Cyclin G2 regulates adipogenesis through PPARγ coactivation.

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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 PPARγ, the key regulator of adipocyte differentiation. The role of cyclin G2 as a novel PPARγ coactivator was further demonstrated by chromatin immunoprecipitation assays, which showed that the protein is present in the PPARγ-responsive element of the promoter of aP2, which is a PPARγ target gene. Luciferase reporter gene assays, showed that cyclin G2 positively regulates the transcriptional activity of PPARγ. 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.

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 (PPARγ). PPARγ 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). PPARγ activity is regulated by fatty acid derivatives or by the antidiabetic drugs, thiazolidinediones. Post-transcriptional modifications, such as phosphorylation also regulates PPARγ activity. MAPK phosphorylation inhibits PPARγ

69 activity (12, 13), whereas cdk7, cdk6, or cdk9 phosphorylation enhances PPARγ activity (14,
70 15) (16). We demonstrate in this study that cyclin G2 is a novel PPARγ interacting protein.
71 Cyclin G2 is up-regulated during early stages of preadipocyte differentiation and positively
72 regulates PPARγ transcriptional activity.
73

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-PPARγ 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-PPARγ A/B, GST-PPARγ DEF, GST-PPARγ bA/B, PPRE-TK-Luc, and the PPARγ expression vector were described previously (17),(18). Gal4-PPARγ-LDB and UAS-TK-Luc reporter were described in (15). Gal4-PPARα-LDB and Gal4-PPARδ-LBD were described previously (19). A pCMV ß-galactosidase vector was used as an internal control for transfection efficiency in mammalian cells.

The cyclin G2 ΔLXXLL point mutations were performed by PCR of pCDNA3-CycG2 with primers GGGGTTCAGCTTTTCGCGGCGTTGAACTTCTACCTGG (for the Nt mutant) and CCAGGTAGAAGTTCAACGCCGCGAAAAGCTGAACCCC (for th Ct mutant), generating the plasmid pCDNA3-ΔLXXLL.

The pCDNA3-Δ1-22 deletion mutant was created by PCR amplification of pCDNA-CycG2-GFP with primers: forward 5'-CAGGGTACCA**CCATGG**GATTGTTGAACTTCTACC-3' and reverse 5'-TCAGCGGCCGCTTATGGTGGAAAGCACAGTGTCTG-3' containing *KpnI* and *NotI* restriction sites respectively. The Δ1-22 deletion mutant was cloned in pCDNA3 at *NotI* and *KpnI* restriction sites. The pCDNA3-Δ223-344 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 Δ223-344 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 manufacter'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 μg/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 μM rosiglitazone. Oil red O staining was performed as described elsewhere (20). Human adipocytes in primary culture were differentiated as previously described (21).

Tranfections. 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 µl Nucleofector solution. For overexpression of cyclin G2, 500 ng of pcDNA3-CycG2-GFP or empty vector were used. For siRNA experiments control (5'-AACGGUUCUGGAUAUAGGCaa-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 N_2 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 37ºC. Mature adipocytes (ADI) were separated by filtration through a 200µm mesh fabric (Spectrum Laboratories, Rancho Domínguez, 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 PPARγ (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 antigoat 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'-CCTCCCGGTAGGCAAACTGGA-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-ΔLXXLL, pCDNA3-Δ1-22, pCDNA3-Δ223-344 and pSG5-PPARγ was performed with [^{35}S]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 *Bl21*, and purified on glutathione-sepharose-4B beads (Amersham Biosciences, Uppsala, Sweden). For in vitro binding GST, GST-cyclin G2, GST-PPARγ A/B, GST-PPARγ DEF, and GST-PPARγ 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 +/- 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 PPARγ 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 PPARγ, 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 PPARγ 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 PPARγ.

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 PPARγ, 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-F422A 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, PPARγ, LPL, adiponectin, Glut4 and aP2 mRNAs (Fig. 2D), further demonstrating the importance of cyclin G2 in adipogenesis.

Cyclin G2 increases PPARγ transcriptional activity

We next aimed to elucidate the molecular mechanisms underlying the effects of cyclin G2 in adipogenesis. Since PPARγ is the master regulator of this process, we tested a potential

functional relation between cyclin G2 and PPARγ. Cotransfection experiments using a PPARγ-responsive luciferase-based reporter construct (PPRE-TK-Luc) and expression vectors for PPARγ and cyclin G2 in COS cells were performed. PPARγ 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 PPARγ agonist rosiglitazone (Fig. 3A, right panel). In order to rule out the possibility that cyclin G2 may increase PPRE-TK-luc activity independently of PPARγ, a new reporter assay was performed using a UAS-TK-luc reporter assay (Fig. 3B). The Gal4-PPARγ-LBD fusion protein was used to drive the activity of the UAS-TK-luc. As shown in figure 3B, the Gal4-PPARγ-LBD fusion protein is able to induce the UAS-TK-Luc activity in the presence of the PPARγ 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 PPARγ in a ligand-independent manner.

Next we wanted to elucidate whether the effects of cyclin G2 were specific for PPARγ, or if cyclin G2 would be rather a general coactivator of nuclear receptors. The fusion proteins Gal4-PPARα-LBD or Gal4-PPARδ-LBD where 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 PPARγ target genes *in-vivo*, we performed chromatin immunoprecipitation (ChIP) experiments in differentiating 3T3-F442A cells. Interestingly, immunoprecipitated chromatin using either a PPARγ or an anti-cyclin G2 antibody contained the PPARγ response element (PPRE) of PPARγ target genes (aP2, LPL or Tmem143) (Fig. 3E). No amplification of the aP2 promoter

was observed when either PPARγ or cyclin G2 were immunoprecipitated from confluent, non-differentiated 3T3-F422A cells which do not express PPARγ or cyclin G2, nor when non-specific IgGs were used to immunoprecipitate the chromatin or when a non PPRE containing region of the aP2 promoter was amplified (Fig 3E). This suggested that cyclin G2 is specifically associated to PPARγ in the PPRE of PPARγ target gene promoters. To further prove this hypothesis re-ChIP experiments were performed. Strikingly, cyclin G2 immunoprecipitation from PPARγ immunoprecipitates also contained the PPRE of the aP2 promoter as demonstrated by PCR amplification. Taken together these results demonstrated that cyclin G2 and PPARγ form a transcriptional complex in the promoters of PPARγ-target genes that results in the activation of the transcription of these genes.

Cyclin G2 interacts with PPARγ

To test the hypothesis that cyclin G2 induces PPARγ activity through a direct interaction with PPARγ, 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 PPARγ antibody indicating that cyclin G2 is associated with PPARγ (Fig. 4A). No PPARγ could be detected when a non-specific IgG was used to immunoprecipitate the proteins (Fig. 4A).

To better characterize the interaction between PPARγ and cyclin G2, cyclin G2 was incubated with GST- PPARγ DEF, AB and b-AB (where "b" contains an additional 30 aa subunit specific to the PPARγ2 transcript) fusion proteins. Cyclin G2 could bind only the GST-DEF fusion protein (Fig. 4B). Interestingly, this region of PPARγ contains the ligand-binding domain. Next, to see if the association between cyclin G2 and the DEF construct of PPARγ could depend on ligand, we performed a similar pull-down assay using GST-cyclin G2 fusion protein and *in-vitro* translated ^{35}S radiolabeled PPARγ in the presence and absence

of the PPARγ ligand pioglitazone. No increase in binding of PPARγ 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 PPARγ. 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 PPARγ (Fig. 4D-E). Surprisingly, a mutant cyclin G2 protein containing point mutations in the two LXXLL motifs was still capable of binding to PPARγ, suggesting that cyclin G2 bound to PPARγ 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 PPARγ 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 PPARγ 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 PPARγ, the master regulator of adipocyte differentiation. The particular role of each of these cyclins and cdks on PPARγ remains to be elucidated. It is likely that increased PPARγ 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 PPARγ 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 PPARγ 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.

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Figure legends

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 PPARγ 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). PPARγ 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 submited 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 asses the expression levels of cyclin G2 and the adipocyte markers PPARγ, 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, PPARγ, LPL, Adipo, Glut4 and aP2 by quantitative real time PCR.

Figure 3: Cyclin G2 stimulates the PPARγ transcriptional activity.

A. Activity of the PPRE-TK-Luc reporter carrying the PPARγ specific response elements measured in COS cells upon transfecting expression vectors for cyclin G2, PPARγ or both plasmids together. The experiments were performed in triplicate in the presence or absence of the PPARγ 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-PPARγ–LBD or both plasmids together in the presence or absence of the PPARγ ligand rosiglitazone.

C. Activity of the UAS-TK-luc reporter measured in COS cells upon transfection of expression vectors for cyclin G2, Gal4-PPARα-LBD or both plasmids together in the presence or absence of the PPARα ligand GW 3276.

D. Activity of the UAS-TK-luc reporter measured in COS cells upon transfection of expression vectors for cyclin G2, Gal4-PPARδ-LBD in the presence or absence of the PPARδ 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 PPARγ, 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 Tmeme143 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 PPARγ, 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 PPARγ during adipogenesis.

A. Coimmunoprecipitation of PPARγ and cyclin G2 from differentiated 3T3-F442A. Extracts were imunoprecipitated with a PPARγ, cyclin G2 or rabbit IgGs and revealed with an anti-PPARγ antibody. One twentieth of the total extract is shown as control input.

B. Schematic representation of the deletion GST-PPARγ 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 PPARγ (lower panel).

C. GST pull-down assay showing the interaction of GST-cycling G2 with *in vitro* translated PPARγ in the presence or absence or the PPARγ 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 PPARγ.