/L)	1.16 (0.69–3.02)	0.41 (0.27–0.63)	<0.001
LDL Cholesterol (mM/L)	1.87 (0.70–2.33)	2.96 (2.53–3.39)	<0.001
HDL Cholesterol, women (mM/L)	0.517 (±0.25)	1.4 (±0.24)	<0.001
HDL Cholesterol, men (mM/L)	0.652 (±0.18)	1.273 (±0.38)	<0.001
Fasting Blood Glucose, FBG (mM/L)	5.9 (5.30–10.60)	5.3 (4.60–5.70)	0.007
Insulin (pM/L)	189 (137–227)	90 (51–114)	<0.001
C-peptide (pM/L)	1300 (1010–1935)	728 (531–961)	<0.001
Insulin sensitivity (HOMA2%S)	30 (21–39)	60 (46–102)	<0.001
Beta-cell function (HOMA2%B)	142 (74–184)	122 (98–152)	0.453
HbA1c (percent)	6.6 (5.7–8.1)	5.4 (5.1–5.7)	<0.001
Leptin, women (ug/L)	0.96 (0.83–1.21)	14.88 (7.45–31.68)	<0.001
Leptin, men (ug/L)	0.98 (0.83–1.34)	3.07 (1.90–5.29)	<0.001
Adiponectin, women (ug/ml)	4.25 (2.95–8.30)	13.08 (6.34–19.61)	0.006
Adiponectin, men (mg/L)	7.45 (3.48–8.34)	10.15 (5.60–12.90)	0.2
Free fatty acids (mM/L)	0.65 (0.46–0.81)	0.76 (0.62–0.94)	0.07
C-reactive protein (high-sensitivity assay) (mg/L)	0.48 (0.32–1.04)	1.09 (0.50–2.21)	0.034

HOMA2%S, insulin sensitivity calculated by the Homeostatic model assessment 2. HOMA2%B, β-cell function. 100% is normal for each.

TABLE 3. Relevant findings on clinical examination and history

	PPAR γ E157D (N = 15)	Normal (N = 43)	P of Fisher's exact test
Marked lipodystrophy ^a	6	0	<0.001
Prominent veins	10	0	<0.001
Muscular appearance	14	3	<0.001
Hepatomegaly	2	0	0.064
Acanthosis nigricans	5	0	<0.001
Hirsutism in women	5	0	<0.001
Xanthomas	3	0	0.015
History of pancreatitis	7	0	<0.001
Median neuropathy	12	6	<0.001

^aIncreased adiposity in the trunk and back with decreased adiposity in the extremities was considered as marked lipodystrophy.

using a gel EMSA. PPRE DNA sequences consist of direct repeats of AGGTCA or TGACCT upstream of coding sequences that are recognized by PPAR γ , the latter regulating gene transcription. As shown in Fig. 3A and B, nuclear lysates from cells expressing either wild-type or E157D PPAR γ 1 produced shifted bands of equal intensity using a malic enzyme PPRE as probe. Similar results were obtained with eight other PPRE sequences (see supplementary Table II). A squelching mechanism whereby the mutation increases the affinity to nonspecific sequences is unlikely because we observed normal binding to PPREs despite an excess of nontarget DNA in EMSA experiments (14).

Transcriptional activity of mutant PPAR γ

To assess the effect of the E157D mutation on PPAR γ 1 transcriptional activity, human adiponectin PPRE was inserted into a minimal TK promoter upstream of the luciferase reporter gene. Although both the wild-type and mutant receptors, when transfected into NIH3T3 cells, activated transcription in response to rosiglitazone treatment, the mutant receptor was not able to drive transcription to the same maximal level (Fig. 3D). This was not due to differences in affinity for the agonist as shown by normalized rosiglitazone dose response curves (Fig. 3E), or to a reduction in protein expression of the mutant receptor (Fig. 3F). Similar results were obtained for three additional PPRE sequences (data not shown). This reduction in transcription is comparable to the reduction we have observed with mutations F388L, Y355X, and P467L, which also cause FPLD3 (7, 15, 16). To confirm findings in a more natural context, lentiviral vectors were used to generate stable NIH3T3 cell lines expressing equal amounts of either the wild-type or E157D PPAR γ 2 (Fig. 3H). The ability of wild-type and mutant PPAR γ 2 to activate transcription from endogenous PPAR γ target genes in response to rosiglitazone was assessed by qRT-PCR. Similar to what was observed in the transient transfection system, E157D PPAR γ 2 was defective in its ability to activate transcription of the endogenous adiponectin gene in response to rosiglitazone (Fig. 3G). This was also true for three other well-characterized PPAR γ target genes that were examined (data not shown).

Assessment of dominant-negative activity of mutant PPAR γ

To determine whether E157D PPAR γ has dominant-negative activity, we performed a competition assay in transfected

cells in which increasing amounts of the mutant receptor were cotransfected with a fixed amount of the wild-type receptor. When increasing amounts of the wild-type PPAR γ alone were introduced into NIH3T3 cells, transcription from a PPRE-driven luciferase reporter increased as expected (Fig. 3I). Addition of a well-characterized dominant-negative PPAR γ mutant (P467L) suppressed transcriptional activity of the wild-type receptor as expected (15). In contrast, E157D PPAR γ failed to suppress wild-type-driven transcription, indicating that this mutant lacks dominant negative activity. Rather, the E157D PPAR γ increased expression of the reporter gene, but failed to reach the wild-type level of transcription, which is consistent with the reduction of transcriptional activity by the E157D mutation.

Microarray experiments

To identify the set of PPAR γ target genes that were misregulated by the E157D receptor, we compared RNA expression profiles generated from NP γ 2 cells expressing either wild-type or mutant PPAR γ 2, with or without a 24 h rosiglitazone (20 μ M) treatment. In the wild-type PPAR γ expressing cells, 528 genes were induced 1.5-fold or more and 54 genes downregulated two-fold or more by rosiglitazone. In the mutant cell line, these numbers were 89 and 5, respectively, confirming that the predominant effect of the mutation is to reduce the transcriptional activity of the receptor. Of the 528 genes upregulated by wild-type PPAR γ , 90% were not induced by E157D PPAR γ ; referred to as the 'loss-of-function' gene set. On the remaining 10% of these genes, the mutant receptor showed similar or only slightly reduced levels of induction compared with the wild-type receptor. The set of 528 stimulated genes were categorized by biological processes enrichment analysis using Genomatix and DAVID software as being involved in adipogenesis, as well as metabolism, biosynthesis, and storage of various organic molecules ($P < 0.01$, supplementary Table III). This gene set contained the classic well-described PPAR γ target genes. Genes in the loss-of-function category notably include genes well known to be involved in PPAR signaling (*Lpl*, *Adipoq*, *Cpt2*, *Fabp5*), the immune system (*Myd88*, *Casp8*, *Vegfa*), hematopoiesis (*Ciartin1*), and metabolism in muscle (*Acadm*, *Ldha*).

Interestingly, we also identified a set of 34 genes that were uniquely induced by the mutant receptor. We refer to this set of genes as E157D 'gain-of-function' genes. Biological process enrichment analysis revealed that these genes were involved

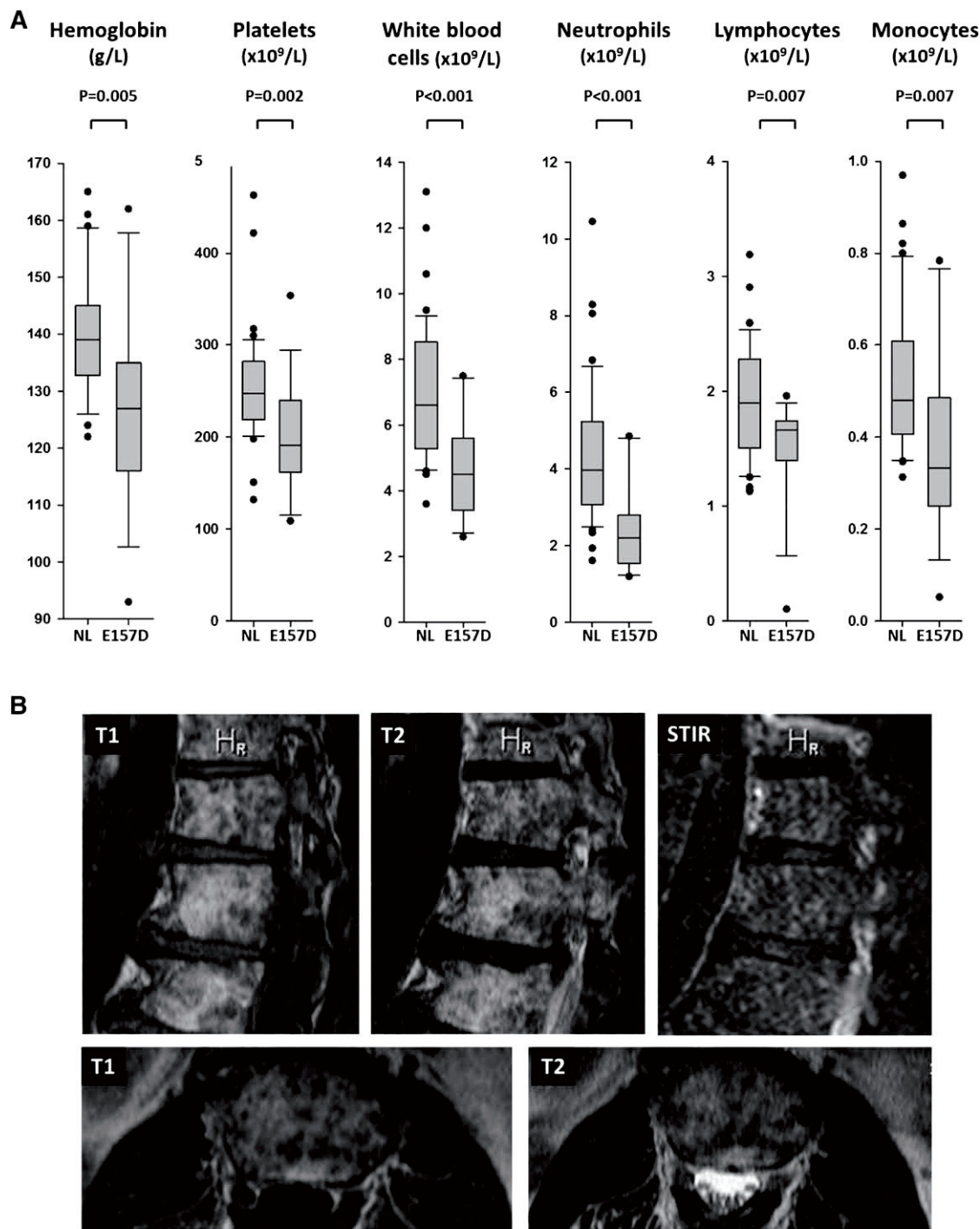


Fig. 2. A: Multiple cytopenias in E157D heterozygotes. Boxes represent the 25th and 75th percentiles with median line. Whiskers represent the 10th and 90th percentiles, and dots represent values beyond these percentiles. Supplementary tables have more details. B: Bone marrow reconversion caused by the E157D PPAR γ mutation. MRI of a patient with the E157D mutation. Diffuse foci across the vertebral marrow hypointense in T1 and T2 weighted MRIs, without hyperintensity on Short TI Inversion Recovery (STIR). Upper images show sagittal cuts through vertebrae T11, T12, L1 and L2. Lower images are transverse cuts across L4.

in nervous system development (*Cck*, *Irx5*, *Sema5a*, *Mtap2*, *Sncg*, and *Sepp1*, $P=0.0007$), and angiogenesis (*Vav3*, *Sema5a*, and *Klf5*, $P=0.0002$) (supplementary Table IV). Notably, the biological processes enriched in this set of genes were mutually exclusive from those in the loss-of-function set described above. None of the gain-of-function genes fell into the adipogenesis or metabolism categories and none of them have

been previously identified as PPAR γ target genes. Conversely, none of the 528 genes upregulated by wild-type PPAR γ fell into the categories of angiogenesis or nervous system development.

Given the possibility that expression the E157D gain-of-function genes in patients could contribute to the atypical clinical characteristics found in affected individuals, we

TABLE 4. Blood count data

	Continuous values			Categorical values (<i>i.e.</i> , number of individuals below thresholds)			
	PPARG E157D	Normal	<i>P</i>	Grading ^a	PPARG E157D (N = 15)	Normal (N = 42)	<i>P</i> of Fisher's exact test
Hemoglobin, women (g/L)	115 (±12)	137 (±9)	<0.001	Grade 2 (80 to <100)	1	0	0.212
				Grade 1 and 2 (<120 ^c)	4	0	<0.001
Hemoglobin, men (g/L) ^b	135 (127–154)	143.5 (139–158)	0.15	Grade 1 (100 to <130 ^c)	3	0	0.028
Platelets (x10 ⁹ /L) ^b	191 (162–240)	248 (219–282)	0.002	Grade 1 (75 to <130 ^d)	2	0	0.066
White blood cells (x10 ⁹ /L) ^b	4.5 (3.40–5.60)	6.6 (5.28–8.53)	<0.001	Grade 2 (2.0 to <3.0)	3	0	0.016
				Grade 1 and 2 (<4.8 ^d)	10	5	<0.001
Neutrophils (x10 ⁹ /L) ^b	2.20 (1.54–2.80)	3.97 (3.06–5.23)	<0.001	Grade 2 (1.0 to <1.5)	3	0	0.016
				Grade 1 and 2 (<2.0 ^d)	6	2	0.003
Lymphocytes (x10 ⁹ /L)	1.48 (±0.48)	1.90 (±0.49)	0.007	Grade 4 (<0.2)	1	0	0.263
				Grade 1 and 4 (<1.2 ^d)	3	3	0.18
Monocytes (x10 ⁹ /L) ^b	0.33 (0.25–0.49)	0.48 (0.41–0.61)	0.007	<0.10 ^d	1	0	0.263

^aAll grades are according to the NCI Common Terminology Criteria for Adverse Events v3.0, where grade 1 for hematological variables correspond to values below the lower limit determined by the laboratory performing the analysis.

^bNot normally distributed.

^cThreshold from the World Health Organization (35).

^dLower limit of the normal determined by the laboratory.

further explored the functionality of this set of genes. To characterize the regulatory elements directing the gain-of-function activity, we examined the DNA sequences of the promoters of these genes for PPRES. We found no difference ($P = 0.8$) in the number of gain-of-function gene promoters containing at least one PPRE (25, or 74%), compared with the loss-of-function genes (324, or 68%). We chose two putative PPRES for further evaluation, located ~4kb upstream of the transcription start site of *Sncg* and in the first exon of *Mtap2*. Both PPRES are aligned and conserved in the mouse and human genomes (**Fig. 4A**). We confirmed the gain-of-function status of these genes by qPCR. Both genes were expressed at higher levels in non-stimulated E157D NPγ2 cells than in wild-type cells, and further induced by rosiglitazone in the mutant but not the wild-type cell line (**Fig. 4B**). Gel-shift analysis of DNA binding revealed that the E157D PPARγ bound to both the *Sncg* and *Mtap2* putative PPRES with much higher affinity than the wild-type receptor (**Fig. 4C, D**). This is in striking contrast to the consensus and adiponectin PPRES, which bind both receptors equally well (**Fig. 4C, D**). These results suggest that these gain-of-function genes contain cryptic PPRES that while inactive in cells containing only the wild-type receptor, are recognized by E157D PPARγ and bestow PPARγ responsiveness to these otherwise non-responsive genes.

DISCUSSION

We have described a family with a severe form of FPLD3 with novel features including muscle pseudohypertrophy and myopathy, neuropathies, multiple cytopenias, and a susceptibility to autoimmune disease. The novel mutation changes an amino acid in the DNA-binding site of PPARγ, and results in decreased activation of a wide array of classical PPARγ target genes while not affecting the binding affinity to their promoters. However, the mutation also leads to increased binding affinity and marked activation to a limited subset of genes with cryptic PPAR response elements.

Although the muscle pseudohypertrophy and myopathy as well as neuropathies have been described in FPLD2, hematological abnormalities such as those presented in this family have never been described in patients with familial partial lipodystrophies. In searching for a molecular link for the hematological system involvement, we note that the expression of PPARγ in hematopoietic cells and its colocalization with the hematopoietic transcription factor PU.1 suggest a role in hematopoiesis (17, 18). Interestingly, thiazolidinediones, which are artificial PPARγ ligands, can cause nondilutional anemia (19) (20), cytopenias, and pancytopenia (21–24). Although our study was not designed to study molecular or cellular mechanisms of the hematological abnormalities, we hypothesize that the multiple cytopenias might be caused either by abnormal bone marrow adipocytes, which are critical to support hematopoiesis (25, 26), or an intrinsic hematopoietic defect causing a decreased survival or inefficient production. With regards to the tendency toward autoimmune diseases in the family, PPARγ is known to counter inflammation and suppress autoimmunity in mice by restricting the differentiation of T helper cells secreting interleukin-17 (27–30). Our study exemplifies how basic investigations in a murine model can be subsequently supported by clinical observations in humans with rare mutations.

The E157D mutation could affect transcriptional activity of PPARγ by inducing conformational changes in the receptor, thus affecting its activity (31). Alternatively, the E157D mutation could disrupt the promoter release of PPARγ necessary for continued transcription. Indeed, the interaction of nuclear receptors with target promoters is a dynamic cyclical process: the receptor is released from the promoter and degraded, allowing a new molecule to bind and initiate a new round of transcription (32). We and others have shown that inhibition of the ubiquitin-proteasome pathway decreases PPARγ activity even while increasing PPARγ abundance (15, 33). Our analysis of gene expression indicates that the E157D mutation renders the receptor largely transcriptionally defective on genes that have been

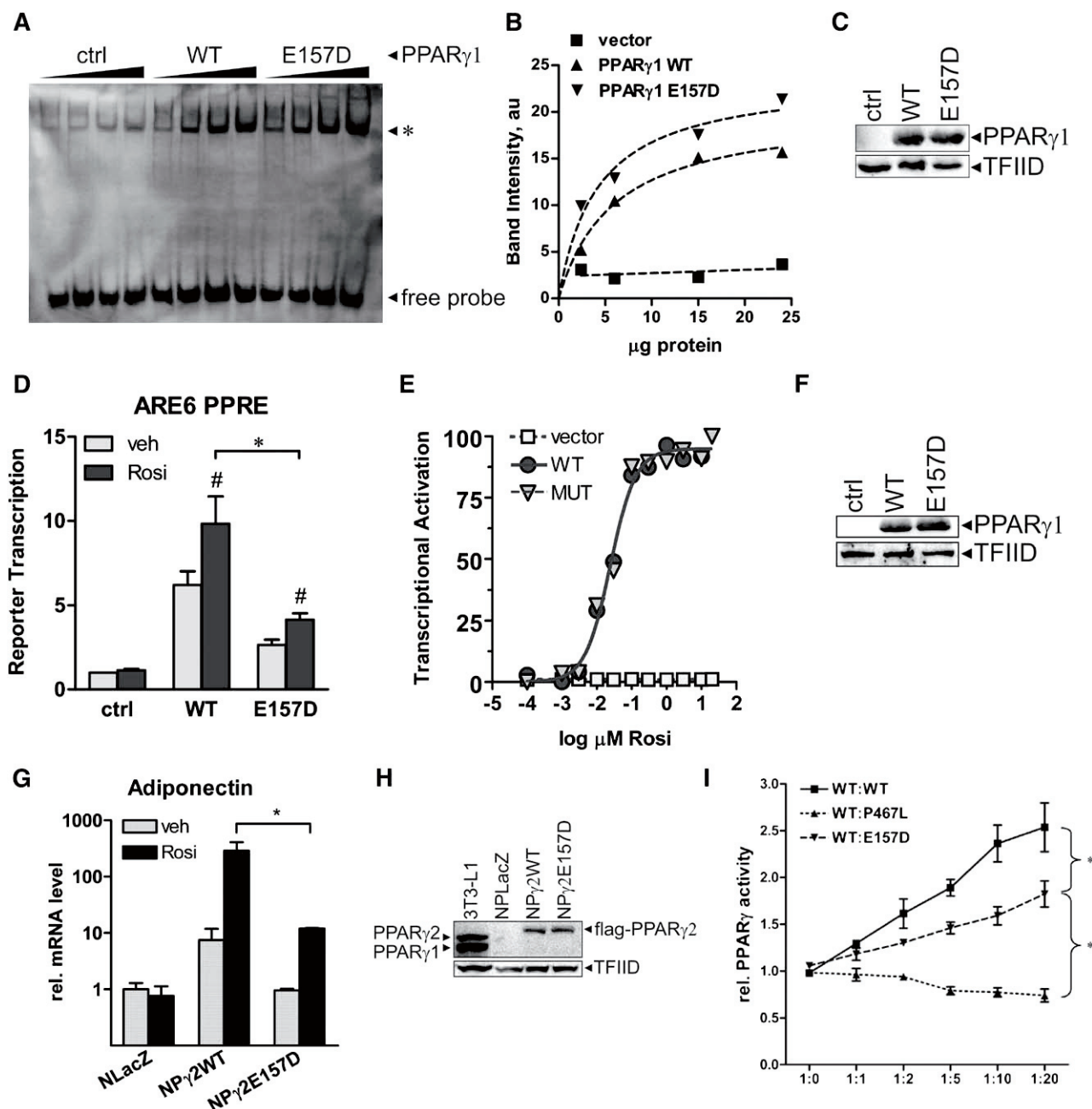


Fig. 3. A: E157D PPAR γ binds DNA as well as wild-type PPAR γ . EMSA showing the DNA binding activity of wild-type (WT) and mutant (E157D) PPAR γ 1 to malic enzyme PPRE. Amounts of nuclear extract were 2, 6, 15, and 24 μ g total protein. Ctrl, empty vector; **, PPAR γ 1-RXR α -PPRE complex. Representative gel from three independent experiments. B: Intensity of shifted bands shown in A. C: For EMSA shown in A, equal levels of wild-type and E157D protein levels in nuclear extracts were confirmed by Western blot. D: E157D PPAR γ is defective in activating the transcription of a reporter gene but responds well to rosiglitazone. Luciferase reporter assay using a minimal promoter containing the ARE6 PPRE in NIH3T3 cells transfected with wild-type and mutant PPAR γ 1. Cells were treated with 20 μ M rosiglitazone for 24 h posttransfection. *, $P < 0.05$; #, $P < 0.05$ versus corresponding vehicle-treated sample. Error bars are the standard errors of three experiments. E: Normalized rosiglitazone dose response curves of wild-type and mutant PPAR γ 1 showing equal sensitivity to agonist activation. Experiment repeated two other times with similar results. F: For reporter assay shown in D and E, equal expression of the wild-type and mutant PPAR γ 1 protein was confirmed on a Western blot. G: Defective transcriptional activity of the E157D mutant PPAR γ 2 on the endogenous target adiponectin in NIH3T3 cells transduced with a lentivirus expressing biologically relevant amounts of PPAR γ 2. Expression of the endogenous adiponectin gene was measured by qRT-PCR after a 24 h treatment with 20 μ M rosiglitazone. Error bars are the standard errors of three experiments. *, $P < 0.05$. H: For endogenous gene expression measurements shown in G, expression levels of the wild-type and mutant PPAR γ 2 in the stable cells lines were determined by Western blot and compared with PPAR γ levels in 3T3L1 adipocytes. I: E157D PPAR γ lacks dominant negative activity and is defective in activating an endogenous target. Competition assay to assess dominant negative effect; NIH3T3 cells were transfected with a malic enzyme PPRE-driven luciferase reporter plasmid and increasing amounts of wild-type or mutant PPAR γ 1. *, $P < 0.001$. Error bars are the standard errors of three experiments.

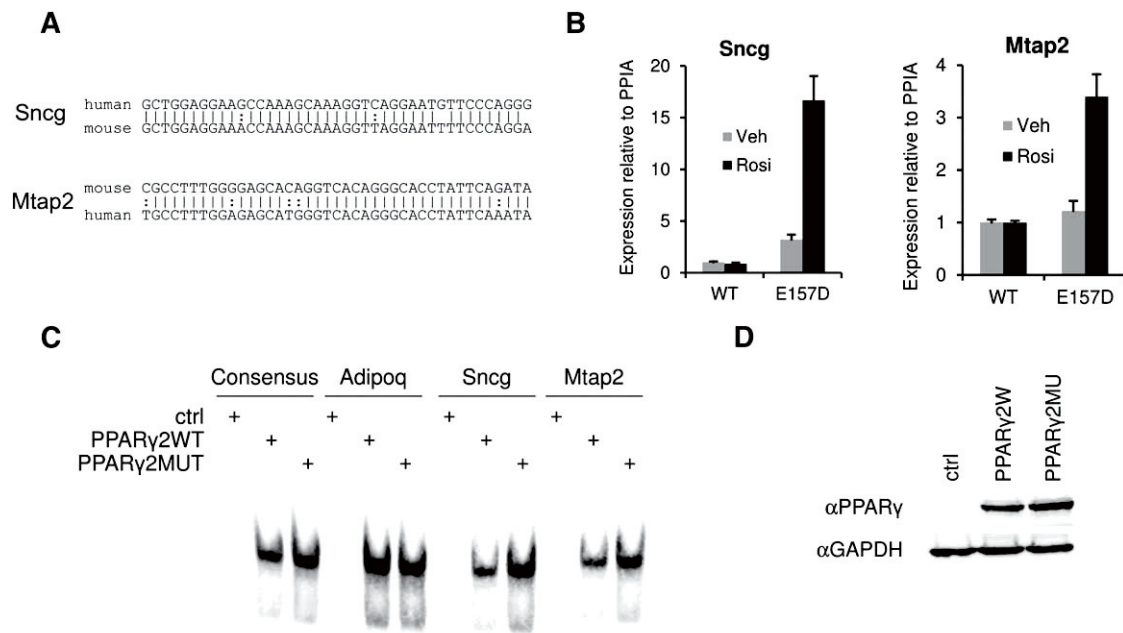


Fig. 4. A: Alignment and conservation of the mouse and human *Sncg* and *Mtap2* genes, putative PPRES are shadowed. B: Characterization of two E157D PPAR γ gain-of-function genes. Mouse *Sncg* and *Mtap2* mRNA in NP γ 2 wild-type (WT) and E157D (MUT) cells were quantified by qPCR after 24 h treatment with DMSO (veh) or 20 μ M rosiglitazone (Rosi). Average and standard error of three independent experiments. C: In vitro analysis of PPAR γ binding to putative PPRES from the mouse *Sncg* and *Mtap2* genes. C:) Gel shift assay with nuclear extracts from Chinese Hamster ovary (CHO) cells transfected with expression plasmids for the indicated PPAR γ 2 allele, and indicated biotinylated PPRE probes. Only the shifted band is shown. Specificity of the shifted band was confirmed by supershift with the M2 flag antibody (Sigma) and competition with a 200-fold molar excess of unlabeled PPRE (not shown). D: Western blot of nuclear extracts used in the gel shift assay. Control treatment was transfected with the empty expression vector.

extensively characterized as PPAR γ targets and are associated with insulin-resistant diabetes and lipodystrophy. The lipodystrophy, hyperlipidemia, and diabetes in the E157D cohort are therefore most likely due to the loss-of-function effect of the mutation on PPAR γ target promoters. Because some of the genes with a loss-of-function are involved in the immune response, hematopoiesis, and metabolism in muscle, we can hypothesize that these genes might be implicated in the novel features found in this family. In addition, the mutant gains transcriptional activity on a smaller set of genes that are not induced by wild-type PPAR γ in our model system or in any previous reports. Some of the unique features of the disease in this family may alternatively be caused by the gain-of-function of the mutant receptor on genes that are not regulated by the wild-type PPAR γ , although the functional ontologies of this set of genes in the mouse cell line do not directly predict the pathology observed in the human cohort. These discrepancies may be related to differential activities of various TZD and nonTZD PPAR γ ligands, an important variable to study in the future. The promoter structure of these genes with the respect to PPRE content is similar to classic PPAR γ target genes; however, both of the two novel PPAR γ binding elements that we identified in this set have a higher affinity for the E157D than the wild-type receptor. Notably, PPAR γ has been shown to bind the site containing the SNCG PPRE in previous chromatin immunoprecipitation followed by high-throughput sequencing

studies (18, 34), suggesting that the mutation allows PPAR γ to activate transcription from some PPRES on which the wild-type has no transcriptional activity. A mechanism of action emerges in which the E157D PPAR γ has increased affinity for weak PPRES in the promoters of genes that have not been previously recognized as PPAR γ targets.

We thus describe a mutation in PPAR γ with a unique postDNA-binding dysfunction on normal PPAR γ targets and an increased activation of alternate genes. Affected individuals, in addition to lipodystrophy and a metabolic syndrome, display heretofore unassociated findings of myopathy, neuropathy, multiple cytopenias, and a susceptibility to autoimmune disease. This supports an important role for PPAR γ in the hematological system and muscle metabolism.

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