

Digenic inheritance of severe insulin resistance in a human pedigree

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Impaired insulin action is a key feature of type 2 diabetes and is also found, to a more extreme degree, in familial syndromes of insulin resistance. Although inherited susceptibility to insulin resistance may involve the interplay of several genetic loci, no clear examples of interactions among genes have yet been reported. Here we describe a family in which five individuals with severe insulin resistance, but no unaffected family members, were compound heterozygous with respect to frameshift/premature stop mutations in two unlinked genes, *PPARG* and *PPP1R3A* these encode peroxisome proliferator activated receptor γ , which is highly expressed in adipocytes, and protein phosphatase 1, regulatory subunit 3, the muscle-specific regulatory subunit of protein phosphatase 1, which are centrally involved in the regulation of carbohydrate and lipid metabolism, respectively. That mutant molecules primarily involved in either carbohydrate or lipid metabolism can combine to produce a phenotype of extreme insulin resistance provides a model of interactions among genes that may underlie common human metabolic disorders such as type 2 diabetes.

As part of an investigation into the etiology of inherited syndromes of severe insulin resistance¹, we identified a European pedigree (family A) with several affected members (Fig. 1). The grandparents (individuals Ii and Iii) had typical late-onset type 2 diabetes with no clinical features of severe insulin resistance. Three of their six children and two of their grandchildren had acanthosis nigricans, a dermatological marker of extreme insulin resistance. All five individuals with acanthosis nigricans had markedly elevated fasting plasma insulin levels, indicative of severe insulin resistance (Fig. 1). Using mutational screening (Fig. 1a), we identified a heterozygous frameshift resulting in a premature stop mutation of *PPARG* (A⁵⁵³ΔAAAiT)fs.185(stop 186) that was present in the grandfather (Ii), all five relatives with severe insulin resistance and one other relative with normal insulin levels (IIvi). Further candidate-gene studies (Fig. 1b) revealed a heterozygous frameshift/premature stop mutation in *PPP1R3A* (C¹⁹⁸⁴ΔAG)fs.662(stop 668) that was also present in this family. In this case, the mutation was present in the grandmother (Iii), in all five individuals with severe insulin resistance and in one other relative (IIIii). Thus, all five family members with severe insulin resistance, and no other family members,

were compound heterozygous with respect to two frameshift mutations of these two unlinked genes. Fasting insulin levels in the singly heterozygous and wildtype family members were within the normal range. By contrast, the compound heterozygotes showed extreme hyperinsulinemia (Fig. 1d) and, to a variable extent, diabetes, hyperlipidemia and hypertension (Fig. 1c and Table 1). As diabetes, hypertension or dyslipidemia were also present in some other members of the kindred, these phenotypes do not seem to require mutations in both *PPARG* and *PPP1R3A*.

We screened our cohort of probands with syndromes of severe insulin resistance ($n = 129$) for the *PPARG* and *PPP1R3A* frameshift mutations. The *PPARG* frameshift mutation was not detected in any other individuals, whereas one European individual carried the same heterozygous frameshift mutation of *PPP1R3A* that was found in family A. This individual (IIi, family B) presented with acanthosis nigricans at age 20 years. He had a body mass index (BMI) of 36.5 kg m⁻² and a fasting insulin level of 437 pmol l⁻¹ (normal <80 pmol/l). He inherited the mutation from his moderately obese father (BMI 30 kg m⁻²), who also has marked hyperinsulinemia (fasting insulin 178 pmol l⁻¹; Fig. 1e). The two other family members who did not carry these mutations were clinically and biochemically normal. Notably, subject IIIi (family B) subsequently lost 40 kg and reduced his BMI to 27 kg m⁻². By that time, his fasting insulin level had fallen to 93 pmol l⁻¹.

The PPAR γ protein is a ligand-inducible transcription factor that regulates target gene transcription as a heterodimer with the retinoid X receptor (RXR)². This heterodimeric complex can be activated synergistically by antidiabetic PPAR γ agonists (thiazolidinediones) and RXR-specific ligands³. The modular structure of PPAR γ consists of a central DNA-binding domain, an amino-terminal activation domain, and a carboxy-terminal ligand-binding domain (Fig. 1a). The frameshift/premature stop mutation reported here is predicted to lead to a mutant receptor that is truncated within the second zinc finger of the DNA-binding domain. This region is common to both the $\gamma 1$ and $\gamma 2$ isoforms of the receptor (Fig. 1a) and is crucial in mediating receptor interaction with PPAR-specific response elements (PPAREs) in target gene promoters. We therefore examined whether the PPAR γ mutants could bind to DNA as heterodimers with RXR, using an electrophoretic mobility shift assay. Unlike

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their wildtype counterparts, neither mutant PPAR γ isoform formed heterodimeric complexes when co-incubated with a radiolabeled probe encoding the acyl-CoA oxidase PPARE (Fig. 2a). Accordingly, and in contrast to wildtype receptors, neither mutant receptor mediated transactivation when cotransfected with a reporter gene containing the PPARE and increasing concentrations of the thiazolidinedione rosiglitazone (Fig. 2b). Moreover, unlike the previously reported, naturally occurring missense PPAR γ mutants (Pro467Leu and Val290Met)¹, the truncated mutant proteins did not show dominant-negative activity when co-expressed with the wildtype receptor (Fig. 2c).

Is a loss-of-function mutation in a single allele of *PPARG* a plausible contributor to insulin resistance? It has been shown that PPAR γ agonists enhance insulin sensitivity⁴, and humans with dominant-negative mutations in *PPARG*¹ and mice with severe

PPAR- γ deficiency⁵ show marked insulin resistance. Heterozygous PPAR- γ -deficient mice seem to be less insulin resistant than their wildtype littermates^{6,7}, however. Although both individuals from family A who carry only the *PPARG* frameshift mutation have fasting insulin levels in the normal range, the co-occurrence of this mutation with the *PPP1R3A* frameshift mutation results in severe insulin resistance. This might seem to conflict with the findings in heterozygous *Pparg* mice. There are several possible explanations for this apparent discrepancy. First, the combination of a genetic defect in muscle glycogen synthesis with the haploid *PPARG* state has not yet been examined in mice. Second, although the particular *PPARG* mutation found in family A does not seem to exert a dominant-negative effect, it might have some properties distinct from a purely null allele. Finally, it is possible that species differences in adipose-tissue biochemistry⁸ may mean

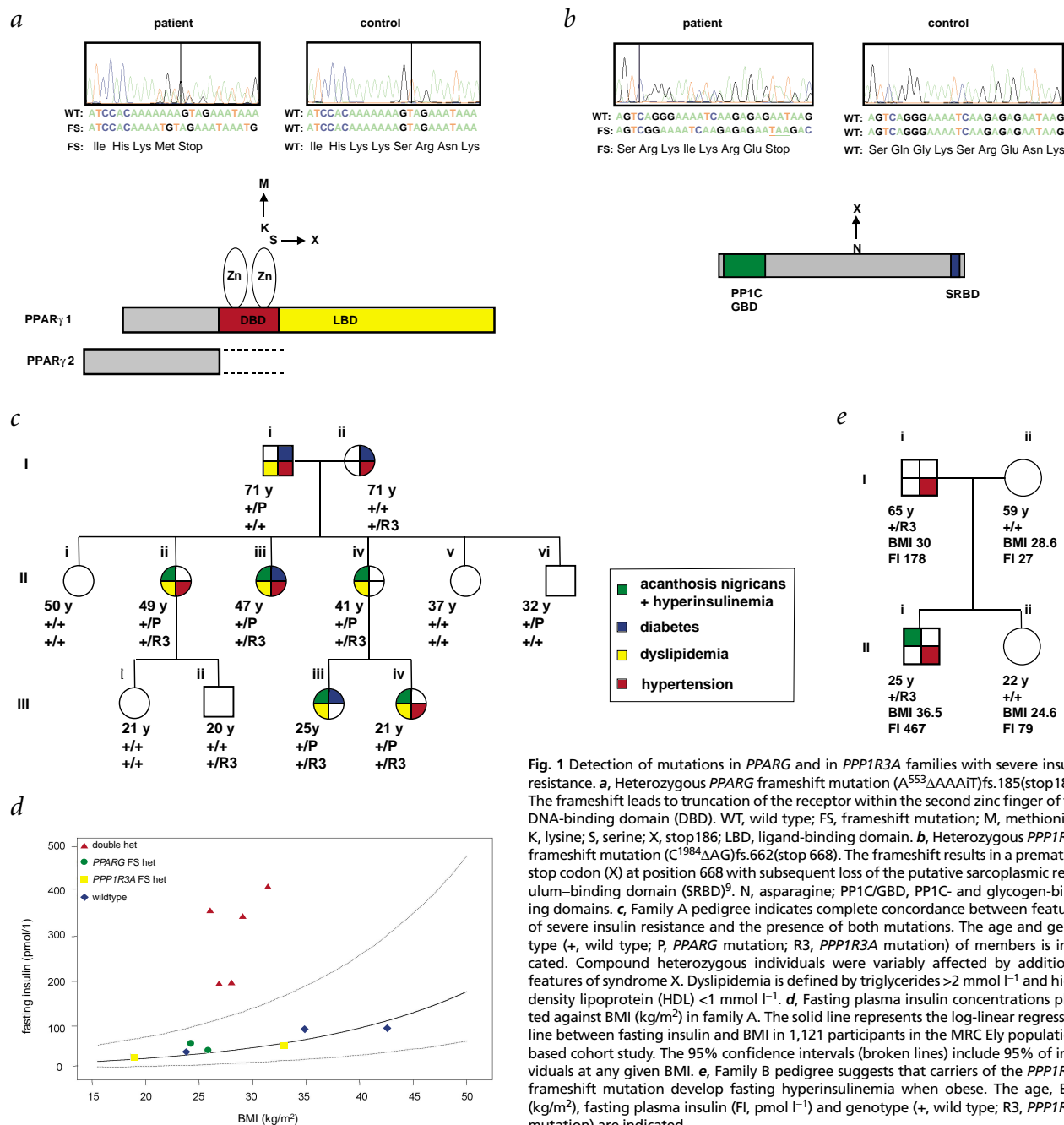


Fig. 1 Detection of mutations in *PPARG* and *PPP1R3A* families with severe insulin resistance. **a**, Heterozygous *PPARG* frameshift mutation (A⁵⁵³ΔAAAT)fs.185(stop186). The frameshift leads to truncation of the receptor within the second zinc finger of the DNA-binding domain (DBD). WT, wild type; FS, frameshift mutation; M, methionine; K, lysine; S, serine; X, stop186; LBD, ligand-binding domain. **b**, Heterozygous *PPP1R3A* frameshift mutation (C¹⁹⁸⁴ΔAG)fs.662(stop 668). The frameshift results in a premature stop codon (X) at position 668 with subsequent loss of the putative sarcoplasmic reticulum-binding domain (SRBD)⁹. N, asparagine; PP1C/GBD, PP1C- and glycogen-binding domains. **c**, Family A pedigree indicates complete concordance between features of severe insulin resistance and the presence of both mutations. The age and genotype (+, wild type; P, *PPARG* mutation; R3, *PPP1R3A* mutation) of members is indicated. Compound heterozygous individuals were variably affected by additional features of syndrome X. Dyslipidemia is defined by triglycerides >2 mmol l⁻¹ and high-density lipoprotein (HDL) <1 mmol l⁻¹. **d**, Fasting plasma insulin concentrations plotted against BMI (kg/m²) in family A. The solid line represents the log-linear regression line between fasting insulin and BMI in 1,121 participants in the MRC Ely population-based cohort study. The 95% confidence intervals (broken lines) include 95% of individuals at any given BMI. **e**, Family B pedigree suggests that carriers of the *PPP1R3A* frameshift mutation develop fasting hyperinsulinemia when obese. The age, BMI (kg/m²), fasting plasma insulin (FI, pmol l⁻¹) and genotype (+, wild type; R3, *PPP1R3A* mutation) are indicated.



that quantitative decrements in PPAR γ function have different metabolic implications for humans and mice.

The PPP1R3A protein is a key molecule in the regulation of glycogen metabolism. Insulin activates glycogen synthase, the rate-limiting enzyme in glycogen synthesis, by promoting its dephosphorylation through the inhibition of kinases (glycogen synthase kinase-3 and protein kinase-A) and the activation of protein phosphatase 1 (PP1)⁹. Insulin activates discrete pools of PP1 in the vicinity of glycogen by facilitating binding of the PP1 catalytic subunit (PP1C) to glycogen-targeting regulatory subunits⁹. These subunits serve as 'molecular scaffolds', bringing PP1C together with its substrates glycogen synthase and phosphorylase in a macromolecular complex, and in the process have significant effects on PP1C activity⁹.

The PPP1R3A regulatory subunit is specific to skeletal and cardiac muscle. The PPP1R3A frameshift mutation is predicted to truncate the protein prematurely (Fig. 1*b*), resulting in the loss of

its C-terminal sarcoplasmic reticulum-binding domain⁹. When transiently expressed in CHO cells, the frameshift-mutant vector produced a detectable protein of the expected reduced size (approximately 83 kD; Fig. 3*a*). In addition, the truncated protein interacted with PP1C with an efficiency similar to that of wildtype PPP1R3A (Fig. 3*b*). Confocal microscopy revealed different intracellular distributions of wildtype and mutant PPP1R3A. A significant fraction of wildtype PPP1R3A localized, as expected, to intracellular membranes, whereas mutant PPP1R3A was almost exclusively cytosolic (Fig. 3*c*).

Is the PPP1R3A frameshift mutation a plausible contributor to a state of insulin resistance? Mice rendered null for *Ppp1r3a* have major defects in muscle glycogen synthesis, although, somewhat unexpectedly, the effects of insulin on this process are maintained in such animals¹⁰. The PPP1R3A frameshift mutation in humans results in a major intracellular mislocalization of the truncated protein and is likely to have phenotypic effects distinct from those

Table 1 • Clinical and biochemical characteristics of frameshift mutation carriers

	Family A					Family B				Normal values		
	Compound heterozygous subjects		FS PPAR γ mutant heterozygotes			FS PPP1R3A mutant heterozygotes						
Fig. 1 reference	lii	liiii	liiv	liiii	liiv	li	liiv	lii	liiii	lii	li	
Age	49	47	41	25	21	71	32	71	20	20	65	
Gender	F	F	F	F	F	M	M	F	M	M	M	
BMI (kg/m ²)	26.8	26	28	31.4	29	24.2	25.8	32.9	18.9	36.5	30	
Blood pressure	190/110	140/80 ^a	130/84	130/70	150/110	170/90 ^a	125/90	170/105 ^a	105/69	135/82 ^a	172/93 ^a	
Measured/predicted body fat ^b (%)	84.3	63.5	83.7	46.8	79.4	n/a	75.2	n/a	n/a	n/a	n/a	100%
Glucose	5.6	6.4	4.4	9.2 ^c	3.9	12 ^c	4.6	4.5	5.2	4.4	6.2	3.5–6.3 mmol/l
Insulin	195	359	197	411	346	61	46	56	31	437	178	<80 pmol/l
Insulin sensitivity (HOMA; %) ^e	27	15	28	14	20	87	115	95	168	13	30	100%
TG	6.1	2.1 ^d	3.4	34.6 ^c	10.1	6.6	1.5	1.1	0.7	1.5	2.4	desirable <2.0 mmol/l
HDL	0.82	0.63 ^d	0.81	0.52	1.04	0.71	1.02	1.84	1.36	0.7	0.91	desirable >0.9 mmol/l
NEFA	1442	202 ^d	526	2532	867	1219	584	933	n/a	n/a	n/a	280–920 μ mol/l
Uric acid	0.31	0.24	0.23	0.23	0.28	0.35	0.31	0.23	0.32	0.17	0.44	0.15–0.35 mmol/l
Leptin	12.1	4.4	8.2	17.3	12.4	1.2	0.9	13.2	0.6	14.6	19.8	μ g/l
IMCL/creatinine ratio (soleus)	19.8	19.1	25.5	28.3	44.9	n/a	28.3	n/a	n/a	n/a	n/a	13.6 \pm 6.6 ^f

All samples were obtained after an overnight fast. ^aMeasurements affected by anti-hypertensive therapy. ^bBody fat was quantified by magnetic resonance imaging (MRI) as described previously²⁷. Predicted body fat²⁸: for women = $(1.48 \times \text{BMI (kg/m}^2) - 7.00)$; for men = $(1.281 \times \text{BMI (kg/m}^2) - 10.13)$. ^cAbnormalities detected at the time of screening. ^dMeasurements affected by lipid-lowering therapy. ^eHOMA (homeostasis model assessment)²⁹ may be influenced by the diabetic status of some individuals. ^fIMCL reference values represent mean \pm s.d. of measurements from 76 control subjects (E.L. Thomas and J.D. Bell, unpublished observations). TG, triglycerides; HDL, high-density lipoprotein; NEFA, non-esterified fatty acids; IMCL, intramyocellular lipid.

the development of muscle insulin resistance in fat-specific *Glut4* knockout mice¹⁵ recently provided *in vivo* evidence of such an interaction between fat and muscle.

The precise mechanism by which loss of a single *PPARG* allele might contribute to maladaptive metabolic cross-talk is not yet known. The generation of a mouse model is currently in progress and will help to reveal the details of such cross-talk. But deficiency of this key transcriptional regulator of adipocyte biology may alter plasma fatty-acid flux or adipokine concentrations¹⁶. Thus, it is notable that plasma leptin levels were below the 25th percentile of healthy BMI and sex-matched normal controls in all compound heterozygous individuals (see Web Table A online). In addition, the levels of intramyocellular lipids (IMCL) were higher in the soleus muscle of compound heterozygous individuals than in controls (mean \pm s.d. IMCL creatine ratio 27.5 ± 10.5 as compared to 13.6 ± 6.6 , $P < 0.05$). Levels of IMCL are correlated with whole-body and muscle-specific insulin sensitivity and are thought to reflect excessive delivery of non-esterified fatty acids from adipose stores to myocytes¹⁷. We hypothesize that in family B, carrying only the *PPP1R3A* mutation, the expanded fat mass of obesity produces the 'second hit' by altering adipose tissue function. This notion is supported by the marked effect of weight loss on fasting hyperinsulinemia in individual III of family B.

The *PPARG* frameshift mutation was not detected in 1,034 UK European individuals (517 diabetics and 517 controls). By contrast, the *PPP1R3A* frameshift mutation was found in two independent case-control studies in a total of 20/1,029 UK individuals with type 2 diabetes and 8/1,033 normoglycemic controls (weighted Mantel-Haenszel odds ratio 2.53; 95% confidence limits 1.06–6.70, $P = 0.03$), indicating that this mutation may result in a predisposition to type 2 diabetes in the general UK population. Given the rarity of this mutation, further large multicenter population genetic studies are required to test this hypothesis.

These findings provide evidence that mutations that, when present alone, have at most subtle effects on different, metabolically relevant tissues can combine to result in extreme disturbances of human insulin action. There has been considerable debate about the relative roles of disturbances of carbohydrate or lipid metabolism in the development of insulin resistance, the metabolic syndrome and type 2 diabetes¹⁸. Our finding that a combination of modest primary defects in both processes can have significant consequences for insulin sensitivity emphasizes the need for an integrated approach to the search for etio-pathogenic pathways in common metabolic diseases.

Methods

Screening of *PPARG* and *PPP1R3A*. Genomic DNA from subjects was randomly pre-amplified in a primer extension pre-amplification (PEP) reaction¹⁹. All coding exons and splice junctions of *PPARG* transcripts and *PPP1R3A* were amplified by PCR from PEP DNA with gene-specific primers (primer sequences are available upon request). We studied PCR products using single-stranded conformation polymorphism analysis and direct sequencing of all abnormal conformers²⁰. We screened for the *PPP1R3A* frameshift mutation in participants in two independent, population-based, case-control studies in East Anglia, UK. The presence of type 2 diabetes was assumed if the onset of diabetes was after the age of 30 y and insulin therapy was not used in the first year after diagnosis. Controls were individually age- and gender-matched to each of the cases. We excluded controls that had glycated hemoglobin (HbA1c) levels $>6.0\%$. We did not detect the *PPARG* frameshift mutation in any individuals from the first population-based cohort.

DNA-binding assays. We assessed receptor binding to DNA in electrophoretic mobility supershift assays as described previously²¹, using ³⁵S-labeled, *in vitro* translated receptors quantified by SDS-PAGE

analysis, and a ³²P-labeled oligonucleotide duplex corresponding to the PPARE derived from the acyl-CoA oxidase gene²².

Transactivation assays. We transfected 293 EBNA cells in 24-well plates with 500 ng of (PPARE)₃TKLUC²³ and 100 ng of receptor expression vector (wild type, frameshift mutants or empty vector pcDNA3) using the calcium phosphate method²¹. Luciferase values were normalized to β -galactosidase activity from the internal control plasmid Bos β gal²¹ and represent the mean \pm s.e.m. of at least three independent experiments, each carried out in triplicate.

Immunofluorescence microscopy. CHO cells were transiently transfected (Fugene) with N-terminal, HA-tagged expression vectors containing wildtype or mutated *PPP1R3A* (pACCMV.pKpA-HA-*PPP1R3A*, gift from P. Cohen²⁴).

Cells were fixed in 3% paraformaldehyde/0.05% glutaraldehyde in 100 mM potassium HEPES/3 mM MgCl₂ buffer (pH 7.5) for 15 min, treated with 0.5% borohydride/PBS for 10 min, and then blocked and permeabilized in 1% BSA/0.1% saponin for 20 min. When permeabilizing cells before fixation, we incubated them for 5 min in 0.05% saponin in 80 mM potassium PIPES/5 mM EGTA/1 mM MgCl₂ (pH 6.8) at room temperature. Cells were labeled with a rat anti-HA (Boehringer; 1:100), followed by Texas Red goat anti-rat (Molecular Probes; 1:200). We collected confocal images using a Leica TCS SP system and processed them using Adobe Photoshop software (Adobe Systems).

Clinical studies. We obtained informed consent from all individuals involved in the study, and ethics committee approval from both the local (Cambridge) and regional ethics committees. We determined IMCL content as described previously²⁵ and measured plasma leptin concentration using an in-house, two-site immunoassay (see Web Note A online).

Note: Supplementary information is available on the Nature Genetics website.

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Competing interests statement

The authors declare that they have no competing financial interests.

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Owing to a copy-editing error that was implemented after the authors returned the corrected proofs, the term 'doubly heterozygous' was substituted with the term 'compound heterozygous' throughout the text and in Table 1. Similarly, 'double heterozygotes' was erroneously substituted with 'compound heterozygotes'. The full-text of the online Letter, including Table 1, has been corrected online. Per company policy, the PDF version has not been corrected; an erratum will be published in an upcoming issue. *Nature Genetics* sincerely regrets these errors.