ARTICLE

Recurrent activating mutations of PPARγ associated with luminal bladder tumors

Natacha Rochel1, Clémentine Krucker2,3, Laure Coutos-Thévenot2,3, Judit Osz1, Ruiyun Zhang2,3,9, Elodie Guyon2,3, Wayne Zita1, Séverin Vanthong1, Oscar Alba Hernandez4, Maxime Bourguet4, Kays Al Badawy1, Florent Dufour2,3, Carole Peluso-Iltis1, Syrine Heckler-Beji1, Annick Dejaegere1, Aurélie Kamoun2,3, Aurélien de Reyniès5, Yann Neuzillet2,3, Sandra Rebouissou2,3,10, Claire Béraud6, Hervé Lang7, Thierry Massfelder8, Yves Allory2,3, Sarah Cianférani4, Roland H. Stote1, François Radvanyi2,3 & Isabelle Bernard-Pierrot2,3

The upregulation of PPARγ/RXRα transcriptional activity has emerged as a key event in luminal bladder tumors. It renders tumor cell growth PPARγ-dependent and modulates the tumor microenvironment to favor escape from immuno-surveillance. The activation of the pathway has been linked to PPARG gains/amplifications resulting in PPARγ overexpression and to recurrent activating point mutations of RXRα. Here, we report recurrent mutations of PPARγ that also activate the PPARγ/RXRα pathway, conferring PPARγ-dependency and supporting a crucial role of PPARγ in luminal bladder cancer. These mutations are found throughout the protein—including N-terminal, DNA-binding and ligand-binding domains—and most of them enhance protein activity. Structure-function studies of PPARγ variants with mutations in the ligand-binding domain allow identifying structural elements that underpin their gain-of-function. Our study reveals genomic alterations of PPARG that lead to pro-tumorigenic PPARγ/RXRα pathway activation in luminal bladder tumors and may open the way towards alternative options for treatment.

https://doi.org/10.1038/s41467-018-08157-y

1 Institut de Génétique et de Biologie Moléculaire et Cellulaire (IGBMC), Institut National de La Santé et de La Recherche Médicale (INSERM), U1258/Centre National de Recherche Scientifique (CNRS), UMR7104/Université de Strasbourg, 67404 Illkirch, France. 2 Institut Curie, PSL Research University, CNRS, UMR144, Equipe Labellisée Ligue contre le Cancer, 75005 Paris, France. 3 Sorbonne Universités, UPMC Université Paris 06, CNRS, UMR144, 75005 Paris, France. 4 Laboratoire de Spectrométrie de Masse BioOrganique, Université de Strasbourg, CNRS, IPHC UMR 7178, 67000 Strasbourg, France. 5 Ligue Nationale Contre le Cancer, Programme Cartes d’Identité des Tumeurs (CIT), 75013 Paris, France. 6 UROLEAD SAS, School of Medicine, 67085 Strasbourg, France. 7 Département de Urologie, Nouvel Hôpital Civil Hôpitaux Universitaires de Strasbourg, Hôpitaux Universitaires de Strasbourg, 67091 Strasbourg, France. 8 INSERM UMR2113, Section of Cell Signaling and Communication in Kidney and Prostate Cancer, INSERM and University of Strasbourg, School of Medicine, Fédération de Médecine Translationnelle de Strasbourg (FMTS), 67085 Strasbourg, France. 9 Present address: Department of Urology, Ren Ji Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai, China. 10 Present address: INSERM, UMR-1162 “Génomique Fonctionnelle des tumeurs solides”, 75010 Paris, France. These authors contributed equally: Clémentine Krucker, Laure Coutos-Thévenot. Correspondence and requests for materials should be addressed to N.R. (email: rochel@igbmc.fr) or to I.B-P. (email: isabelle.bernard-pierrot@curie.fr)
PARy (peroxisome proliferator-activated receptor gamma) is a transcription factor of the nuclear receptor family that functions predominantly as a heterodimer with RXRs. PARy is a key regulator of glucose homeostasis and adipogenesis\(^\text{12,13}\). In addition to its well established role in adipocyte differentiation, it has also been shown to be involved in differentiation in several tissues including the urothelium\(^\text{3}\). Its role in cancer is far less understood. Historically, PARy was considered to be a tumor suppressor\(^\text{4}\), but several studies showed that it displays pro-tumorigenic effects in neuroblastoma, metastatic prostate cancer, and bladder carcinoma\(^\text{4-8}\).

In bladder cancer, the fourth most frequent cancer in men in industrialized countries, PARy focal amplifications, resulting in PARy and PARy target genes overexpression were observed in 12–17% of the muscle-invasive bladder carcinomas (MIBC) and in 10% of the non-muscle-invasive bladder carcinomas (NMIBC)\(^\text{9,10}\). These copy number alterations of PARy are associated with luminal tumors, a subtype of bladder tumors accounting for 75% of non-muscle-invasive bladder carcinomas (NMIBCs)\(^\text{3,9,10}\) and 60% of muscle-invasive bladder carcinomas (MIBCs)\(^\text{11}\). The luminal MIBCs have been shown to display a PARy activation signature\(^\text{7,12}\). We previously demonstrated that PARy overexpression induces an oncogenic addiction in these tumors by showing that the loss of PARy expression inhibited bladder cancer cell viability\(^\text{7}\), most notably observed in cell lines presenting PARy gain or amplification\(^\text{6,8}\). Recurrent mutations of RXRs (S242FY) have also been identified in 5% of MIBCs and the luminal subgroup of MIBC tumors is enriched in these mutations\(^\text{8,13}\). These RXR mutations are gain-of-function mutations that promote ligand-independent activation of PARy signaling pathway\(^\text{13,14}\). They drive proliferation of urothelial organoids in a tumor suppressor loss context\(^\text{14}\), render bladder tumor cell growth PARy-dependent\(^\text{14}\) and promote immune evasion in MIBCs\(^\text{11}\). Hyper-activation of PARy signaling, either due to PARy gene amplification or an RXR hotspot mutation (S427 F/Y), can be pharmacologically inhibited with PARy-selective inverse agonists that decrease the viability of PARy-dependent bladder cancer cells. This highlights PARy as a therapeutic target in luminal bladder tumors\(^\text{8}\).

Given this crucial role of the PARy/RXRα pathway in bladder tumors, in this work, we search for other genetic alterations that could drive its activation in both NMIBC and MIBC. By sequencing PARy and RXRs in 359 tumors and studying publicly available data for 455 MIBC, we identify eight recurrent mutations of PARy associated with luminal tumors. Functional analyses reveal that five of these mutations enhance the transcripational activity of PARy and that the activation of PARy pathway confers a PARy-dependence to the cells. Biochemical analyses show that PARy mutations favor the recruitment of coregulators. Finally, by a structure-function analysis of three mutations affecting the ligand-binding domain of PARy, we demonstrate that these mutations promote the activation of PARy active state accounting for the recruitment of coactivators. Our study provides additional genetic evidence for a protumorigenic role of PARy in bladder cancer and strengthens the importance of the PARy/RXRα pathway in luminal bladder cancer.

**Results**

**Recurrent mutations of PARy and RXRα in bladder tumors.** Using the conventional Sanger’s method, we sequenced the PARy and RXRα exons in 359 bladder tumors (199 of which were NMIBCs) from our CIT series of tumors (carte d’identité des tumeurs) and from a bank of samples collected at Strasbourg hospital, and in 25 bladder cell lines (Supplementary Table 1). We detected PARy mutations in 3.9% of the tumors (14/359) and in one cell line, and RXRα mutations in 1.7% of the tumors (6/338), all of which being MIBC, and in one cell line (Fig. 1a and Supplementary Tables 2 and 3). We also analyzed publicly available deep sequencing data from The Cancer Genome Atlas\(^\text{3}\) genomic databases (405 MIBC samples)\(^\text{10}\) (http://cancergenome.nih.gov) and from the Dana Farber & Memorial Sloan Kettering (MSKCC) cohort\(^\text{15}\) (50 MIBC samples) (Supplementary Table 1). We detected a comparable PARy mutation rate, 3.1% of the tumors (14/455), but a higher RXRα mutation rate, 6.1% of the tumors (28/455) (Fig. 1a and Supplementary Tables 2 and 3) in these tumor series compared to the previous tumor series analyzed. This higher mutation rate was mostly due to the absence of RXRα mutation in NMIBC in the CIT series of tumors. PARy mutations were not mutually exclusive with either RXRα mutations (\(p = 1\) and \(p = 0.2105\) for conventional and next-generation sequencing, respectively) or PARy amplification (\(p = 1\) and \(p = 1\) for conventional and next-generation sequencing, respectively), as shown by Fisher’s exact test analyses. Two mutations (E3K and D7N) were specific to the PARy isoform (NM_015869) (Supplementary Table 2). We therefore numbered all mutations relative to this isoform, which is 28 amino acids longer than the PARy1 isoform (NM_138712) at the N-terminal end. The PARy2 isoform is expressed principally in adipocyte cells, as well as in urothelial cells, whereas PARy1 is ubiquitously expressed. As we aimed to identify genetic alterations that could activate the PARy/RXRα pathway, we focused on recurrent mutations that were likely to be gain-of-function mutations. Of the 21 unique PARy mutations identified here in bladder tumors, 6 were recurrent and occurred in both MIBC and NMIBC (Fig. 1b and Supplementary Table 2). Using publicly available data from the COSMIC (http://cancer.sanger.ac.uk/cosmic) or cBioPortal (http://www.cbioportal.org/) databases, we determined whether the 21 PARy mutations identified here were also present in other bladder tumors or in other tumor types. From this analysis, we found a seventh recurrent mutation (E3K) in bladder tumors (Fig. 1b and Supplementary Tables 2 and 4). Interestingly, four of these seven recurrent mutations and one unique mutation (M280I) identified in bladder tumors were also reported in other type of tumors in these databases, strengthening their likelihood to be activating mutations (Fig. 1b and Supplementary Table 4). We therefore considered in total of eight recurrent mutations of PARy for further studies (Fig. 1b, c). These recurrent PARy mutations (\(p = 0.015\)) and PARy amplifications (\(p = 1.5 \times 10^{-5}\)) were significantly enriched in bladder tumors presenting a PARy activation signature, which were mostly luminal tumors (\(p = 2.2 \times 10^{-16}\)), as shown by Fisher’s exact test. These mutations affect different major functional domains of the PARy protein: the N-terminal domain (E3K) including the area around the EGFR phosphorylation sites (S112) (P113S), the DNA-binding domain (R164W, R168K), the ligand-binding domain (S249L, T475M) including the area spatially close to the CDK5 phosphorylation site, S273 [M280I, I290M] (Fig. 1b, c). Visualization of recurrent mutations in the three-dimensional (3D) structure of the PARy/RXRα heterodimer revealed that the most frequent recurrent mutation of PARy(T475M) and the hotspot mutation of RXRa (S427Y/F) affected residues that co-localized spatially at the PARy/RXRα dimer interface, suggesting that both these mutations would affect heterodimer formation (Fig. 1c). Accordingly, RXRa (S427Y/F) mutations have been shown to enhance RXRa interaction with PARy and activation of the PARy/RXRα pathway\(^\text{13}\).
of the protein in HEK293FT cells, using a luciferase reporter gene containing three copies of the DR1 sequence of PPARγ DNA response element (PPRE) arranged in tandem and linked to the thymidine kinase promoter (PPRE-3×-TK)\(^{16}\). The PPARγ P113S, R168K, S249L, M280I, I290M, and T475M mutant proteins had significantly higher levels of transcriptional activity than the wild type (WT) in the absence of exogenous ligand (two to six times higher), whereas the R164W mutant, which is located in the DNA-binding domain, had levels of activity similar to that of the WT (Fig. 2a). Similar results were obtained in the presence of 1 µM rosiglitazone, a synthetic PPARγ ligand (Supplementary Fig. 1A). Consistent with these findings, overexpression of the PPARγ mutants P113S, S249L, M280I, I290M, and T475M, in 5637 cells, in the absence of exogenous PPARγ ligand, significantly enhanced the expression of several known PPARγ target genes (FABP4, ACSL5, and PLIN2) relative to the WT, as shown by RT-qPCR (Fig. 2b and Supplementary Fig. 1B, C). However, overproduction of both the R164W and R168W mutant proteins had an impact on PPARγ target gene expression similar to that of the WT protein (Fig. 2b). Similar results were obtained in presence of 500 nM rosiglitazone (Supplementary Fig. 1D). By combining the results of these two different approaches to measure PPARγ transcriptional activity, we clearly showed that five of the seven recurrent PPARγ mutations analyzed were gain-of-function mutations. The remaining two mutations, R164W and R168K, had no clear apparent
**Fig. 2** Transcriptional activity of recurrent PPARγ mutants. **a** A reporter plasmid containing the firefly luciferase gene under the control of a PPRE-X3-TK promoter was co-expressed in HEK293FT cells with a pcDNA3 vector encoding wild-type (WT) or mutant (P113S, R164W, R168K, S249L, M280I, I290M, T475M) PPARγ2. Renilla luciferase, expressed under the control of the CMV promoter, was used to normalize the signal. The data shown are the means ± SD of one representative experiment conducted in sextuplicate. The results for each mutant were compared with those for the WT in Dunnett’s multiple comparisons test, *0.01 < p < 0.05; ****p < 0.0001. **b** 5637 cells were transiently transfected with a pcDNA3 vector encoding WT or mutant (P113S, R164W, R168K, S249L, M280I, I290M, T475M) PPARγ. The expression of all PPARγ forms was checked by western blotting and quantified by RT-qPCR (Supplementary Fig. 1B). The effect of WT PPARγ2 expression on three PPARγ target genes was evaluated by RT-qPCR (Supplementary Fig. 1C). The expression of PPARγ target genes was normalized against PPARγ expression and is expressed as percentage of stimulation relative to the expression induced by WT PPARγ. The data are presented as the mean ± SD of four independent experiments. The results for each mutant were compared with those for the WT in Dunnett’s multiple comparisons test: *0.01 < p < 0.05; **0.001 < p < 0.01; ***0.0001 < p < 0.001; ****p < 0.0001. **c** PPARγ knockdown with three different siRNAs in RT4 cells harboring the PPARγ T475M mutation. PPARγ expression was evaluated by western blotting (lower left panel) 96 h after transfection, living cells were counted (upper left panel) 24, 48, 72, and 96 h after transfection. Data are presented as means ± SD of three independent experiments performed in duplicate. The results for each mutant were compared with those for the WT in Dunnett’s multiple comparisons test: *0.01 < p < 0.05; **0.001 < p < 0.01; ***0.0001 < p < 0.001; ****p < 0.0001. The expression levels of three PPARG target genes were assessed by RT-qPCR for two independent experiment at 48 and 96 h after transfection (right panel). Data for each experiment are represented. **a-d** Source data are provided as a Source Data file.

**Effect on PPARγ activity in these two assays. Interestingly, the most frequent mutation, T475M, induced a slightly higher increase in the ligand-independent transcriptional function of PPARγ as compared to any of the recurrent mutations considered (Fig. 2a, b). We used the RT4 cell lines harboring the PPARγ-T475M mutation to demonstrate that, like PPARG WT amplification in SD48 and UMUC9 cells7 and RXRα-S427F mutation in HT11978,13 cells, PPARγ mutations render tumor cell growth PPARγ-dependent and regulate PPARγ target genes expression. Indeed, PPARγ depletion with siRNAs significantly inhibited, in a time-dependent manner, the growth of RT4 cells (Fig. 2c, left panel) and the expression of several known PPARγ target genes (FABP4, ACSL5, and PLIN2) (Fig. 2c, right panel).
PPARγ LBD mutations favor its interactions with coregulators.

We then used biochemical and biophysical analysis to understand how three mutations located in the ligand-binding domain of PPARγ (M280I, I290M and T475M) promote PPARγ activity. As bacterially expressed PPARγ may contain fatty acids, the purified PPARγ LBD WT and mutants (Supplementary Figs. 2 and 3) were analyzed by native electrospray mass spectrometry (Supplementary Fig. 4). The results indicated the absence of any bound ligands. The addition of the potent full PPARγ agonist, GW1929, an N-aryl tyrosine derivative and coactivator peptide led to the formation of ternary complexes for all constructs (Supplementary Fig. 4, right panel). We characterized the interaction between the ligand-binding domains of the PPARγ WT and mutants and the WT RXRs monomer by analytical ultracentrifugation (Supplementary Fig. 5A), which showed that as for the WT, all mutant proteins were able to form heterodimer with RXRs. To further quantify the interaction between monomeric PPARγ and RXRs ligand-binding domains, we used surface plasmon resonance (SPR) (Fig. 3a and Supplementary Fig. 5B, C). PPARγ T475M, localized at the dimer interface, exhibits an increase by 2-fold of the binding affinity to RXRα compared to PPARγ WT (Fig. 3a), as a consequence of a slower dissociation rate of the dimer (Supplementary Fig. 5B, C). In RXRα the S427F mutation is also spatially localized at the PPARγ/RXRα dimer interface, and it has been shown to similarly stabilize the heterodimer. In contrast, PPARγ M280I shows similar affinity for RXRα as PPARγ WT.

The functional profile of PPARγ, analogous to many other nuclear receptors, is determined by the selective use of transcriptional coregulators that ultimately control the transcriptional output of the target genes. The recruitment of coactivators by PPARγ is primarily determined by the interaction of

**Fig. 3** Effect of the PPARγ mutations on RXRs and coactivator interactions. **a** Representative data set used for SPR (surface plasmon resonance) analysis of the interactions of PPARγ WT or mutants with RXRα showing enhanced interaction between PPARγ T475M and RXRα. Report point 4 s before injection stop was used for the analysis. Equilibrium responses are plotted as a function of total protein concentration and fit to simple 1:1 binding isotherms. Data analysis by 1:1 kinetic model and mean kinetic parameters and equilibrium dissociation constants are reported in Supplementary Fig. 4. **b** Effect of PPARγ mutations on the PGC1α peptide interaction. The binding affinity of the PGC1α NRI motif for the purified WT and mutant PPARγ LBDs, as determined by microscale thermophoresis. Unlabeled PPARγ LBD protein was titrated into a fixed concentration of fluorescently labeled PGC1α peptide in the absence of ligand, in the presence of three equivalents of rosiglitazone (top right) or of three equivalents of GW1929 (bottom left). Isotherms averaged over three consecutive measurements and fitted according to the law of mass action to yield the apparent Kd. Each plot is representative of at least two independent experiments performed with different batches of protein preparation. **c** Mammalian two-hybrid analysis reveals increased interaction of PPARγ mutants (M280I, I290M and T475M) with MED1 coactivator domain. pG5-Firefly luciferase reporter plasmid was co-expressed with VP16-PPARG (WT or mutants) and with GAL4-DNA-binding domain-fused MED1. Renilla luciferase, expressed under the control of the CMV promoter, was used to normalize the signal. The data shown are the means ± SD of one representative experiment conducted in quadruplicate. Results (means ± SD) of three independent experiments are also provided as Supplementary Fig. 16. The results for each mutant were compared with those for the WT in Dunnett’s multiple comparisons test, *0.01 < p < 0.05; **0.001 < p < 0.01. a-c Source data are provided as a Source Data file.
coactivator LXXLL motifs with the receptor LBD. To analyze the functional consequences of the mutations in the LBD, coactivator peptide recruitment by the LBD was monitored. We measured the interaction between monomeric WT or mutant forms of PPARγ and a fluorescently labeled coactivator peptide of PGC1α (PPARGC1A), by MicroScale Thermophoresis. In the absence of ligand, the three mutations enhanced the interaction with coactivator peptide compared to PPARγ WT (Fig. 3b). The addition of a full agonist, rosiglitazone (Fig. 3b), enhanced the interaction between PPARγ and PGC1α coactivator peptide for PPARγ I290M and T475M, but not for PPARγ M280I. The addition of another potent full agonist of PPARγ, GW1929, enhanced yet again the interaction for all mutants, as well as the WT (Fig. 3b). Consistent with the functional data (Fig. 2a, b), monomeric PPARγ T475M had the highest affinity for the coactivator peptides (Fig. 3b). This strongest structural stabilizing effect of T475M mutant on PPARγ-coactivator peptide complex was also observed by ion mobility mass spectrometry which showed significantly improved gas phase stability in collision induced experiments of T475M compared to WT and other PPARγ mutants (Supplementary Figs. 6 and 7). Mammalian two-hybrid assay in HEK293FT cells using VP16-fused PPARγ (WT, M280I, I290M, and T475M), GAL4-DNA-binding domain-fused coactivator MED1 and pG5-LUC reporter confirmed that in the context of full protein, the three mutations enhanced interaction with MED1 coactivator domain compared to PPARγ WT (Fig. 3c). Together, these data suggest that the three mutations considered, M280I, I290M and T475M, promote the adoption of an agonist conformation by PPARγ in the absence of ligand, thereby enhancing coactivator interaction.

**PPARγ LBD mutations stabilize an active conformation.** To gain structural insight into the mechanism responsible for the increases in activity and coactivator interaction, we analyzed the structures of the PPARγ LBD T475M, M280I and I290M mutants (Supplementary Table 5). The PPARγ T475M LBD mutant was crystallized in its apo form and in complex with GW1929 and the PGC1α coactivator peptide (Fig. 4a and Supplementary Fig. 8). In both functional states, PPARγ T475M crystallized as a homodimer. More than 150 crystal structures of the PPARγ LBD have been deposited in the Protein Data Bank (www.rcsb.org)19, and in many of them, the PPARγ LBD crystallized as a homodimer displaying the canonical dimer interface observed in the heterodimer complex. Of these structures, the apo protein and some
complexes with partial agonists display one monomer in an active conformation and one monomer in an inactive conformation with a different positioning of helix 12. Helix 12 is key regulatory structural element in the activation function 2 interacting with coregulators. These 2 conformations are generally designated as fully active and inactive. Although these helix 12 conformations observed in the crystal structures are influenced to some degree by crystal packing, they reflect the dynamic character of helix 12 in absence or in presence of a partial agonist ligand. This was further confirmed by solution structural methods. Interestingly, in contrast to the structures of WT PPARγ in its apo form, PPARγ T475M apo displays an agonist conformation in both homodimer LBDs, indicating a stabilization of helix 12 in an active conformation even in absence of any agonist ligand (Supplementary Fig. 8).

In a similar way, the holo PPARγ T475M-GW19129-PGC1α ternary complex crystallized as a homodimer (Fig. 4a) and the structure agrees with the crystal structure of another N-aryl tyrosine derivative complex, GI262570. The GW19129 ligand binds in a U-shaped conformation with the carboxyl group of GW19219 making hydrogen bonds with S317, H351, H427, and Y501 (Supplementary Fig. 9). The pyridinyl tail is directed towards the β-sheet and its nitrogen atom makes an H-bond with a bound water molecule. The benzophenone attached to the N-aryl tyrosine, also present in GI262570, forms additional hydrophobic interactions, not available to rosiglitazone, explaining their increased PPARγ binding affinity and enhanced coactivator interaction. The mutated residue T475M interacts with the C-terminal Y505 (Fig. 4a) stabilizing helix 12 into the active conformation and leading to a more stable interaction with the coactivator peptide. Interestingly, a similar interaction stabilizing PPARγ in the active agonist conformation was observed in the crystal structure of a heterodimer complex of holo WT PPARγ and the RXRα S427F mutant. A similar observation was made by molecular dynamics (MD) simulations of the WT and T475M PPARγ LBD (Fig. 4b). Starting from a previously determined crystal structure of the WT protein, the point mutation was modeled into the WT structure. Separate 100 ns simulations of monomeric WT and T475M mutant PPARγ LBDs in both apo form and in complex with rosiglitazone were performed. We found that the T475M mutation decreased the structural flexibility of the protein, particularly that of the C-terminal H12 helix (Fig. 4b), through direct interaction between the M475 and the C-terminal Y505, as observed in the structure of PPARγ T475M in complex with GW1929. In the WT structure, the analogous interaction (between T475 and Y505) does not appear; the M475-Y505 interaction formed during the molecular dynamics simulations (Supplementary Fig. 10). The decrease in the flexibility of the C-terminal H12 helix in the T475M mutant led to a lower flexibility of the coactivator peptide, resulting in a more stable complex. Free energies of coactivator interaction were calculated by the MM/GBSA method on 100 structures taken from the MD simulations at regular intervals. This analysis yielded binding energy estimates of −43 ± 7 kcal/mol and −48 ± 5 kcal/mol for WT PPARγ and for the T475M mutant, respectively, consistent with the trends observed in the experimental data of coactivator interaction. In this application, the MM/GBSA binding energies are used to rank interactions and not to calculate absolute binding-free energies.

As the T475M mutation is located at the dimer interface, we modeled the structure of the heterodimer formed by PPARγ T475M (Supplementary Fig. 11). The results suggest that T475M will strengthen the heterodimer through interactions with L430 and S427 in contrast to WT, in agreement with the experimental data. Molecular dynamics simulations of a PPARγ-T475M/RXRα heterodimer model showed that the heterodimer is more structurally stable than the WT and, as observed in the monomer simulations, the coactivator peptide displayed reduced flexibility (Supplementary Fig. 11C). Thus, our structural analysis of PPARγ T475M indicates that the mutation stabilizes PPARγ helix 12 in the active conformation as well as the heterodimer with RXRα, leading to a stronger binding to coactivators. This mechanism may explain PPARγ-dependent transcription program activation.

The two other mutants of interest here are localized in the Ω loop of the PPARγ LBD; M280I is in the helix 2 of the Ω loop and I290M is in the loop between helix 2 and helix 3. The Ω loop is often poorly defined in the electron density maps of the published structures of PPARγ, adopting varying conformations depending on the nature of the ligand. It is thought to serve as a gate to the ligand-binding pocket and also serve as an alternative ligand-binding site for some ligands. The PPARγ M280I LBD crystallized in complex with GW1929 and PGC1α peptide as a homodimer (Fig. 5a) and PPARγ I290M in complex with GW1929 as a monomer (Supplementary Fig. 12B). The conformation and interactions of GW1929 in the PPARγ LBDs is similar in both complexes (Supplementary Fig. 9A). While helix 2 is similarly positioned in all complexes, the flexible loop connecting helix 2 and helix 3 shows different conformations and is partially resolved in some complexes. The M280I mutant stabilizes helix 3, notably through specific interactions with V305 (Fig. 5a), that, in turn stabilizes PPARγ in the agonist conformation. Recall that the coactivator interaction is via the activation function 2 coactivator surface composed of helix 3, 4, 5, and helix 12. For the I290M mutant, the surrounding loop connecting helix 2 and helix 3 shows some flexibility as indicated by the poor density of some side chains residues and higher temperature factors. However, the structural analysis of I290M-GW1929 complex indicates that I290M interacts more strongly with I369, which contacts the ligand (Supplementary Fig. 12B) and may stabilize the β-sheet, as well as helix 3. Following the protocol used in the previously described simulations, molecular dynamics simulations of the M280I and I290M LBDs were carried out. From the average structure calculated over the last 10 ns of each simulation, we found interactions that stabilized the Ω loop region via helix 3 and further stabilization of H12 activation function 2 for both the M280I and the I290M mutants. In the case of M280I mutant, I280 formed a stable interaction with V305 of H3, the N-terminal end of H3. This interaction was also seen in the crystal structure of the M280I mutant described above. In the I290M variant, M290 forms an interaction with Phe315 of H3, which interacts with His494 at the N-terminal end of H12. Stabilization of H12 leads to a stabilization of the coactivator peptide, as indicated by the decreased atomic fluctuations shown in Fig. 5b, which shows the rms fluctuations mapped onto the 3D surface of the proteins complexes. The free energies of coactivator binding to M280I and I290M were also estimated by the MM/GBSA method on structures taken from the MD simulations at regular intervals. The binding energies, −40 ± 7 kcal/mol and −45 ± 7 kcal/mol for M280I and I290M, respectively, are consistent with the trends observed in the experimental data of coactivator interaction. In particular, the mutant I290M shows an enhanced binding free energy with respect to the WT.

We used hydrogen/deuterium exchange coupled to mass spectrometry (HDX–MS) to investigate complementary structural information about the effect of T475M and M280I mutations on PPARγ structure. Of note, due to lack of reproducibility in HDX–MS data acquisition on I290M, these data were excluded from the present study. HDX–MS is a powerful readout to monitor protein conformational dynamics at peptide resolution, by monitoring the D exchange of amide hydrogens with deuterated solvent. In order to evidence PPARγ point mutation...
effects on secondary structures of PPARγ/PGC1α complexes, we performed pairwise comparison of deuterium uptakes of PPARγ WT/PGC1α to PPARγ mutant/PGC1α (Supplementary Fig. 13). For this, relative fractional uptakes (RFU) of PPARγ mutants/PGC1α were subtracted from the PPARγ WT/PGC1α RFU and the significance of the RFU difference plots was validated using the MEMHDX software (p < 0.01, Supplementary Fig. 13). Overall, PPARγ mutant proteins presented a lower level of deuterium uptake compared to WT when bound to PGC1α, highlighting the fact that PPARγ mutant/PGC1α complexes present several regions less flexible or less exposed to solvent than PPARγ WT/PGC1α complex (Supplementary Fig. 14).

Comparison of PPARγ T475M/PGC1α to PPARγ WT/PGC1α RFUs revealed that different regions of the T475M mutant presented a significant lower deuterium uptake (Supplementary Fig. 14, upper panel), including H2, H2', N-terminal H3, H6, N-terminal H8, helix H10, and the H12 helix. These regions are thus more protected from deuterium exchange for PPARγ T475M compared to PPARγ WT, which might be in favor of a decreased conformational flexibility of PPARγ T475M compared to PPARγ WT, in agreement with MD simulation data.

For PPARγ M280I a significantly different behavior was observed compared to PPARγ T475M (Supplementary Fig. 14, middle panel). Decreased deuterium uptake was observed around the mutation site (from the C-terminal H1 towards the mutation site and until H3, including the Ω loop). In addition, the β-sheet region, H6 helix, the loop between H8 and H9 and finally the region encompassing H10, H11, and H12 helices showed also significant decrease in deuterium uptake, which might correspond to a decreased conformational flexibility. Our HDX–MS data for the PPARγ M280I are consistent with MD simulations highlighting a stabilization of the Ω loop, H3 and H12 helices in the M280I mutant protein, compared to WT. Altogether, HDX–MS data are thus in agreement with our structural analysis of the structural elements involved in agonist conformation stabilization of the T475M and M280I mutant proteins.

**Discussion**

Taken together, our study highlighted the existence of recurrent driver gain-of-function PPARγ mutations in a particular type of cancer: luminal bladder tumors. Frequent PAX8-PPARγ fusions have already been reported in follicular thyroid tumors (30% of these tumors), but these proteins, which also have a proto-oncogenic role, are likely to act in a dominant negative manner. The P113S mutation enhanced PPARγ activity, probably by inhibiting S112 phosphorylation by MAP kinases, as shown for the only gain-of-function mutation of PPARγ reported to date, P113G, which is associated with obesity. We have also identified two recurrent mutations affecting the DNA-binding domain of PPARγ, but the functional impact of these mutations was not as clear as for the other mutants and requires further investigation. However, we clearly showed that the three recurrent mutations affecting the ligand-binding domain of PPARγ altered the
conformation and structural dynamics of the protein, stabilizing helix H12 and so promoting the adoption of the active form by the receptor even in the absence of ligand. These mutations also favor the recruitment of coactivators. Such effects have already been described for mutations affecting the ligand-binding domain of another hormone receptor, ER, in breast cancer, these mutations being found exclusively in hormone-resistant metastases, not in primary tumors. Mutations affecting the ligand-binding domain of the androgen receptor have also been observed in metastatic or castration-resistant prostate cancers and these mutations enable the protein to bind other ligands. Our demonstration that the activation of PPARγ by point mutation, like PPARγ amplification or RXRa mutation, confers bladder cancer cells a PPARγ dependency, provides additional genetic evidence for a pro-tumorigenic role of PPARγ in bladder cancer and strengthens the importance of the PPARγ/RXXra pathway in luminal bladder cancer. RXRs hotspot mutations appear to be specific to bladder cancer. By contrast, the PPARγ gain-of-function mutations identified here are also observed in other types of cancer. In particular, T475M mutations are observed in four different cancer types (Supplementary Table 4). Moreover, other recurrent mutations not identified in bladder cancer have been observed in different types of cancer, including melanoma and prostate carcinoma (Supplementary Table 6), and these mutations may also likely be activating mutations. The activation of PPARγ by mutations may, therefore, result in PPARγ-dependence not only in bladder cancer, but also in other types of cancer, reinforcing the importance of developing new pharmacological approaches targeting the ligand-independent activity of PPARγ in tumors. Such treatments would also favor the immune response in bladder tumors.

A better understanding of the molecular basis of the pro-oncogenic activity of PPARγ in bladder luminal tumors might also make it possible to propose alternative therapeutic options for targeting this pathway without directly inhibiting PPARγ, which could lead to diabetes or lipodystrophy.

Methods

Human bladder samples. We used DNA extracted from 359 bladder tumors. The flash-frozen tumor samples were stored at –80°C immediately after transurethral resection or cystectomy. All tumor samples contained more than 80% tumor cells, as assessed by the hematoxylin and eosin (H&E) staining of histological sections adjacent to the samples used for DNA extraction. All subjects provided informed consent and the study was approved by the institutional review boards of the Henri Mondor, Foch, Institut Gustave Roussy and Strasbourg Hospitals.

Sanger sequencing. The coding exons and splice junctions of PPARγ and RXRa were amplified from genomic DNA by PCR with gene-specific primers available on request and sequenced by the Sanger method. For tumors, the somatic status of the identified mutation was confirmed by sequencing normal DNA from blood. Sequencing was performed for 25 bladder cell lines: 5637, BFTC-905, CAL29, 5637, BFTC-905, CAL29, 5637, BFTC-905, CAL29, 5637, BFTC-905, CAL29, 5637, BFTC-905, CAL29, 5637, BFTC-905, CAL29, 5637, BFTC-905, CAL29, 5637, BFTC-905, CAL29, 5637, BFTC-905, CAL29, 5637, BFTC-905, CAL29, 5637, BFTC-905, CAL29, 5637, BFTC-905, CAL29, 5637, BFTC-905, and 5637, BFTC-905. In accordance with the manufacturer’s instructions, and the results obtained were normalized with the Renilla luciferase signal obtained with the pRL-SV40 plasmid.

For PPARγ overexpression in the 5637 cell line, we used six-well plates, 250,000 cells seeded per well. These cells were transfected 24 h later with 2.5 µg of pcDNA3-PPARγ2 (WT or mutated), 50 ng PPRE-X3-TK-luc and 6 ng pRL-SV40 (Promega), in the presence of the Fugene HD transfection reagent (Promega), in accordance with the manufacturer’s protocol. Luciferase activity was determined 48 h later, with the Dual-Luciferase Reporter assay system (Promega), following the manufacturer’s instructions, and the results obtained were normalized with the Renilla luciferase signal obtained with the pRL-SV40 plasmid.

For PPARγ knockdown, we used siRNA targeted to the 3’UTR of PPARγ. Cells were transfected with two-well plates at a density of 300,000 cells/well. Cells were transfected with 20 µM siRNA in the presence of Lipofectamine RNAi Max reagent (Invitrogen), in accordance with the manufacturer’s protocol. siRNAs were purchased from Qiagen. For the control siRNA, we used a control targeting luciferase (SI03650535). We used three PPARγ siRNAs designed to knockdown the expression of all known mRNAs isoforms. The sense-strand sequences were: PPARγ siRNA#2: GACAAUAACCAUAGCUUATT, PPARγ siRNA#5: GGCACUUUGGAAUAUUAUATT and PPARγ siRNA#6: CCGAGAAGAACCGAUUGUATT.

Cells were detached from the plates with trypsin 96 h after transfection, counted using a hemocytometer and lysed in Laemmli buffer (50 mM Tris-HCl pH 7.5, 250 mM NaCl, 1% SDS) supplemented with protease inhibitors and phosphatase inhibitors (Roche) 48 h after transfection.

For immunoblotting, the 5637 and RT4 cell lines were used to seed six-well plates at a density of 300,000 cells/well. Cells were transfected with 20 µM siRNA in the presence of Lipofectamine RNAi Max reagent (Invitrogen), in accordance with the manufacturer’s protocol. siRNAs were purchased from Qiagen. For the control siRNA, we used a control targeting luciferase (SI03650535). We used three PPARγ siRNAs designed to knockdown the expression of all known mRNAs isoforms. The sense-strand sequences were: PPARγ siRNA#2: GACAAUAACCAUAGCUUATT, PPARγ siRNA#5: GGCACUUUGGAAUAUUAUATT and PPARγ siRNA#6: CCGAGAAGAACCGAUUGUATT.

Cells were detached from the plates with trypsin 96 h after transfection, counted using a hemocytometer and lysed in Laemmli buffer (50 mM Tris–HCl (pH 7.5), 250 mM NaCl, 1% SDS) supplemented with protease inhibitors and phosphatase inhibitors (Roche).

For immunoblotting, the 5637 and RT4 cell lines were used to seed six-well plates at a density of 300,000 cells/well. Cells were transfected with 20 µM siRNA in the presence of Lipofectamine RNAi Max reagent (Invitrogen), in accordance with the manufacturer’s protocol. siRNAs were purchased from Qiagen. For the control siRNA, we used a control targeting luciferase (SI03650535). We used three PPARγ siRNAs designed to knockdown the expression of all known mRNAs isoforms. The sense-strand sequences were: PPARγ siRNA#2: GACAAUAACCAUAGCUUATT, PPARγ siRNA#5: GGCACUUUGGAAUAUUAUATT and PPARγ siRNA#6: CCGAGAAGAACCGAUUGUATT.

Plasmid constructs. The pcDNA3-PPARγ2 and PPRE-X3-TK-luc were generously provided by Pr. Chatterjee (Institute of Metabolic Science, IMS, Cambridge) and Bruce Spiegelman (Addgene plasmid #10151), respectively. We used pcDNA3.1-PPARγ2 and the QuickChange II Site-Directed Mutagenesis Kit (Agilent Technologies) according to the manufacturer’s protocol, to generate all the mutations. Mutations were confirmed by DNA sequencing. The GAL4-DNA-binding domain cloning vector pM and the activation-domain cloning vector p2 (part of the Mammalian Matchmaker Two-Hybrid Assay Kit (BD Biosciences Clontech). The construct pM-MEDI (510–787) expressing the Gal4 DBD-MEDI nuclear receptor interacting domain was provided by Lieve Verlinden (KU Leuven, Belgium).

Cell culture and transfection. The HEK293FT human cell line and the RT4 and 5637 human bladder tumor-derived cell lines were obtained from DSMZ (Heidelberg, Germany). HEK293FT cells were cultured in DMEM, whereas 5637 and RT4 cells were cultured in RPMI. Media were supplemented with 10% fetal calf serum (FCS). Cells were incubated at 37°C, under an atmosphere containing 5% CO2.

For reporter gene assays, HEK293FT cells were plated in 96-well plates (30,000 cells per well) and transfected with 30 ng pcDNA3-PPARγ2 (WT or mutated), 50 ng PPRE-X3-TK-luc and 6 ng pRL-SV40 (Promega), in the presence of the Fugene HD transfection reagent (Promega), in accordance with the manufacturer’s protocol. Luciferase activity was determined 48 h later, with the Dual-Luciferase Reporter assay system (Promega), following the manufacturer’s instructions, and the results obtained were normalized with the Renilla luciferase signal obtained with the pRL-SV40 plasmid.

For PPARγ overexpression in the 5637 cell line, we used six-well plates, 250,000 cells seeded per well. These cells were transfected 24 h later with 2.5 µg of pcDNA3-PPARγ2 (WT or mutated), 50 ng PPRE-X3-TK-luc and 6 ng pRL-SV40 (Promega), in the presence of the Fugene HD transfection reagent (Promega), in accordance with the manufacturer’s protocol. Luciferase activity was determined 48 h later, with the Dual-Glo® Luciferase Assay System (Promega), according to the manufacturer’s instructions, and the results obtained were normalized with the Renilla luciferase signal obtained with the pRL-SV40 plasmid.

Immunoblotting. The 5637 and RT4 cell lines were clarified by centrifugation.

The protein concentration of the supernatants was determined with the BCA protein assay (Thermo Scientific). 10 µg of proteins were resolved by SDS-PAGE in a 4–15% polyacrylamide gels, electrotransferred onto Biorad nitrocellulose membranes and analyzed by incubation with primary antibodies against PPARγ (Abcam #ab41928, used at 1/1000) and β-actin (Sigma Aldrich #A2228, used at 1/25,000), Horseradish peroxidase-conjugated anti-mouse IgG (Cell Signaling Technology # 7074, used at 1/3000) was used as the secondary antibody. Protein loading was checked by staining the membrane with Amido Black after electro-blotting. Uncropped scans of the western blot are supplied Supplementary Fig. 15.

Real-time reverse transcription-quantitative PCR. Reverse transcription was performed with 1 µg of total RNA, and a high-capacity cDNA reverse transcription kit (Applied Biosystems). cDNAs were amplified by PCR in a Roche real-time thermal cycler, with the Roche Taqman master mix (Roche) and Taqman probe/primer pairs as follows: PLIN2:

Forward primer—TCTGGAATGACGGAATCTACAC; Reverse primer—GGCTGTTGCAAGACATGC; Roche Taqman probe no. 57

ACSL5:

Forward primer—TGTCACCTTGCATTTCACACTT; Reverse primer—TCCAGTCCCCAGTAACTGTA; Roche Taqman probe no. 83
Table 5. GW1929 and side chains of the mutated residues could be modeled overnight incubation at 22 °C after induction with 1 mM IPTG at an OD600 of ~0.8. Relative gene expression was analyzed by the delta delta Ct method, with TBP and TBPΔ as references.

Biochemistry. The sequences encoding the ligand-binding domains of the His-flPPARγ (231–505) and His-mRXRα (228–467) receptors were inserted into pET15b. Point mutations were made by the QuikChange II XL Site-Directed Mutagenesis kit (Agilent), in accordance with the manufacturer’s instructions.

The corresponding proteins were produced in Escherichia coli BL21 DE3 by overnight incubation at 22 °C after induction with 1 mM IPTG at an OD600 of ~0.8. Solubility assessed by Ni-NTA chromatography followed by exclusion chromatography on a Superdex 200 (GE) column equilibrated in 25 mM Tris–HCl, pH 8.0, 200 mM NaCl, 5% glycerol, and 1 mM TCEP. The proteins were concentrated to 3–6 mg/ml with an Amicon Ultra 10 kDa MCWO. Purity and homogeneity of all proteins were assessed by SDS and Native PAGE and for PPAR proteins by denaturing and native electrospray ionization mass spectrometry (Supplementary Figs. 3 and 4).

Crystallization, data collection, and structure refinement. Crystallization trials were performed with either apo protein or with the protein in complex with a threefold excess of GW1929 or a threefold excess of GW1929 and PGC1α peptide. The crystallization experiments were performed by sitting drop vapor diffusion at 290 K, mixing equal volumes (200 nl) of protein at 5 mg/ml and reservoir solution. For all crystal structures, the data were indexed and integrated with XDS and scaled with AIMLESS 57,38. The structure was solved by molecular replacement in PHASER 39 and refined with PHENIX 40 and BUSTER 41 with TLS refinement, followed by iterative model building in COOT 42.

Crystals of PPARγ M280I-GW1929-PGC1α were grown in 20% PEG 5500, 0.2 M sodium fluoride, 0.3 M sodium bromide, 30 mM sodium iodide, 0.1 M sodium Hepes/MOPS pH 7.5, transferred to artifical mother liquor containing 15% glycerol and flash-cooled in liquid nitrogen. X-ray diffraction data were collected at PX1 beamline of the SOLEIL synchrotron with a wavelength of 0.9724 Å. The data were indexed and integrated with XDS and scaled with AIMLESS 57,38. The structure was solved by molecular replacement in PHASER 39 and refined with PHENIX 40 and BUSTER 41 with TLS refinement, followed by iterative model building in COOT 42.

Crystals of PPARγ T475M apo were grown in 0.5 M ammonium sulphate, 0.1 M PIPES pH 7, 0.9 M disodium tartrate. Crystals were transferred to artificial mother liquor containing 15% glycerol and flash-cooled in liquid nitrogen. X-ray diffraction data were collected at the ID23-1 beamline of ESRF with a wavelength of 0.9724 Å. The final structure was refined to Rwork and Rfree values of 15.9% and 19.88%, respectively, with excellent geometry (98.23% of residues in favored region of the Ramachandran plot, 2.01% in the allowed region, and 0.0% outliers).

Crystals of PPARγ T475M-GW1929-PGC1α were grown in 25% PEG 3500, 0.2 M ammonium sulphate, 0.1 M Bis-Tris pH 6.5, transferred to artificial mother liquor containing 15% glycerol and flash-cooled in liquid nitrogen. X-ray diffraction data were collected at the ID23-1 beamline of ESRF with a wavelength of 0.9724 Å. The final structure was refined to Rwork and Rfree values of 15.9% and 19.88%, respectively, with excellent geometry (98.23% of residues in favored region of the Ramachandran plot, 2.01% in the allowed region, and 0.0% outliers).

Crystals of PPARγ WT-GW1929-PGC1α were grown in 20% PEG 5000 MME, 0.1 M Bis-Tris pH 6.5. Crystals were transferred to artificial mother liquor containing 15% glycerol and flash-cooled in liquid nitrogen. X-ray diffraction data were collected at the ID23-1 beamline of ESRF with a wavelength of 0.9724 Å. The final structure was refined to Rwork and Rfree values of 19.5% and 23.48%, respectively, with good geometry (96.05% of residues in favored region of the Ramachandran plot, 3.77% in the allowed region, and 0.19% outliers).

Crystals of PPARγ WT-GW1929-PGC1α were grown in 20% PEG 5000 MME, 0.1 M Bis-Tris pH 6.5. Crystals were transferred to artificial mother liquor containing 15% glycerol and flash-cooled in liquid nitrogen. X-ray diffraction data were collected at the ID23-1 beamline of ESRF with a wavelength of 0.9724 Å. The final structure was refined to Rwork and Rfree values of 19.5% and 23.48%, respectively, with good geometry (96.05% of residues in favored region of the Ramachandran plot, 3.77% in the allowed region, and 0.19% outliers).

Data collection and refinement statistics are provided in Supplementary Table 5. GW1929 and side chains of the mutated residues could be modeled with confidence in all chains and all complexes as shown into the Polder omit maps 43, displaying reduced model bias and exclusion of solvent molecules (Supplementary Figs. 9A and 12A). All structural figures were prepared with PyMOL (www.pymol.org/).

Molecular dynamics simulations. hPPARγ PDB:2PRG 40 was used for all calculations. For the WT holo form, we used chain A, the ligand and coactivator peptide segment from the crystal structure. For the WT apo form, we used the same structure, but we removed the ligand and the coactivator peptide from the structure. For the corresponding mutant forms, the point mutation was built into this experimental crystal structure with CHARMM program version c57b144. All forms were subjected to the PROPKA program 42 to determine the protonation states of titratable residues. These calculations indicated that the H217, H266, H323, H449, and H466 residues of chain A were protonated on the N-5e, but that H425 could be considered doubly protonated. We modified the PDB file accordingly to incorporate the correct protonation states. Hydrogen atom positions were positioned with the HBUILD module of the CHARMM program. The parameters for rosiglitazone were determined in a previous study 77. MD simulations were performed with NAMD software and the CHARMM all-atom force field, version 36 49. Crystal water molecules associated with the hPPARγ protein were retained. Energy minimization was performed for the protein, in 700 steps, with the steepest descent (SD) algorithm in CHARMM software. Non-bonded interactions were treated with a 14-Å cutoff distance, using switch and shift functions for van der Waals and electrostatic forces, respectively. The protein was then placed in a cubic TIP3P explicit water box of dimensions 80 × 80 × 80 Å. Chloride and sodium counter ions were added to the water box to neutralize the charge of the system and additional ions were added to yield a final concentration of 0.15 M. MD simulations were initiated with two rounds of energy minimization and heating while the atomic coordinates of the protein–ligand complexes were fixed. In the first phase, water was minimized by 1000 steps of conjugate gradient (CG) and then heated to 600 K. In the second phase, the water was minimized by 250 steps of CG, followed by heating to 300 K. The constraints on the protein were then removed and the entire system was subjected to energy minimization through 2000 steps of CG and subsequent heating to 300 K. The system was equilibrated for 150 ps. With a 1 fs time step, the production phase extended over a total of 107 ns. This protocol was used for all forms studied. The root-mean-square deviation (RMSD) and root-mean-square fluctuation (RMSF) were calculated using the CHARMM program and the coactivator peptide binding energies were estimated with an MM/GBSA procedure using the CHARMM program combined with in-house programs.

Microscale thermophoresis. Measurements were performed with a Monolith NT.115 instrument (NanoTemper Technologies GmbH, Munchen, Germany). The PPAR complexes were prepared in 20 mM Tris pH 8.0, 200 mM NaCl, 1 mM TCEP, 0.05% Tween 20. Each measurement consists of 16 reaction mixtures where each was incubated in 1:5 serial dilutions of PPAR and RXR proteins into a 1:1 molar ratio. The concentration of the stock solution was 100 μM. The SPR was performed at 50 μM PPAR and 50 μM RXR. The measurement protocol used was the PDB:2PRG structure. For the corresponding mutant forms, the point mutation was built into this experimental crystal structure with CHARMM program version c57b144. All structural figures were prepared with PyMOL (www.pymol.org/).

Analytical ultracentrifugation. Sedimentation velocity experiments were performed at 4 °C, with a Beckman Optima XL-A analytical ultracentrifuge equipped with absorbance optics and an An50-Ti rotor in a buffer containing 20 mM Tris, pH 8.0, 300 mM NaCl, 5 mM Glycerol, 1 mM TCEP. For this experiment, the heterodimer was formed by mixing the monomeric PPARγ and RXRα proteins into a 1:1 molar ratio and the concentration of the stock solution was 100 μM. The sedimentation velocity analysis was conducted at 165,500 x g. Values were normalized to standard conditions by correcting for buffer density and viscosity. Sedimentation coefficient distributions were calculated from the sedimentation velocity data using the SEDFIT software program (www.analyticalultracentrifugation.com).

Surface plasmon resonance. The SPR measurements were performed by Biacore T100 sensitivity enhanced T200 equipment (GE Healthcare) using CMS series S sensor chip. The RRXa LBD monomer was immobilized on the chip surface using standard amino-coupling protocol in 10 mM Na-acetate buffer pH 5.5. The resulting immobilized RRXa was in the range of 400–500 response units. The running buffer was 50 mM Tris pH 7.5, 150 mM NaCl, 1 mM TCEP, 0.005% Tween 20. For regeneration, sodium chloride solution containing 200 mM N-ethylmaleimide was flowed through the chip. The interactions of the RRXa LBD with the LBDs of PPAR WT, T475M and M280I mutants were analyzed in the manner of dose response using twofold dilution series of PPAR LBDs ranging from 1 to 250 nM. The association phase was 120 s and the dissociation phase was 120 s. After subtracting the reference and buffer signal, the data were fit to a steady state binding model and 1:1 kinetic model to define the Kd and Kd,eff using the Biacore T200 Evaluation software (GE Healthcare).

Mass spectrometry analysis. Prior to mass spectrometry analysis, PPARγ and all the different mutant proteins were buffer exchanged against 200 mM of ammonium.
acetate at pH 6.8, using five cycles of concentration/dilution with a micro-
concentrator (Vivaspin, 10-kD cutoff, Sartorius, Göttingen, Germany). All
the samples were diluted either in H2O/ACN/HCOOH (denaturing MS conditions)
or in 200 mM AcONH4 (native MS conditions) to a final concentration of 5 μM
and infused with an automated chip based nanoelectrospray device (Triversa
Nanomate, Advion Bioscience, Ithaca, USA) operating in the positive ion mode, coupled
to a Synapt G2 HDMS mass spectrometer (Waters, Manchester, UK).
Under denaturing mass spectrometry conditions, the backing pressure, the cone
voltage and the extraction voltage of the mass spectrometer were set to 1.47 mbar,
20 V, and 4 V, respectively. The trap cell was operating under a constant Ar
pressure of 9 × 10–3 mbar (2 ml/min). The voltage within the trap cell and the
trap DC bias were set to 7 V and 2 V, respectively. In this case, the travel wave-
based helium cell and ion mobility cell were used as ion guides under 1.2 × 10–4 mbar
and 8 × 10–5 mbar, respectively. In the transfer cell, ions were transmitted
using an acceleration voltage of 7 V under a constant pressure of 10–6 mbar.
The pressure within the time of flight analyzer was kept at 6.4 × 10–7 mbar. For
native mass spectrometry analysis, the cone voltage, the extraction voltage and the
backing pressure of the mass spectrometer were set to 20 V, 5 V, and 6 mbar,
respectively, in order to improve the transmission of the “native-like” molecular ions
and avoiding ion heating. Ions were efficiently trapped with a constant Ar flow
rate of 4.5 mll/min (leading to a final pressure in the trap cell of 8.8 × 10–7 mbar)
and 4 V and then they were pulse driven with a trap DC bias of 2 V. During
mass spectrometry analysis, the pressure within the helium cell and ion mobility cell
were kept at 1.2 × 10–4 and 8 × 10–5 mbar, respectively. Ions were transferred from the ion
mobility cell to the analyzer by applying a constant voltage of 2 V under a constant
pressure of 10–6 mbar in the transfer cell. Finally, ions were analyzed within the time
of flight analyzer under high vacuum conditions (6.3 × 10–7 mbar) in order to
ensure highly accurate mass measurements. Mass spectra recorded with Synapt
G2 platform were analyzed with Masslynx 4.1 (Waters, Manchester, UK).

Native IM-MS. Ion mobility experiments were performed on the Synapt G2
HDMS (Waters, Manchester, UK). The backing pressure, the cone voltage and the
extraction cone was set to 6 mbar, 40 V, and 5 V, respectively. The traveling wave-
based ion trap was filled with a continuous Ar flow of 6 ml per min and the trap
collision energy was set to 4 V. Ions were pulse-driven with a trap DC bias of 60 V
and subsequently separated in the TWIMs (Traveling Wave Ion Mobility Spec-
trometry) cell under a constant N2 pressure of 5.36 mbar (N2 flow rate of 25 ml per
min). The IM wave velocity and height were set to 1250 m/s, and 40 V, respectively.
The other mass spectrometer parameters not described in this section remained
the same as those used during the native mass spectrometry analysis. IM data were
calibrated as described previously. Briefly, the mobilities of two native calibrants
(Cytochrome C and β-lactoglobulin) were used to determine the rota-
tionally averaged collision cross section (CCS) of the ion of interest. Masslynx 4.1
was used to perform IM data interpretation. Reported TWCCS2N values corre-
spond to the average TWCCS2N measurement performed in triplicate under the
same experimental conditions.

Collision-induced unfolding (CIU) experiments. Prior to IM separation, ions
were progressively activated in the trap cell by increasing the acceleration voltage
in 5 steps from 0 to 60 V. Ion mobility data at each individual voltage were acquired
during 1 min, and finally compiled to give rise to the CIU fingerprints. The arrival
time distribution (ATD) of each individual charge state of the protein complexes
were extracted with the open-source CIU-Suite software50. CIU experiments have
time distribution (ATD) of each individual charge state of the protein complexes
calibrated as described elsewhere. Briefly, the arrival time fingerprints. The arrival
time distribution (ATD) of each individual charge state of the protein complexes
were extracted with the open-source CIU-Suite software50. CIU experiments have
time distribution (ATD) of each individual charge state of the protein complexes
calibrated as described elsewhere. Briefly, the arrival time fingerprints. The arrival
time distribution (ATD) of each individual charge state of the protein complexes
were extracted with the open-source CIU-Suite software50. CIU experiments have
time distribution (ATD) of each individual charge state of the protein complexes
calibrated as described elsewhere. Briefly, the arrival time fingerprints. The arrival
time distribution (ATD) of each individual charge state of the protein complexes
were extracted with the open-source CIU-Suite software50. CIU experiments have
time distribution (ATD) of each individual charge state of the protein complexes
calibrated as described elsewhere. Briefly, the arrival time fingerprints. The arrival
time distribution (ATD) of each individual charge state of the protein complexes
were extracted with the open-source CIU-Suite software50. CIU experiments have
time distribution (ATD) of each individual charge state of the protein complexes
calibrated as described elsewhere. Briefly, the arrival time fingerprints. The arrival
time distribution (ATD) of each individual charge state of the protein complexes
were extracted with the open-source CIU-Suite software50. CIU experiments have

References
1. Tontonoz, P. & Spiegelman, B. M. Fat and beyond: the diverse biology of
PPARgamma. Annu. Rev. Biochem. 77, 289–312 (2008).
2. Ahmadian, M. et al. PPARgamma signaling and metabolism: the good, the
bad and the future. Nat. Med. 19, 557–566 (2013).
3. Varley, C. L. et al. Role of PPARgamma and EGR signalling in the
urothelial terminal differentiation programme. J. Cell. Sci. 117, 2029–2036
(2004).
4. Peters, J. M., Shah, Y. M. & Gonzalez, F. J. The role of peroxisome proliferator-
activated receptors in carcinogenesis and chemoprevention. Nature
Rev. Cancer 12, 181–195 (2012).
5. Lee, J. I., Drakaki, A., Iliopoulos, D. & Struhl, K. MIR27 targets
PPARgamma to inhibit growth, tumor progression and the inflammatory
response in neuroblastoma cells. Oncogene 31, 3818–3825 (2012).
6. Ahmad, I. et al. Sleeping Beauty screen reveals Pparg activation in metastatic
prostate cancer. Proc. Natl Acad. Sci. USA 113, 8292–8295 (2016).
7. Biton, A. et al. Independent component analysis uncovers the landscape of
the bladder tumor transcriptome and reveals insights into luminal and basal
subtypes. Cell Rep. 9, 1235–1245 (2014).
8. Goldstein, J. T. et al. Genomic activation of PPAR gamma reveals a candidate
therapeutic axis in bladder cancer. Cancer Res. 77, 6987–6998 (2017).
9. Cancer Genome Atlas Research, N. Comprehensive molecular
characterization of urothelial bladder carcinoma. Nature 507, 315–322
(2014).
10. Robertson, A. G. et al. Comprehensive molecular characterization of
malignant invasiveness. Cell 171, 540–556 (2017).
11. Hedegaard, J. et al. Comprehensive transcriptional analysis of early-stage
urothelial carcinoma. Cancer Cell 30, 27–42 (2016).
12. Choi, W. et al. Identification of distinct basal and luminal subtypes of
malignant invasiveness bladder cancer with different sensitivities to frontline chemotherapy.
Cancer Cell 25, 150–162 (2014).
13. Korpal, M. et al. Evasion of immunosurveillance by genomic alterations
of PPARgamma/RXRalpha in bladder cancer. Nat. Commun. 8, 103 (2017).
14. Halstead, A. M. et al. Bladder-cancer-associated mutations in RXRA activate
peroxisome proliferator-activated receptors to drive urothelial proliferation.
Nat. Metabol. 6, 1424 (1999).
15. Van Allen, E. M. et al. Somatic ERCC2 mutations correlate with cisplatin
sensitivity in muscle-invasive urothelial carcinoma. Cancer Discov. 4, 110–1153 (2014).
16. Kuns, J. B., Wright, H. M., Wright, M. & Spiegelman, B. M. ADD1/SP1/RBP1
activates PPARgamma through the production of endogenous ligand.
Proc. Natl Acad. Sci. USA 95, 4333–4337 (1998).
17. Liberato, M. V. et al. Medium chain fatty acids are selective peroxisome
activators and pan-PPAR partial agonists. PLoS ONE 7, e36297 (2012).
18. Brown, K. K. et al. A novel N-arlyl tyrosine activator of peroxisome
proliferator-activated receptor-gamma reverses the diabetic phenotype of
the Zucker diabetic fatty rat. Diabetes 48, 1415–1424 (1999).
19. Berman, H. M. et al. The protein data bank. *Nucleic Acids Res.* **28**, 235–242 (2000).
20. Nolte, R. T. et al. Ligand binding and co-activator assembly of the peroxisome proliferator-activated receptor-gamma. *Nature* **395**, 137–143 (1998).
21. Ohashi, M., Oyama, T. & Miyachi, H. Different structures of the two peroxisome proliferator-activated receptor gamma (PPARgamma) ligand-binding domains in homodimeric complex with partial agonist, but not full agonist. *Bioorg. Med. Chem. Lett.* **25**, 2639–2644 (2015).
22. Chrisman, I. M. et al. Defining a conformational ensemble that directs activation of PPARy. *Nat. Commun.* **9**, 1794 (2018).
23. Gampe, R. T. et al. Asymmetry in the PPARalpha/PPARgamma crystal structure reveals the molecular basis of heterodimerization among nuclear receptors. *Mol. Cell* **5**, 545–553 (2000).
24. Hamuro, Y. et al. Hydrogen/deuterium-exchange (H/D-Ex) of PPARgamma LBD in the presence of various modulators. *Protein Sci.* **15**, 1883–1892 (2006).
25. Kollman, P. A. et al. Calculating structures and free energies of complex molecules: combining molecular mechanics and continuum models. *Acc. Chem. Res.* **33**, 889–897 (2000).
26. Genest, D. et al. Ligand-escape pathways from the ligand-binding domain of PPARalpha receptor as probed by molecular dynamics simulations. *Eur. Biophys. J.* **37**, 369–379 (2008).
27. Hughes, T. S. et al. An alternate binding site for PPAR gamma ligands. *Nat. Commun.* **5**, 3571 (2014).
28. Capelli, D. et al. Structural basis for PPAR partial or full activation revealed by a novel ligand binding mode. *Sci. Rep.* **6**, 34792 (2016).
29. Houndrel, V. et al. MEMHDX: an interactive tool to expedite the statistical validation and visualization of large HDX-MS datasets. *Bioinformatics* **32**, 3413 (2016).
30. Raman, P. & Koenig, R. J. Pax-8-PPAR-gamma fusion protein in thyroid cancer. *Nat. Rev. Endocrinol.* **10**, 616–623 (2014).
31. Ristow, M., Muller-Wieland, D., Pfeiffer, A., Krone, W. & Kahn, C. R. Obesity associated with a mutation in a gene regulator of adipocyte differentiation. *N. Engl. J. Med.* **339**, 953–959 (1998).
32. Robinson, D. R. et al. Activating ER2 mutations in hormone-resistant metastatic breast cancer. *Nat. Genet.* **45**, 1446–1451 (2013).
33. Shukla, G. C., Plaga, A. R., Shankar, E. & Gupta, S. Androgen receptor-related dihydroxyvitamin D explained by vitamin D receptor-coactivator interaction. *J. Clin. Invest.* **116**, 581–589 (2006).
34. Emsley, P. & Cowtan, K. Coot: model-building tools for molecular graphics. *Acta Crystallogr. D Biol. Crystallogr.* **67**, 1566–1573 (2005).
35. Kabesch, W. Xds. *Acta Crystallogr., D Biol. Crystallogr.* **66**, 125–132 (2010).
36. Evans, P. Scaling and assessment of data quality. *Acta Crystallogr., D Biol. Crystallogr.* **62**, 72–82 (2006).
37. Collaborative Computational Project, N. The CCP4 suite: programs for protein crystallography. *Acta Crystallogr., D Biol. Crystallogr.* **50**, 766–773 (1994).
38. McCoy, A. J., Grosse-Kunstleve, R. W., Storoni, L. C. & Read, R. J. Likelihood-enhanced fast translation functions. *Acta Crystallogr., D Biol. Crystallogr.* **61**, 458–464 (2005).
39. Adams, P. D. et al. PHENIX: a comprehensive Python-based system for macromolecular structure solution. *Acta Crystallogr., D Biol. Crystallogr.* **66**, 213–220 (2010).
40. Smart, O. S. et al. Exploiting structure similarity in refinement: automated NCS and target-structure restraints in BUSTER. *Acta Crystallogr., D Biol. Crystallogr.* **68**, 368–380 (2012).
41. Emsley, P. & Cowtan, K. Coot: model-building tools for molecular graphics. *Acta Crystallogr., D Biol. Crystallogr.* **60**, 2126–2132 (2004).
42. Liebschner, D. et al. Polder maps: improving OMIT maps by excluding bulk solvent. *Acta Crystallogr., D Biol. Crystallogr.* **73**, 148–157 (2017).
43. Brooks, B. R. et al. CHARMM: the biomolecular simulation program. *J. Comput. Chem.* **30**, 1543–1614 (2009).
44. Olson, M. H., Sturtevant, C. R., Rostkowski, M. & Jensen, J. H. PROPKA3: Consistent Treatment of Internal and Surface Residues in Empirical pKa Predictions. *J. Chem. Theory Comput.* **7**, 525–537 (2011).
45. Brunger, A. T. & Karplus, M. Polar hydrogen positions in proteins: empirical energy placement and neutron diffraction comparison. *Proteins* **4**, 148–156 (1988).
46. Fidelak, J. et al. Dynamic correlation networks in human peroxisome proliferator-activated receptor-gamma nuclear receptor protein. *Eur. Biophys. J.* **39**, 1503–1512 (2010).
47. Phillips, J. C. et al. Scalable molecular dynamics with NAMD. *J. Comput. Chem.* **26**, 1781–1802 (2005).
48. Mackrell, A. D. et al. All-atom empirical potential for molecular modeling and dynamics studies of proteins. *J. Phys. Chem. B* **102**, 3586–3616 (1998).
49. Phillips, J. C. et al. Scalable molecular dynamics with NAMD. *J. Comput. Chem.* **26**, 1781–1802 (2005).
50. Eschweiler, J. D., Rabuck-Gibbons, N. J., Tian, Y. & Ruotolo, B. T. CIUSuite: a quantitative analysis package for collision induced unfolding measurements of gas-phase protein ions. *Anal. Chem.* **87**, 11316–11322 (2015).
51. Ozs, J. et al. Solution structures of PPARgamma2/RXRalpha complexes. *PPAR Res.* **2012**, 701412 (2012).

**Acknowledgements**

This work was supported by a grant from Ligue Nationale Contre le Cancer (C.K., L.C.-T., E.G., R.Z., S.R., Y.N., Y.A., F.R., I.B.-P.) as an associated team (Equipe labellisée), the "Carte d'Identité des Tumeurs" program initiated, developed and funded by Ligue Nationale Contre le Cancer, by a "PL-Bio" project funded by INCA (2016–146), the French Ministry of Education and Research, the CNRS, and the Institut Curie. The project was also supported by the Centre National de la Recherche Scientifique, the Institut National de la Santé et de la Recherche Médicale and the University of Strasbourg. We acknowledge the support and the use of resources of the French Infrastructure for Integrated Structural Biology FRISBI ANR-10-INBS-05, the Instruct-ERIC and the French Proteomic Infrastructure ProFI ANR-10-INBS–08–03. We would like to thank the staff of Proxima 1 at SOLEIL as well as of ID23 and ID30A at ESRF for assistance in using the beamlines. We also thank Alastair McEwen (IGMBC) for help in X-ray data collections and structural refinement and Camille Kostmann (IGMBC) for assistance in SPR. We would like to thank Simone Benhamou (IGR) and Thierry Lebret (Foch Hospital) for their help in setting up the CIT series of bladder tumors. We thank GIS IBiSA and Region Alsace for financial support in purchasing a Synapt G2 HDMS instrument. We acknowledge the Strasbourg University High Performance Computing Center and their staff for providing access to computing resources and for their support. We also acknowledge GENCSI (Grand Equipment National de Calcul Intensif) for computing resources and the Equipex Equip@Meso project (Programme Investissements d’Avenir). MB was supported by a fellowship from the Region Alsace. OA-H acknowledges the IdEx program of the University of Strasbourg for funding his postdoctoral fellowship.

**Author contributions**

N.R., R.H.S. F.R. and I.B.-P. designed the study. H.L., C.B. and T.M. provided and characterized the tumors from the Strasbourg cohort. C.K., R.Z. and E.G. sequenced PPAR and RXRA and analyzed the data together with L.C.-T., A.K. and A.d.R. who carried out the bioinformatics analysis. A.K., A.d.R. and Y.A. classified the tumors according to pathological status and to the different subgroups identified by the TCGA. C.K., L.C.-T., F.D., S.R., Y.N. and I.B.-P. performed the functional studies and analyzed the data. J.O., W.Z., C.P.-I. and S.H.-B. performed the biochemical, biophysical, and structural studies, and J.O. and N.R. analyzed the data. S.V. and K.A. performed the molecular dynamics, and A.D. and R.H.S. analyzed the MD data. O.A.H. and M.B. performed the mass spectrometry experiments, O.A.H., M.B. and S.C. analyzed the mass spectrometry data. N.R., C.K., L.C.-T., R.H.S., F.R. and I.B.-P. wrote the manuscript. All authors made comments on the manuscript.

**Additional information**

Supplementary Information accompanies this paper at https://doi.org/10.1038/s41467-018-08157-y.

**Competing interests:** The authors declare no competing interests.

Reprints and permission information is available online at http://npg.nature.com/reprintsandpermissions/

**Journal peer review information:** *Nature Communications* thanks the anonymous reviewers for their contribution to the peer review of this work. Peer reviewer reports are available.

**Publisher’s note:** Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.