

Cyclin G2 regulates adipogenesis through PPAR gamma coactivation.

Victor Aguilar, Jean-Sébastien Annicotte, Xavier Escote, Joan Vendrell,
Dominique Langin, Lluís Fajas

► **To cite this version:**

Victor Aguilar, Jean-Sébastien Annicotte, Xavier Escote, Joan Vendrell, Dominique Langin, et al.. Cyclin G2 regulates adipogenesis through PPAR gamma coactivation.. *Endocrinology, Endocrine Society*, 2010, 151 (11), pp.5247-54. <10.1210/en.2010-0461>. <inserm-00519587>

HAL Id: inserm-00519587

<http://www.hal.inserm.fr/inserm-00519587>

Submitted on 20 Sep 2010

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Cyclin G2 regulates adipogenesis through PPAR gamma coactivation

Victor Aguilar¹, Jean-Sébastien Annicotte¹, Xavier Escote², Joan Vendrell², Dominique Langin³, Lluis Fajas^{1*}

¹ IRCM, Institut de recherche en cancérologie de Montpellier INSERM : U896, Université Montpellier I, CRLC Val d'Aurelle - Paul Lamarque F-34298 Montpellier,FR

² Endocrinology and Diabetes Unit University Hospital of Tarragona, Research Department. Pere Virgili Institute, 43007 Tarragona,,ES

³ I2MR, Institut de médecine moléculaire de Rangueil INSERM : U858, IFR31, IFR150, Université Paul Sabatier - Toulouse III, Institut Louis Bugnard I, avenue Jean Poulhes BP 84225 31432 TOULOUSE CEDEX 4,FR

* Correspondence should be addressed to: Lluis Fajas <lluis.fajas@inserm.fr >

Abstract

Summary

Cell cycle regulators such as cyclins, cyclin dependant kinases (CDKs) or Rb play important roles in the differentiation of adipocytes. In the present paper we investigated the role of cyclin G2 as a positive regulator of adipogenesis. Cyclin G2 is an unconventional cyclin which expression is up regulated during growth inhibition or apoptosis. Using the 3T3-F442A cell line we observed an up-regulation of cyclin G2 expression at protein and mRNA levels throughout the process of cell differentiation, with a further induction of adipogenesis when the protein is transiently overexpressed. We show here, that the positive regulatory effects of cyclin G2 in adipocyte differentiation are mediated by direct binding of cyclin G2 to PPAR γ , the key regulator of adipocyte differentiation. The role of cyclin G2 as a novel PPAR γ coactivator was further demonstrated by chromatin immunoprecipitation assays, which showed that the protein is present in the PPAR γ -responsive element of the promoter of aP2, which is a PPAR γ target gene. Luciferase reporter gene assays, showed that cyclin G2 positively regulates the transcriptional activity of PPAR γ . The role of cyclin G2 in adipogenesis is further underscored by its increased expression in mice fed a high fat diet. Taken together, our results demonstrate a novel role for cyclin G2 in the regulation of adipogenesis.

MESH Keywords 3T3-L1 Cells ; Adipocytes ; cytology ; metabolism ; Adipogenesis ; genetics ; Animals ; Cells, Cultured ; Cyclin G2 ; genetics ; metabolism ; Fluorescent Antibody Technique ; Immunoprecipitation ; Male ; Mice ; Mice, Inbred C57BL ; PPAR gamma ; genetics ; metabolism ; Reverse Transcriptase Polymerase Chain Reaction ; Transfection ; Up-Regulation

Author Keywords adipogenesis ; cyclin G2 ; PPAR γ

Introduction

Cyclins are key components of cell cycle that associate in complexes with the catalytic protein kinase termed cyclin dependent kinases (CDKs) thereby enabling their activation. Activated CDKs will promote cell cycle transition through phosphorylation of specific targets such as the tumor suppressor Rb. While the role of cyclins in the control of cell division is widely characterized, less is known about their role in the control of metabolism and cell differentiation in adipose tissue. Cyclin G2 is a member of the cyclin G family of proteins comprising also cyclin G1 and cyclin I (1), (2), (3). These cyclins were named unconventional since no known active CDK partner was described so far. More recently, however CDK5 was found to be a partner of cyclin I and cyclin G1 (4) (5) and cyclin G associated kinase (GAK) as a cyclin G1 partner (6). In contrast to the mRNA expression of cyclin G1 and I that do not fluctuate with cell cycle (1), (3), (7), cyclin G2 expression fluctuates during the cell cycle with a peak level of expression in the late S/early G2 phase (1), (2). Furthermore, Cyclin G2 is also atypically up-regulated during cell cycle arrest or apoptosis (1), (8), (9).

In addition to its potential role in cell differentiation, cyclin G2 (and not cyclin G1) has two putative Nuclear Receptor box (NR box) and is therefore an interesting target for interaction with nuclear receptors. These observations prompted us to characterize the potential interaction of cyclin G2 with the nuclear receptor peroxisome proliferator-activated receptor gamma (PPAR γ). PPAR γ plays a central role in the adipocyte differentiation process, through regulation of the expression of adipocyte-specific genes, such as aP2, fatty acid transport protein-1 (FATP-1), or lipoprotein lipase (LPL), which are involved in lipid storage and control of metabolism (10) (11). PPAR γ activity is regulated by fatty acid derivatives or by the antidiabetic drugs, thiazolidinediones. Post-transcriptional modifications, such as phosphorylation also regulates PPAR γ activity. MAPK phosphorylation inhibits PPAR γ activity (12, 13), whereas cdk7, cdk6, or cdk9 phosphorylation enhances PPAR γ activity (14, 15) (16). We demonstrate in this study that cyclin G2 is a novel PPAR γ interacting protein. Cyclin G2 is up-regulated during early stages of preadipocyte differentiation and positively regulates PPAR γ transcriptional activity.

Material and methods

Materials

All chemicals, except if stated otherwise, were purchased from Sigma Chemical Co. (St. Louis, MO). Pioglitazone was provided by Takeda Pharmaceutical company (Osaka, Japan). GW3276 and GW 61072 were provided by Biomol (France). Rosiglitazone was purchased from Molekula (Lisses, France). Anti-cyclin G2 antibody was purchased from Santa Cruz Biotechnology (N-19 antibody) or Abcam (Abcam, Cambridge, United Kingdom), anti-PPAR γ E-8 antibody, were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Plasmids and Oligonucleotides

pCDNA3-CycG2-GFP and pGEX4T1-CycG2 vector was a gift from Dr Horne M. GST-PPAR γ A/B, GST-PPAR γ DEF, GST-PPAR γ bA/B, PPRE-TK-Luc, and the PPAR γ expression vector were described previously (17),(18). Gal4-PPAR γ -LDB and UAS-TK-Luc reporter were described in (15). Gal4-PPAR α -LDB and Gal4-PPAR δ -LDB were described previously (19). A pCMV β -galactosidase vector was used as an internal control for transfection efficiency in mammalian cells.

The cyclin G2 Δ LXXLL point mutations were performed by PCR of pCDNA3-CycG2 with primers GGGGTTTCAGCTTTTCGCGGGCGTTGAACTTCTACCTGG (for the Nt mutant) and CCAGGTAGAAGTTCAACGCCGCGAAAAGCTGAACCC (for the Ct mutant), generating the plasmid pCDNA3- Δ LXXLL.

The pCDNA3- Δ 1-22 deletion mutant was created by PCR amplification of pCDNA-CycG2-GFP with primers: forward 5'-CAGGGTACCACCATGG GATTGTTGAACTTCTACC-3' and reverse 5'-TCAGCGGCCGCTTATGGTGGAAAGCACAGTGTCTG-3' containing KpnI and NotI restriction sites respectively. The Δ 1-22 deletion mutant was cloned in pCDNA3 at NotI and KpnI restriction sites. The pCDNA3- Δ 223-344 deletion mutant was created by PCR amplification of pCDNA-CycG2-GFP with primers: forward 5'-CAGGGATCCTACCATGGAGGATTTGGGGGCCAA-3' and reverse 5'-TCAGCGGCCGCTTAAATTTCCAGCAGTTCCACGG-3' containing BamHI and NotI restriction sites respectively. The Δ 223-344 deletion mutant was cloned in pCDNA3 at BamHI and NotI restriction sites. All plasmids used subsequently were confirmed by DNA sequencing.

RNA Isolation, Reverse Transcription, and Real-Time PCR

Total RNA from cells was isolated with TRIzol reagent (Invitrogen) as described by the manufacturer. Reverse transcription was performed using 500ng total RNA, random primers and MMLV enzyme (Invitrogen). mRNA expression was measured by quantitative real-time PCR (Q-PCR) with Power SYBR Green master mix using a 7300 ABI PRISM sequence detector system (Applied Biosystems) according to the manufacturer's recommendations. Q-PCR was performed using gene-specific oligonucleotides under the following conditions: 2 min at 50 °C, 10 min at 95 °C followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Ribosomal protein 18s was used as an internal control. The sequence of the primers used is available upon request.

Cell Culture, Cell Differentiation

COS and 3T3-F442A cells were grown in DMEM supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μ g/ml streptomycin in a humidified atmosphere of 5% CO₂ at 37°C. 3T3-F442A were grown to confluence and after two days differentiation was induced by switching cells to DMEM, 10% serum, 50 ng/ml insulin and 1 μ M rosiglitazone. Oil red O staining was performed as described elsewhere (20). Human adipocytes in primary culture were differentiated as previously described (21).

Transfections

Transfections of COS cells were performed using the Jet PEI reagent (Qbiogene, Irvine, CA). Electroporation of 3T3-F442A was conducted with cells induced to differentiate for five days. Cells were suspended by mild trypsinisation and electroporated using Amaxa kit, according to manufacturer's recommendations (Cell line Nucleofector kit L; Amaxa). Briefly, about 2 million cells were collected and resuspended in 100 μ l Nucleofector solution. For overexpression of cyclin G2, 500 ng of pCDNA3-CycG2-GFP or empty vector were used. For siRNA experiments control (5'-AACGGUUCUGGAUUAUAGGCaa-3'; Ambion) or mice cyclin G2 (5'-CAGCGACACUGAAUUCUUUtt-3', Ambion) siRNAs were delivered into adipocytes (250 nmol/l). Adipocytes were then reseeded into 12-well plates.

Protein Expression Assays

Human samples of WAT (anterior abdominal wall, and omental) samples were obtained during abdominal elective surgical procedures (cholecystectomy or surgery for abdominal hernia) at Hospital Universitari Joan XXIII (Tarragona, Spain) and Hospital Sant Pau i Santa Tecla (Tarragona, Spain). All subjects were of Caucasian origin. Informed written consent was obtained, and the experimental protocol was approved by the ethics committee of the hospital. Adipose tissue samples were collected, washed in PBS, immediately frozen in liquid N₂ and stored at -80°C.

White adipose tissue from mice origin were removed from C57BL/6 mice and pieces were placed into special centrifuges containing ceramic beads (Roche, Penzberg, Germany) with 1 mL of pre-chilled TEG buffer (10 mM Tris-HCl, pH 7.4, 1.5 mM EDTA, and 10% glycerol) containing protease inhibitors cocktail (Sigma). Tubes were subjected to oscillation made by the MagNA Lyser machine at 7 500 r/min for 15 seconds. The mixture was then sonicated, and the cellular debris were pelleted by centrifugation at 13,000 g for 10 min at 4°C. For cellular extracts, cells were homogenized in a lysis buffer containing 10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.1% Nonidet P-40 (MP Biomedicals, Aurora, OH), 1 mM dithiothreitol and protease inhibitor cocktail (Sigma). Lysates were centrifuged at 13,000 g for 10 min at 4°C and resuspended in lysis.

Protein concentrations were determined by the Bradford method (Bio-Rad, Hercules, CA). SDS-PAGE and electrotransfer were performed as described elsewhere. Human adipocytes in primary culture were differentiated as previously described (15). The membranes were blocked 1 h in blocking buffer (TBS, 0.5% Tween 20, 5% skimmed milk). Filters were first incubated overnight at 4 °C with the indicated primary antibodies, and then for 1 h at room temperature with a peroxidase conjugate secondary antibody. The complex was visualized with enhanced chemiluminescence (Interchim, Montluçon, France).

Adipose tissue fractionation

Fresh adipose tissue was finely diced into small pieces (10–30 mg), washed in 1x PBS and incubated in Medium 199 (Gibco) plus 4% BSA and 2 mg/mL of collagenase Type I (Sigma) for 1 h in a shaking water bath at 37°C. Mature adipocytes (ADI) were separated by filtration through a 200µm mesh fabric (Spectrum Laboratories, Rancho Dominguez, CA, USA) and by centrifugation for 5 min at 1500g. The mature adipocytes were removed from the top layer and the pellet consisted of stromal vascular fraction (SVF) cells. Cells were washed 4 times in 1xPBS.

Immunofluorescence in 3T3-F442A Cells

Cells were grown on coverslips. After fixation and permeabilization with 100% methanol, cells were incubated with antibodies directed against cyclin G2 and PPAR γ (Santa Cruz Biotechnology, Inc.). Preparations were then incubated with a combination of Texas Red-conjugated antimouse IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) or fluorescein isothiocyanate-conjugated anti-goat IgG (Santa Cruz Biotechnology, Inc.).

Coimmunoprecipitation and ChIP Assays

For coimmunoprecipitation assays, whole-cell extracts were precleared with protein G-agarose beads (Sigma) during 30 min at room temperature, and an aliquot of the precleared lysates was saved as input. Extracts were then centrifuged (5 min at 3000 rpm), and supernatants were immunoprecipitated with the indicated specific antibodies overnight at 4°C, rabbit IgGs (Sigma) were used as negative control (mock). Immunoprecipitates were then washed twice with IP buffer (150 mM NaCl, 1% Nonidet P-40, 50 mM Tris/HCl (pH 8), and protease inhibitor cocktail) and three times with washing buffer (0.25 M KCl in PBS) and subjected to SDS-PAGE electrophoresis.

ChIP assays were performed as described previously (22). Chromatin reimmunoprecipitation (Re-ChIP) assays were performed as described previously (23). Briefly, proteins were formaldehyde cross-linked to DNA in confluent 3T3-F442A preadipocytes before induction of differentiation or in cells induced with differentiation medium for 7 days. Proteins were then immunoprecipitated using the indicated antibodies or rabbit IgGs as mock control. DNA was extracted from the immunoprecipitates, and PCR amplification was performed using promoter-specific oligonucleotide primers to amplify the PPRE region in mouse aP2 promoter: 5' -GAGCCATGCGGATTCTTG-3' and 5'-CCAGGAGCGGCTTGATTGTTA-3', a non PPRE region in the aP2 promoter: 5' -CAGCCCCACATCCCCACAGC-3' and 5'-GGATGCCCAACAACAGCCACAC-3', a PPRE region in mouse LPL promoter: 5' -CCTCCCGGTAGGCAAACCTGGA-3' and 5'-CCACTGCACAGCTGTTTAAGTGACTGG-3', a PPRE region in mouse Tmem143 promoter: 5'-GTGGGGGATTGCATGGCCCA-3' and 5'-TCCTGGCCAGTCTCCCTCCC-3'.

Pull-Down Assays

In vitro translation of pCDNA3-CycG2-GFP, pCDNA3- Δ LXXLL, pCDNA3- Δ 1-22, pCDNA3- Δ 223-344 and pSG5-PPAR γ was performed with [³⁵S]methionine (PerkinElmer, Boston, MA) in a TNT-coupled transcription-translation system, as described by the manufacturer (Promega Corp.). GST fusion or GST alone were expressed in BI21 , and purified on glutathione-sepharose-4B beads (Amersham Biosciences, Uppsala, Sweden). For in vitro binding GST, GST-cyclin G2, GST-PPAR γ A/B, GST-PPAR γ DEF, and GST-PPAR γ bA/B deletion mutants were incubated with the different labeled protein in 1 ml binding buffer containing 300 mM NaCl, 0.5 % Triton-X-100, 50 mM Tris (pH 8), and 2 mM EDTA at room temperature for 1 h. Beads were washed five times with the same buffer. The proteins were visualized by autoradiography after SDS-PAGE.

Animals

Male C57BL/6 mice were purchased at 6 weeks of age from Charles River Laboratory. Animals were maintained according to European Union guidelines for use of laboratory animals. In vivo experiments were performed in compliance with the French guidelines for experimental animal studies (Agreement No. B-34-172-27). For the diet-induced obesity model, 6-week-old C57BL/6 mice were fed ad libitum with a high fat diet (HFD) including 45% from fat (TestDiet) for 8 weeks or 20 weeks.

Statistical analysis

Data are presented as mean \pm s.e.m.; statistical analyses was performed using unpaired Student's t -test. Differences were considered statistically significant at $P < 0.05$.

Results

Cyclin G2 expression is up-regulated during adipocyte differentiation

When hormonally stimulated, confluent 3T3-F442A preadipocytes undergo differentiation. We correlated the expression of the mRNA of cyclin G2, aP2 and PPAR γ during differentiation of these cells. Cyclin G2 expression was undetectable, as measured by QPCR analysis during the early stages and was robustly induced starting at day 3 of differentiation. High levels of expression could still be measured when cells reached the differentiated stage of adipocytes (Fig. 1A). Strikingly, this pattern of expression was almost identical to the expression of PPAR γ , which is the master regulator of adipocyte differentiation. Furthermore, the expression of the adipocyte marker aP2 was induced at the same stage than cyclin G2 (Fig. 1A). A similar expression pattern of cyclin G2 was observed by western blot analysis (Fig. 1B). Moreover, increased cyclin G2 expression was also observed during differentiation into adipocytes of primary human preadipocytes (Fig. 1C). Interestingly we observed that cyclin G2 and PPAR γ appeared to be co expressed in the same cells, as assessed by immunofluorescence analysis (Fig. 1D). The relevance of cyclin G2 expression in adipose tissue was further suggested by the increased expression of this protein in mice fed high fat diet compared to mice fed normal chow diet (Fig. 1E-F).

We further characterized in human visceral adipose tissue (VAT, omental) and subcutaneous adipose tissue (SAT, anterior abdominal wall) samples the cyclin G2 expression in stromal versus mature adipocyte fraction. As shown in Fig 1G , the expression of cyclin G2 is mainly detected in the adipocyte fraction. These results suggested a role of cyclin G2 in the regulation of adipogenesis, which may involve PPAR γ .

Cyclin G2 overexpression stimulates, whereas its inhibition impairs adipogenesis

To further assess the role of cyclin G2 during adipogenesis, we overexpressed cyclin G2 in differentiating cells. In order to clearly differentiate the effects of cyclin G2 overexpression on cell cycle regulation, and the effects on differentiation we forced ectopic expression of cyclin G2, as described in the methods section in 3T3-F442A cells induced to differentiate for 5-days, which are already permanently quiescent. Oil Red O staining indicated an increase in lipid accumulation in cells overexpressing cyclin G2, compared to cells transfected with control empty vector (Fig. 2A). Ectopic expression of cyclin G2 was monitored by Q-PCR, and was correlated with the degree of differentiation (Fig. 2B). Moreover, increased differentiation was consistent with higher expression of PPAR γ , lipoprotein lipase (LPL), adiponectin and aP2 adipocyte markers (Fig. 2B). These results suggested that cyclin G2 promotes adipogenesis, independent of its role in cell cycle regulation.

To further elucidate the role of cyclin G2 during adipocyte differentiation we genetically silenced cyclin G2 expression using siRNA techniques. Differentiated 3T3-F442A cells were electroporated with either a siRNA sequence against the mouse cyclin G2 transcript or an irrelevant siRNA, and were compared for their ability to express markers of adipocyte differentiation. After 4 days in differentiation media, normal lipid accumulation was observed in control cells whereas a significant decrease in lipid accumulation was observed in cyclin G2 knockdown cells as assessed by Oil Red O staining (Fig. 2C). Differentiated cyclin G2 knockdown cells express significantly reduced levels of cyclin G2, PPAR γ , LPL, adiponectin, Glut4 and aP2 mRNAs (Fig. 2D), further demonstrating the importance of cyclin G2 in adipogenesis.

Cyclin G2 increases PPAR γ transcriptional activity

We next aimed to elucidate the molecular mechanisms underlying the effects of cyclin G2 in adipogenesis. Since PPAR γ is the master regulator of this process, we tested a potential functional relation between cyclin G2 and PPAR γ . Cotransfection experiments using a PPAR γ -responsive luciferase-based reporter construct (PPRE-TK-Luc) and expression vectors for PPAR γ and cyclin G2 in COS cells were performed. PPAR γ induced 3.4-fold luciferase activity, which was further enhanced up to 8-fold in the presence of cyclin G2 (Fig. 3A , left panel). As expected these effects were increased in the presence of the PPAR γ agonist rosiglitazone (Fig. 3A , right panel). In order to rule out the possibility that cyclin G2 may increase PPRE-TK-luc activity independently of PPAR γ , a new reporter assay was performed using a UAS-TK-luc reporter assay (Fig. 3B). The Gal4-PPAR γ -LBD fusion protein was used to drive the activity of the UAS-TK-luc. As shown in figure 3B , the Gal4-PPAR γ -LBD fusion protein is able to induce the UAS-TK-Luc activity in the presence of

the PPAR γ ligand rosiglitazone. Similarly to what observed in the PPRE reporter-based assay, cyclin G2 coexpression resulted in a substantial additional increase in promoter activity (Fig. 3B). These results suggested that cyclin G2 induces the transcriptional activity of PPAR γ in a ligand-independent manner.

Next we wanted to elucidate whether the effects of cyclin G2 were specific for PPAR γ , or if cyclin G2 would be rather a general coactivator of nuclear receptors. The fusion proteins Gal4-PPAR α -LBD or Gal4-PPAR δ -LBD were able to induce the UAS_TK-Luc reporter activity in the presence of their ligands (GW 3276 and GW 61072 respectively) as expected, whereas no further reporter activity was observed by cotransfection with cyclin G2 (Fig. 3C and D).

Finally, to demonstrate that cyclin G2 could regulate the expression of PPAR γ target genes in-vivo , we performed chromatin immunoprecipitation (ChIP) experiments in differentiating 3T3-F442A cells. Interestingly, immunoprecipitated chromatin using either a PPAR γ or an anti-cyclin G2 antibody contained the PPARE of PPAR γ target genes (aP2, LPL or Tmem143) (Fig. 3E). No amplification of the aP2 promoter was observed when either PPAR γ or cyclin G2 were immunoprecipitated from confluent, non-differentiated 3T3-F442A cells which do not express PPAR γ or cyclin G2, nor when non-specific IgGs were used to immunoprecipitate the chromatin or when a non PPARE containing region of the aP2 promoter was amplified (Fig 3E). This suggested that cyclin G2 is specifically associated to PPAR γ in the PPARE of PPAR γ target gene promoters. To further prove this hypothesis re-ChIP experiments were performed. Strikingly, cyclin G2 immunoprecipitation from PPAR γ immunoprecipitates also contained the PPARE of the aP2 promoter as demonstrated by PCR amplification. Taken together these results demonstrated that cyclin G2 and PPAR γ form a transcriptional complex in the promoters of PPAR γ -target genes that results in the activation of the transcription of these genes.

Cyclin G2 interacts with PPAR γ

To test the hypothesis that cyclin G2 induces PPAR γ activity through a direct interaction with PPAR γ , cell extracts from differentiated 3T3-F442A adipocytes were immunoprecipitated with an anti-cyclin G2 antibody. Consistent with the ChIP analysis (Fig. 3B) a 55-kDa protein was recognized by immunoblot analysis with a PPAR γ antibody indicating that cyclin G2 is associated with PPAR γ (Fig. 4A). No PPAR γ could be detected when a non-specific IgG was used to immunoprecipitate the proteins (Fig. 4A).

To better characterize the interaction between PPAR γ and cyclin G2, cyclin G2 was incubated with GST-PPAR γ DEF, AB and b-AB (where "b" contains an additional 30 aa subunit specific to the PPAR γ 2 transcript) fusion proteins. Cyclin G2 could bind only the GST-DEF fusion protein (Fig. 4B). Interestingly, this region of PPAR γ contains the ligand-binding domain. Next, to see if the association between cyclin G2 and the DEF construct of PPAR γ could depend on ligand, we performed a similar pull-down assay using GST-cyclin G2 fusion protein and in-vitro translated ³⁵S radiolabeled PPAR γ in the presence and absence of the PPAR γ ligand pioglitazone. No increase in binding of PPAR γ to cyclin G2 was observed upon incubation with pioglitazone, suggesting a ligand independent interaction. (Fig. 4C). Finally we wanted to determine the cyclin G2 binding domain to PPAR γ . We focused the studies in two LXXLL motifs in the cyclin G2 protein. Deletion of the C-terminal part of cyclin G2 abolished binding to PPAR γ (Fig. 4D–E). Surprisingly, a mutant cyclin G2 protein containing point mutations in the two LXXLL motifs was still capable of binding to PPAR γ , suggesting that cyclin G2 bound to PPAR γ through a non-identified motif in the C-terminal part of the protein (Fig. 4F).

Discussion

Cyclins and cdks ultimately translate external signaling into a transcriptional response, which is the final step of the regulatory cascade (24) (25). This makes cyclins and cdks good candidates for the control of the cross talk that exists between proliferative stimuli and metabolic, transcriptional response. Adipogenesis is a paradigm of this particular system, which involves two major events: preadipocyte proliferation and adipocyte differentiation (26). Both processes are tightly regulated and the cross talk between them determines the final adipocyte phenotype of the cell. We and others have shown that some cyclins and cdks are associated with functions not directly linked to cell cycle progression. We have already demonstrated the participation of cyclin D3, and cdk4 in metabolism. We recently showed that cyclin D3 (16), cdk4 (27), and cdk9 (28) are adipogenic factors with strong effects on whole metabolism through regulation of PPAR γ activity. These are illustrative examples of how cell cycle regulatory proteins can also modulate metabolic processes. When looking at the expression of other members of the cyclin/cdk family, we found that cyclin G2 was up-regulated during the adipocyte differentiation process, and in fully differentiated adipocytes, suggesting that this cyclin could also be involved in the regulation of adipogenesis and adipocyte function. We demonstrate here that cyclin G2 is a positive factor for adipocyte differentiation since its overexpression and inhibition respectively results in stimulation and abrogation of adipogenesis. Furthermore we show that the stimulatory effects of cyclin G2 are mediated through the activation of PPAR γ transcriptional activity by a direct protein interaction.

Recent reports consistently point to cyclin G2 as a mediator of cell cycle inhibition during responses to diverse growth inhibitory signals, such as heat shock, oxidative stress, hypoxia and differentiation, further supporting the hypothesis that G2 has cell cycle inhibitory functions (29), (30). The effects of cyclin G2 on adipogenesis are not likely mediated, however by cell cycle regulation. This is demonstrated by the finding that cells that have already permanently exit cell cycle (day 5 of differentiation) are still sensitive to both overexpression and inhibition of cyclin G2. Other arguments also support the cell cycle independent role of cyclin G2, and other cyclins

and cdks in the control of differentiation of adipocytes. Interestingly, despite an opposite role of cyclin D3 or cdk4 in the one hand, and cyclin G2 on the other hand in the control of cell cycle, all of them share the ability to promote adipogenesis. This is the result of the interaction with PPAR γ , the master regulator of adipocyte differentiation. The particular role of each of these cyclins and cdks on PPAR γ remains to be elucidated. It is likely that increased PPAR γ activity in response to different stimuli is mediated by distinct cyclins and cdks. This would explain the pleiotropic effects of these cyclins. Concerning cyclin G2 some particular conditions are known to activate cyclin G2. This includes heat shock, ER stress, and oxidative stress (31). Strikingly heat shock also induces PPAR γ expression (32), and oxidative and ER stress have been correlated with increased adipocyte differentiation (33), (34). Cyclin G2 could therefore trigger adipocyte differentiation in response of these, and likely other adipogenic stimuli.

In summary we have established a link between cyclin G2 and the control of adipogenesis through PPAR γ interaction. The regulatory mechanism of cyclin G2 on adipocytes can also take place in adult differentiated tissue, since a prolonged HFD increased the protein expression in adipose tissue. This opens the possibility that cyclin G2 can also be implicated in adipose tissue general metabolism.

Acknowledgements:

This work was supported by grants from FRM, INCA, and ANR.

The members of Fajas' lab are acknowledged for discussions. This work was supported by funds of Inserm, Fondation pour la Recherche Medicale, and ANR.

Footnotes:

Disclosure summary : the authors have nothing to disclose.

References:

1. Horne MC, Donaldson KL, Goolsby GL, Tran D, Mulheisen M, Hell JW, Wahl AF. 1997; Cyclin G2 is up-regulated during growth inhibition and B cell antigen receptor-mediated cell cycle arrest. *J Biol Chem*. 272 : 12650 - 12661
2. Bates S, Rowan S, Vousden KH. 1996; Characterisation of human cyclin G1 and G2: DNA damage inducible genes. *Oncogene*. 13 : 1103 - 1109
3. Nakamura T, Sanokawa R, Sasaki YF, Ayusawa D, Oishi M, Mori N. 1995; Cyclin I: a new cyclin encoded by a gene isolated from human brain. *Exp Cell Res*. 221 : 534 - 542
4. Brinkkoetter PT, Olivier P, Wu JS, Henderson S, Krofft RD, Pippin JW, Hockenbery D, Roberts JM, Shankland SJ. 2009; Cyclin I activates Cdk5 and regulates expression of Bcl-2 and Bcl-XL in postmitotic mouse cells. *J Clin Invest*.
5. Seo HR, Kim J, Bae S, Soh JW, Lee YS. 2008; Cdk5-mediated phosphorylation of c-Myc on Ser-62 is essential in transcriptional activation of cyclin B1 by cyclin G1. *J Biol Chem*. 283 : 15601 - 15610
6. Kanaoka Y, Kimura SH, Okazaki I, Ikeda M, Nojima H. 1997; GAK: a cyclin G associated kinase contains a tensin/auxilin-like domain. *FEBS Lett*. 402 : 73 - 80
7. Tamura K, Kanaoka Y, Jinno S, Nagata A, Ogiso Y, Shimizu K, Hayakawa T, Nojima H, Okayama H. 1993; Cyclin G: a new mammalian cyclin with homology to fission yeast Cig1. *Oncogene*. 8 : 2113 - 2118
8. Martinez-Gac L, Marques M, Garcia Z, Campanero MR, Carrera AC. 2004; Control of cyclin G2 mRNA expression by forkhead transcription factors: novel mechanism for cell cycle control by phosphoinositide 3-kinase and forkhead. *Mol Cell Biol*. 24 : 2181 - 2189
9. Bennin DA, Don AS, Brake T, McKenzie JL, Rosenbaum H, Ortiz L, DePaoli-Roach AA, Horne MC. 2002; Cyclin G2 associates with protein phosphatase 2A catalytic and regulatory B' subunits in active complexes and induces nuclear aberrations and a G1/S phase cell cycle arrest. *J Biol Chem*. 277 : 27449 - 27467
10. Fajas L, Fruchart JC, Auwerx J. 1998; Transcriptional control of adipogenesis. *Current Opinions in Cell Biology*. 10 : 165 - 173
11. Rosen ED, Spiegelman BM. 2001; PPAR γ : a nuclear regulator of metabolism, differentiation, and cell growth. *J Biol Chem*. 276 : 37731 - 37734
12. Hu E, Kim JB, Sarraf P, Spiegelman BM. 1996; Inhibition of adipogenesis through MAP kinase-mediated phosphorylation of PPAR γ . *Science*. 274 : 2100 - 2103
13. Adams M, Reginato MJ, Shao D, Lazar MA, Chatterjee VK. 1997; Transcriptional activation by peroxisome proliferator-activated receptor gamma is inhibited by phosphorylation at a consensus mitogen-activated protein kinase site. *J Biol Chem*. 272 : 5128 - 5132
14. Compe E, Drane P, Laurent C, Diderich K, Braun C, Hoesjmakers JH, Egly JM. 2005; Dysregulation of the peroxisome proliferator-activated receptor target genes by XPD mutations. *Mol Cell Biol*. 25 : 6065 - 6076
15. Iankova I, Petersen RK, Annicotte JS, Chavey C, Hansen JB, Kratchmarova I, Sarraf D, Benkirane M, Kristiansen K, Fajas L. 2006; Peroxisome proliferator-activated receptor gamma recruits the positive transcription elongation factor b complex to activate transcription and promote adipogenesis. *Mol Endocrinol*. 20 : 1494 - 1505
16. Sarraf DA, Iankova I, Abella A, Assou S, Miard S, Fajas L. 2005; Cyclin D3 promotes adipogenesis through activation of peroxisome proliferator-activated receptor gamma. *Mol Cell Biol*. 25 : 9985 - 9995
17. Gelman L, Zhou G, Fajas L, Raspe E, Fruchart JC, Auwerx J. 1999; p300 interacts with the N- and C-terminal part of PPAR γ 2 in a ligand-independent and -dependent manner, respectively. *J Biol Chem*. 274 : 7681 - 7688
18. Fajas L, Egler V, Reiter R, Hansen J, Kristiansen K, Debril MB, Miard S, Auwerx J. 2002; The retinoblastoma-histone deacetylase 3 complex inhibits PPAR γ and adipocyte differentiation. *Dev Cell*. 3 : 903 - 910
19. Seimandi M, Lemaire G, Pillon A, Perrin A, Carlan I, Voegel JJ, Vignon F, Nicolas JC, Balaguer P. 2005; Differential responses of PPAR α , PPAR δ , and PPAR γ reporter cell lines to selective PPAR synthetic ligands. *Anal Biochem*. 344 : 8 - 15
20. Hansen JB, Petersen RK, Larsen BM, Bartkova J, Alsner J, Kristiansen K. 1999; Activation of peroxisome proliferator-activated receptor gamma bypasses the function of the retinoblastoma protein in adipocyte differentiation. *J Biol Chem*. 274 : 2386 - 2393
21. Mazzucotelli A, Viguier N, Tiraby C, Annicotte JS, Mairal A, Klimcakova E, Lepin E, Delmar P, Dejean S, Tavernier G, Lefort C, Hidalgo J, Pineau T, Fajas L, Clement K, Langin D. 2007; The transcriptional coactivator peroxisome proliferator activated receptor (PPAR) γ coactivator-1 alpha and the nuclear receptor PPAR alpha control the expression of glycerol kinase and metabolism genes independently of PPAR gamma activation in human white adipocytes. *Diabetes*. 56 : 2467 - 2475
22. Takahashi Y, Rayman JB, Dynlacht BD. 2000; Analysis of promoter binding by the E2F and pRB families in vivo: distinct E2F proteins mediate activation and repression. *Genes Dev*. 14 : 804 - 816
23. Annicotte JS, Blanchet E, Chavey C, Iankova I, Costes S, Assou S, Teyssier J, Dalle S, Sartet C, Fajas L. 2009; The CDK4-pRB-E2F1 pathway controls insulin secretion. *Nat Cell Biol*. 11 : 1017 - 1023
24. Matsushima H, Roussel MF, Ashmun RA, Sherr CJ. 1991; Colony-stimulating factor 1 regulates novel cyclins during the G1 phase of the cell cycle. *Cell*. 65 : 701 - 713
25. Malumbres M, Barbacid M. 2005; Mammalian cyclin-dependent kinases. *Trends Biochem Sci*. 30 : 630 - 641

- 26 . Fajas L . 2003 ; Adipogenesis: a cross-talk between cell proliferation and cell differentiation . *Annals of Medicine* . 35 : 79 - 85
- 27 . Abella A , Dubus P , Malumbres M , Rane SG , Kiyokawa H , Sicard A , Vignon F , Langin D , Barbacid M , Fajas L . 2005 ; Cdk4 promotes adipogenesis through PPAR γ activation . *Cell Metab* . 2 : 239 - 249
- 28 . Iankova I , Petersen RK , Annicotte JS , Chavey C , Hansen JB , Kratchmarova I , Sarruf D , Benkirane M , Kristiansen K , Fajas L . 2006 ; PPAR γ Recruits the P-TEFb Complex to Activate Transcription and Promote Adipogenesis . *Mol Endocrinol* .
- 29 . Gajate C , An F , Mollinedo F . 2002 ; Differential cytostatic and apoptotic effects of ecteinascidin-743 in cancer cells. Transcription-dependent cell cycle arrest and transcription-independent JNK and mitochondrial mediated apoptosis . *J Biol Chem* . 277 : 41580 - 41589
- 30 . Arachchige Don AS , Dallapiazza RF , Bennin DA , Brake T , Cowan CE , Horne MC . 2006 ; Cyclin G2 is a centrosome-associated nucleocytoplasmic shuttling protein that influences microtubule stability and induces a p53-dependent cell cycle arrest . *Exp Cell Res* . 312 : 4181 - 4204
- 31 . Murray JI , Whitfield ML , Trinklein ND , Myers RM , Brown PO , Botstein D . 2004 ; Diverse and specific gene expression responses to stresses in cultured human cells . *Mol Biol Cell* . 15 : 2361 - 2374
- 32 . Bernabucci U , Basirico L , Morera P , Lacetera N , Ronchi B , Nardone A . 2009 ; Heat shock modulates adipokines expression in 3T3-L1 adipocytes . *J Mol Endocrinol* . 42 : 139 - 147
- 33 . Basseri S , Lhotak S , Sharma AM , Austin RC . 2009 ; The chemical chaperone 4-phenylbutyrate inhibits adipogenesis by modulating the unfolded protein response . *J Lipid Res* . 50 : 2486 - 2501
- 34 . Lee H , Lee YJ , Choi H , Ko EH , Kim JW . 2009 ; Reactive oxygen species facilitate adipocyte differentiation by accelerating mitotic clonal expansion . *J Biol Chem* . 284 : 10601 - 10609

Figure 1

Cyclin G2 expression is increased during adipocyte differentiation

A. Quantification of mRNA expression levels by real time PCR of cyclin G2 at the indicated times of differentiation in mouse 3T3-F442A adipocytes or human primary adipocytes (C). Results were normalized by the expression levels of 18s mRNA. B. Protein expression of cyclin G2 during the indicated time points of differentiation. D. Comparative analysis of PPAR γ and cyclin G2 expression by immunofluorescence in 3T3-F442A adipocytes during differentiation. Days of differentiation indicated are confluent (D0) early differentiation (D3) and terminally differentiated (D8). PPAR γ expressing cells are labeled in red, cyclin G2 expressing cells in green and nuclei were visualized by Hoechst staining. E. Representative protein expression of cyclin G2 in mouse subcutaneous fat pads after 8 weeks normal diet (ND) or high fat diet (HFD). F. Densitometry analysis of cyclin G2 expression in subcutaneous fat pads of mice submitted to ND (n=7) or HFD (n=7). Images were analysed by ImageJ software. G. Expression of cyclin G2 in stromal (SVF) and adipocyte (ADI) fraction of human WAT.

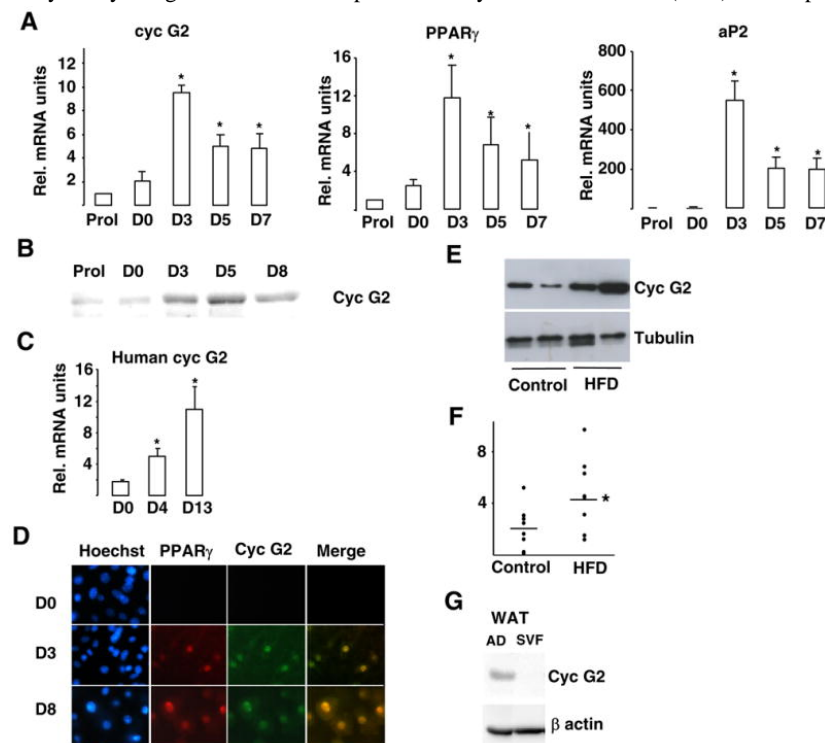


Figure 2

Cyclin G2 regulates adipogenesis

A. Representative micrographs of oil red O staining of 3T3-F442A cells during differentiation. Cells were either transfected with an expression vector of cyclin G2 (pCDNA3-cyclin G2) or empty vector (pCDNA3) five day after induction of differentiation. Oil red O staining was conducted at day 9 post differentiation. B. mRNA of adipocyte cells described in (A), at 7 days of differentiation was analyzed to assess the expression levels of cyclin G2 and the adipocyte markers PPAR γ , lipoprotein lipase (LPL), adiponectin (Adipo), Glut 4 and aP2 by quantitative real time PCR. C. Representative micrographs of oil red O staining of 3T3-F442A cells at day 9 of differentiation. Cells were either transfected with control or mice cyclin G2 siRNAs at day 5 after induction of differentiation. D. mRNA of differentiating cells described in (C) at seven days of differentiation was analyzed to assess the expression levels of cyclin G2, PPAR γ , LPL, Adipo, Glut4 and aP2 by quantitative real time PCR.

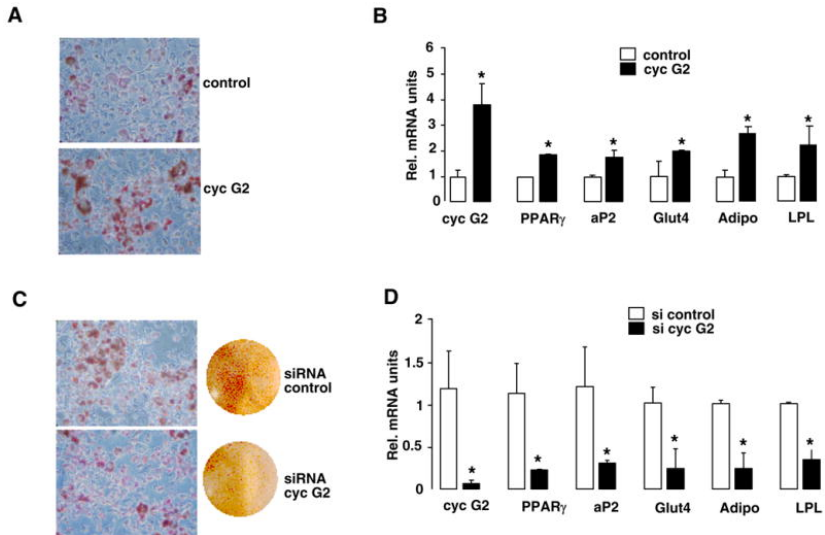


Figure 3

Cyclin G2 stimulates the PPAR γ transcriptional activity

A. Activity of the PPRE-TK-Luc reporter carrying the PPAR γ specific response elements measured in COS cells upon transfecting expression vectors for cyclin G2, PPAR γ or both plasmids together. The experiments were performed in triplicate in the presence or absence of the PPAR γ agonist rosiglitazone (10^{-4} M) and were normalized for β -galactosidase activity. B. Activity of the UAS-TK-luc reporter measured in COS cells upon transfection of expression vectors for cyclin G2, Gal4-PPAR γ -LBD or both plasmids together in the presence or absence of the PPAR γ ligand rosiglitazone. C. Activity of the UAS-TK-luc reporter measured in COS cells upon transfection of expression vectors for cyclin G2, Gal4-PPAR α -LBD or both plasmids together in the presence or absence of the PPAR α ligand GW 3276. D. Activity of the UAS-TK-luc reporter measured in COS cells upon transfection of expression vectors for cyclin G2, Gal4-PPAR δ -LBD in the presence or absence of the PPAR δ ligand GW 61072. E. ChIP assay demonstrating binding of cyclin G2 to the aP2 promoter. Cross-linked chromatin from either confluent 3T3-F442A preadipocytes (lower panel) or 3T3-F442A adipocytes differentiated during 6 days (upper panels) was incubated with antibodies against PPAR γ , cyclin G2 or with purified rabbit IgGs as control. Immunoprecipitates were analyzed by PCR using primers specific for the promoter region containing a PPRE of aP2, LPL and Tmem143 genes. The input included in the PCR was conducted with 20 % of the total chromatin. A region of the aP2 promoter outside the PPRE was amplified as negative control. F. Chip and Rechip assay. Cross-linked chromatin from 3T3-F442A adipocytes differentiated during 6 days was incubated with antibodies against cyclin G2. The immunoprecipitated chromatin was incubated with antibodies against PPAR γ , or with purified rabbit IgGs as control. Immunoprecipitates were analyzed by PCR using primers specific for the aP2 promoter region containing a PPRE.

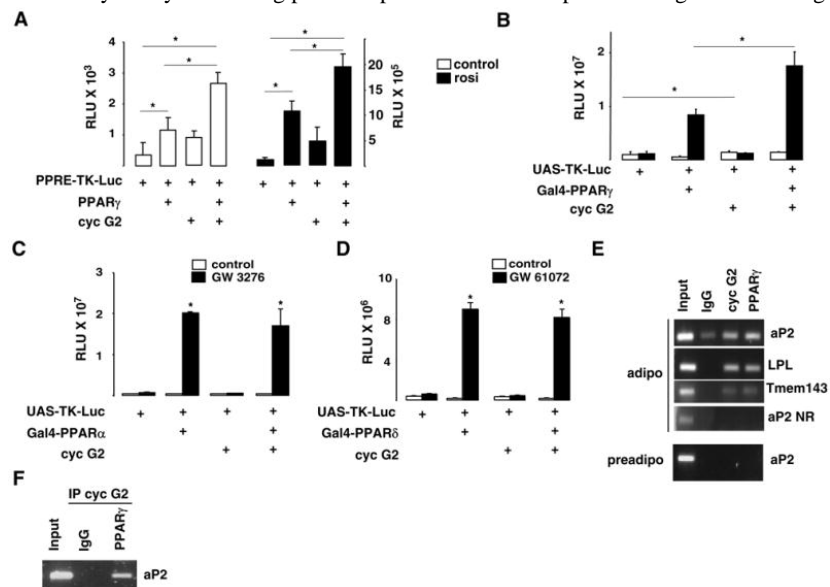


Figure 4

Cyclin G2 interacts with PPAR γ during adipogenesis

A. Coimmunoprecipitation of PPAR γ and cyclin G2 from differentiated 3T3-F442A. Extracts were immunoprecipitated with a PPAR γ , cyclin G2 or rabbit IgGs and revealed with an anti-PPAR γ antibody. One twentieth of the total extract is shown as control input. B. Schematic representation of the deletion GST-PPAR γ constructs used in the subsequent experiments (upper panel). GST pull-down assay showing the interaction of in vitro translated cyclin G2 with the GST-DEF domain of PPAR γ (lower panel). C. GST pull-down assay showing the interaction of GST-cyclin G2 with in vitro translated PPAR γ in the presence or absence of the PPAR γ drug co-activator pioglitazone used at 100 nM. D. Schematic representation of the deletion GST-cyclin G2 constructs used in the subsequent experiments. Mutations in the LXXLL motifs are indicated. E-F. GST pull-down assay showing the interaction of in vitro translated cyclin G2 constructs as represented in D, with the GST-DEF domain of PPAR γ .

