FEBS Letters 478 (2000) 4–8 FEBS 23933

A 45 kDa protein related to PPARγ2, induced by peroxisome proliferators, is located in the mitochondrial matrix

François Casas[a,*], Lionel Domenjoud[b], Pierrick Rochard[a], Renée Hatier[c], Anne Rodier[a], Laetitia Daury[a], Arnaud Bianchi[a], Pascaline Kremarik-Bouillaud[b], Philippe Becuwe[b], Jean-Marie Keller[b], Hervé Schohn[b], Chantal Wrutniak-Cabello[a], Gérard Cabello[a], Michel Dauça[b]

[a] *UMR Différenciation Cellulaire et Croissance (INRA-UMII-ENSAM), Unité d'Endocrinologie Cellulaire, Institut National de la Recherche Agronomique (INRA), place Viala, 34060 Montpellier Cedex 1, France*
[b] *Laboratoire de Biologie Cellulaire du Développement, EA 2402 'Prolifératéurs de Peroxysomes', Université Henri Poincaré-Nancy I, Faculté des Sciences, P.O. Box 239, 54506 Vandoeuvre-lès-Nancy Cedex, France*
[c] *Laboratoire de Microscopie Electronique and EP CNRS 616, Université Henri Poincaré-Nancy I, Faculté de Médecine, P.O. Box 184, 54505 Vandoeuvre-lès Nancy Cedex, France*

Received 11 May 2000; revised 28 June 2000; accepted 30 June 2000

Edited by Vladimir Skulachev

Abstract Besides their involvement in the control of nuclear gene expression by activating several peroxisome proliferator-activated receptors (PPARs), peroxisome proliferators influence mitochondrial activity. By analogy with the previous characterization of a mitochondrial T3 receptor (p43), we searched for the presence of a peroxisome proliferator target in the organelle. Using several antisera raised against different domains of PPARs, we demonstrated by Western blotting, immunoprecipitation and electron microscopy experiments, that a 45 kDa protein related to PPARγ2 (mt-PPAR) is located in the matrix of rat liver mitochondria. In addition, we found that the amounts of mt-PPAR are increased by clofibrate treatment. Moreover, in EMSA experiments mt-PPAR bound to a DR2 sequence located in the mitochondrial D-loop, by forming a complex with p43. Last, studies of tissue-specific expression indicated that mt-PPAR is detected in mitochondria of all tissues tested except the brain in amounts positively related to p43 abundance.

Key words: Mitochondrion; Peroxisome proliferator-activated receptor; Peroxisome proliferator; Triiodothyronine

1. Introduction

Thyromimetic effects of peroxisome proliferators (PPs) have been reported earlier [1–3]. As thyroid hormone, they are potent hypolipidemic agents [4,5]. In rats, several PPs induce the expression of enzymes regulated by thyroid hormone, such as malic enzyme, mitochondrial glycerol-3-phosphate dehydrogenase, glucose-6-phosphate dehydrogenase, and S14 [5]. Last, as thyroid hormone, PPs affect mammalian mitochondrial activity [6–8]. Triiodothyronine (T3) is considered to be the major regulator of mammalian mitochondrial biogenesis [9]. Interestingly, PPs also alter mitochondrial morphology and enzyme composition [10–12]. In addition, they increase mitochondrial mRNA and rRNA levels [1].

Recently, we provided evidence that a 43 kDa c-Erb A α1 protein (p43) located in the mitochondrial matrix of rat liver [13], is a mitochondrial transcription factor [14]. These data underlined the occurrence of a novel endocrine pathway exerted at the mitochondrial level, involving a truncated nuclear receptor acting as a transcription factor.

On the basis of these observations, in the present study, we searched for the presence of protein(s) related to PPAR in mitochondria. We report here the occurrence in the mitochondrial matrix of a 45 kDa protein related to PPARγ2 (mt-PPAR) whose abundance is upregulated by clofibrate, and which specifically binds to a DR2 sequence located in the D-loop of the mitochondial genome.

2. Materials and methods

2.1. Animals and mitochondrial preparation

Male Wistar rats (body weights ranging from 200 to 300 g) received a daily intramuscular injection of 0.56 nmol clofibrate during 7 days. Rats of the same bred and same body weight receiving only the clofibrate vehicle were used as a control. Liver mitochondria were prepared by differential centrifugations and purity of mitochondrial preparations was tested as previously described [13]. Membrane and matrix extracts were collected as previously described [15].

2.2. Production of antibodies raised against several PPAR isoforms

For immunization we used the following sequences of human PPAR peptides coupled to bovine serum albumin and subcutaneously injected into rabbits using standard procedures: anti-PPARα (SSGSFGFTEYQYE, amino acids 45–57 of human PPARα); anti-PPARδ (EGAPELNGGPQHAL, amino acids 24–37 of human NUC1); anti-PPARγ1/γ2 (EMPFWPTNFGISSVD, amino acids 7–21 of human PPARγ1, 35–49 of human PPARγ2); anti-PPARγ2 (GETLGDSPIDPESDS, 2–16 of human PPARγ2); anti-PPARs (DAALHPLLQEIYRDMY, amino acids 453–468 of rat PPARα). The anti-PPARγ was provided by Affinity Bioreagents Inc. (Interchim).

2.3. Immunoprecipitation and Western blotting

Immunoprecipitations of mitochondrial proteins were performed with anti-PPARγ2 and anti-PPARs antisera. Purified mitochondrial extracts (200 μg) were mixed with 1 μl antiserum and 300 μl binding buffer (HEPES 50 mM, NaCl 400 mM, Nonidet P40 1%, Aprotinin 1 μg/ml, phenylmethylsulfonyl fluoride 100 μg/ml), during 3 h at 4°C. Samples were incubated with 30 μl protein G–Sepharose for 1 h at 4°C, and washed four times with binding buffer. Immunoprecipitation specificity was assessed in each case by using the related pre-immune rabbit serum.

Proteins were electrophoresed (SDS–PAGE), transferred to a PDVF membrane and detected by a chemiluminescent Western blot procedure (ECF kit, Amersham). In Western blot experiments,

*Corresponding author. Fax: (33)-4-67 54 56 94.
E-mail: casasf@ensam.inra.fr

0014-5793/00/$20.00
PII: S0014-5793(00)01814-7

PPARs proteins used as control were synthesized in vitro using reticulocyte lysate (Promega) and [^{35}S]methionine with the following vector: pSG5-*m*PPARα, pSG5-*m*PPARδ, pSG5-*m*PPARγ1 and pBSIIK-*h*PPARγ2 (gift of Prof. W. Wahli, Lausanne, Switzerland).

2.4. Gel mobility shift assays

Gel mobility shift assays were performed as reported previously [14], using a ^{32}P-labeled oligonucleotide probe corresponding to the mitochondrial DR2 sequence identified in the rat mitochondrial genome (GTCA**AGGCAT**GA**AGGTCA**GCAC) [14]. Specificity of DNA binding was tested by addition of cold related or unrelated probe as described [14].

3. Results

3.1. A 45 kDa protein related to PPARγ2 is detected in the mitochondrial matrix

In order to test the possibility that the influence of PPs at the mitochondrial level could, at least partly, involve a direct target located in the organelle, we searched for the presence of PPAR-related proteins in rat liver mitochondria. In this purpose, Western blot analysis using six antisera (four antibodies were directed against different sequences of PPARγ) raised against the four PPAR isoforms were performed in highly purified rat liver mitochondrial extracts. PPAR proteins synthesized in rabbit reticulocyte lysate were used as control. In all cases, related pre-immune sera were tested and did not detect any mitochondrial protein (data not shown). Whereas no signal was observed with antisera specifically raised against PPARα and δ (Fig. 1A,B), a 45 kDa protein was detected using two other antibodies raised against sequences respectively located in the hinge region and the amino-terminus of both PPARγ1 and γ2 (Fig. 1D,E). Further experiments using an antiserum raised against another amino-terminal sequence to PPARγ2 isoform also led to the detection of a 45 kDa protein (Fig. 1C). Altogether, this set of data indicated that a 45 kDa protein displaying three different sequences immunologically related to the nuclear receptor PPARγ2 is located in mitochondria (mt-PPAR). However, the molecular weight of this protein is clearly less when compared to that of PPARγ2 (56 kDa). Therefore, we tested the possibility that mt-PPAR could lack a part of the carboxy-terminus domain of PPAR isoforms. In agreement with this hypothesis, Western blot experiments using an antiserum raised against a carboxy-terminal sequence well conserved in all PPAR isoforms failed to detect a mitochondrial protein (Fig. 1F).

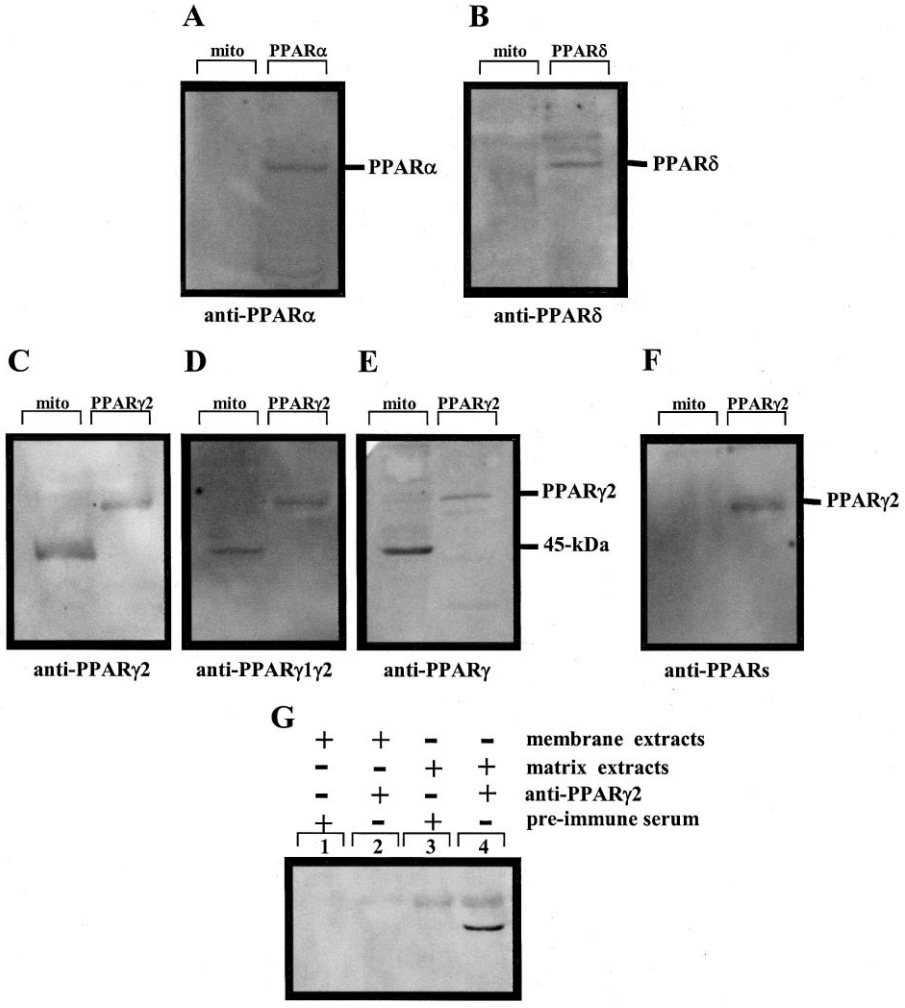

Fig. 1. Immunodetection of a protein related to PPARγ2 in rat liver mitochondrial matrix. A–F, Western blot analysis of mitochondrial proteins (100 μg). PPARα, PPARδ or PPARγ2 proteins synthesized in rabbit reticulocyte lysate were used as controls. Western blotting using specific antibodies previously described; A: anti-PPARα; B: anti-PPARδ; C: anti-PPARγ2; D: anti-PPARγ1γ2; E: anti-PPARγ; F: anti-PPARs. G: By immunoprecipitation experiment, the 45 kDa protein was specifically detected by the anti-PPARγ2 antibody in liver mitochondrial matrix. Mito: mitochondrial extracts.

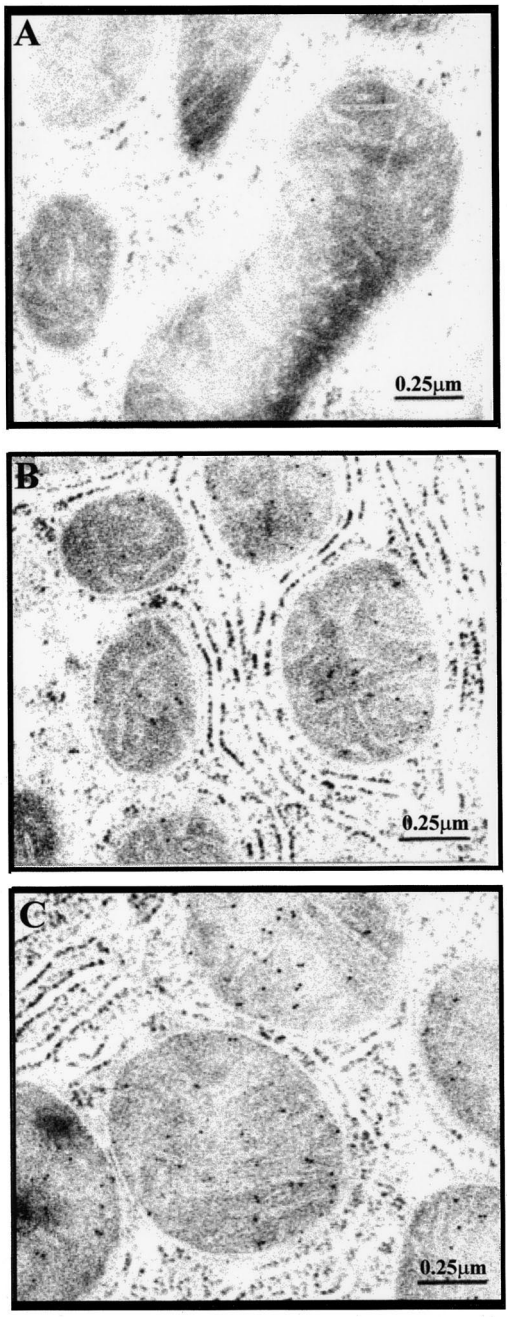

Fig. 2. Observation by electron microscopy of a protein related to PPARγ2 in rat liver mitochondria. A: Using the preimmune serum, no labeling is observed in untreated rat liver. B: A specific mitochondrial labeling appeared when anti-PPARγ2 antibody was used. C: Influence of clofibrate treatment on anti-PPARγ2 staining. Bar: 0.25 μm.

Immunoprecipitation experiments using antiserum raised against PPARγ2 were performed with mitochondrial membrane or matrix preparations. Reciprocal contamination of membrane and matrix extracts was assessed by cytochrome oxidase (inner membrane) and citrate synthase (matrix) activities (data not shown). Our results confirmed our previous Western blot data, and brought evidence that mt-PPAR was located in the mitochondrial matrix (Fig. 1G). In well agreement with this set of results, electron microscopy studies performed with the antiserum raised against the specific PPARγ2

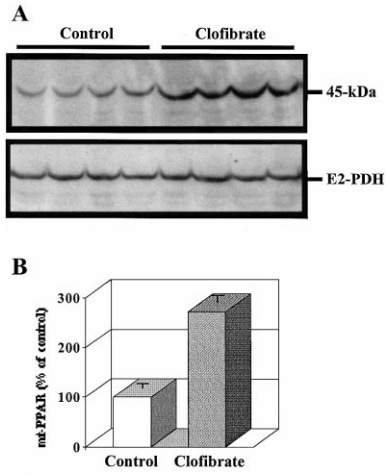

Fig. 3. mt-PPAR abundance is increased by a rat clofibrate treatment. A: Western blot analysis of mitochondrial proteins (100 μg) using four different rat liver mitochondrial extracts and anti-PPARγ2 or anti-E2PDH antibody (for normalization). B: Quantification of the signal intensities was carried out with a phosphorimager (Molecular Dynamics) after normalization of mitochondrial proteins by E2-PDH.

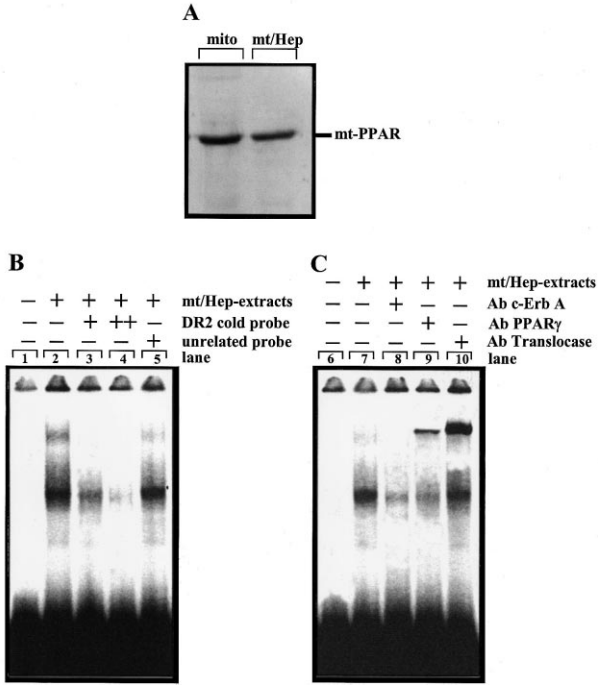

Fig. 4. As p43, a T3 mitochondrial receptor, a mitochondrial protein related to PPARγ2 binds to a DR2 sequence located in the D-loop. A: mt-PPAR is detected by Western blot with a specific PPARγ2 antibody in highly purified mitochondrial extracts used for EMSA experiments. B,C: EMSA experiments performed with highly purified mitochondrial extracts and a ^{32}P-labeled DR2 probe corresponding to the mitochondrial sequence. B: When indicated an excess of cold DR2 or unrelated probe was added to assess the binding specificity (+: 100 fold excess; ++: 200 fold excess). C: When indicated, antibodies raised against c-Erb A, PPARγ2 or ANT (adenine nucleotide translocator) were incubated for 15 min with highly purified mitochondrial extracts prior to the addition of the labeled probe. Mito: mitochondrial extracts; mt/Hep: mitochondrial extracts passed onto heparin agarose column.

sequence allowed us to directly observe the occurrence of a PPARγ2-related protein in mitochondria of rat liver (Fig. 2). Interestingly, rat clofibrate treatment induced an increase in the number of gold particles present in the organelle (Fig. 2C vs. 2B).

3.2. The 45 kDa protein related to PPARγ2 is induced by a clofibrate treatment

In order to confirm this clofibrate influence, we performed a Western blot using the antiserum raised against the specific PPARγ2 sequence and highly purified liver mitochondrial extracts from untreated or clofibrate treated rats. Mt-PPAR abundance was assessed by scanning (phosphorimager) and normalization relatively to E2-PDH, a mitochondrial invariant. We observed that clofibrate induced an up to two fold increase in the mitochondrial amounts of mt-PPAR (Fig. 3).

3.3. mt-PPAR specifically binds to a mitochondrial DNA sequence located in the D-loop

PPAR isoforms have been shown to stimulate transcription after binding to repetitions of the AGGTCA consensus sequence occurring in the promoter of target genes. We have previously identified several related motifs in rat mitochondrial genome, inducing binding of p43, a mitochondrial truncated form of the T3 nuclear receptor c-Erb A α1 [14]. Interestingly, one of these motifs, a DR2 sequence, has been previously described to be a PPRE [16,17]. In gel retardation assays using highly purified mitochondrial protein extracts prepared on heparin agarose columns which contain mt-PPAR (Fig. 4A), we observed that a mitochondrial protein complex bound to a probe corresponding to this DR2 sequence (Fig. 4B, lane 2). In addition, this binding was efficiently competed by an excess of the corresponding cold probe (Fig. 4B, lanes 3 and 4), whereas an unrelated probe was inefficient (Fig. 4B, lane 5), thus demonstrating binding specificity. As previously reported [13,14], an antibody raised against c-Erb A strongly inhibited binding of the mitochondrial complex to the DR2 probe, thus indicating that p43 is a major element of such a complex (Fig. 4C, lane 8). Interestingly, preincubation of mitochondrial preparations with an antibody raised against the PPARγ1/γ2 proteins also inhibited the binding of this complex (Fig. 4C, lane 9), whereas non-related antibodies (raised against ADP/ATP translocator) did not (Fig. 4C, lane 10). This set of data clearly suggests that p43 and mt-PPAR occur in the same protein complex.

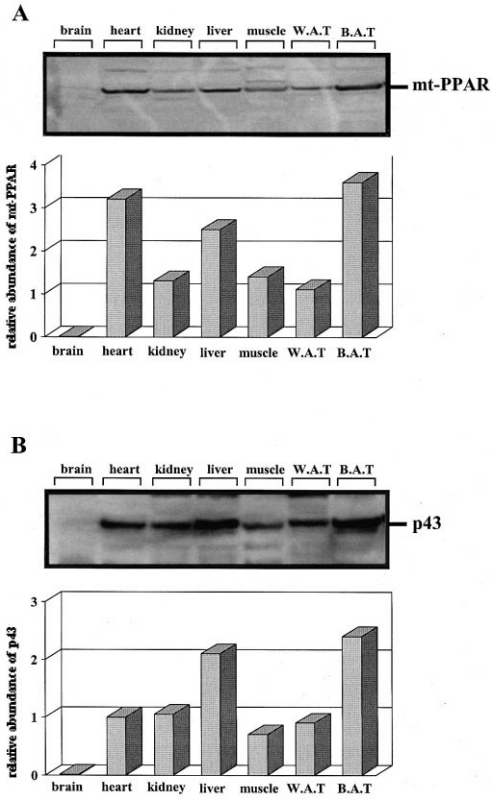

Fig. 5. mt-PPAR and p43 levels in mitochondria extracted from different tissues. A,B: Western blot (100 μg of mitochondrial proteins per lane) using anti-PPARγ2 or anti-RHTII antibodies; detection was performed by chemiluminescence. Quantification of signal intensities was carried out with a phosphorimager (Molecular Dynamics). W.A.T: white adipose tissue; B.A.T: brown adipose tissue.

3.4. mt-PPAR is detected in mitochondria extracted from different rat tissues

In Western blot experiments using the antiserum specifically raised against PPARγ2, we studied the relative mitochondrial amounts of mt-PPAR in several tissues. As shown in Fig. 5A, important levels of this protein were detected in heart and brown adipose tissue mitochondria; mt PPAR was also recorded in slightly lower amounts in liver, whereas kidney, muscle and white adipose tissue displayed more reduced levels. Strikingly, mt-PPAR was not detected in brain mitochondria. These data underline that expression of this protein displays a strong tissue-specificity, very close to p43 distribution in several tissues (Fig. 5A,B).

4. Discussion

Several studies brought evidence that PPs regulate mitochondrial activity, in a way displaying strong similarities with thyroid hormone action. In particular, as T3, they affect mitochondriogenesis [10–12] and mitochondrial genome expression [1]. These observations raised the question of the occurrence of a possible cross-talk between the thyroid hormone and PP pathways. We have previously shown that T3 directly acts at the mitochondrial level [13,14]. These data led us to test the possibility that mitochondrial PPAR-related proteins could directly mediate a part of PPs influence upon the organelle activity.

In line with this hypothesis, we detected a 45 kDa protein immunologically related to PPAR in the mitochondrial matrix of rat liver (mt-PPAR). As this protein was detected by all antisera raised against sequences shared by PPARγl and PPARγ2, and by an antiserum specifically recognizing the γ2 isoform, it was more closely related to PPARγ2. In addition, in agreement with the reduced molecular weight of the mitochondrial protein relatively to PPARγ2 (45 vs. 56 kDa), the mitochondrial protein lacks the carboxy-terminal end of the nuclear protein. Interestingly mt-PPAR amounts in the organelle are increased by clofibrate treatment.

A major question raised by our data is the function of this mitochondrial protein. In gel mobility shift experiments, we found that mt-PPAR specifically binds to a direct repeat sequence of the AGGTCA motif (DR2) located in the mitochondrial DNA D-loop, a region which contains the pro-

moters of transcription. Moreover, we observed that mt-PPAR and p43 probably occur in a common complex. In addition, we have recorded similar patterns of tissue expression for mt-PPAR and p43. In particular, neither p43 nor mt-PPAR were detected in brain mitochondria, whereas they were strongly expressed in brown adipose tissue. In conclusion, as mt-PPAR mitochondrial amounts were increased by clofibrate, these data suggest the occurrence of a direct cross talk between T3 and PPs at the mitochondrial level.

Acknowledgements: We acknowledge Prof. W. Wahli for the gift of pSG5-mPPARα, pSG5-mPPARβ, pSG5-mPPARγ and pBSIIK-hPPARγ2; D. Baudoin-Malaprade, J. Chanel and A. Stoekel for their skilful assistance. This work was supported by Institut National de la Recherche Agronomique (INRA), and by grants from Association de la Recherche contre le Cancer (ARC).

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