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Abstract

In addition to their role in cell cycle progression, new data reveals an emerging role of D-type cyclins in transcriptional regulation and cellular differentiation processes. Using 3T3-L1cell lines to study adipogenesis, we observed an up regulation of cyclin D3 expression throughout the differentiation process. Surprisingly, cyclin D3 was only minimally expressed during the initial stages of adipogenesis where mitotic division is prevalent. This seemingly paradoxal expression led us to investigate a potential cell cycle independent role for cyclin D3 during adipogenesis. We show here a direct interaction between cyclin D3 and the nuclear receptor PPARγ. Our experiments reveal cyclin D3 to act as a ligand dependent PPARγ coactivator which, together with its CDK partner, phosphorylates the A-B domain of the nuclear receptor. Over-expression and knockdown studies with cyclin D3 had marked effects on PPARγ activity and subsequently on adipogenesis. ChIP assays confirm the participation of cyclin D3 in the regulation of PPARγ target genes. We show that cyclin D3 -/- mice are protected from diet induced obesity, display smaller adipocytes, have reduced adipogenic gene expression and are insulin sensitive. Our results indicate that cyclin D3 is an important factor governing adipogenesis and obesity.

Introduction

Our understanding of the molecular mechanisms which orchestrate adipocyte differentiation have been greatly advanced by the use of preadipocytes cell lines, such as 3T3-L1 cells, capable of undergoing adipogenesis (14). Upon reaching confluence, proliferating preadipocytes become growth arrested by contact inhibition. These growth-arrested preadipocytes reenter the cell cycle after hormonal induction, arrest proliferation again, and finally undergo terminal adipocyte differentiation. The peroxisome proliferator-activated receptor γ (PPARγ), a ligand inducible transcription factor, has been identified as a major regulator of terminal adipocyte differentiation (10, 37). PPARγ, upon activation by either fatty acid derivatives or antidiabetic thiazolidinediones, drives the expression of several adipocyte-specific genes, such as the fatty acid binding protein (aP2), thus transforming the cell into the characteristic lipid rich adipocyte (42). Subsequent studies have demonstrated that ectopic expression of PPARγ further induces adipocyte differentiation (43). This pivotal role of PPARγ in adipocyte differentiation is also highlighted by the phenotype observed in humans with mutations in the PPARγ gene and by PPARγ deficient mice which are essentially void of white adipose tissue (8).

D-type cyclins were first characterized for their ability to coordinate cell cycle progression through the G1 phase. Three D cyclins (cyclins D1, D2, and D3) bind and activate cyclin dependent kinases 4/6 (CDK4/6), directing the phosphorylation of RB, as well as pRB related proteins p107 and p130. (4, 18, 28). This phosphorylation event disrupts the pRB repressor complexes leading to derepression of E2F transcription factors and induction of E2F target genes which are required for S phase entry (6).

In addition to their defined role as part of the core cell cycle machinery, a new potential for D cyclins has emerged in other cellular processes including transcriptional control and differentiation. Cyclin D1 can bind and repress the activity of several transcription factors including b-Myb (15), MyoD (34, 40), and DMP1 (17). Although less well explored, a CDK independent role for cyclin D3 has also been reported including inhibition of granulocyte differentiation (19). More recent studies have attributed cyclin D3 with the ability to bind and activate certain transcription factors such as the human activating transcription factor 5 (hATF5) (25). In the case of cyclin D3-/- mice it has been found that they fail to undergo development of immature T lymphocytes (39).

Recently our lab explored a link between the molecular processes governing adipocyte differentiation and the molecular machinery involved in cell cycle progression. These studies have established key cell cycle regulators including the RB protein and the E2F transcription factor family as fundamental regulators of adipogenesis through their modulation of PPARγ expression and activity (9, 11). Other recent studies have linked loss of cyclin dependent kinase inhibitors with obesity in mice (30). The notion that adipogenesis is regulated by proteins of the cell cycle is not unexpected since early stages of 3T3-L1 adipogenesis (days 1-2) are marked by active rounds of mitotic clonal expansion. An active cell cycle during the initial stages of adipogenesis is considered a prerequisite for terminal adipocyte differentiation (days 3-6) since CDK and MEK-1 (mitogen-activated protein kinase-1) inhibitors which prevent mitotic clonal expansion also block the differentiation process (41). Following a few rounds of mitotic division,

CDK inhibitors mediate cell cycle exit which sets the stage for PPARγ driven terminal adipocyte differentiation (29).

Because D-type cyclins represent a link between cell cycle progression, cell differentiation and transcriptional regulation, we wanted to explore their potential role during adipogenesis. We show here that cyclin D3 expression is up regulated during terminal stages of adipogenesis and functions as a ligand dependent coactivator of PPARγ capable of phosphorylating the A-B domain of the nuclear receptor. Knockdown of cyclin D3 diminished PPARγ activity and adipogenesis whereas cyclin D3 overexpression had the opposite effect. Consistent with these findings we show that cyclin D3 null mice are protected from diet induced obesity, have reduced adipocyte size and increased sensitivity to insulin.

Materials and methods

Chemical reagents and antibodies. Pioglitazone was provided by Takeda Pharmaceutical Company (Osaka, Japan) and rosiglitazone was purchased from Interchim (Montlucon, France). All were purchased from Sigma (St. Louis, MI). Anti-cyclin D3 (sc-6283)), PPARγ (sc-7273), PPARγ (sc-7196), and actin (sc-1615) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-BrdU antibody was bought from Dako A/S (Glostrup, Denmark)

Cell culture, transfections, and protein extracts. Cos and 3T3-L1 cells were grown in DMEM with 10% fetal bovine serum. In differentiation studies MDI (0.5 mM 3 Isobutyl-1-methylxanthine, 10 μg/ml insulin, and 1 μM dexamethasone) was added for 2 days. From day 3 on, 10 μg/ml insulin, and in certain cases 10^{-6} M pioglitazone was added. Nuclear extracts and Oil Red O staining were prepared as described (35) with the exception of cells being incubated with Oil-Red-O solution for only 90 seconds. For reporter assays, cells were transfected with 10ng of PPARγ and 300ng of cyclin D3 expression vectors using Lipofectamine (Life technologies, Rockville, MD). Luciferase and β-gal activity was measured as described (35). Stable 3T3-L1 cell lines were carried out by transfection of the pcDNA3-cycD3 vector and the control empty vector followed by selection with neomycin (500μg/ml) for 15 days.

Pull-Down, Coimmunoprecipitation, and chromatin immunoprecipitation. In-vitro translation of pSG5-PPARγ and pcDNA3-cycD3 was done using ^{35}S-methionine (Amersham, Orsay, France) in a TNT coupled reticulocyte lysate (promega, Madison, WI). Pull-down, immunoprecipitation assays were performed as described (9). ChIP

assays were performed using three 10cm plates per point according to the Upstate ChIP assay kit (Lake Placid, NY). Oligonucleotides used to amplify the mouse aP2 promoter are 5'-CCCAGCAGGAATCAGGTAGC-3' and 5'-AGAGGGCGGAGCAGTTCATC.

RNA isolation, quantitative real-time PCR and Northern Blot. RNA isolation was carried out using Rneasy Mini Kit (Qiagen Sciences, Maryland, USA) according to the manufacturers instructions. Reverse transcription of total RNA was performed at 42°C using MMLV reverse transcriptase enzyme and random hexanucleotide primers (Invitrogen, Carlsbad, CA), followed by 15 minute inactivation at 70°C. Quantitative PCR was carried out by real time PCR using a LightCycler and the DNA double-strand-specific SYBR Green I dye for detection (Roche, Basel, Switzerland). Results were normalized to GAPDH levels. Oligonucleotide sequences used for qRT-PCR are available upon request. A melting temperature of 60°C was used for all of the primers used above. Northern blot analysis was performed as described (23).

SiRNA against cyclin D3. To target cyclin D3 expression, siRNA sequences were designed against the 5' –CAGCGGGAGATCAAGCCGCACAT-3' sequence of the mouse cyclin D3 transcript. Oligonucleotides coding for a hairpin siRNA
were cloned into pRNAT-U6.1/Neo vector (GenScript Piscataway, NJ) as described by the manufacturer. Stable 3T3-L1cell lines expressing the pRNAT-U6.1/Neo αD3 and the pRNAT-U6.1/Neo-control vector were created by Lipofectamine transfection of the respective vectors followed by selection with neomycin (500μg/ml) for 14 days.

Western blot analysis and immunofluorescence. SDS-PAGE and electro-transfer were performed as described (38). The membranes were blocked at RT for 45 min in PBS, 0.5% Tween-20, 5% milk and incubated overnight at 4°C with the indicated

antibodies followed by 1 hour with a peroxidase conjugated secondary antibody at RT. The complex was visualized with a 4-chloro-1-naphtol reagent. For all immunofluorescence experiments cells were grown on cover slips and fixed with methanol at 4°C for 10 minutes. For BrdU incorporation, cells were additionally treated with 1.5 N HCL for 10 min at RT. After incubation with the indicated antibodies, cells were incubated with a combination of Texas red-conjugated anti mouse IgG and FITC-conjugated anti-rabbit IgG.

Plasmids and mutant constructs. pcDNA3 vector was purchased from Stratagene (La Jolla, CA). The pcDNA3-cyc D3 expression vector was created by excising the cyclin D3 insert from the pBABE-cyc D3 vector (gift from B. Amati) at BamH1/EcoR1restriction sites followed by ligation into the pcDNA3 vector. The TK-Luc, PPRE-TK-Luc, UAS TK-Luc, GST- PPARγ DE, GST- PPARγ b-AB, GST- PPARγ AB, gal4-PPARγ and PPARγ2 expression vectors have been previously described (13, 35). Cyclin D3 LxxLL point mutations were performed by PCR of pcDNA3-cyc D3 with the following primers GATCCCTGCCAGGAATTCTGTGAGCTCATC (for the Ct mutant), and GATGAGCTCACAGAATTCCTGGCAAGGGATC (for the Nt mutant). The Δ 1-129 deletion mutant was created by PCR amplification of pcDNA3-cyc D3 with the following primers, forward CGGGATCCCGCAAGTGGGACCTGGCTGCTGTGAT, and reverse, CGGAATTCCGGCGGCCCCTCCTCTGCTTAGTGG containing BamH1 and EcoR1 restriction sites respectively. WTcycD3, LxxLL point mutations and Δ 1-129 deletion mutants were cloned into pGex4T1 vector at BamH1/EcoR1 restriction sites . Δ 148-293

deletion mutant was created by digesting WTcycD3-pGex4T1 with PpuMI followed by ligation.

Kinase assays. Kinase assays were performed using 100 ng of an active cyclin D3/ CDK6 kinase (Upstate, Charlottesville, Virginia) and 250 ng of recombinant PPARγ protein as substrate (Active Motif, Carlsbad, CA). Reactions were performed in kinase buffer (25mM Tris/HCl pH7,5, 150mM NaCl, 10mMMgCl2, 1mM DTT) in the presence of 40 mM ATP and 8 mCi γ-^{33}P ATP for 30 minutes at 37 °C. The reaction was stopped by boiling the samples for 5 minutes in the presence of denaturing sample buffer. Samples were then subjected to SDS-PAGE electrophoresis, gels were then dried in a gel dryer for 1h at 80 °C and exposed to an X-ray film overnight.

Animal experiments. The cyclin D3 KO mice were a generous gift from P. Sicinski with whom their generation has been previously described (39). Animals were maintained according to EU guidelines for use of laboratory animals. Sections from WAT were fixed in 4% formaldehyde and stained with H&E. IPGTT and insulin sensitivity tests were performed as described (33). Cyclin D3 -/- and age matched WT mice were fed a lipid rich diet (58% fat, 25% carbohydrates, and 16% protein) for 8 weeks. All experiments were performed with 6 age and gender matched mice for each group.

Results

Cyclin D3 expression is up-regulated during adipogenesis. When hormonally stimulated, confluent 3T3-L1 preadipocytes reenter the cell cycle before they undergo differentiation (14). We correlated the expression of cyclin E, cyclin D3 and PPARγ during differentiation of 3T3-L1s. Protein levels of cyclin E were observed to increase after 1 day of differentiation coincident with cell cycle entry (Fig. 1A). After 2 days of differentiation cyclin E expression drops as differentiating cells exit from the cell cycle and the expression of adipogenic markers such as PPARγ is switched on. (Fig. 1A). Surprisingly, cyclin D3 protein levels were undetectable during the early stages of differentiation and were strongly induced during later stages after the cells had already exited from the cell cycle and began to express PPARγ. A similar expression pattern was observed by immunofluorescence microscopy (Fig. 1B). Interestingly we observed that cyclin D3 and PPARγ appeared to be co expressed in the same cells. To obtain further evidence of the expression pattern of cyclin D3 during adipogenesis we performed Northern blot analysis on differentiating 3T3-L1's. Such analysis revealed a potent increase in cyclin D3 mRNA throughout adipogenesis (Fig. 1C). This result was confirmed by quantitative real time PCR analysis (qRT-PCR), which revealed a three-fold increase in cyclin D3 mRNA throughout adipogenesis (Fig. 1D). Consistent with protein expression (Fig 1A), mRNA levels for cyclin E increase during days 1-2 and decreased thereafter, whereas expression of PPARγ increased strongly from day 1 through the end of differentiation. To determine the relative expression of cyclin D3

mRNA *in-vivo*, we performed qRT-PCR, comparing cyclin D3 mRNA expression in mouse white adipose tissue, brown adipose tissue, muscle, and liver. Interestingly we observed strongly elevated cyclin D3 expression in white adipose tissue as compared to other tissues (Fig. 1E), suggesting a possible role of this protein in adipose tissue biology. To determine whether cyclin D3 expression during adipogenesis was associated with an active cell cycle, we incubated differentiating 3T3-L1 cells with BrdU to mark proliferating cells. After a 24-hour incubation at day 5 of differentiation, cells were co-labeled with Brdu and cyclin D3 antibodies and visualized by fluorescence microscopy. We observed that only a limited percentage of cyclin D3 positive cells had incorporated BrdU (less than 12%) (Fig. 1F). These results suggest a cell cycle independent role for cyclin D3 during adipogenesis.

Cyclin D3 inhibition impairs adipogenesis. To elucidate the role of cyclin D3 during adipogenesis, we silenced cyclin D3 expression using siRNA techniques. 3T3-L1 cell lines stably expressing a vector coding for a hairpin siRNA sequence against the mouse cyclin D3 transcript or an irrelevant siRNA were compared for their ability to differentiate into adipocytes. After 6 days in differentiation media, normal lipid accumulation was observed in control cells whereas a dramatic decrease in lipid accumulation was observed in cyclin D3 knockdown cells as assessed by Oil Red O staining (Fig. 2A-B). Differentiated cyclin D3 knockdown cells express significantly reduced levels of cyclin D3 and slightly reduced PPARγ protein compared to control cells (Fig. 2B). Q-RT-PCR performed on differentiated 3T3-L1 cells revealed a dramatic reduction of adipogenic gene markers, including adiponectin, aP2, and LPL (lipoprotein

lipase), and a modest reduction of PPARγ expression when cyclin D3 was knocked down (Fig. 2C) further demonstrating the importance of cyclin D3 in adipogenesis.

Cyclin D3 overexpression stimulates adipogenesis. Since knockdown of cyclin D3 was inhibiting adipogenesis, we next tested whether overexpressing cyclin D3 could accelerate adipocyte differentiation. For this we created stable cell lines expressing the pcDNA3-cyclin D3 or control vector. After just 4 days in differentiation media, we observed increased lipid accumulation, increased PPARγ protein expression and up regulation of adipogenic mRNA markers as assessed by q-RT-PCR in cells over expressing cyclin D3 compared to control (Fig. 3A-3C). Immunofluorescence performed on differentiated 3T3-L1's (day 5) overexpressing cyclin D3 reveals a positive correlation between the level of cyclin D3 expression and that of PPARγ in individual cells (Fig 3D), further suggesting that cyclin D3 is an adipogenic factor.

Cyclin D3 stimulates PPARγ transcriptional activity. In light of our above observation showing co-expression of cyclin D3 and PPARγ in the same cells (Fig. 3D), we decided to explore a potential functional relationship between the two proteins. To test whether cyclin D3's stimulatory role during adipogenesis may be mediated though activation of PPARγ, we performed cotransfection experiments using a PPARγ responsive, luciferase-based, reporter construct (PPRE-TK-Luc) and expression vectors for PPARγ2 and cyclin D3. A 3.5 fold induction of luciferase activity was observed upon transfection of PPARγ2 in the presence of the PPARγ agonist pioglitazone (Fig. 4A). This induction was further enhanced up to 5.5 fold by co-transfection of cyclin D3. Transfection of cyclin D3 alone stimulated the PPARγ response element over two fold. No effects of either PPARγ or cyclin D3 were observed on the parental reporter vector

TK-luc which does not contain a PPRE (Fig. 1A, right panel). No induction of the PPRE-TK-Luc was observed after transfecting expression vectors coding for cyclin D1 and D2 (data not shown). We next tested whether the cotransfection of expression vectors coding for siRNA against cyclin D3 could attenuate the PPARγ mediated luciferase response. Cotransfection of siRNA vector against cyclin D3 reduced PPARγ mediated luciferase activation by approximately 2 fold (Fig. 4B). Together, these results suggest that cyclin D3's stimulatory role during adipogenesis is likely the result of its ability to modulate PPARγ activity.

Cyclin D3 physically interacts with PPARγ. To test whether the induction of PPARγ activity in the presence of cyclin D3 is the consequence of an interaction between PPARγ and cyclin D3, nuclear extracts from Cos cells transfected with cyclin D3 and PPARγ expression vectors were immunoprecipitated with an anti- PPARγ antibody. A 33-kDa protein was recognized by a cyclin D3 antibody indicating that cyclin D3 is associated with PPARγ (Fig. 5A, top panel). We performed the same immunoprecipitation on endogenous PPARγ from differentiated 3T3L1's (day 5) and also revealed an association between the two proteins (Fig. 5A, bottom panel). To identify the PPARγ domain responsible for the interaction with cyclin D3, GST-PPARγ DEF, AB and b-AB (where "b" contains an additional 30 aa subunit specific to the PPARγ2 transcript) fusion proteins were incubated with *in-vitro* translated ^{35}S radiolabled cyclin D3. We found that cyclin D3 binds to all three constructs of PPARγ (Fig. 5B). Next, to see if the association between cyclin D3 and the DEF construct of PPARγ which contains the ligand binding region of the receptor, could depend on ligand, we performed the same pull-down assay in the presence and absence of the PPARγ

ligand rosiglitazone. Interestingly, we observed a strong enhancement of the interaction between cyclin D3 and DEF-PPARγ construct in the presence of rosiglitazone (Fig. 5C). To see if the ligand dependent interaction between cyclin D3 and PPARγ DEF could also be observed using full length. PPARγ, we incubated GST-cyclin D3 with full length *in-vitro* translated ^{35}S radiolabled PPARγ. We observed no interaction enhancement between cyclin D3 and PPARγ in the presence of ligand, (Fig. 5D), possibly due to the masking of the ligand dependent effect by the additional contribution of the AB domain. We next set out to identify the region of cyclin D3 responsible for the interaction with PPARγ. Upon amino acid sequence screening of cyclin D3, we identified two LxxLL nuclear receptor interaction motifs located at the N and C terminal regions of the transcript (Fig. 5E). To test the contribution of these LxxLL motifs on the interaction with PPARγ, we performed site specific mutagenesis converting the second L to I and performed GST pull down with purified full length PPARγ. Despite mutations of both LxxLL sites, the interaction with PPARγ was not disrupted indicating that these sites do not contribute to the interaction with PPARγ (Fig. 5E, lane 4). Next, we created two deletion mutants of cyclin D3, Δ 1-149 which lacks the cyclin box (CDK binding unit), and deletion mutant Δ 148-293. GST pull-down assays reveal that amino-acid region 1-149 of cyclin D3 is required for the interaction with PPARγ (Fig. 5E).

To demonstrate that cyclin D3 could regulate the expression of PPARγ target genes *in-vivo*, we performed chromatin immunoprecipitation (ChIP) experiments on differentiating 3T3-L1 cells. As expected, when chromatin of cells after 5 days of differentiation was immunoprecipitated with an anti PPARγ antibody, we observed amplification of the region of the aP2 promoter containing the PPARγ response element

(PPRE). Immunoprecipitation of cyclin D3 in the same conditions also resulted in amplification of the aP**2** promoter (Fig. 5F, center panel). No amplification of the aP2 promoter was observed when either PPARγ or cyclin D3 were immunoprecipitated from confluent , non-differentiated 3T3Ll cells which do not express PPARγ nor cyclin D3 (Fig 5F, top panel). Binding of cyclin D3 and PPARγ was specific to the PPARγ binding site of the aP2 enhancer since no amplification was observed of a promoter region located outside the PPRE (Fig 5F, bottom panel). To see if the recruitment of cyclin D3 to PPARγ target genes is dependent on the presence of PPARγ, we performed additional ChIP experiments on NIH 3T3 cells, which do not express PPARγ, transfected or not with PPARγ2. We show that cyclin D3 is targeted to the PPRE of the aP2 promoter only when PPARγ is introduced into the cells by transfection (Fig. 5G). The recruitment of cyclin D3 to the aP2 promoter was however not found to depend on ligand (data not shown). The results of these ChIP assays demonstrate that cyclin D3 is recruited to the promoter of PPARγ target genes during adipogenesis and that this recruitment is dependent on the presence of PPARγ.

The cyclin D3/CDK6 complex phosphorylates PPARγ. Because cyclin D3 together with its cyclin dependent kinase partners CDK4/6 constitutes an active kinase that phosphorylates RB during the cell cycle, we wanted to investigate whether cyclin D3 could also participate in PPARγ phosphorylation. To test this hypothesis we performed *in-vitro* kinase assays using an active cyclin D3/CDK6 kinase complex and purified PPARγ protein as substrate. Kinase assays resulted in the apparition of a 60-kD band corresponding to the recombinant PPARγ molecular weight (Fig. 6A, lane 3). As a control, the kinase assay was performed in the absence

of PPARγ (Fig. 6A lanes 1 and 2). Next, to investigate which domain of PPARγ is phosphorylated by cylinD3/CDK6, we performed the same kinase assays on GST-PPARγ DEF and AB domains. The AB domain, but not the DEF domain was found to be phosphorylated by cyclinD3/CDK6 (Fig. 6B). To further investigate the functional significance of the PPARγ-AB domain in the activation by cyclin D3, we performed cotransfection experiments using a chimeric gal4 PPARγ lacking the AB domain and whose activity is measured via the UAS-TK-Luc reporter construct. As expected, when chimeric gal4 PPARγ is introduced into the system in the presence of ligand, a strong induction of luciferase activity was observed. However, unlike experiments performed with full length PPARγ, cotransfection of cyclin D3 with PPARγ, failed to increase the luciferase response (Fig. 6C). These results suggest that the AB domain of PPARγ is important for its activation by cyclin D3, possibly due the pressence of important phosphorylation sites. Next, to see if CDK6 could associate on the PPRE of the aP2 promoter and thereby contribute to the adipogenic process, we performed ChIP assays of CDK6 on differentiated 3T3L1s. PCR analysis of CDK6 immunoprecipitations confirmed its presence on the PPRE of the aP2 promoter (Fig. 6D), further suggesting a role for CDK6 during adipogenesis.

Cyclin D3 null mice display a compromised adipose tissue phenotype. We have shown that cyclin D3 and PPARγ are expressed during the same time in the differentiation process and that cyclin D3 binds PPARγ and activates its transcriptional potential. To determine whether the activating effect of cyclin D3 could apply to *in-vivo* models, we analyzed the adipose tissue phenotype of cyclin D3 -/- mice. Cyclin D3 -/- mice showed normal weight gain and initial examination of fat tissue mass revealed no

significant differences in weight between cyclin D3 -/- and WT mice (data not shown and Fig. 7A). Histological analysis of epididymal fat pads uncovered that adipocytes from cyclin D3 -/- mice were significantly smaller than WT mice (Fig. 7B and 7C), suggesting that cyclin D3 is important for normal adipocyte differentiation. Interestingly this difference was observed in mice at five months of age but was absent in three-month old mice whose adipocytes were still of premature size in both cyclin D3 -/- and WT groups (data not shown). Gene expression analysis of white adipose tissue taken from five month old mice demonstrated a marked decrease in adipogenic markers aP2, LPL, and PPARγ (Fig. 7D). We next analyzed the effect of challenging cyclin D3-/- mice with a high fat diet. After feeding the mice a lipid rich diet for 8 weeks, we observed a 30 % decrease in weight gain in cyclin D3-/- mice compared to their WT littermates (Fig. 7E). Because adipocyte size is also known to affect glucose homeostasis, we measured both glucose tolerance and insulin sensitivity in cyclin D3 -/- mice. Initial glucose measurements indicated that cyclin D3 -/- mice have a 30% decrease in fasting glucose levels (Fig 7F). Intraperitoneal glucose tolerance test (IPGTT), revealed that cyclin D3 -/- cleared glucose more efficiently than WT mice (Fig. 7G). Consistent with this observation, glucose decreased over two times more efficiently in cyclin D3 -/- compared to WT mice after insulin injection, indicating that the absence of cyclin D3 improves insulin sensitivity (Fig. 7H). Taken together, these *in–vivo* studies confirm our *in-vitro* data and suggest a crucial role for cyclin D3 in adipose tissue development.

Discussion

The results presented in this study establish a new role for cyclin D3 as a PPARγ co-factor. We show that cyclin D3 is preferentially expressed in adipose tissue and that its expression is strongly induced during terminal stages of 3T3-L1 adipogenesis. We have identified cyclin D3 as a PPARγ co-activator capable of phosphorylating its AB domain. The essential role that cyclin D3 plays during adipogenesis was highlighted by the observation that silencing its expression strongly inhibited adipogenesis whereas its over-expression promoted adipogenesis. Finally, we show that cyclin D3 -/- mice have a compromised adipose tissue phenotype. Our finding that cyclin D3 -/- mice display reduced adipocyte size, stunted adipogenic gene expression, resistance to high fat weight gain, and are insulin sensitive is reminiscent of the phenotype observed in PPARγ +/- mice (21). This observation is consistent with the hypothesis that the phenotype observed in cyclin D3 -/- mice is due to reduced PPARγ activity.

Over 20 years ago it was discovered how the ability of cyclins to bind and induce their CDK partners was dependent on their fluctuating expression pattern during the cell cycle (7). Here we show that the ability of cyclin D3 to bind PPARγ and help drive adipogenesis is also dependent on its differential expression pattern during adipogenesis. Strikingly the stimulatory function of cyclin D3 during adipogenesis seems to fall deliberately outside its cell cycle role as evidenced by its protein expression pattern; cyclin D3 is almost undetectable during the mitotic clonal expansion phase of adipogenesis and then its expression is strongly induced during the non cycling terminal differentiation stage. Interestingly, an up regulation of cyclin D3 expression has also been

documented in other differentiation processes including during hematopoiesis (12, 26) and colon development (3) (incidentally PPARγ is also strongly induced during the later (23). In the present study, we not only demonstrate an up regulation of cyclin D3 during adipocyte differentiation but also identify PPARγ as a functional partner through which cyclin D3 mediates its pro-adipogenic effects.

The stimulatory effects of cyclin D3 on PPARγ could be the direct result of phosphorylation of PPARγ (Fig. 4C). Regulation of PPARγ activity by phosphorylation has already been documented. While some studies have linked PPARγ phosphorylation with its activation (2), others have shown PPARγ phosphorylation inhibits its activity as is the case with MAP kinase mediated phosphorylation (16). Other cyclin/CDK complexes are able to phosphorylate nuclear receptors. This includes cyclin A/CDK2 driven phosphorylation and activation of the estrogen receptor alpha (36), the progesterone receptor (46), and the glucocorticoid receptor (20).

The notion that cyclin D3 functions as a PPARγ co-activator during adipogenesis is not completely unexpected. Several studies have emerged showing regulation of nuclear receptor biology by D-type cyclins. Cyclin D1 can activate estrogen receptor alpha transcription through a direct interaction with the ligand binding domain of the receptor (47). On the other hand cyclin D1, was shown to repress transcriptional activity of the thyroid hormone receptor (24), and the androgen receptor (31). Moreover, cyclin D1 can interact with several cofactors including SRC1, (47), P/CAF (27), HDAC 3 (24, 31) and TAF250 (1). While less well explored, cyclin D3 has also been implicated in the regulation of nuclear receptors including activation of the retinoic acid receptor (5). In

addition, cyclin D3 can bind the SRC family coactivator GRIP-1 thereby disrupting its association with transcriptional regulators (22).

Recently, Pestell and colleagues reported that cyclin D1 represses PPARγ expression (45). In addition to inhibiting PPARγ promoter activity, they show that cyclin D1 retards adipogenesis and correlate this block with reduced PPARγ expression and activity. Remarkably they show that mouse embryonic fibroblasts (MEFs) from cyclin D1 -/- mice have elevated levels of PPARγ even prior to inducing differentiation. Our lab and others have observed cyclin D1 expression to rapidly decrease after the mitotic clonal expansion phase of 3T3-L1 differentiation ((32) and data not shown), consistent with the finding that PPARγ inhibits cyclin D1 expression (44). Taken together, this information has allowed us to develop a model on how D-type cyclins are orchestrating the molecular events taking place during adipocyte differentiation. Before adipogenesis is induced and immediately after its induction, elevated levels of cyclin D1 blocks immature expression of PPARγ. After a couple of rounds of mitotic clonal expansion, cyclin D1 levels rapidly shoot down thereby releasing its inhibitory effect on PPARγ expression and poising the cell for terminal differentiation. Once PPARγ protein is induced, cyclin D3 expression is increased allowing it to bind and activate PPARγ. Such a model is dependent on the timely expression profiles of both cyclin D1 and D3 and highlights their distinctive cell cycle independent roles during adipogenesis.

In the present study we have established a link between the cell cycle machinery and adipogenesis. The metabolic response needed for growth and or calorie storage is under the direct control of extra-cellular nutrients, growth factors, and hormones. Growth stimuli including glucose, insulin, and glucocorticoids are known to have an immediate

mitogenic effect, however the pathways by which these nutrients initiate the metabolic response are poorly understood. Here we shed new light on this question by showing how cyclin D3 can functionally bind and activate the master regulator of adipogenesis, PPARγ. Thus we propose cyclin D3 as a type of metabolic sensor linking external nutritional stimuli with the metabolic growth response.

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Figure legends:

Figure 1. Expression pattern of cyclin D3 during adipogenesis.

(A) Western blot analysis of whole-cell extracts prepared at the indicated days of 3T3-L1 adipocyte differentiation. The proteins detected by cyclin E, cyclin D3, and PPARγ antibodies are indicated.

(B) Analysis of cyclin D3 and PPARγ protein expression by immunofluorescence during the first 4 days of 3T3-L1 differentiation. Cells expressing PPARγ are labeled in red (Texas red) whereas cells expressing cyclin D3 are labeled in green (FITC). Nuclei were stained with the Hoechst reagent (blue staining).

(C) Northern blot analysis of cyclin D3 mRNA expression during adipocyte differentiation, 28s is used as a loading control.

(D) Relative cyclin E, cyclin D3, and PPARγ gene expression measured by qRT-PCR during 3T3-L1 differentiation.

(E) Relative expression of cyclin D3 mRNA as measured by qRT-PCR in different mouse tissues (WAT= white adipose tissue, BAT=brown adipose tissue, MUS= muscle, and LIV= liver). Results were normalized to the expression of GAPDH.

(F) Analysis of cyclin D3 protein expression and BrdU by immunofluorescence at day 5 of differentiation. After a 24 hour incubation with BrdU cells which have incorporated BrdU are labeled in red while cells expressing cyclin D3 are labeled green, nuclei are visualized with Hoechst staining.

Figure 2. Cyclin D3 knockdown inhibits 3T3-L1 adipogenesis.

(A) Micrographs of Oil Red O-staining of cyclin D3 silenced 3T3-L1's (siRNA-cycD3) and control (siRNA-mock) after 6 days of differentiation (top panel). Middle panel confirms knockdown of cyclin D3 by immunofluorescence assay. Nuclei are stained in blue (Hoechst).

(B) Western blot showing knockdown of cyclin D3 expression in 3T3-L1 cells expressing a stable vector coding for a siRNA sequence directed against mouse cyclin D3 and corresponding PPARγ protein expression.

(C) QRT-PCR showing gene expression of the adipogenic markers adiponectin, aP2, LPL, and PPARγ in knockdown vs. control differentiation experiments. Results are expressed as fold repression as compared to control.

Figure 3. Cyclin D3 over expression stimulates 3T3-L1 adipogenesis.

(A) Western blot showing cyclin D3 and PPARγ expression in 3T3-L1 cells overexpressing cyclin D3 and control cells.

(B) Oil Red O micrographs taken from stable 3T3-L1 cells overexpressing cyclin D3 (pcDNA3-cycD3) and control (pcDNA3-) after 4 days of differentiation.

(C) Gene induction change of adipogenic markers as measured by qRT-PCR resulting from cyclin D3 overexpression after 4 days of differentiation.

(D) Immunofluorescence showing correlation between PPARγ expression intensity and cyclin D3 in differentiated (day5) 3T3L1s overexpressing cyclin D3.

Figure 4. Cyclin D3 stimulates 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 D3, PPARγ or both plasmids together. The experiments were performed in triplicate in the presence or absence of the PPARγ agonist pioglitazone (10^{-6}M) and were normalized for β-gal activity. The same transfections were performed using a TK-luc vector which does not contain the PPRE.

(B) Knockdown of cyclin D3 by transfection of expression vectors coding for siRNA against cyclin D3 abrogates PPARγ transcriptional activity.

Figure 5. Cyclin D3 interacts with PPARγ

(A) Co-immunoprecipitation of PPARγ and cyclin D3 from Cos cells transfected with the expression vectors for both proteins (top panel) and in 5 day differentiated 3T3L1 cells (bottom panel). Whole cell extracts were immunoprecipitated with either PPARγ or mock (no antibody) and blots were revealed by an anti-cyclin D3 antibody.

(B) GST-pull down assay showing details of the PPARγ -cyclin D3 interaction. In vitro translated ^{35}S-radiolabled cyclin D3 protein was incubated with GST- PPARγ AB (residues 1-146), b-AB (contains an additional 30 aa specific for PPARγ 2 transcript), GST- PPARγ DEF (residues 203-477), and GST alone.

(C) The same experiment was performed using the DEF domain in the presence and absence of rosiglitazone, 10^{-6}M.

(D) GST-pull down between GST-cyclin D3 and full length *in-vitro* translated ^{35}S-radiolabled PPARγ protein in the presence and absence of rosiglitazone 10^{-6}M.

(E) GST-fusion wild type cyclin D3 (WTD3), deletion, and point mutations were constructed and tested for their ability to bind full length recombinant PPARγ. After washing beads, PPARγ was revealed by western blot**.**

(F) Chromatin immunoprecipitation (ChIP) assays demonstrating binding of cyclin D3 to the region of the aP2 promoter carrying the PPARγ responsive element. Cross-linked chromatin from either confluent (top panel) or from 5 day differentiated 3T3-L1s (bottom panel) was incubated with antibodies against PPARγ, cyclin D3, or non-specific antibody (mock). Immunoprecipitates were analyzed by PCR using primers specific for the mouse aP2 promoter containing the PPRE and by primers amplifying a region outside the PPRE (negative control). A sample representing 0.1% of total chromatin was included in the PCR (Input).

(G) The same experiment was performed on NIH-3T3 cells transfected or not with PPARγ.

Figure 6. Cyclin D3/CDK6 phosphorylates PPARγ.

(A) In-vitro kinase assay showing phosphorylation of purified full length PPARγ by cyclin D3/CDK6 complex (lane 3). As a control, assays were performed in the absence of PPARγ (lane 1) or cyclin D3/CDK6 (lane 2).

(B) The same kinase assay was performed using PPARγ-AB and DEF domain fusion proteins.

(C) Activity of the UAS-TK-Luc reporter carrying the gal4 specific response element measured in Cos cells upon transfecting expression vectors for cyclin D3, and chimeric gal4 PPARγ containing a gal 4 DBD and lacking the AB domain. The experiments were

performed in triplicate in the presence or absence of the PPARγ agonist pioglitazone and were normalized for β-gal activity.

(D) ChIP assays showing association of CDK6 with the PPRE of the aP2 promoter in differentiated 3T3-L1s (day 5). Cross-linked chromatin was incubated with antibodies against PPARγ, cyclin D3, CDK6 or non-specific antibody (mock). Immunoprecipitates were analyzed by PCR using primers specific for the mouse aP2 promoter containing the PPRE and by primers amplifying a region outside the PPRE (negative control). A sample representing 0.1% of total chromatin was included in the PCR (Input).

Figure 7. Analysis of adipose tissue phenotype in cyclin D3 deficient mice.

(A) Epididymal fat pad weight relative to total body weight for cyclin D3 -/- and +/+ mice.

(B-C) Histological analysis of epididymal WAT from cyclin D3 -/- and +/+ mice and corresponding histograms of adipocyte cell size.

(D) Gene expression profiles of pertinent adipogenic genes from WAT measured by qRT-PCR.

(E) Weight gain curves for cycD3 -/- and +/+ mice fed a lipid rich diet for 8 weeks. Each group was composed of 6 animals weighed on a weekly basis. Weight gain is adjusted to total initial body weight.

(F) Fasting plasma glucose levels in cyclin D3 -/- and +/+ mice.

(G) IPGTT measuring the glucose levels at different times after intraperitoneal injection of glucose in cyclin D3 -/- and +/+ mice. (H) Glucose clearance after intraperitoneal

injection of insulin (0.75 IU/kg) as a measure of insulin sensitivity in cyclin D3 -/- and +/+ mice.