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Summary

Cell cycle regulators, such as E2F1 and Retinoblastoma (RB) play crucial roles in the control of adipogenesis, mostly through the control of the transition between preadipocyte proliferation and adipocyte differentiation. Cyclin-dependent kinase 4 (cdk4) is a serine/threonine kinase that works in complex with D-type cyclins to phosphorylate RB mediating the commitment of the cells to enter cell cycle in response to external stimuli. Since cdk4 is an upstream regulator of the E2F-RB pathway we hypothesized that cdk4 is a good target for new factors that regulate adipogenesis. In this study we found that cdk4 inhibition impairs adipocyte differentiation and function. Disruption of cdk4 or activating mutations in cdk4 in primary mouse embryonic fibroblast, results in reduced and increased adipogenic potential respectively of these cells. We show that the effects of cdk4 are not limited to the control of differentiation but cdk4 also participates in adipocyte function through activation of PPARγ.
**Introduction**

Cell proliferation and differentiation have been considered to be mutually exclusive events; however, a close relationship has been established between both cell processes during the adipocyte differentiation program (reviewed in (Fajas, 2003)). Re-entry into cell cycle is one of the key events taking place in early adipogenesis, since inhibition of DNA synthesis at this stage blocks differentiation (Patel and Lane, 2000; Reichert and Eick, 1999). Like in most cells, the transition from growth arrested preadipocytes into S-phase likely depends on the reactivation of the G1 cyclins/cdkks and the retinoblastoma protein pRB-E2F pathway that controls the G1/S transition of the cell cycle. Association of E2Fs with proteins of the pRB family facilitates active repression through recruitment of histone deacetylases (Brehm et al., 1998; Magnaghi-Jaulin et al., 1998). Upon re-entry into cell cycle of these growth arrested preadipocytes, the members of the retinoblastoma family, are phosphorylated by the cyclin/cdk holoenzymes, releasing the E2F complex, resulting in the activation of the E2F target genes (Richon et al., 1997). We have previously shown that cell cycle regulators, such as E2F1 and RB play crucial roles in the control of adipogenesis, mostly through the control of the transition between preadipocyte proliferation and adipocyte differentiation. E2F1 positively regulates the expression of peroxisome proliferator-activated receptor gamma (PPARγ) (Fajas et al., 2002b), which is the master regulator of adipocyte differentiation, whereas RB inhibits PPARγ activity through direct protein-protein interaction (Fajas et al., 2002a). PPARγ is a ligand-activated transcription factor belonging to the nuclear receptor superfamily. PPARγ is preferentially expressed in the adipose tissue and upon activation by fatty acids derivatives or antidiabetic thiazolidinediones, PPARγ drives the expression of several adipocyte-specific genes, such as the fatty acid binding protein (aP2) (for review see (Debril et al., 2001)). Since cyclin-dependent kinase 4 (cdk4), which mediates the commitment of the cells to enter cell cycle in response to external stimuli (reviewed in (Ortega et al., 2002)) is an upstream regulator of the E2F-RB pathway we hypothesized that cdk4 would be a good target in the search for new factors that
regulate adipogenesis. Cdk4-/- mice develop insulin-deficient diabetes due to reduced β-cell pancreatic mass. These mice have reduced body weight and are smaller (Rane et al., 1999) (Tsutsui et al., 1999). Furthermore, mice expressing an activating mutation of cdk4 (R24C) that cannot bind the cell cycle inhibitor P16INK4a show pancreatic hyperplasia and increased body weight.

We show in this study that cdk4 participates in adipose tissue biology, not only through the control of the clonal expansion phase of adipogenesis, but also regulating PPARγ activity and terminal differentiation and function of adipocytes.

**Results**

**Cdk4 is expressed and active in non-proliferating mature adipocytes**

While evaluating the expression of cell cycle regulators implicated in the transition between preadipocyte proliferation and adipocyte differentiation, we found that cdk4 protein was expressed at similar levels at all stages of differentiation of 3T3-L1 preadipocytes as measured by immunofluorescence assays (Fig.1A). Interestingly, we observed changes in the cellular localization of cdk4 by confocal microscopy. At confluence (day 0) cdk4 expression was mainly cytoplasmic, whereas differentiated adipocytes (day 5) expressed mainly nuclear cdk4 (Fig. 1A). Interestingly, nuclear colocalization of cdk4 and PPARγ could be observed by confocal microscopy in differentiated 3T3-L1 adipocytes (Fig. 1B). Differentiation of the cells was quantified by measuring the mRNA expression of the adipogenic marker aP2 (Fig. 1C). Similar to 3T3L1 cells, no significant changes in the expression of cdk4 mRNA were observed during differentiation of primary human preadipocytes whereas PPARγ expression was increased (Fig. 1D). Consistent with these experiments we found that cdk4 was expressed in mature adipocytes of mouse adipose tissue as assessed by immunohistochemical analysis of mouse adipose tissue sections (Fig. 1E). These results suggested a role of cdk4 in adipose tissue biology and differentiation.

To further assess the participation of cdk4 in differentiated adipocytes, kinase activity experiments were performed. Immunoprecipitated cdk4 from either differentiated
3T3-L1 adipocytes (Fig. 1F, lane 2) or freshly prepared primary mice adipocytes (Fig. 1F, lane 5) was able to phosphorylate a purified recombinant retinoblastoma protein in vitro, indicating that cdk4 was indeed active in adipocytes (Fig. 1F). No phosphorylation of RB was observed when rabbit antiserum was used to immunoprecipitate the extracts (Fig. 1F, lanes 3, 4, 6). Cdk4 was also inactive in quiescent, non-differentiated 3T3-L1 preadipocytes (Fig. 1F, lane 1). Since cdk4 activity is related to the control of cell cycle, we next evaluated DNA synthesis in 3T3-L1 adipocytes by BrdU incorporation assays. As expected most of cdk4-expressing 3T3-L1 preadipocytes incorporated BrdU indicating that these cells were proliferating (Fig. 1G). In contrast, only a small proportion of cdk4-expressing 3T3-L1 adipocytes were positive for BrdU incorporation (Fig. 1G), demonstrating that cdk4 was expressed in non-proliferating cells and suggesting that the detected cdk4 activity in differentiated cells could be independent of the control of cell cycle (Fig. 1F-G).

**Inhibition of cdk4 activity impairs adipogenesis**

Participation of cdk4 in adipogenesis was further studied. Differentiation of 3T3-L1 cells was induced with a typical hormonal mix either in the absence or in the presence of the specific cdk4 inhibitor 2-Bromo-12,13-dihydro-5H-indolo(2,3-a)pyrrolo[3,4]carbazole (IDCX) (Zhu et al., 2003). Oil red-O staining indicated that IDCX was a potent inhibitor of 3T3-L1 adipocyte differentiation (Fig. 2A). This was consistent with a decrease in the expression of the adipogenic markers PPARγ and aP2 in the presence of the inhibitor (Fig. 2B). Similar results were observed when the cdk4 inhibitor I3M was used (data not shown). The inhibition of cdk4 kinase activity in these cells was demonstrated by in vitro kinase assays (data not shown).

After hormonal induction, 3T3-L1 preadipocytes re-enter cell cycle before terminally differentiating into adipocytes. This clonal expansion phase (days 1 and 2) is required for the differentiation process of the 3T3-L1 cells. It would be therefore plausible that cdk4 participates in adipogenesis by inducing the proliferative clonal expansion phase. This was consistent with the observation that cdk4 inhibition with IDCX
abrogated the clonal expansion of hormonally induced 3T3-L1 cells as analysed by BrdU incorporation experiments (Fig. 2C, day 1). Furthermore, the expression of the cell cycle markers cyclin E and cyclin B was decreased in the presence of IDCX, consistent with the BrdU incorporation experiments (Fig. 2D). To further elucidate whether participation of cdk4 was limited to the control of the proliferative phase of the adipocyte differentiation process, we inhibited cdk4 activity with IDCX after completion of clonal expansion at day 2. Interestingly, adipocyte differentiation was also inhibited under these conditions as assessed by oil-red-O staining (Fig. 2E) and by the expression of adipocyte marker genes (Fig. 2F). BrdU incorporation studies demonstrated that the cells were not proliferating at the time of IDCX incubation (Fig. 2G). These results suggested that active cdk4, in addition to its role in the control of cell cycle during the clonal expansion phase, is also required during terminal differentiation in a cell cycle independent manner.

A second cellular model was next used to further demonstrate the implication of cdk4 in adipocyte differentiation. F442A cells were induced to differentiate into adipocytes in the absence or in the presence of IDCX. Lipid incorporation (data not shown), as well as the expression of adipocyte markers indicated that cdk4 inhibition resulted in decreased adipocyte differentiation when compared with F442A differentiated in the absence of IDCX (Fig. 2H). As for 3T3-L1 cells, F442A preadipocytes re-entered cell cycle after insulin induction of differentiation. Surprisingly, IDCX did not prevent re-entry into cell cycle of differentiating cells as measured by BrdU incorporation (Fig. 2I) and expression of cyclin E and cyclin B (Fig. 2J). Yet, IDCX decreased differentiation to a similar level to what observed in 3T3-L1 cells, suggesting that, in F442A cells, cdk4 inhibition prevents adipogenesis independently of the control of cell cycle.

**Cdk4 overexpression enhances adipogenesis.**

To test the hypothesis that cdk4 is a positive factor for adipocyte differentiation, a 3T3-L1 stable cell line constitutively expressing cdk4 was generated by retroviral
infection (pBabe-cdk4). Oil red-O staining indicated an increased capacity to differentiate into adipocytes of pBabe-cdk4 cells compared to 3T3-L1 cells infected with an empty retrovirus (pBabe) 4 days after induction of adipogenesis (Fig. 3A). Overexpression of cdk4 could not bypass, however, the requirement of differentiation medium in order to differentiate (data not shown). Interestingly, 3T3-L1 cells infected with a retrovirus expressing a kinase-dead cdk4 mutant (pBabe -K35M) lost their capacity to differentiate (Fig. 3A), suggesting that the positive effects on adipogenesis are mediated by the kinase activity of cdk4. Cdk4 protein was highly expressed, as expected, in pBabe-cdk4 and pBabe-K35M cells, compared to pBabe 3T3-L1 cells (Fig. 3B). Consistent with the oil red-O assays, real time PCR analyses showed increased levels of aP2 up to 2-fold in pBabe-cdk4-3T3L1 cells, whereas no such increase was observed in cells infected with pBabe-K35M (Fig. 3B).

Mice deficient for cdk4 have decreased body weight (Rane et al., 1999) (Tsutsui et al., 1999), decreased fat mass and smaller adipocytes (unpublished results). In contrast, mice expressing an hyperactive cdk4 mutant have increased weight (Rane et al., 1999), increased fat mass and bigger adipocytes (unpublished results). The phenotype of cdk4-/- and cdk4R24C mice suggest a defect in adipose tissue homeostasis. However, cdk4-/- mice are hypoinsulinemic as a result of diabetes, and cdk4 R24C mice develop pancreatic islet β-cell hyperplasia with increased production of insulin. It could be therefore possible that the effects on adipogenesis in these mice are secondary to insulin signaling and not directly related to adipose tissue development. To test this notion, we compared the capacity of cdk4-/- or cdk4R24C primary isolated MEFs to differentiate into adipocytes in vitro in response to hormone stimulation. Adipocytes were scored using oil red-O staining to detect lipid droplets and mRNA expression of adipogenic markers was quantified. Consistent with a direct effect of cdk4 in adipogenesis, the capacity of cdk4-/- MEFs to differentiate into adipocytes was totally inhibited (Fig. 3C). Conversely, hormonally stimulated cdk4R24C MEFs showed a robust increase in their capacity to differentiate into adipocytes in vitro, when compared to wild type MEFs (Fig. 3C). Furthermore, when
expression of cdk4 was rescued by retroviral infection cdk4-/ MEFS could normally differentiate, demonstrating that inhibition of differentiation in cdk4-/ MEFS was directly the result of the lack of cdk4 (Fig. 3C). Re-expression of cdk4 in cdk4-/ MEFS was verified by QPCR analysis (Fig. 3D). Gene expression analysis of the adipose tissue specific gene aP2 further demonstrated the decreased and increased number of cdk4-/ and cdk4R24C MEF’s-derived adipocytes respectively, as well as the increased number of adipocytes in rescued cdk4-/ MEFS (Fig. 3E). Taken together, these data suggest that cdk4 directly stimulates adipogenesis.

**Cdk4 participates in adipocyte biology**

In addition to its participation in adipocyte differentiation, we next wanted to analyse whether cdk4 have a role in adipocyte biology. First, we performed glucose uptake experiments in fully differentiated 3T3-L1 adipocytes in response to cdk4 inhibitors. 24 hours IDCX treatment inhibited in a dose-dependent manner the incorporation of radioactive glucose into adipocytes in response to insulin (Fig. 4A). Interestingly, basal glucose uptake was increased upon incubation of high doses of IDCX when compared to cells treated with vehicle only (Fig. 4A). To exclude the possibility that the observed differences in the response of the cells to insulin were the result of a distinct stage of differentiation of the cells, the expression of aP2 mRNA was measured, and no differences were found (Fig. 4B).

Consistent with decreased insulin sensitivity the expression of genes such as Glut-4, insulin receptor (IR), insulin receptor substrate-1 (IRS1), IRS2, and PI3K, which are key proteins in glucose transport and glucose homeostasis, was also decreased in the presence of IDCX (Fig. 4C). Strikingly, Glut-1 mRNA expression was increased upon incubation with IDCX, which was consistent with the increased basal glucose uptake observed under these conditions.

Finally, the expression of genes implicated in lipogenesis was analysed. Fatty acid synthase (FAS), and phosphoenol pyruvate carboxy kinase (PEPCK) mRNA expression was decreased in IDCX treated adipocytes (Fig. 4D), which was
consistent with decreased lipid load in these cells. No changes in the expression of genes implicated in lipolysis was observed in these cells (data not shown). These results suggested that cdk4 inhibition impaired adipocyte function decreasing lipogenesis and glucose transport and metabolism in these cells.

**Cdk4 activates PPARγ through direct interaction**

PPARγ plays a crucial role in the adipocyte differentiation process. Any factor modulating PPARγ activity has major effects on adipogenesis. We therefore tested the ability of cdk4 to activate PPARγ in transient transfection experiments in COS cells, using a PPARγ-responsive luciferase reporter (PPRE-TK-Luc), and PPARγ and cdk4 expression vectors. A 3-fold induction of luciferase activity was observed upon transfection of limiting concentrations of PPARγ in the presence of pioglitazone. This induction was significantly increased up to 6-fold by cotransfection of cdk4 (Fig. 5A), suggesting that cdk4 activates PPARγ. Interestingly, the cdk4 K35M kinase-dead mutant was not able to increase PPARγ activity (Fig. 5A), which was consistent with the lack of effects of this mutant on adipogenesis (Fig. 3A). Furthermore, cdk4 inactivation by the specific cdk4 inhibitor IDCX resulted in the attenuation, in a dose-dependent manner, of PPARγ activation by pioglitazone (from 3- to 1-fold; Fig. 5B). Consistent with these results we found that either pioglitazone or rosiglitazone treatment of cdk4-/MEFs in which cdk4 expression was rescued by retroviral infection resulted in a 3-fold induction of the PPARγ target aP2 mRNA, whereas only a 1,5-fold induction was observed when cdk4-/MEFs infected with empty retrovirus were treated (Fig. 5C, left panel). PPARγ was equally expressed in both cell lines (Fig. 5C, right panel). Next, to test whether the increase in PPARγ activity in the presence of cdk4 was the consequence of an interaction with PPARγ, total cell extracts from PPARγ- and cdk4-transfected COS cells were immunoprecipitated with an anti-PPARγ antibody. A 33 kD protein was recognized in the immunoprecipitates by an anti-cdk4 antibody, indicating that cdk4 interacted in vivo with PPARγ (Fig. 5D). Furthermore, when extracts from cdk4- expressing differentiated 3T3-L1 adipocytes
were immunoprecipitated using an anti PPARγ antibody, endogenous cdk4 protein was associated to PPARγ (Fig. 5E). Deletion experiments in the cdk4 protein indicated that the K35 aminoacid in the ATP binding domain of cdk4 was not implicated in the PPARγ interaction. However, deletion of aa 203-295 abolished binding to PPARγ (Fig. 5F). Interestingly, GST-pull down experiments indicated that cdk4 is able to bind both, the AB domain of PPARγ and the DEF domain, which contain the ligand-independent and the ligand-dependent transactivation domains respectively (Fig. 5G).

Finally, to further prove that cdk4 is associated with PPARγ and that it activates PPARγ-mediated transcription, chromatin immunoprecipitation studies of the aP2 promoter were performed in both non-differentiated and differentiated 3T3-L1 adipocytes. A 200 bp fragment of the mouse aP2 promoter, containing the binding site of PPARγ was amplified by PCR when anti-cdk4, anti-PPARγ, or anti-acetylated histone H4 antibodies were used to immunoprecipitate chromatin from differentiated 3T3-L1 cells (Fig. 5H). No amplification product was observed when immunoprecipitated chromatin from confluent, non-differentiated 3T3-L1 preadipocytes was used as template (data not shown). The results of the ChIP assays demonstrate that the complex cdk4-PPARγ is present in the promoter of PPARγ target genes. Moreover, the presence of acetylated histone H4 on the PPARγ binding site of the aP2 promoter suggests that in the presence of cdk4 this promoter is active. These results suggest that the positive effects of cdk4 on adipogenesis are the result of cdk4-mediated increase of PPARγ activity through cdk4 kinase activity.

Discussion

Cdk4 is the catalytic subunit of the cyclin D-cdk holoenzyme. The kinase activity of this complex is induced in response to extracellular signals, including growth factors, and translate signals from extracellular environment into cell cycle activation (Matsushime et al., 1991). We have recently shown that the transcription factor E2F1, which is the effector of the cyclin/cdk pathway in cell cycle regulation,
mediates the transition between preadipocyte proliferation and adipocyte differentiation through activation of the expression of PPARγ, which is the master regulator of adipogenesis. The most studied role of cdk4 is phosphorylation of the retinoblastoma family proteins, that repress E2F1 activity, thereby facilitating the release of E2F1 from this complex, resulting in the activation of E2F1 target genes. Since cdk4 is an upstream regulator of the E2F1/RB pathway it is therefore likely that cdk4 also participates in adipocyte differentiation. Consistent with this, we found that cdk4 activity is required for adipogenesis. We propose that cdk4 plays a dual role in this process (Fig. 6). On the one hand, cdk4 participates in the clonal expansion phase of adipocyte differentiation phosphorylating RB and therefore facilitating activation of E2Fs complexes, which will trigger the transcription of PPARγ and likely other factors implicated in terminal differentiation. This is so far consistent with the known functions of cdk4. On the other hand we found, surprisingly that participation of cdk4 was not limited to the control of cell cycle and E2F1 activity in the proliferative phase of adipocyte differentiation, but we found that cdk4 has also a positive, cell cycle-independent role in terminal differentiation and function of adipocytes. This is supported by three main observations. First, cdk4 is expressed in fully differentiated adipocytes in both human and mice adipose tissue (Fig. 1 and (Phelps and Xiong, 1998)). These differentiated adipocytes do not express E2F1 (Fajas et al., 2002b) and are not proliferating (Fig. 1). Furthermore, cdk4 activity can be detected in differentiated 3T3-L1 adipocytes and in mice adipose tissue (Fig. 1 and (Phelps and Xiong, 1998)). Second, inhibition of cdk4 activity with specific cdk4 inhibitors, impairs adipocyte differentiation regardless of the inhibitor is added before or after the proliferative phase of adipogenesis (Fig. 2). Moreover, cdk4 inhibitors do not block the proliferative phase of F442A preadipocyte differentiation process, and yet inhibit adipogenesis in this system. We cannot explain, at present, why IDCX did not affect the clonal expansion phase of F442A cells. Finally, cdk4 interacts with PPARγ, resulting in increased transactivation activity of PPARγ (Fig. 5). Furthermore, cdk4 can be found in the PPARγ-responsive element of the aP2 promoter (Fig. 5).
We can conclude from our results that cdk4 not only regulates adipocyte differentiation, but also participates in adipocyte biology. Cdk4 might act as a sensor of homeostatic signals. Nutrition and the subsequent hormonal signaling, such as insulin, results in the activation of preadipocyte differentiation and lipogenic pathways in already pre-existing adipocytes. In support of this hypothesis, the experiments in F442A cells show that inhibition of cdk4 in these cells results in decreased lipogenesis rather than differentiation. As a proof of the participation of cdk4 in adipocyte biology, we observed decreased insulin sensitivity, reduced glucose uptake, and decreased expression of genes implicated in lipogenesis and insulin signaling such as IRS1 and 2, Glut4, PI3K, FAS, or PEPCK in 3T3-L1 adipocytes in which cdk4 activity was inhibited. In contrast with this, we found that cdk4 inhibition resulted in increased Glut1 mRNA expression and enhanced basal glucose uptake in 3T3-L1 adipocytes. We cannot explain at present how cdk4 regulates basal glucose uptake and further studies are required.

The role of cdk4 as a growth signal sensor is underscored in cdk4-/- mice, which have impaired post-natal pancreatic β-cell growth resulting in severe atrophy and diabetes (Mettus and Rane, 2003; Rane et al., 1999). Furthermore, cdk4-/- and D-type cyclins -/- mice show reduced body size, stressing the importance of cyclin D/cdk4 complex in the control of tissue growth (Kozar et al., 2004; Mettus and Rane, 2003; Rane et al., 1999). In contrast, cdk4R24C mice have increased body weight (Rane et al., 1999). Cdk4R24C mice express a mutant cdk4 protein (R24C) which is not inhibited by the cdk inhibitor p16, rendering cdk4 hyperactive (Zuo et al., 1996). Supporting our results of the participation of cdk4 in adipose tissue development is the observation that cdk4-/- mice have reduced adipose tissue mass and adipocyte size, whereas cdk4R24C mice have increased fat mass and adipocyte size (unpublished results). Unfortunately a more in deep metabolic analysis of this mice is constrained by their diabetic status. We have, however demonstrated that primary embryonic fibroblasts from cdk4-/- mice have lost their capacity to differentiate into adipocytes (Fig. 3). Most importantly, when we analysed MEFs from mutant...
cdk4R24C mice, which express hyperactive cdk4 we found that these cells had increased adipogenic potential.

The positive role of cdk4 in adipogenesis is likely the result of its interaction with PPARγ (Fig. 5). Strikingly, the decreased size of adipocytes observed in cdk4-/- mice (our unpublished results) is reminiscent to what is observed in PPARγ +/- mice adipocytes (Barak et al., 1999; Kubota et al., 1999). It is also evocative of the decreased cdk4 activity observed upon energy restriction (Jiang et al., 2003), a situation where lipogenesis is inhibited and PPARγ activity is also decreased. Interaction of PPARγ with cdk4 is a new mechanism to activate PPARγ. This broadens the range of signals that can activate the transcriptional activity of PPARγ further refining the response of the receptor, in conjunction with PPARγ ligands, to accommodate to the cellular needs. Several members of the cdk family have been shown to phosphorylate and modulate the activity of other members of the nuclear receptor superfamily, which also act as mediators in a variety of signalling pathways through integrating diverse phosphorylation events (reviewed in (Rochette-Egly, 2003)). Of particular interest is the phosphorylation and activation of the glucocorticoid receptor (Krstic et al., 1997), the estrogen receptor alpha (Rogatsky et al., 1999), and likely the progesterone receptor (Zhang et al., 1997) by cdk2. Together with our results on regulation of PPARγ activity and adipogenesis by cdk4, these studies clearly support a role for cdks in hormonal signaling.

The effects of cdk4 on PPARγ activity could be the result of a direct phosphorylation of PPARγ. When we tested this hypothesis we found that PPARγ was not phosphorylated in vitro by cdk4 in several experimental conditions (data not shown), suggesting that other mechanisms are mediating cdk4 activation of PPARγ. We know from our results that the kinase activity of cdk4 is, however, required to activate PPARγ and promote adipogenesis. One interesting hypothesis to explain the kinase activity requirement could be the phosphorylation of PPARγ cofactors by cdk4. In this scenario, PPARγ would recruit cdk4 to the coactivator complex. Alternatively, the effect of cdk4 could be the result of the phosphorylation and inactivation of the
RB protein, which it has been shown to be a PPARγ repressor (Fajas et al., 2002a), or the inactivation of other PPARγ corepressors in differentiated adipocytes.

Since cdk4 is the catalytic subunit of type D cyclins, participation of cyclin Ds in adipogenesis is likely. Interestingly cyclin D3 KO mice are lean and smaller than wt mice (Sicinska et al., 2003). Furthermore, expression of cyclin D3 is increased during adipogenesis (Phelps and Xiong, 1998; Reichert and Eick, 1999). Adipose tissue phenotype in cyclin D3 -/- mice is currently under study in our lab. On the other hand, cdk4 activity can be modulated by the cdk inhibitors p21 and p27. In support of our results showing a positive role of cdk4 in adipogenesis, p21-/-, p27-/-, and p21/p27 -/- mice had up to 4-fold increases in body fat percentage compared to wild-type mice (Naaz et al., 2004). This suggests that the release of cdk4 inhibition, such as expected in p21-/- or p27 -/- mice, might result in increased cdk4 activity, and therefore increased adiposity.

Adipose tissue growth, such as that observed during obesity, is the result of both hypertrophy (increase in size) and hyperplasia (increase in number) of adipocytes (Brook et al., 1972). From our results we can conclude that participation of cdk4 in these processes is likely. Obesity and associated pathologies are the second most common preventable cause of death. Discovery of new targets may have a major impact on the associated metabolic syndromes. Identification of cdk4 as an adipogenic factor might result in new therapeutic strategies for the treatment of obesity. Specific cdk4 inhibitors which have no effects on cell cycle progression, but retain its ability to inhibit lipogenesis could be used for the treatment of obesity. This would eliminate the undesired side effects of cdk4 inhibitors. Furthermore, cdk4 should be therefore considered as a new target for the search of genetic mutations predisposing to obesity.

**Material and methods**
Materials: All chemicals, except if stated otherwise, were purchased from Sigma (St-Louis, MO). Pioglitazone was a kind gift of Takeda Pharmaceuticals (Osaka, Japan). Cdk4 inhibitor was purchased from Calbiochem (San Diego, CA). Antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) except for the anti-BrdU antibody, which was from Dako A/S (Glostrup, Denmark) and the anti-acetylated histone H4 (Lys 9) antibody, which was from Upstate Biotechnology (Lake Placid, NY). Deoxy-D-[2-3H]glucose (2-DG; 26 Ci/mmol) was from Perkin Elmer (Boston, MA, U.S.A). Purified porcine insulin was a gift from Eli Lilly (Indianapolis, IN). The cdk4 kinase mutant (K35M) was a gift of Dr. M. Serrano (CNIO, Madrid, Spain).

RNA isolation, quantitative real time PCR, : RNA isolation has been previously described (Hansen et al., 1999). Reverse transcription of total RNA was performed at 37°C using the M-MLV reverse transcriptase (Invitrogen SARL, France) and random hexanucleotides primers, followed by a 5 min inactivation at 95°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 then normalized to b-actin levels. The primer pairs used in this study are available upon request.

Cell culture. Cdk4-/-, cdk4R24C/R24C and wild type MEFs were obtained as previously described (Humbert et al., 2000a; Humbert et al., 2000b). MEFs, COS, Phoenix and 3T3-L1 cells were grown in DMEM, 10% foetal bovine serum (FBS). MEFs and 3T3-L1 cells were differentiated with DMEM, 10% serum, 0.5 mM 3-Isobutyl-1-methylxanthine (IBMX), 10 mg/ml insulin, 1mM dexamethasone and 100nM pioglitazone for 2 days. From the day 3 on, cells were incubated with DMEM, 10% serum, 10 mg/ml insulin, and 100nM pioglitazone. Oil-red-O staining is described elsewhere (Ramirez-Zacarias et al., 1992). 3T3-F442A preadipocytes were cultured in DMEM containing foetal calf serum (10%), penicillin (200 units/ml), and streptomycin (50 mg/l) at 37°C in a humidified atmosphere of 7% CO2. For adipocyte differentiation, the medium was supplemented with insulin (50 nmol/l) once cells reached confluence, Medium was renewed every 2 days until the end of the
experiment. Human preadipocytes were obtained from subcutaneous abdominal adipose tissue donors in agreement with French laws on biomedical research. Separation and differentiation of preadipocytes was previously described (Hauner et al., 1989). Glucose transport experiments were performed as previously described (Carpene et al., 1993).

**Retroviral construction and infection.** Wild type cdk4 and the kinase cdk4 mutant were cloned in a pBabe Puro vector (cloning details are available upon request). Virus production and infection of 3T3-L1 and MEF cells was performed as described previously (Kim and Spiegelman, 1996). Infection efficiency was estimated at 90%. Pooled clones were differentiated as described.

**Immunofluorescence in 3T3-L1 cells:** Cells were grown on cover-slips. For BrdU incorporation, cells were incubated 4h in the presence of BrdU and an additional treatment of the cells with 1.5N HCl for 10 minutes at 21 °C was performed. After fixation and permeabilization with 100% methanol, cells were incubated with antibodies directed against cdk4 (Santa Cruz Biotechnology, CA), PPARg (Santa Cruz Biotechnology, CA) or BrdU (Dako A/S, Glostrup, Denmark). Preparations were then incubated with a combination of Texas Red-conjugated anti-rabbit IgG and FITC-conjugated anti-mouse IgG.

**Mice.** 12 weeks mice were used. C57BL/6J mice were maintained according to EU guidelines for use of laboratory animals. Mice had ad libitum access to water and regular rodent chow (DO4, UAR, France). For in vivo cell proliferation assays twelve weeks-old mice were intraperitoneally injected with BrdU (50 mg / Kg body weight). After 18 hours, mice were sacrificed and epidydimal white adipose tissue (WAT) was excised and fixed in 4% formaldehyde.

**Histological and immunofluorescence analysis in tissue sections:** WAT was fixed as described and paraffin-embedded. Sections were cut and stained with haematoxylin and eosin, or treated with 1.5N HCl before incubation with BrdU (Dako A/S, Glostrup, Denmark) specific antibody. Preparations were then incubated with a Texas Red-conjugated anti-mouse IgG. BrdU positive cells were counted.
Transfections and plasmids. PcDNA3 vector was purchased from Stratagene (La Jolla, CA). The pcDNA3-ckd4 expression vector was a gift from Dr. C. Sardet. The PPARg promoter-reporter vector has been previously described (Fajas et al., 1997; Fajas et al., 1998). All transfections were performed using the Lipofectamine Plus reagent (GIBCO Life Technologies, Rockville, MD). Luciferase and b-gal activity was measured as described (Fajas et al., 1997).

Protein expression assays: Whole cell extracts were prepared as described (Fajas et al., 1997). SDS-PAGE and electrotransfer was performed as described (Rocchi et al., 2001). The membranes were blocked 1h in blocking buffer (PBS 0,5% Tween-20, 5% skimmed milk). Filters were first incubated overnight at 4°C with the indicated primary antibodies, and then for 1h at room temperature with a peroxidase conjugate secondary antibody. The complex was visualized with ECL reagament.

Co-immunoprecipitation, pull-down, and chromatin immunoprecipitation (ChIP) assays: For co-immunoprecipitation assays whole cell extracts were precleared with protein A-agarose beads (Roche) during 30 minutes at room temperature, an aliquote of the precleared lysates was saved as input. Extracts were then centrifuged (5 minutes at 3000rpm) and supernatants were immunoprecipitated with the indicated specific antibodies during 2h at room temperature, 2-4 mg of rabbit IgG (Sigma, St-Louis, MO) were used as negative control (mock). Immunoprecipitates were then washed twice with IP buffer (150mM NaCl, 1% NP40 in 50mM Tris/HCl buffer pH8) and three times with washing buffer (0,25M KCl in PBS) and subjected to SDS-PAGE electrophoresis. Chromatin immunoprecipitation assays were performed as described previously (Takahashi et al., 2000). Briefly, proteins were formaldehyde cross-linked to DNA in confluent 3T3-L1 preadipocytes before induction of differentiation or in cells induced with differentiation medium for 7 days. Proteins were then immunoprecipitated using the indicated antibodies, rabbit IgGs were used as mock, DNA was extracted from the immunoprecipitates and PCR amplification was performed using promoter-specific oligonucleotide primers. For pull down assays in vitro translation of PPARg and cdk4 was performed with 35S-methionine
(Perkin Elmer, Boston, MA) in a TNT-coupled transcription-translation system following manufacturers instructions (Promega, Madison, WI). GST fusion or GST alone were expressed in Escherichia Coli and purified on glutathione- sepharose beads (Amersham Biosciences, Uppsala, Sweden). For in vitro interactions GST, GST-PPARg-AB, GST-PPARg-EDF, GST-cdk4 and GST-cdk4 deletions (c-del positions 1-609, n-del positions 109-912) were incubated with the labeled proteins in 200 ml binding buffer containing 150mM NaCl, 1% Nonidet P-40 and 50mM Tris pH 8 at room temperature for 2 hr. Beads were washed three times with buffer containing 0.25M KCl and 0.1% Tween 20 in PBS, followed by three washes with the same buffer without Tween 20. The protein complexes were analyzed by autoradiography after SDS-PAGE electrophoresis.

**Kinase assays.** Immunoprecipitated cdk4 from transfected COS cells, 3T3-L1 cells or WAT was used as kinase. cdk6/cycD3 kinase (Upstate, Charlottesville, Virginia) was used as positive control. Immunoprecipitated cdk4 was washed once with kinase buffer (25mM Tris/HCl pH7.5, 150mM NaCl, 10mM MgCl2, 1mM DTT) in the presence of a protease inhibitor cocktail (Sigma, St-Louis, MO) and phosphatase inhibitors (5 mM Na4P2O7, 50 mM NaF, 1 mM vanadate). Kinase assay was performed during 30 minutes at 37 °C in the presence of 40 mM ATP and 8 mCi g-33P ATP and the substrate reaction substrate; recombinant PPARg protein (Active Motif, Carlsbad, CA) or Rb protein (Santa Cruz, CA). Reaction was stopped boiling the samples during 5 minutes in the presence of denaturing sample buffer. samples were then subjected to SDS-PAGE electrophoresis, gels were then dry in a gel dryer for 1h at 80 °C and exposed to an X-ray film.

**Statistical analysis:** Data are presented as means ± S.E.M. Group means were compared by factorial analysis of variance (ANOVA). Upon significant interactions, differences between individual group means were analyzed by Fisher’s protected least squares difference (PLSD) test. Differences were considered statistically significant at \( P < 0.05 \).
**Acknowledgements**

Nadège Servant and Jacques Teyssier are acknowledged for technical assistance. Johan Auwerx, Claude Sardet, Laurent Le Cam and various members of Fajas lab are acknowledged for support and discussion. This work was supported by grants of INSERM (Avenir), FRM, Alfediam, and ARC. Anna Abella is supported by a grant of Inserm Poste Vert. P. Dubus is supported by the Inserm (CreS 4CR03G).

**References**


Figure legends

Figure 1. Cdk4 is expressed during adipogenesis and in fully differentiated human and mouse adipocytes.

A. Analysis of cdk4 and PPARγ co-expression by immunofluorescence in 3T3-L1 cells induced to differentiate. Days of differentiation indicate confluent (day 0), re-entry into cell cycle (day 1), and terminally differentiated (day 5) cells. PPARγ expressing cells are labelled in green whereas cdk4 expressing cells are in red. Nuclei were visualized with Hoechst staining.

B. Detailed analysis by confocal microscopy of cdk4 (red) and PPARγ (green) expression in a representative single cell.

C. Quantification of the mRNA expression by real-time PCR of the adipogenic marker aP2 at the indicated times of differentiation. Results were normalized by the expression of the β-actin mRNA.

D. Quantification of the expression of cdk4 and PPARγ mRNA during differentiation of human primary preadipocytes at days 0 or at day 13 of differentiation.

E. Immunohistochemical analysis of cdk4 expression in histological sections of mouse adipose tissue. Red arrows indicate adipocytes expressing cdk4.

F. Cdk4 activity in adipocytes. SDS-PAGE autoradiography showing phosphorylated purified RB by immunoprecipitated cdk4 from confluent, non differentiated 3T3-L1 preadipocytes (d0, lane 1), differentiated 3T3-L1 adipocytes (d7, lane 2), or from primary mouse adipocytes freshly prepared from white adipose tissue (WAT, lane 5). Immunoprecipitates with rabbit antiserum were used as negative control (mock, lanes 3, 4, and 6). Purified cdk6/cycD3 complex was used instead of the immunoprecipitates as positive control (lane 7).

G. Quantification of cdk4 protein expression (right axis) and BrdU incorporation (left axis) by immunofluorescence in proliferating (day -1) or post-confluent 3T3-L1 cells stimulated with differentiation medium for 7 days as described under experimental procedures. Percentage of cdk4 expressing 3T3-L1 cells, which are BrdU positive at...
the indicated days of differentiation. Results are the mean of at least 500 cells in two independent experiments.

**Fig 2. Cdk4 inhibitors impair adipogenesis.**

A-G. Representative micrographs of oil-red-O staining of 3T3-L1 cells differentiated in vitro for 7 days in the presence or absence of the indicated concentration of the specific cdk4 inhibitor IDCX added either at the induction of differentiation (A) or two days after induction (E). mRNA of differentiated cells was analysed for the expression of the adipocytes markers aP2 and PPARγ by quantitative PCR in response to IDCX added either before (B) or after (F) the clonal expansion phase. Results were normalized by the expression of the β--actin RNA. Cell cycle status of the cells was analysed by quantification of BrdU incorporation either in the absence or presence of 0.5 μM IDCX added before (C) or after (G) the clonal expansion phase. mRNA expression of cyclin E and cyclin B was quantified at different times of adipocyte differentiation (d0, d1, d2, d6) in the absence or in the presence of IDCX added before the clonal expansion phase (D).

H-J. Analysis of the expression of adipocyte markers aP2 and PPARγ 6 days after initiation of differentiation of F442A cells in the absence or presence of IDCX (H). Cell cycle status of the cells was analysed by quantification of BrdU incorporation either in the absence or presence of 0.5 μM IDCX added at initiation of differentiation (I). mRNA expression of cyclin E and cyclin B was also quantified at different times of F442A adipocyte differentiation (d0, d1, d2, d6) in the absence or in the presence of IDCX added before the clonal expansion phase (J).

**Fig 3. Cdk4 overexpression increases adipocyte differentiation**

A. Micrographs of oil red-O staining of 3T3-L1 adipocytes retrovirally infected with either and empty vector (pBabe), a retrovirus encoding for cdk4 (pBabe-cdk4), or a retrovirus encoding for kinase-dead cdk4 mutant (pBabe-K35M) 4 days after induction of adipogenesis.
B. mRNA quantification by real-time PCR of the adipocyte marker aP2 and cdk4 mRNA. Results were normalized by the expression of the β-actin RNA.

C. Oil-red-O staining of MEFs from either wild type (cdk4+/+), cdk4-deficient (Cdk4-/-) or hyperactive cdk4 R24C mutant mice induced to differentiate into adipocytes for 7 days. Cells were infected with retrovirus expressing either an empty vector (pBabe) or retrovirus expressing cdk4 (pBabe-cdk4) as indicated.

D. Quantification of the mRNA expression by real time PCR of cdk4 in cells used in C. Results were normalized by the expression of the β–actin RNA in all subsequent experiments.

E. aP2 mRNA expression analysed by real time PCR in the same cells used in C.

Figure 4. Participation of cdk4 in adipocyte biology.

A. Glucose uptake experiments in 3T3-L1 adipocytes treated with increasing dose of IDCX. The values represent the fold-induction of radioactive glucose uptake by insulin load of the cells relative to basal conditions of cells treated with vehicle.

B. Quantification of aP2 mRNA expression of cells used in A.

C. Quantification of mRNA expression in 3T3-L1 adipocytes of genes involved in glucose transport and metabolism in response to 1 µM of IDCX.

D. Analysis by real time PCR of the expression of genes implicated in lipogenesis in 3T3-L1 adipocytes treated with 1µM IDCX.

Figure 5. Cdk4 interacts with PPARγ and activates PPARγ-target genes

A. Relative luciferase activity as determined after transfection of COS cells with the PPARγ-responsive reporter construct J3-TK-Luc and treated with the PPARγ agonist pioglitazone. Cells were transfected with an expression vector for PPARγ in the absence or presence of increasing concentrations of either a cdk4 expression vector, or a cdk4 mutant (K35M) vector. Results were normalized for the expression of a β-gal reporter. Values are the mean of 3 independent experiments. An asterisk depicts statistically significant differences.
B. Same transfection as in A using J3TK-Luc and PPARγ vectors. Cells were incubated with increasing concentrations of the specific cdk4 inhibitor IDCX (1-5 µM). Results were normalized for the expression of a β-gal reporter. Values are the mean of 3 independent experiments. An asterisk depicts statistically significant differences.

C. Quantitative real time PCR showing mRNA expression levels of aP2 (left panel) in the absence or in the presence of 10^{-6}M pioglitazone or 10^{-6}M rosiglitazone. Expression levels of PPARγ are shown in the right panel.

D. Coimmunoprecipitation of PPARγ and cdk4 from COS cells transfected (lanes 2,4,6) or not (NT, lanes 1,3,5) with PPARγ and cdk4 expression vectors. Extracts are immunoprecipitated with either an anti-PPARγ antibody (PPARγ, lanes 5-6) or preimmune serum (Mock, lane 3-4), and revealed by an anti-cdk4 antibody. One half of the input is shown as a control (lanes 1-2).

E. Coimmunoprecipitation of PPARγ and cdk4 from differentiated 3T3-L1 adipocytes expressing cdk4. Immunoprecipitation with anti PPARγ (lane 3) or IGGs (lane 2) was followed by western blot anti cdk4. Input is indicated in lane 1.

F-G. GST-pull down assays showing details of the PPARγ-cdk4 interaction. In vitro translated 35S-radiolabeled PPARγ (E) or cdk4 protein (F) were incubated respectively with different deletion mutants of cdk4 (E) or PPARγ (F). Mutants are described under the material and methods section.

H. Chromatin immunoprecipitation (ChIP) assay demonstrating binding of cdk4 to the PPARγ-binding site in the aP2 promoter. Cross-linked chromatin from 3T3-L1 cells differentiated during 7 days was incubated with antibodies against acetylated histone H4 (lane 3), actin (lanes 4), PPARγ (lane 5), cdk4 (lane 6) or without any antibody (mock, lane 2). Immunoprecipitates were analyzed by PCR using primers specific for the aP2 promoter. As a control, a sample representing 1% of the total chromatin was included in the PCR (input, lane 1).

Figure 6. Schematical representation of cdk4 functions during adipogenesis.
During the clonal expansion phase, cdk4 activates E2F proteins, which were inhibited by RB. E2F activation will facilitate the expression of PPARγ, which in concerted action with other transcription factors will trigger the expression of adipocyte-specific genes. During the terminal differentiation phase, cdk4 will directly activate PPARγ through phosphorylation and inactivation of RB or other PPARγ corepressors. Alternatively, cdk4 could also activate through phosphorylation PPARγ coactivators.
Abella et al., figure 1

**Panel A:** Immunofluorescence images showing Hoechst, cdk4, and PPAR expression over different days of differentiation.

**Panel B:** Day 5 images showing cdk4 and PPAR expression.

**Panel C:** Graphs showing aP2 mRNA expression over days of differentiation.

**Panel D:** mRNA expression levels of cdk4 and PPAR over days of differentiation.

**Panel E:** Image of WAT with cdk4 highlighted.

**Panel F:** Western blot analysis of ppRB expression in cdk4 and Mock conditions.

**Panel G:** Bar graphs showing BrdU and cdk4+ cell percentages over differentiation days.
Abella et al., figure 2

**A**

Control

IDCX (before clonal expansion)

IDCX (after clonal expansion)

**B**

A

mRNA rel. units

0.5
1.0
1.5
2.0
2.5
mRNA rel. units

M

IDCX

0
0.5
1.0
1.5
2.0
2.5

**C**

% BrdU

cont. IDCX

(d0 d1 d2)

cont. IDCX

(d2 d6)

**D**

mRNA rel. units

control IDCX

d0 d1 d2 d6

d0 d1 d2 d6

d0 d1 d2 d6

d0 d1 d2 d6

d0 d1 d2 d6

d0 d1 d2 d6

**E**

Control

IDCX (after clonal expansion)

**F**

mRNA rel. units

0.5
1.0
1.5
2.0
2.5
mRNA rel. units

M

IDCX

0
0.5
1.0
1.5
2.0
2.5

**G**

% BrdU

cont. IDCX

(d2 d6)

cont. IDCX

(d2 d6)

**H**

mRNA rel. units

control IDCX

d0 d1 d2 d6

d0 d1 d2 d6

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d0 d1 d2 d6

d0 d1 d2 d6

**I**

% BrdU

control IDCX

d0 d1 d2 d6

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d0 d1 d2 d6

d0 d1 d2 d6

d0 d1 d2 d6

d0 d1 d2 d6

**J**

mRNA rel. units

control IDCX

cyclin E

d0 d1 d2 d6

cyclin E

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cyclin E

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cyclin E

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cyclin E

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Abella et al., figure 3

A

3T3-L1

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MRNA rel. units

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Abella et al., figure 4

A

Ratio basal (nmol2DG/mg protein)

Control
Insulin

0 0.5 1 2 M IDCX

B

aP2 mRNA rel. units

0 0.5 1 2 M IDCX

C

mRNA rel. units

Control
IDCX

Glut-4 IR IRS1 IRS2 PI3K Glut-1

D

mRNA rel. units

Control
IDCX

FATP FAS PEPCK

HAL author manuscript inserm-00144639, version 1
**A**

J3-TK-Luc + PPAR (ng)

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J3-TK-Luc + PPAR

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ChIP

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<td>PPAR</td>
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<tr>
<td>cdk4</td>
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<tr>
<td>aP2 promoter</td>
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</table>
**Hormonal induction**

Growth arrest → Clonal expansion → Terminal differentiation

**Cdk4**

- RB → E2F
- C/EBP
- ADD1/SREBP1

**PPIA1/SREBP1**

- RB → Corepressors
- Coactivators

- PPAR → C/EBP
- aP2, LPL, FAS, PEPCK

Abella et al., figure 6