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PPARγ lipodystrophy mutants reveal inter-molecular interactions required for enhancer activation

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Peroxisome proliferator-activated receptor γ (PPARγ) is the master regulator of adipocyte differentiation, and mutations that interfere with its function cause lipodystrophy. PPARγ is a highly modular protein, and structural studies indicate that PPARγ domains engage in several intra- and inter-molecular interactions. How these interactions modulate PPARγ’s ability to activate target genes in a cellular context is currently poorly understood. Here we take advantage of two previously uncharacterized lipodystrophy mutations, R212Q and E379K, that are predicted to interfere with the interaction of the hinge of PPARγ with DNA and with the interaction of PPARγ ligand binding domain (LBD) with the DNA-binding domain (DBD) of the retinoid X receptor, respectively. Using biochemical and genome-wide approaches we show that these mutations impair PPARγ function on an overlapping subset of target enhancers. The hinge region-DNA interaction appears mostly important for binding and remodelling of target enhancers in inaccessible chromatin, whereas the PPARγ-LBD:RXR-DBD interface stabilizes the PPARγ:RXR:DNA ternary complex. Our data demonstrate how in-depth analyses of lipodystrophy mutants can unravel molecular mechanisms of PPARγ function.

The nuclear receptor peroxisome proliferator-activated receptor γ (PPARγ), encoded by the PPARG gene, is the master regulator of adipocyte differentiation and function and an important regulator of whole-body lipid metabolism and insulin sensitivity. Agonists include several unsaturated fatty acids and lipid metabolites as well as various synthetic compounds such as insulin-sensitizing thiazolidinediones. The importance of PPARγ in human adipocyte biology and physiology is underscored by the finding that many cases of familial lipodystrophy, a syndrome characterized by repartitioning of adipose tissue causing severe insulin resistance, type 2 diabetes mellitus (T2DM) and dyslipidemia, are caused by heterozygous point mutations in this transcription factor (FPLD3; OMIM 604367).

PPARγ regulates transcription of its target genes by binding as a heterodimer with members of the retinoid X receptor (RXR) subfamily to PPAR-response elements (PPREs), which are degenerate repeats of 5’-AGGTCA-3’ spaced by one nucleotide. Here RXR occupies the 3’ half site, while PPAR binds to the 5’ half site and its 5’ extension. In adipocytes, the PPARγ:RXR heterodimer cooperates with the CCAAT/
enhancer-binding protein α (C/EBPα) in the activation of many target genes. PPARγ and C/EBPα bind to many of the same target enhancers, in some cases in a highly interdependent manner. For most of these interdependent binding sites, C/EBPα acts as the leading transcription factor facilitating PPARγ binding; however, PPARγ can also act as a leading factor facilitating C/EBPα binding.

Similar to other nuclear receptors, PPARγ is a modular protein composed of two highly structured and evolutionary conserved domains, the DNA-binding domain (DBD) and the ligand-binding domain (LBD), as well as two mostly unstructured and less conserved domains, the N-terminal A/B-domain and the hinge region. While in vitro experiments indicate that the different domains of PPARγ can execute their functions independently, several results point to intramolecular and intermolecular interactions being important for transactivation by the full-length protein. Early cell-based studies indicate intramolecular communication between the ligand-dependent transactivation function in the LBD and the ligand-independent transactivation function in the N-terminus and showed that phosphorylation of Ser112 in the N-terminal domain can affect ligand binding. Furthermore, studies of the structure of the DNA-bound PPARγ:RXR heterodimer using either X-ray crystallography or small-angle X-ray scattering (SAXS) indicate that several different intermolecular interfaces (DNA-protein and protein-protein) are directly involved in regulating PPARγ activity. Furthermore, these studies also indicate that heterodimerization involves several domains of both PPARγ and RXR. X-ray crystallography revealed a compact, "closed" conformation of the DNA-bound heterodimer with three dimerization interfaces involving (i) the RXRα LBD (helix 10) and the PPARγ LBD (helix 10 and 11); (ii) the RXRα DBD and the PPARγ DBD (bridged by the DNA); and (iii) the RXRα DBD and the ligand-binding domain (LBD) and indicated positions of the two mutations. Bottom: Alignment of the amino acid sequence surrounding PPARγ E379K and R212Q between human PPAR subtypes and PPARγ between different species. Residue positions of E379 and R212 are highlighted in green and blue, respectively.

Fig. 1 | Identification of PPARγ2 E379K and R212Q. a Family pedigree of index patient 1. Each family member is numbered for identification. The proband is indicated by an arrow. Squares and circles indicate males and females, respectively. Phenotypes are elaborated by color segments showing the presence of specific features. Gray symbols denote individuals that were not available for DNA analysis. Deceased individuals are indicated by a diagonal line through the symbol. DNA sequence analysis showing the heterozygous E379K mutation. The chromatogram shows both alleles from the patient (left panel) in comparison with corresponding genomic DNA from a non-affected individual (right panel). For tracing, the nucleotide and amino acid sequences are shown. b Family pedigree of index patient 2, harboring a heterozygous R212Q mutation. See description of panel a for details on representation. c Top: Schematic representation of domains in PPARγ2: N-terminal A/B-domain, DNA-binding domain (DBD), hinge region, and ligand-binding domain (LBD) and indicated positions of the two mutations. Bottom: Alignment of the amino acid sequence surrounding PPARγ E379K and R212Q between human PPAR subtypes and PPARγ between different species. Residue positions of E379 and R212 are highlighted in green and blue, respectively. d Crystal structure of PPARγ:RXRα heterodimer bound to DNA (PPARγ in red; RXRα in gray; PDB entry 3DZY). E379 (in green) at the heterodimerization interface with RXRα DBD and R212 (in blue) in the hinge region of PPARγ are encircled. Both amino acid residues are indicated in stick format. Protein Database entry 3DZY. The figure is generated by open-source software PyMOL (www.pymol.org). A similar DNA-bound conformation based on SAXS was proposed by Bernardes et al.
conformation of the DNA-bound heterodimer lacking the PPAR

β interactions were supported by analogy with the RAR heterodimer. Regardless of these differences in structural approaches, there is evidence that PPAR may modulate the ability of PPAR to activate target enhancers in cooperation with other transcription factors.

Here, we investigate the genome-wide epigenomic and transcriptional effects of two previously uncharacterized FPLD3-associated PPAR mutations, i.e., PPAR-R212Q in the hinge region and PPAR-R379K in the LBD, both predicted to interfere with intermolecular interactions of the ternary PPAR:RXR:DNA complex. We show that both mutations impair the adipogenic capacity of PPAR as well as the activation of an overlapping subset of enhancers that are characterized by being highly dependent on PPAR for chromatin remodeling. These findings provide mechanistic insights into the function of PPAR and emphasize the importance of PPAR as the leading transcription factor in a subset of target enhancers.

**Results**

**Identification of the FPLD3-associated PPAR mutations E379K and R212Q**

FPLD3-associated PPAR mutations present a valuable tool to unravel the complex intermolecular interactions (both protein-protein and protein-DNA) required for optimal enhancer activation by PPAR as they invariably result in loss of function. We selected two previously uncharacterized FPLD3-associated PPAR mutations that are both predicted to affect intermolecular interactions based on structural studies. The first represents a novel heterozygous

### Fig. 2 | E379K and R212Q mutants destabilize PPAR:RXR binding to DNA in vitro.

**a** U2OS cells were transiently cotransfected with expression vectors encoding PPAR-WT or mutants and different reporter constructs as indicated, in the absence or presence of 1µM rosiglitazone. Activation of the reporter is expressed as fold induction over that with empty vector (control). Data are presented as mean values ± SEM, with individual data points indicated with circles. **b** Expression of the different PPAR proteins transiently overexpressed in U2OS cells, as assessed by western blot. The arrow indicates PPAR, and the asterisk indicates a non-specific band. Control, empty vector control; WT, wild-type. Three independent experiments were performed and similar results were obtained. **c** Expression of the different FLAG-tagged PPAR proteins stably overexpressed in U2OS cells, as assessed by western blot using a FLAG-tag antibody. Control, empty vector control; WT, wild-type. Three independent experiments were performed and similar results were obtained. **d** DNA affinity purification-mass spectrometry analysis of Cidec PPRE interactors. Forward and reverse experiments were performed using oligonucleotides containing the Cidec PPRE motif or a mutant version (Cidec dead), followed by dimethyl labeling and mass spectrometry analysis. Log2 ratios (L2FC) of all identified and quantified proteins (from nuclear extracts) in both experiments were plotted against each other. Proteins binding equally well to both oligonucleotides centered around log2(ratio) ~ 0 and are marked in light gray. Proteins binding significantly better to the Cidec PPRE motif or the Cidec dead motif were determined by outlier statistics. These proteins are marked in red. **e** DNA affinity purification followed by western blot analysis were performed using oligonucleotides containing the Cidec PPRE motif, the Cidec dead motif and the synthetic PPRE motif. Pulldowns were performed using nuclear extracts containing the different FLAG-tagged PPAR proteins. Three independent experiments were performed, and similar results were obtained. Source data for panel a–c and e are provided in the Source Data file.

PPAR LBD (helix 6). This latter PPAR:RXR LBD:DBD interaction, which was supported by hydrogen-deuterium (H/D) exchange mass spectrometry and mutagenesis studies, may critically depend on DNA binding, as it was not observed in the absence of DNA. Furthermore, it should be noted that a more elongated, “open” conformation of the DNA-bound heterodimer lacking the PPAR:RXR LBD:DBD interaction was reported in other SAXS studies, in this case supported by FRET studies. More recently, LBD:DBD interdomain interactions were supported by analogy with the RAR:RXR heterodimer. Regardless of these differences in structural approaches and data interpretation, it is currently poorly understood how complex intermolecular interactions may modulate the ability of PPAR to activate target enhancers in cooperation with other transcription factors in the context of chromatin.

Here, we investigate the genome-wide epigenomic and transcriptional effects of two previously uncharacterized FPLD3-associated PPAR mutations, i.e., PPAR-R212Q in the hinge region and PPAR-E379K in the LBD, both predicted to interfere with intermolecular interactions of the ternary PPAR:RXR:DNA complex. We show that both mutations impair the adipogenic capacity of PPAR as well as the activation of an overlapping subset of enhancers that are characterized by being highly dependent on PPAR for chromatin remodeling. These findings provide mechanistic insights into the function of PPAR and emphasize the importance of PPAR as the leading transcription factor in a subset of target enhancers.

**Results**

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FPLD3-associated PPAR mutations present a valuable tool to unravel the complex intermolecular interactions (both protein-protein and protein-DNA) required for optimal enhancer activation by PPAR as they invariably result in loss of function. We selected two previously uncharacterized FPLD3-associated PPAR mutations that are both predicted to affect intermolecular interactions based on structural studies. The first represents a novel heterozygous
Missense FPLD3 mutation (Fig. 1a), substituting a highly conserved glutamic acid at position 379 in helix 6 in the LBD of PPARγ with a lysine (E379K; Fig. 1a, c). Genotyping showed the same mutation in seven additional family members, all of whom have derangements in lipid and glucose metabolism, whereas it was absent in family members without metabolic derangements (Fig. 1a). Crystallography and SAXS of the DNA-bound PPARγ:RXRα heterodimer have indicated that E379 is located at the heterodimerization interface between PPARγ LBD and RXRα DBD (Fig. 1d)\(^1\),\(^2\). An alternative conformation has been proposed in other SAXS studies, where E379 points outwards from the ternary complex (Supplementary Fig. 1)\(^3\).

The second heterozygous mutation we selected results in the substitution of a highly conserved arginine with glutamine (R212Q; Fig. 1b, c). Also, in this case, we identified this mutation in a patient with clear lipodystrophy features (Fig. 1b). Germline transmission could not be established as family members were unavailable for genotyping, but R212Q and R212W have previously been reported in FPLD3 patients by others\(^4\),\(^5\). Arginine 212 is located in the hinge region and forms interactions with the minor groove of the DNA helix immediately 5′ of the PPRE (referred to as the 5′ extension or 5′ upstream region; 5′ UR), and substitution of arginine with a glutamine residue may reduce these (Fig. 1d and Supplementary Fig. 1)\(^3\),\(^6\).

Taken together, we identified two previously uncharacterized lipodystrophy mutations that are predicted to interfere with two interaction interfaces of PPARγ that remain functionally poorly understood.

E379K and R212Q mutants destabilize PPARγ:RXR binding to DNA in vitro

The fact that E379 and R212 residues are located in predicted, but functionally elusive interaction interfaces of PPARγ:RXR and PPARγ:DNA, respectively, prompted us to study the transcriptional and epigenomic effects of the E379K and R212Q lipodystrophy mutants in detail. We first investigated the effect of these two mutations on the ability of PPARγ to activate transcription in reporter assays in U2OS cells, which express negligible levels of endogenous PPARγ\(^7\).

Cells were transiently transfected with expression plasmids encoding WT or mutant PPARγ and reporter constructs regulated by well-established PPRE located in the promoter-proximal enhancers of the adipocyte genes. We, therefore, introduced the R212Q and E379K mutants in detail. We recently developed a model system based on immortalized PPARγ−/− mouse embryonic fibroblasts stably transduced to express high levels of coxakie adenoviral receptor (CAR) for efficient uptake of adenoviruses (PPARγ−/− MEF-CARS)\(^8\). We have previously shown that short-term exposure of these cells to adenoviruses expressing PPARγ leads to the activation of PPARγ-target enhancers and induction of many adipocyte genes. We, therefore, introduced the R212Q and E379K mutations into the corresponding sites in murine PPARγ and titrated the adiponuclear vectors to express similar levels of WT and mutant PPARγ in PPARγ−/− MEF-CAR cells (Fig. 4a, b). Following 2 h of exposure to adenovirus, the medium was removed, and cells were stimulated with an adipogenic cocktail. Interestingly, after seven days of incubation, cells transduced with WT PPARγ accumulate lipids and express adipocyte marker genes, whereas differentiation is much less efficient in cells transduced with the mutant PPARγ (Fig. 4c, d). These findings

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indicate that the ability of PPARγ to drive adipocyte differentiation is impaired by both mutations but most markedly by the R212Q mutation.

E379K and R212Q mutants compromise acute activation of a subset of target genes

To obtain molecular insights into the mechanisms underlying the reduced adipogenic potential of the PPARγ mutants (Fig. 4), we compared the ability of adenovirally expressed WT and mutant PPARγ to acutely activate gene expression in PPARγ−/− MEF-CAR cells in the presence of rosiglitazone (Fig. 5a). In this setting, PPARγ-WT expression changes the expression of 399 genes (DESeq2, false discovery rate (FDR) < 5% and fold change PPARγ-WT vs Control ≥ 1.5 or ≤ −1.5), with 277 genes being induced and 122 genes being repressed compared to control cells (Fig. 5b and Supplementary Tables 2, 3). For further analyses, we focused on the genes induced by PPARγ-WT, as the mechanisms underlying PPARγ-mediated gene repression are less well understood and likely indirect22,23. A expected, genes induced by PPARγ-WT are enriched in adipocyte-related GO-categories (e.g., lipid droplet organization, lipid storage, cellular triglyceride homeostasis, fat cell differentiation), and genes that contribute most to both PC1 and PC2 in the principal component analysis (PCA), are primarily genes related to adipocyte biology (Fig. 5c and Supplementary Table 4). Notably, and in line with the adipogenic effect (Fig. 4), cells expressing PPARγ-R212Q are closer to control cells in the PCA plot compared with cells expressing PPARγ-E379K (Fig. 5c), indicating that the R212Q mutant more dramatically affects the ability of PPARγ to acutely activate target genes (Fig. 5a).
helical structure (gray box). Right panel: Far-UV CD spectra of PPARγ WT (red) and PPARγ E379K (green) recorded at 25 °C in 20 mM NaH2PO4/Na2HPO4 (pH 7.4). Dashed vertical line indicated the minimum at 222 nm for α-helix structure. a HEK293T cells were transiently cotransfected with expression vectors encoding WT or mutant PPARγ, WT or mutant RXRα, and the LAP-PPRE-minimal promoter-reporter, in the absence or presence of 1 μM rosiglitazone. Activation of the reporter is expressed as fold induction over empty vector (control). Data are presented as mean values + SEM, with individual data points indicated with circles. n = 3 biologically independent experiments. One-way ANOVA with Tukey’s multiple comparisons was used to compare cells transfected with mutant vs. WT; *p < 0.05. b–d Overexpression of the different PPARγ and RXRα proteins in HEK293T cells, as assessed by western blot analyses using a PPARγ- or RXRα-specific antibody. The arrows indicate PPARα or RXRα, and the asterisk indicates an unknown non-specific band. Control, empty vector control; WT, wild-type. Three independent experiments were performed, and similar results were obtained. Source data for panel b–d are provided in the Source Data file.

The majority of the 277 PPARγ-WT induced genes, including the well-known PPAR target genes Kruellalike Factor 1 (Klf11) and Uncoupling protein 2 (Ucp2) were induced at comparable levels by the WT and mutant PPARα proteins, and one gene (Pyruvate Dehydrogenase Kinase 4 (Pdk4)) was more induced by the mutants (Fig. 5d–f), demonstrating that mutant proteins retain some degree of functionality. Interestingly, however, 98 and 140 PPARγ-target genes are less induced by the E379K and R212Q mutant PPARα, respectively (FDR < 5% and fold change mutant vs. WT < −1.25, Fig. 5d–f), with an overlap of 81 genes (Fig. 5g). This set of mutation-sensitive PPARγ-target genes includes several classic PPARγ-target genes like fatty acid binding protein 4 (Fabp4), Lpl, Cidec, and CCAAT/Enhancer-binding protein alpha (Cebpa) (Fig. 5c–f and Supplementary Table 5). Notably, most genes significantly affected by only the E379K mutation showed a tendency to be affected by the R212Q mutation, and vice versa (Fig. 5h). Together, the results indicate that the PPARγ mutations E379K and R212Q, which affect different domains of the PPARγ protein and different interaction interfaces, diminish the induction of a largely overlapping subset of PPARγ-target genes, while simultaneously retaining activation potential for other target genes. Notably, mutation-sensitive target genes are generally more induced upon PPARγ-WT expression compared to target genes that are changing less than 25% comparing activation by mutant and WT PPARγ (insensitive genes) (Fig. 5i).
Fig. 5 | The E379K and R212Q mutants display partial deregulation of PPARγ-target genes. a Experimental outline showing the timing of virus transduction, ligand stimulation (1 μM Rosiglitazone) and harvest of PPARγ−/− MEF-CAR cells to investigate acute transcriptional changes upon introduction of WT and mutant PPARγ2s. b Identification of PPARγ2-target genes from RNA-seq data. PPARγ2-WT induced (red dots) and repressed (light gray dots) genes are defined using DESeq2 with Benjamin–Hochberg correction (padj. < 0.05, two-sided) and increasing or decreasing by a fold change >1.5 compared to control cells, respectively. Black dots represent unaffected genes. L2FC, log2 fold change. Top-15 most induced and repressed genes are indicated, with genes known to be involved in adipocyte biology marked in bold. c Variance in RNA-seq data (n = 2 independent biological experiments). Identification of d E379K-sensitive and e R212Q-sensitive PPARγ2-target genes. Red dots represent genes more induced by mutant compared to WT PPARγ2 (padj.(mut vs. WT) < 0.05, FC (mut vs. WT) > 1.25). Green and blue dots represent genes less induced by E379K and R212Q compared to WT, respectively (padj.(mut vs. WT) < 0.05, FC (mut vs. WT) < 1.25). Statistical significance was determined by DESeq2 using Benjamin–Hochberg correction, two-sided test. L2FC, log2 fold change. Top-15 less induced genes are indicated. Genes known to be involved in adipocyte biology are marked in bold. f Bar plots indicating RNA-seq based expression of selected PPARγ2-target genes. Bars represent mean of independent biological replicates (n = 2), dots indicate individual replicates. g Venn diagram representing overlap of genes that are significantly downregulated by PPARγ2-E379K and R212Q. h Correlation of sensitivity to the E379K and R212Q mutations relative to WT (L2FC, log2 fold change). Top-10 most affected genes for each mutant vs. WT is indicated. i Boxplot displaying induction levels for genes affected by one or both mutations (sens., n = 157) or genes changing less than 25% (insens., n = 50). L2FC, log2 fold change. Data are presented as notch, median; box, first and third quartiles; whiskers, 1.5 times the interquartile range. Statistical significance was determined by two-sided unpaired two-samples Wilcoxon–Mann–Whitney test. Source data for panel b–i are provided in the Source Data file.
E379K and R212Q mutants affect recruitment of PPARγ to a subset of PPARγ-target enhancers

The finding that R212Q and E379K mutant proteins compromise transactivation of only a subset of target genes and that this subset is mostly shared between the mutants is intriguing and indicates that a subcategory of enhancers may be particularly sensitive to PPARγ mutations.

HA-PPARγ chromatin immunoprecipitation sequencing (ChIP-seq) of PPARγ−/− MEF-CAR cells transduced with adenoviral vectors expressing PPARγ for 2 h followed by treatment with rosiglitazone for 6 h revealed 41,830 PPARγ-WT binding sites (four-fold enrichment above local background, \( p < 0.0001 \)) (Fig. 6a). To evaluate the importance of PPARγ at these binding sites, we performed H3K27 acetylation (H3K27Ac) and Med1 ChIP-seq, which are well-established proxies for enhancer activity. Notably, for the majority of PPARγ binding sites, PPARγ recruitment is not associated with significant changes in H3K27Ac or Med1 occupancy within the time frame of the experiment (Fig. 6b). Thus, PPARγ recruitment leads to increased H3K27Ac at only 1285 sites and Med1 recruitment at 789 PPARγ binding sites (FDR < 0.1), (Fig. 6b), with 501 sites displaying an increase in both marks...
**Fig. 7** | PPARγ target enhancers display different sensitivity to mutations dependent on basal enhancer activity. a MA-plots of PPARγ2-E379K (left) and PPARγ2-R212Q (right) binding relative to PPARγ2-WT binding (L2FC, log2 fold change). Gained and lost binding is defined by padj. (mut vs. WT)< 0.05 (DESeq2 with Benjamini–Hochberg correction, two-sided) and the binding intensity (mut vs. WT) increasing or decreasing by at least 25%, respectively. b Correlation of sensitivity to E379K mutation and R212Q mutation (L2FC, log2 fold change). c Selected enhancers are indicated. Venn diagram of enhancers with mutation-sensitivity to E379K mutation and R212Q mutation (left) (L2FC, log2 fold change). **d** UCSC Genome Browser track showing HA-PPARγ2 ChIP-seq at the Fabp4 locus (left) and Cidec locus (right). PPARγ-target enhancers are highlighted. e Boxplot of log odds motif score for the de novo PPRE within groups of enhancers. Significance was assessed by two-sided pairwise Wilcoxon rank sum tests with Benjamini–Hochberg correction, a, versus insensitive enhancer group (insens., n = 519); b, versus E379K-only sensitive enhancer group (n = 520); c, versus R212Q-only sensitive enhancer group (n = 109); dual-sensitive group (n = 405), *p = 0.0005, *p = 5.7e-5. f Boxplot of basal Med1 (left) and H3K27ac (right) ChIP-seq signal within enhancer groups. Significance was assessed as in panel e. *p = 4.8e-10, *p = 3.3e-10, *p = 2.4e-14, *p = 0.046. g Boxplot of PPARγ2-WT induced changes in Med1 and H3K27ac ChIP-seq signal within enhancer groups (L2FC, log2 fold change). Significance was assessed as in panel e. *p = 0.0037, *p = 1.4e-11, *p = 4.3e-16, *p = 9.4e-12, *p = 0.049. Data in boxplots are presented as notch, median; box, first and third quartiles; whiskers, 1.5 times the interquartile range. Source data for panels a–c and e–f are provided as a Source Data file.
To investigate how the E379K and R212Q mutations affect the ability of PPARγ to bind to chromatin and activate enhancers, we repeated the PPARγ2-WT-induced chromatin remodeling at PPARγ-target enhancers (94 sites defined in panel c). Linear models fitting the data are indicated with dashed lines. Enhancers that significantly lose ATAC-seq signal upon R212Q mutation are marked with dark blue (DESeq2 with Benjamini–Hochberg correction, two-sided test, padj <0.05, L2FC(R212Q vs. WT) < 0). Selected enhancers are indicated. g Mutation-induced changes in remodeling capacity within enhancer groups. Significance was assessed as in panel d. *p = 2.3e−6, **p = 6.8e−9, †p = 8.4e−16. h PPARγ2-facilitated changes in C/EBPα-binding at target enhancers gaining C/EBPα-binding functional PPARγ binding sites, where PPARγ acts as a key driver of enhancer activity.

(Fig. 6c). Based on this, we defined ‘PPARγ-target enhancers’ as the 1573 putative enhancers that gain H3K27ac and/or Med1 in response to PPARγ binding. Importantly, PPARγ-target enhancers display higher average PPARγ occupancy and a stronger PPRE motif, compared with the other binding sites (Fig. 6d, e), and the top-scoring de novo motif found within PPARγ-target enhancers resembles the JASPAR PPRE (Fig. 6f). Taken together, this indicates that PPARγ-activated sites are pairwise Wilcoxon rank sum tests with Benjamini–Hochberg correction, a, versus control; b, versus WT expression; c, versus E379K-expression, *p = 2.3e−6, **p = 6.8e−9, †p = 8.4e−16.
binding and enhancer activation at PPARγ-target enhancers (Fig. 6g).
Taken together, these results indicate that only a small subset of PPAR
binding sites are functionally important PPARγ-target enhancers, and
that PPARγ recruitment to these on average is sensitive to mutations
that destabilize the PPARγ-RXR heterodimer binding to DNA.

To better understand how the two mutations affect the recruit-
ment of PPARγ to target enhancers, we analyzed how mutations affect
the binding of PPARγ to each of the PPARγ-target enhancers. Out of the
1573 target enhancers (Fig. 6c), E379K decreased binding to 925
enhancers, while R212Q decreased binding to 514 enhancers (Fig. 7a).
Interestingly, the reduced ability of the mutant PPARγ proteins to
engage with chromatin defines clear subclasses of enhancers, where
405 enhancers display reduced binding of both mutants, whereas 520
and 109 enhancers are selectively less bound by E379K and R212Q,
respectively (Fig. 7b). Dual-sensitive enhancers are highly enriched in
the vicinity of dual and R212Q-only sensitive genes compared to
insensitive genes, and R212Q-only sensitive enhancers are more often
found in the vicinity of dual and R212Q-only sensitive genes (Fig. 7c).
These findings indicate that the decreased binding of the mutants to
target enhancers is functionally linked to their reduced activation
potential. For instance, binding of PPARγ to the well-known PPREs
controlling expression of the mutation-sensitive genes Fabp4, Cidec,
and Lpl is compromised by the mutations, while binding of
PPARγ to the Ucp3 intronic PPRE controlling expression of the
mutation-insensitive gene Ucp2 is unaffected by the mutations
(Fig. 7d and Supplementary Fig. 6).

Functional characterization of the different subclasses of enhan-
cers showed that enhancers that primarily are sensitive to R212Q
(R212Q-only) are characterized by strong PPRE motifs (Fig. 7e), low
levels of enhancer activity in the non-transduced cells (Fig. 7f) and a
high fold increase in enhancer activity (Med1 recruitment) in response
to PPARγ-WT (Fig. 7g). This indicates that these represent enhancers
that are highly dependent on PPARγ for activation. In contrast,
enhancers primarily sensitive to E379K are characterized by a weaker
PPRE (Fig. 7e), high levels of enhancer activity in the non-transduced
cells (Fig. 7f), and less fold increase in Med1 recruitment in response to
PPARγ-WT, indicating that these are enhancers where PPARγ plays a
more modest role. The enhancers that are sensitive to both mutations
generally share many characteristics of the R212Q-only enhancers,
including strong PPRE motifs (Fig. 7e) and dependency on PPARγ for
activation (Fig. 7f, g). Finally, PPARγ-target enhancers that are insen-
sitive to mutations are more similar to E379K by having high levels of
enhancer activity in the absence of PPARγ (Fig. 7f) and by being more
modestly activated by PPARγ (Fig. 7g). Consistent with that, receiver-
operating characteristic (ROC) analysis indicates that activity of the
PPARγ-target enhancer in the non-transduced cells is the best pre-
dictor of mutation sensitivity (Supplementary Fig. 7).

Taken together, PPARγ-target enhancers with low activity in the
absence of PPARγ are, on average sensitive to both mutations, espe-
cially the R212Q mutation, whereas PPARγ-target enhancers, which are
already active prior to expression of PPARγ are primarily sensitive to
the E379K mutation.

E379K and R212Q mutants decrease the remodeling capacity of
PPARγ
Local chromatin structure constitutes a major determinant of the
ability of transcription factors to bind to DNA, and we hypothesized
that chromatin accessibility could discriminate between mutation-
sensitive and insensitive sites. We therefore determined chromatin
accessibility in non-transduced PPARγ−/− MEF-CARS using an assay for
transposase-accessible chromatin sequencing (ATAC-seq). Notably,
examination of a 3 kb window centered around the PPARγ peak shows
that R212Q-only and dual-sensitive PPARγ-target enhancers have low
chromatin accessibility (i.e., are nucleosome-rich) in chromatin prior
to expression of PPARγ, whereas insensitive and E379K-only sensitive
enhancers are already accessible in non-transduced cells (Fig. 8a and
Supplementary Fig. 8). These findings are consistent with the notion
that the R212Q-sensitive enhancers are inactive in the non-transduced
state and highly dependent on PPARγ for activation.

To examine the ability of PPARγ to drive the remodeling of target
enhancers, we assessed chromatin accessibility before and after
transduction with Ad-mPPARγ-2-WT. Whereas PPARγ expression does
not affect average chromatin accessibility at all PPARγ binding sites,
it specifically increases chromatin accessibility at PPARγ-target enhan-
cers, with 94 enhancers significantly gaining accessibility (FDR < 0.05)
by ATAC-seq (Fig. 8b, c). Notably, R212Q-sensitive enhancers gain
more accessibility than insensitive and E379K-only enhancers (Fig. 8d),
consistent with the higher gain in activity at these enhancers (Fig. 7g).

The finding that R212Q-sensitive enhancers are relatively inac-
cessible and inactive prior to expression of PPARγ indicates that the
R212 residue, possibly through its direct interaction with the minor
groove in DNA10−13, is important for the ability of PPARγ to bind to and
remodel inactive PPARγ-target enhancers. To directly assess this, we
compared the ability of PPARγ-WT and the R212Q and E379K mutants
to remodel target enhancers. While both mutations significantly
diminish PPARγ-induced remodeling, the R212Q mutation has the
most dramatic effect (Fig. 8e, f). Intriguingly, however, the R212Q
mutation also leads to a slightly decreased remodeling and activation
of target enhancers, where PPARγ binding is not affected by the mutation
(insensitive and E379K-only enhancers) (Fig. 8g and Supplementary
Fig. 9). Taken together, these findings indicate that the R212Q muta-
tion in PPARγ compromises the ability to bind to enhancers in closed
chromatin and induce the remodeling and activation of these enhan-
cers, but that the mutation also affects the activation of enhancers
independent of the level of PPARγ binding. Similarly, the E379K
mutation affects the remodeling and activation of R212Q-only enhan-
cers (Fig. 8g and Supplementary Fig. 9), suggesting that this mutation
also modestly affects the activation of enhancers independent of
PPARγ binding.

We have previously shown that the two adipocyte master regu-
lators, PPARγ and C/EBPα, can potentiate the binding of each other to
shared binding sites in closed chromatin in PPARγ−/− MEF-CAR cells6. Of
to interest to this work, we showed that the ability of PPARγ2 to act as a
leading factor facilitating C/EBPα binding appeared to be dependent on
a strong PPRE, indicating that PPARγ requires a strong PPRE to bind
to DNA in nucleosome-embedded chromatin. Interestingly, by com-
paring these data with the current study, we found that dual-sensitive
PPARγ target enhancers, which are 1) most inactive in the absence of
PPARγ (Fig. 7f); 2) most sensitive to PPARγ-induced remodeling
(Fig. 8d); and 3) most sensitive to PPARγ mutations (Fig. 8g), generally
are enhancers where PPARγ act as the leading factor for C/
EBPα (Fig. 8h).
whereas recruitment is negatively affected by the E379K mutation but not the R212Q mutation when the motif score is decreasing. Importantly, for target enhancers in inaccessible chromatin, the ability of both mutants to bind to target enhancers is highly dependent on the motif strength.

Taken together, these results show that the R212 residue is important for the ability of PPARγ to recruit to sites in closed chromatin, whereas the binding to nucleosome-free accessible target sites is much less affected by this mutation. Interestingly, however, the remodeling capacity of PPARγ-R212Q is compromised independently...
of motif score and PPARγ recruitment, suggesting that PPARγ-R212 is particularly important for the ability of PPARγ to remodel chromatin (Fig. 9c). The E379K mutation also displays the greatest effect on PPARγ binding to inaccessible sites; however, in addition, it decreases binding of PPARγ to accessible target sites in a motif-strength-dependent manner. This suggests that the motif becomes more important for the formation of the ternary PPARγ:RXR:DNA complex when the PPARγ:RXR heterodimer is destabilized by the E379K mutation. Collectively, these data reveal novel mechanisms contributing to the stabilization of the binding of the PPARγ:RXR dimer to DNA, which is particularly important in enhancers where PPARγ acts as the leading factor.

**Discussion**

Structure-based studies have indicated extensive intermolecular interactions in the DNA-bound PPARγ:RXR heterodimer \(^{[18-19]}\), but how these interactions translate into gene activation in a cellular context, i.e. in cooperation with other transcription factors and in the context of chromatin, is currently poorly understood. Here we employed two previously uncharacterized FPLD3-associated PPARγ mutants, predicted to be involved in intermolecular interactions in the PPARγ:RXR:DNA complex, to probe the molecular mechanisms underlying enhancer activation by PPARγ. We show that the R212Q and E379K mutations in PPARγ lead to reduced adipogenic potential and impaired activation of a subset of PPARγ target genes. Consistent with this, genome-wide analyses of the transcriptional and epigenomic properties of the mutants demonstrate that the mutations only affect the activation of a subset of PPARγ-target enhancers and that a major determinant of sensitivity to these mutations is low chromatin accessibility. Interestingly, we show that the two mutations affect enhancer activity by distinct mechanisms, thereby providing insight into how PPARγ engages with its target sites. Furthermore, as important intra- and intermolecular interactions have been reported in other nuclear receptors (e.g. RARγ \(^{[20]}\) and GR\(^{[21]}\)), it will be of interest to investigate whether disease-causing mutations in nuclear receptors other than PPARγ have similar effects \(^{[12]}\).

Based on the crystal structure, the R212Q mutation is expected to decrease the ability of the PPARγ hinge to engage in interactions within the minor groove of the DNA helix immediately 5' of the PPRE (referred to as the 5' extension or 5' upstream region; 5' UR)\(^{[13]}\). Previous studies based on selected target sites indicated that the 5' extension is required for PPAR:RXR binding to suboptimal direct repeats and that this sequence provides specificity of the PPRE towards the PPAR:RXR heterodimer \(^{[13]}\). Consistent with a role for the interaction between the PPARγ hinge and the 5' extension of the PPRE in chromatin, genome-wide profiling of PPARγ occupancy by ChIP-seq has shown that the 5' extension is enriched in natural PPARγ:RXR binding sites \(^{[13]}\). Here we show that the R212Q mutation interferes with the ability of PPARγ to recruit to and remodel target sites in nucleosome-rich chromatin, whereas there is little effect on binding to accessible nucleosome-free target sites. This indicates that the interaction between the PPARγ hinge and the 5' extension of the PPRE is particularly important for the ability of PPARγ to bind to target sites in nucleosome-rich chromatin. In addition, this interaction appears to enhance chromatin remodeling capacity independent of PPARγ binding, possibly by increasing intermolecular interactions in the PPAR:RXR:co-factor complex, or by interfering with the interaction between DNA and nucleosomes.
Furthermore, our data support the existence of a functional intermolecular interaction.

**E379K mutation affects PPARα target sites, including dual-sensitive target sites.** Thus, whereas the E379K mutation leads to a more dramatic loss of remodeling capacity at target sites, the R212Q mutation leads to a more general destabilization of the PPARα:RXR heterodimer. This is consistent with the observation that the E379K mutation affects PPARα binding to more target enhancers than the R212Q mutation.

The finding that the R212Q mutation has the greatest effect on enhancer activity and target gene expression compared with the E379K mutation despite affecting PPARα recruitment to fewer enhancers is intriguing. Most likely, this is due to the fact that the R212Q mutation leads to a more dramatic loss of remodeling capacity at target sites, including dual-sensitive target sites. Thus, whereas the E379K mutation affects PPARα binding to many enhancers through destabilization of the PPARα:RXR heterodimer, the R212Q mutation more severely compromises the ability of PPARα to act as a facilitating transcription factor driving remodeling (Fig. 10). Since the need for PPARα to act as a leading transcription factor is highly dependent on which other transcription factors are expressed, one would expect that the phenotypic result of the R212Q mutation is highly context-dependent.

Taken together, our comprehensive investigations of the functional and biochemical properties of two PPARα FPLD3-associated mutants highlight the importance of intermolecular interactions for transcriptional activation. Our data indicate that the interaction between the hinge region of PPARα and the S' extension of the PPRE is particularly important for the ability of PPARα to bind to and remodel enhancers in inaccessible chromatin. Furthermore, our data support the existence of a functional interface between the RXR-DDB and helix 6 in the LBD of PPARα and indicate that this interaction plays a role in the general stabilization of the PPARα:RXR:DNA ternary complex. Overall, this work underscores the importance of intermolecular interactions in PPARα functions and indicates that the subtle molecular defects in these interactions are sufficient to cause FPLD3.

**Methods**

The study was conducted according to the 1964 declaration of Helsinki and its later amendments or comparable ethical standards. The study was approved by the Ethics Committee of University Hospitals Leuven (File S57866) and the Ethics Committee NedMec (File 22–891). Written informed consent for study participation and publication was obtained from the index patients.

**Index subjects and DNA sequence analysis**

Both index patient 1 and 2 displayed typical features of partial lipodystrophy: a clear excess of subcutaneous fat on the face, neck, trunk, and abdomen with a lack of subcutaneous fat on the extremities. Additional clinical features are indicated in Fig. 1a, b. Genomic DNA was extracted from peripheral-blood leukocytes in venous blood samples using QIAamp DNA Blood Mini Kit according to the manufacturer’s instructions (Qiagen Hilden, Germany). We sequenced all coding exons of PPARG, AKT2, CIDEC, and LMNA (exon 8–9), including intron-exon boundaries of the index patients. Primers are available upon request. In index patient 1 we identified a heterozygous mutation in exon 6 of the PPARG gene corresponding to c.1135 G > A and p.E379K in reference sequences NM_015869 and NP_056953.2, respectively. This variant is absent in the variation databases NHLBI GO Exome sequencing Project (ESP) (Exome Variant Server), 1000Genomes (Consortium, 2015), Single Nucleotide Polymorphism Database (dbSNP), and The Genome Aggregation Database (gnomAD). Amplification and Sanger sequencing of exon 6 and flanking regions were performed to genotype family members. In index patient 2, we identified a heterozygous mutation in exon 5 of PPARG corresponding to c.635 G > A and p.R212Q in reference sequences NM_015869 and NP_056953.2, respectively. Genotype data from family members were not available. The same variant was previously reported independently in a female subject with FPLD3 but not functionally characterized.

**Cell culture**

The murine PPARα−/− MEF-CAR cell line, human osteosarcoma cell line U2OS, and the human embryonic kidney cell line HEK293T were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Gibco) 4.5 g/L D-glucose supplemented with 10% fetal bovine serum (Gibco), and 100 μg penicillin/ml and 100 μg streptomycin/ml (Invitrogen).

**Luciferase-based reporter assays**

The pG3-lmlppre-Luc2 (lipoprotein lipase; Lpl) reporter construct was generated by the insertion of one copy of the mouse Lpl −160 bp PPRE upstream of the minimal promoter and one copy downstream of the luciferase gene in a pG3 minimal promoter-luciferase 2 (Luc2) backbone (Supplementary Fig. 2). The pG3-lmlppre-Luc2 was used as a template to generate Lpl 5 upstream region (5' UR) mutant, CIDEC and synthetic PPRE reporters using QuickChange mutagenesis kit (Stratagene) following the instructions provided by the manufacturer. The CIDEC 5'UR mutant reporter was generated in the same way, based on the CIDEC reporter. Primer sequences are given in Supplementary Table 1a. The reporter construct S5Gal4E1BTATA-pG3 has previously been described. The CIDEC (promoter)-pG3 luciferase reporter was a kind gift of Dr. P.F. Marrero. pCDNA3.1 expression vectors for hPPARγ and hRXRα were used to generate the hPPARα mutants E379K, R212Q and R212W and the hRXRα K175E mutant, respectively, using the QuickChange mutagenesis kit (Stratagene). Gal4DBD-hPPARγAF2 WT, pGEX-PPAR-LBD WT, pGEX-RXRα, pGEX-SMRT, and pGEX-5SRC1 have been described previously and mutants thereof were generated as described above.

For luciferase reporter assays U2OS and HEK293T cells were seeded in 24-wells plates and transiently transfected using PEI. Each well was cotransfected with a reporter construct (1 μg), 2 ng Renilla, and PPAR and/or expression constructs (10 ng). The next day, media were removed and fresh media were added with or without 1 μM Rosiglitazone, as indicated in the figure legends. After incubation, cells were washed twice with phosphate-buffered saline (PBS) and harvested in lysisbuffer (Promega), and assayed for luciferase activity according to the manufacturer’s protocol (Promega Dual-Luciferase Reporter Assay System) and for Renilla to correct for transfection efficiency. The relative light units were measured by a Centro LB 960 luminometer (Berthold Technologies, Bad Wildbad, Germany). The results are averages of at least three independent experiments assayed in duplicate ±SEM. To compare three or more groups, an ordinary one-way analysis of variance (ANOVA) was performed with a Tukey’s multiple comparison test to compare the mean of each group with that of every other group. A statistically significant difference was defined as a p-value <0.05.
Western blot analysis
Protein expression of the transfected U2OS and HEK293T cells and of adenovirally transduced PPARγ MEF-CARS was determined by western blotting. For this, lysates were boiled in Laemmli sample buffer for 5 min at 95 °C. Samples were subjected to SDS-PAGE and transferred to a Millipore membrane (Millipore). Anti-PPARγ (sc-7196; RRID: AB_654710), anti-Gal4 DBD (sc-510; RRID: AB_627655), anti-FABP4 (sc-18661; RRID: AB_2231568), anti-RRXs (sc-533; RRID: AB_2184874), anti-tubulin (Sigma–Aldrich T9026; RRID: AB_477593), anti-LpL (sc-12486; anti-FLAG-HRP (Sigma–Aldrich) were used for detection. Western blot analysis of eluted proteins was performed using the FLAG epitope tag for detection.

DNA affinity purifications
Lentiviral vectors for stable expression of PPARγ in U2OS cells were generated by replacing beta-catenin with the PPARγ2 expression cassette in pLV-CMV-FLAG-betaCatenin-Ires-PURO, a kind gift of Dr. J. de Rooij. Lentiviral particles were produced in HEK293T cells. After lentiviral transduction, stably transduced U2OS cells were selected and maintained on 2 µg/ml puromycin. Nuclear extracts from U2OS were prepared as described previously. Oligonucleotides containing either the CIDEK wt or CIDEK dead motif (Integrated DNA Technologies) with 5' biotinylation of the forward strand (Supplementary Table 1) were annealed using a 1.5 x molar excess of the reverse strand. For DNA affinity purifications, 500 pmol of DNA oligonucleotides were immobilized using 20 µl of Streptavidin-Sepharose bead slurry (GE Healthcare, Chicago, IL). Then, 500 µg of nuclear extract and 10 µg of non-specific competitor DNA (5 µg polydADT, 5 µg polydIdC) were added to each pulldown. After extensive washing, samples were prepared for mass spectrometry analysis or western blotting.

Peptide analyses by NMR and CD spectroscopy
Peptides were purchased from TAG Copenhagen A/S (Denmark), purified to ≥ 95% by reverse-phase HPLC and identities confirmed by mass spectrometry. For circular dichroism (CD) spectroscopy, samples of 100 µM WT-PPARγ376–385 and E379K-PPARγ376–385 peptides were prepared in 20 mM Na2HPO4 /NaH2PO4 (pH 7.4). Far-UV CD spectra of the peptides and of a matched buffer sample were recorded from 260 to 190 nm on a Jasco J-810 spectropolarimeter in a 0.1 cm Quartz cuvette (Hellma, Suprasil®) with a Peltier controlled temperature set to 25 °C. Data acquisition was carried out with a scanning speed, 10 nm/ min; data pitch, 0.1 nm; accumulations, 10; bandwidth, 1 nm; response time, 2 s. After subtraction of the buffer spectrum, the spectra were smoothed by Fast Fourier Transformation. Helical percentage was calculated using (θ M–θ H)/θ M × 100%. All chemical shifts were referenced to internal DSS directly (δH = 4.79 ppm) or indirectly (δC, δN) as described. Secondary chemical shifts of Co were calculated using the random coil set from Kjærgaard et al.

Peptide analyses by NMR and CD spectroscopy
To assign the resonances of the WT-PPARγ376–385 and E379K-PPARγ376–385 peptides, NMR experiments were recorded on 2.3 mM PPARγ376–385 in 20 mM Na2HPO4 /NaH2PO4 (pH 7.4) at 25 °C on a Bruker Avance NEO 800 MHz spectrometer equipped with a 5 mm CPTXO CryoProbe C/N-H-D. The following spectra were acquired at natural isotopic abundance: 1H (zgssp), 1H, 15N HSQC (hsqcetfip3gp), 1H, 13C HSQC (hsqcetpsisp2.2), 2D TOCSY (dipspi2esgph, mixing time 80 ms) and 2D ROESY (roeseysgph, mixing time 250 ms). The spectra were transformed using TopSpin 3.6.2 and analyzed manually in CcpNmr Analysis 2.5.0. All chemical shifts were referenced to internal DSS directly (δH = 4.79 ppm) or indirectly (δC, δN) as described. Secondary chemical shifts of Co were calculated using the random coil set from Kjærgaard et al.

NR-Coregulator Interaction analyses
Rosetta pLysS competent bacteria (Novagen, EMD Chemicals Inc., Darmstadt, Germany) were transformed with GST expression plasmids. GST-fusion protein expression was induced with 1 mM IPTG and purified on Glutathione-Sepharose beads (Amersham Biosciences).

For NR-Coregulator interaction profiling, GST-fusion proteins were eluted from glutathione-Sepharose beads (Amersham, Buckinghamshire, UK) using elution buffer (20 mM glutathione, 100 mM Tris pH 8.0, 10 mM DTT) and alkylated with 50 mM iodoacetamide. Proteins were digested on beads with 0.25 µg of trypsin for 2 h. After the elution of peptides from beads, an additional 0.1 µg of trypsin was added, and digestion was continued overnight. Peptides were labeled on Stage tips using dimethyl labeling. Each pulldown was performed in duplicate and label swapping was performed between duplicates to avoid labeling bias. Matching light and heavy peptides were combined and analyzed on an Orbitrap Exploris (Thermo) mass spectrometer with acquisition settings described previously. Raw mass spectrometry data were analyzed with MaxQuant 1.6.0.1 by searching against the UniProt curated database (Proteome Center, Stockholm, Sweden). Protein ratios obtained from MaxQuant were used for outlier calling. An outlier cutoff of 1.5 interquartile ranges in two out of two replicates was used. Western blot analysis of eluted proteins was performed using the FLAG epitope tag for detection.

Modeling of PPARγ-RXRα 3D structures
To analyze the impact of the E379K mutation in PPARγ, which is located in a functionally less well-characterized region than the R212Q mutation in the hinge region, we used the HADDOCK2.2 web server. As the starting point for the modeling we used the crystal structure of the intact PPARγ-RXRα nuclear receptor complex with DNA (PDB ID 3DZY) (isomorph I). Both WT and E379K (E351K isomorph I) mutant structures were subjected to a short refinement in explicit solvent using the refinement interface of the HADDOCK server. The mutation was introduced by changing the residue name in the PDB file and letting HADDOCK rebuild any missing atoms.

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Samples were boiled at 95 °C for 5 min and loaded on a 10% SDS-polyacrylamide gel. Proteins were stained with Coomassie Brilliant Blue, and gels were fixed (20% methanol and 10% acetic acid for 10 min) and dried. Radioactive signals were analyzed with a Storm 820 Imager (Molecular Dynamics).

Protein complementation assays (PCA) Heterodimerization between PPARγ and hRXRα was analyzed in live cells with the NanoBiT® PPI System (Promega). To generate a protein fusion pair displaying effective complementation of the split luciferase, the Large BiT (LgBiT; 18 kDa) of luciferase was fused to the C-terminus of PPARγ, while the Small BiT (SmBiT; 1.3 kDa) was fused to the N-terminus of hRXRα (Supplemental Fig. 3a). Mutants were generated using the QuickChange mutagenesis kit (Stratagene). Mutants previously shown to have partially or completely lost heterodimerization capacity (R425C and I464R, respectively) were included as controls.

HEK293T cells were seeded in 96-well plates and transfected with plasmids encoding PPARγ2-LgBiT (50 ng/well) or mutant versions, and SmBiT-hRXRα (50 ng/well) using Xtreme gene (Sigma–Aldrich). After 48 h, cells were washed with PBS and diluted substrate (Nano-Glo® Live Cell Assay System; Promega) was added according to the manufacturer’s protocol. The relative light units were measured by a Centro LB 960 luminometer (Berthold Technologies, Bad Wildbad, Germany). The results are averages of at least three independent experiments assayed in triplicate ±SEM. To compare three or more groups, an ordinary one-way analysis of variance (ANOVA) was performed with a Tukey least significant difference test to compare the mean of each group with that of every other group. A statistically significant difference was defined as a p-value <0.05. After luminescence analysis, cells were lysed in RIPA buffer, triplicates were pooled and subjected to Western blot analysis, as described above.

Adenovirus generation and purification Recombinant adenoviruses containing a hemagglutinin (HA)-tagged mouse PPARγ2 were generated using AdEasy cloning system (Stratagene). The Ad-hA-mPPARγ2-WT was used as a template to generate the mutations R212Q and E379K using QuickChange mutagenesis kit (Stratagene) following the instructions provided by the manufacturer. Successful mutagenesis was verified by Sanger sequencing. The plasmids were linearized and transfected into the HEK293 cell line for amplification and purification using CsCl gradients.

Adenoviral delivery of PPARγ in PPARγ−/− MEF-CAR cells PPARγ−/− MEF-CAR cells were transduced with adenoviral vectors expressing HA-tagged mPPARγ2-WT, mPPARγ2-R212Q or mPPARγ2-E379K. After two hours of virus exposure, the medium was removed, and cells were treated with medium containing either vehicle dimethyl sulfoxide (DMSO) or 1 μM rosiglitazone (Alexis) for another 6 hours before harvest for RNA and protein expression analyses and for ChIP- and ATAC-seq.

RNA analysis RNA extraction, cDNA synthesis, and qPCR were performed as described previously. For RNA-seq analysis, RNA from two independent experiments was prepared in triplicate. After qPCR validation, the triplicates were pooled and prepared for sequencing according to the manufacturer’s instructions (Illumina).

Samples were sequenced on Illumina NovaSeq 6000 using NovaSeq Control Software v1.3.0, and the quality of sequenced reads was assessed using FastQC (https://qubeshub.org/resources/fastqc). Reads were aligned to the mouse genome (mm10) using STAR with default settings. HOMER makeTagDirectory was used to generate tag directories for all conditions on primarily aligned reads. HOMER analyzeRepeats.pl was used to count reads within exons of the mm10 genome with the settings -count exons, -condenseGenes, -noCondensing, -noadj. DESeq2 was used to identify differentially regulated genes between conditions.

ChIP-seq ChIP was performed as previously described. Samples were sonicated using the Covaris ME220 sonicator (Covaris, Woburn, MA, USA); peak power 75, duty factor 26.66%, 500 cycles/burst for 7.5 minutes. Antibodies directed against hemagglutinin (HA, Abcam Ab9110; RRID: AB_307019), H3K27ac (Abcam, Ab4729; RRID: AB_2118291), and MED1 (Bethyl Laboratories, A300–793A; RRID: AB_577241) were used for immunoprecipitation. For ChIP-seq, the ChIP reactions were scaled to obtain 10 ng DNA. After deconvolution, the DNA that had undergone ChIP was prepared for sequencing according to the manufacturer’s protocol (Illumina). ChIP-seq was performed on two independent experiments.

Alignment and construction of tag directories. ChIP-seq libraries were sequenced on the Illumina NovaSeq 6000 using NovaSeq Control Software v1.4.0 and v1.6.0, and the quality of sequenced reads was assessed using FastQC (https://qubeshub.org/resources/fastqc). Reads were aligned to the mouse genome (mm10) using STAR with default settings: outFilterMismatchNmax 2, alignIntronMax 1, outFilterMatchNmin 25. Picard Tools (Broad Institute http://broadinstitute.github.io/picard/) were used to deduplicate primary aligned reads. HOMER makeTagDirectory was used to generate tag directories for all conditions, allowing only one read per position per length of the read (-tbp 1). Due to the uneven sequencing depth of H3K27Ac ChIP-seq samples, one tag directory was down-sampled to 20 mill. reads.

Identification of PPARγ WT binding sites. Putative PPARγ binding sites were defined as follows using the HA-PPARγ ChIP-seq data. First, replicates from HA-PPARγWT treated cells were pooled. Homer findPeaks was used to call putative PPARγ sites using settings -style factor, -localSize 20000, and the pooled tag directory from control samples as reference. Peaks were extended to 500 bp centered around the peak center. Finally, artifact regions were discarded based on the ENCODE Consortium blacklist for the mm10 genome. Peaks with a tag count of fewer than 35 tags were filtered away.

Identification of sites differentially bound by PPARγ, Med1, or acetylated at H3K27. PPARγ, Med1 and H3K27Ac ChIP-seq tag counts were quantified in the above defined PPARγWT binding regions using HOMER annotatePeaks with no normalization of sequencing depth (-noadj). Prior to counting H3K27Ac ChIP-seq tags the PPARγ binding sites were extended ±1.5 kb of the peak center to capture more histone ChIP-seq signal, as histone modifications are generally distributed broadly across transcription factor binding sites. Next, we used DESeq2 to (1) call sites that gain Med1 and/or H3K27Ac signal upon PPARγWT expression (denoted PPARγ-target enhancers); (2) call sites that were differentially bound by PPARγ, Med1, or acetylated when
comparing WT and mutant PPARγ; and (3) obtain PPARγ, Med1, and H3K27Ac counts normalized to the total tag directory size.

**Motif analysis.** HA-PPARγWT binding sites were scanned for the PPRE motif from the JASPAR database in a region of 200 bp around the peak center and the motif score was extracted using HOMER annotatePeaks. If more than one PPRE motif was identified within a PPARγ-binding region, the motif with the highest motif score was used for further analyses.

De novo motif search was made using Homer findMotifsGenome with motif length of 15–17 bases and searched within ±100 bp of peak center of PPARγ-target enhancers. The de novo motif was hereafter used to scan all PPARγ-binding sites using HOMER annotatePeaks.

To analyze the motif score within sections of the motif, position files of PPARγ-binding sites were used to extract the sequence from the FASTA sequence of the mm10 reference genome. The de novo motif was extracted within these sequences using Homer2 find, where the threshold for calling a motif was set to ~2. The motif score was hereafter calculated within sections; 5 extension (base 1–4), PPARγ-half site (base 5–10), and RXR-half site (base 12–17).

**ATAC-seq**

ATAC-seq was made in two biological replicates. 100,000 cells were centrifuged 500 × g for 5 min, 4 °C. The cells were washed once in 200 μl DPBS and resuspended in 50 μl tagmentation mix (33 mM Tris-acetate, pH 7.8, 66 mM potassium acetate, 10 mM magnesium acetate, 16% dimethylformamide, 0.01% digitonin and 2.5 µl Trs (Nextera, Illumina)). Tagmentation was performed for 30 min at 37 °C, shaking at 800 rpm. Tagmented DNA was purified using Qiagen PCR purification kit according to the protocol of the manufacturer. Purified tagmented DNA was prepared for sequencing using dual-unique index primers (Illumina TG Nextera XT Index Kit) and Q5 High fidelity DNA polymerase (NEB) under the following PCR conditions: 72 °C 5 min; 98 °C 1 min; 10 cycles 98 °C 10 s, 63 °C 30 s, 72 °C 20 s; 10 °C hold, purified in accordance with the protocol of the manufacturer (Illumina) and sequenced on NovaSeq 6000 using NovaSeq Control Software v1.6.0.

**Enrichment analysis**

The number of enhancers in the vicinity of PPARγ-target genes relative to the number of enhancers in the vicinity of non-PPARγ-target genes was determined using BedTools. Two hundred genes not regulated by PPARγ were randomly selected 10 times, and the average number of enhancers was used for normalization.

**Reporting summary**

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

**Data availability**

The sequence datasets generated in this study have been deposited at NCBI GEO under accession code GSE199426. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD036589. Processed data used in the preparation of this manuscript are detailed in the Source Data files provided with the manuscript. There are no restrictions on data access. Source data are provided with this paper.

**Code availability**

Codes and scripts used to process and analyze data have been deposited to GitHub: https://github.com/mstahlmadsen/PPARgamma-lipodystrophy-mutants-reveal-intermolecular-interactions-required-for-enhancer-activation.

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Author contributions
M.S.M., M.F.B., M.R.M., A.K., A.B., C.G., E.G.K.T., D.W., M.E.G.K., M.G.K., N.H., and J.M.R.P. designed and performed experiments and analyzed the data, M.S.M. and M.R.M. performed computational analyses, A.M.J.J.B. and B.K. performed detailed protein structure analyses, H.M. and D.C. performed patient identification and detailed clinical assessment, E.K., S.M., S.K., F.C.P.H., H.M., S.W.O.M., M.V., B.K., D.C., and K.N.N. supervised the study, M.S.M., M.F.B., S.M., and E.K. wrote the manuscript. S.M. and E.K. were responsible for the overall project management and co-supervised the research.

Competing interests
The authors declare no competing interests.

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