Chapter 2
Emerging Roles of Cell Cycle Regulators in Adipocyte Metabolism

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Introduction

Cells can adapt their growth and metabolism to their needs and the extracellular signals they receive. Some stimuli, such as stress or nutrients, are not only proliferative but also metabolic signals, suggesting a close crosstalk between these two biological processes. Indeed, the response to a metabolic signal can require the activation of transcription factors and of signaling molecules that result in the inhibition of cell cycle progression and ultimately cell proliferation.

The cell cycle is a finely tuned process (Fig. 2.1) the progression of which is orchestrated by holoenzymes that are composed of regulatory subunits (i.e., cyclins) and catalytic subunits, i.e., cyclin-dependent kinases (Cdks). Cdks, which belong to the family of the serine/threonine kinases, are activated by phosphorylation and dephosphorylation events and interact with specific cyclins to form heterodimers (Malumbres and Barbacid 2005). The cyclin D-Cdk4 and cyclin D-Cdk6 complexes act during the G1 phase, whereas the cyclin E-Cdk2 complex regulates the G1/S transition. The main substrates of the cyclin-Cdk complexes are proteins of the retinoblastoma (known also as «pocket protein») family (i.e., pRb, p107 and p130) that regulate the G1/S transition of the cell cycle. Phosphorylation of retinoblastoma proteins by cyclin-Cdk complexes

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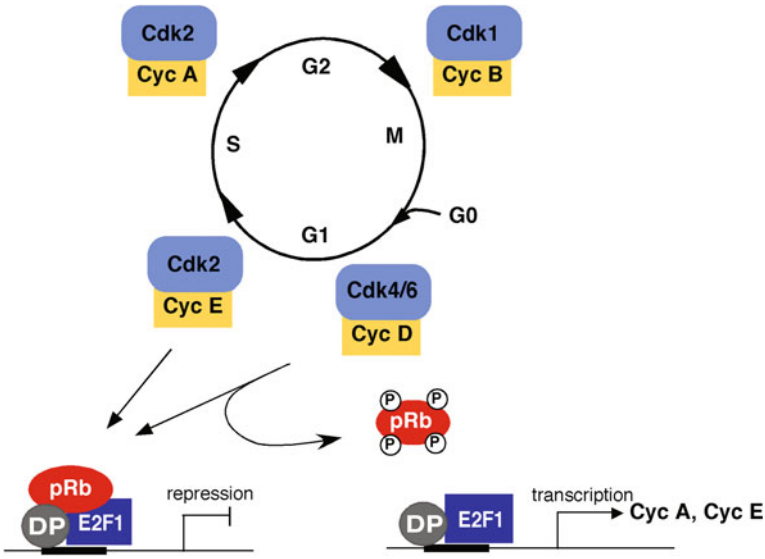

Fig. 2.1 The cell cycle machinery. During the G1/S transition, the Cdk4-cyclin D complex is activated by proliferative stimuli and then it phosphorylates pRb, p107, and p130. This phosphorylation allows the dissociation of the repressive complex constituted by the "pocket" proteins and E2F transcription factors. Once liberated, E2F factors can transactivate genes that promote cell cycle progression

abolishes their repressive effect on the E2F/DP transcription factors, thus allowing the transcription of genes required for cell cycle progression.

Indeed, E2F factors (E2F1-8) regulate the expression of genes involved in cell cycle progression, apoptosis, and DNA synthesis. The association of E2F proteins with proteins of the retinoblastoma family represses transcription following the recruitment of histone deacetylases (HDAC) (Brehm et al. 1998; Magnaghi-Jaulin et al. 1998) and of lysine/arginine methyl-transferases (Fabbrizio et al. 2002) and consequently the cell cycle is blocked in the G0/G1 phase.

Besides their established role in the control of cell proliferation and cell death, these cell cycle players are also key regulators of cell metabolism and of lipid and glucose homeostasis.

Cell Cycle Regulators in Adipose Tissue Physiology

Adipocyte Differentiation

Adipocytes are the main component of adipose tissue (AT). The elucidation of the molecular mechanisms underlying adipocyte differentiation is essential for understanding the physiology of AT.

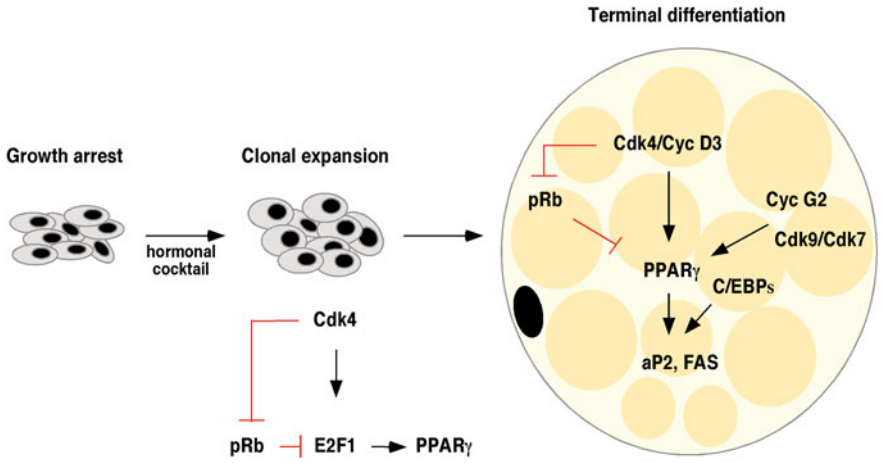

Fig. 2.2 Participation of cell cycle regulators in adipocyte differentiation. During the phase of clonal expansion, the Cdk4-pRb-E2F1 signaling pathway regulates the expression of genes involved in cell cycle entry. During the phase of terminal differentiation, the cell cycle regulators can influence the adipocyte biology by regulating directly the expression/activity of the key adipocyte transcription factors C/EBPα and PPARγ

Proliferation and differentiation are two extremely interconnected biological processes. During the development of the AT, adipocyte differentiation (adipogenesis) includes a proliferative step followed by a phase of differentiation, in which all the specific adipocyte markers are induced (Fajas 2003). Adipogenesis thus requires a very intimate crosstalk between cell cycle regulation and metabolic control.

The pre-adipocytic 3T3-L1 cell line is an experimental model used to identify molecular mechanisms involved in adipogenesis. In response to a hormonal cocktail, growth-arrested 3T3-L1 pre-adipocytes will re-enter the cell cycle (Fig. 2.2). This phase is called clonal expansion and cannot be dissociated from terminal differentiation. Indeed, blocking the phase of clonal expansion with DNA synthesis inhibitors fully arrests adipocyte differentiation (Richon et al. 1997). It has been shown that the Cdk-cyclin-E2F-Rb signaling cascade plays a major role during adipocyte differentiation. E2F1 is strongly upregulated during the first phases of adipogenesis, followed by the expression of its target genes, such as cyclin D1 and cyclin E (Richon et al. 1997). Moreover, E2F1 regulates adipocyte differentiation also by modulating the expression of peroxisome proliferator-activated receptor (PPAR)γ, the main transcription factor of adipogenesis (Fajas et al. 2002). However, other members of the E2F family seem to be involved in the negative regulation of adipogenesis. For instance, E2F4 inhibits the expression of PPARγ during terminal adipocyte differentiation (Fajas et al. 2002; Landsberg et al. 2003).

The retinoblastoma proteins (pRb, p107, and p130) regulate the activity of E2F transcription factors and play an important role during adipocyte differentiation as well. However, the findings are contradictory. pRb acts positively on terminal adipocyte differentiation by binding directly to the transcription factor CCAAT/

enhancer binding protein (C/EBP)β and facilitating its transactivation (Chen et al. 1996). Conversely, during cell cycle arrest in the G1 phase, pRb negatively regulates E2F1. Moreover, pRb can also act negatively during adipogenesis by forming a complex with PPARγ and HDAC3 on the promoter of target genes and thus blocking their induction (Fajas et al. 2002).

The cyclin-Cdk complexes, which regulate the activity of E2F factors by phosphorylating retinoblastoma proteins, also participate in the fine-tuning of adipogenesis. Cdk4 is an important regulator of adipocyte differentiation. We have shown that inactivation of Cdk4 blocks adipocyte differentiation, whereas the Cdk4 mutant R24C, which cannot be inhibited by $p16^{INK4a}$, increases the adipogenic potential of 3T3-L1 cells (Abella et al. 2005). Cdk4 directly regulates PPARγ by activating the transcription of its target genes through their direct phosphorylation.

Cdk7 and Cdk9 also are involved in adipocyte differentiation. Differently from other Cdks that act directly on cell cycle progression, these two kinases regulate transcription via phosphorylation of the RNA polymerase during the cell cycle. Cdk9 favors the transcription of PPARγ-target genes by direct interaction with and phosphorylation of PPARγ (Iankova et al. 2006). Conversely, Cdk7 phosphorylates PPARγ to inhibit its transcriptional activity (Helenius et al. 2009).

To carry out their activity, Cdks are associated with cyclins, which are the regulatory subunits of the Cdk/cyclin complexes. Cyclin D1 and D3 modulates adipogenesis in opposite ways. Both cyclins interact with PPARγ but, different from cyclin D3, cyclin D1 negatively regulates adipogenesis by recruiting HDACs on the promoter of PPARγ-target genes (Fu et al. 2005; Sarruf et al. 2005). On the other hand, cyclin G2, like cyclin D3, acts as a cofactor of PPARγ, thus favoring its transcriptional activity and consequently also adipocyte differentiation (Aguilar et al. 2010).

During the cell cycle, Cdk activity is also finely regulated by their physiological inhibitors, the CDKIs (CIP, KIP, and INK4). It is thus not surprising to see that CDKIs modulate adipocyte differentiation as well. The expression of p21/CIP and p27/KIP1 during adipocyte differentiation is very controversial. Nevertheless, disruption of p21 activity in a 3T3-L1 cell model (p21 knockdown by RNA interference) or in p21-/- MEFs (primary mouse embryonic fibroblasts) inhibits adipocyte differentiation, thus making p21 a pro-adipogenic factor (Inoue et al. 2008).

Overall, the studies carried out using the 3T3-L1 cell line show that many regulators of the cell cycle play a crucial role also in the process of adipocyte differentiation, either by modulating the cell cycle during the clonal expansion phase, or by regulating key adipogenic transcription factors.

Adipocyte Biology

Many works have described the key role of cell cycle players during adipogenesis, whereas very few studies have focused on the involvement of these regulators in adipocyte biology. We have reported that some cell cycle regulators are

expressed in mature adipocytes, suggesting a role in the biology of adipocytes. Cdks could control the activity of enzymes required for the adipocyte functions, such as lipolysis or lipid synthesis. Indeed, we have shown that, in adipocytes, Cdk4 regulates glucose transport upon stimulation by insulin. Inhibition of Cdk4 by a pharmacological inhibitor blocks the glucose transporter and decreases the expression of genes involved in the signaling cascades of insulin and of the glucose transporter GLUT4 as well as of lipogenic genes, such as fatty acid synthase (FAS) and phosphoenol pyruvate carboxy kinase (PEPCK) (Abella et al. 2005). Similarly, Cdk5 plays a major role in glucose transport. Silencing of Cdk5 by siRNA or its pharmacological inhibition impairs the transport of glucose stimulated by insulin. Indeed, following its insulin-dependent activation, Cdk5 phosphorylates the protein E-Syt1, a partner of the GLUT4 transporter, thus favoring the transport of glucose (Lalioti et al. 2009; Muruais et al. 2009).

Moreover, in yeast, Kohlwein's group has shown that Cdk1 phosphorylates the lipase tgl4, which is an ortholog of adipose triglyceride lipase (ATGL), the key enzyme of lipolysis (Kurat et al. 2009). These results suggest that Cdks might also control lipolysis.

Although data on the involvement of the cell cycle regulators in the physiology of adipocytes are scarce, it seems that these factors could act directly on the biological functions of adipocytes, such as lipolysis and glucose transport.

Cell Cycle Regulators and AT Physiopathology

As cell cycle regulators are now considered novel major players in the control of lipid and glucose metabolism, they could also be involved in the development of associated pathologies, such as obesity, diabetes, and cancer.

Obesity and Type 2 Diabetes

Obesity is a major health problem. It is characterized by adipose mass accumulation resulting in an increase of the size and number of adipocytes. During weight gain, adipocytes become bigger (hypertrophy) and accumulate lipids. Since adipocytes cannot indefinitely accumulate lipids, they will then recruit adipocyte precursors to form new mature adipocytes. This process is called hyperplasia.

Type 2 diabetes is the most frequent complication of obesity. This pathology is characterized by fasting hyperglycemia due to the association of insulin resistance with destruction of pancreatic beta cells that produce insulin.

In the mouse, specific genetic ablation of cell cycle regulators in the AT has allowed demonstrating their importance in the physiopathology of AT, particularly during the development of hyperplasia (Table 2.1).

Table 2.1 Adipose tissue phenotype of cell cycle regulators knockout mice

Gene	Metabolic phenotype	References
Cyclin D3	Resistance to high fat diet, small adipocytes	(Sarruf et al. 2005)
Cdk4	Decrease body weight and adipose tissue mass	(Abella et al. 2005; Rane et al. 1999)
pRb	Resistance to high fat diet, switch from white to brown adipocyte	(Dali-Youcef et al. 2007)
P107	Decrease adipose tissue mass	(Scime et al. 2005)
P21	Increase adipose tissue mass by hyperplasia	(Naaz et al. 2004)
P27	Increase adipose tissue mass by hyperplasia	(Naaz et al. 2004)

Genetic ablation of cyclin D3 in the mouse protects these animals against obesity induced by a lipid-rich diet (Sarruf et al. 2005). Their adipocytes are smaller and express very low levels of adipocyte markers, such as aP2 and PPARγ, in comparison to wild-type controls. The presence of smaller adipocytes improves the general metabolism of these mice. Indeed, cyclin D3-/- mice are more glucose-tolerant and more sensitive to insulin in comparison to wild-type mice.

Since Cdks carry out the catalytic activity of the Cdk-cyclin complexes, it is not surprising that genetic ablation of cyclins or Cdks leads to similar phenotypes. In Cdk4-/- mice the adipose mass is reduced (Abella et al. 2005). Conversely, transgenic mice that express Cdk4R24C, the active mutant of Cdk4 (R24C), have increased body weight (Rane et al. 1999). These effects on the physiology of the AT are due, in part, to the direct effects of Cdk4 on PPARγ during adipocyte differentiation and also to the effects of Cdk4 on insulin secretion from the pancreas.

The metabolic phenotype of Cdk5-/- mice is unfortunately not available, but Cdk5 seems to play a primordial role in insulin resistance. Indeed, Nohara's group has shown that the effects of tumor necrosis factor (TNF)α on insulin resistance, particularly on glucose transport, are mediated through activation of Cdk5 (Nohara et al. 2011). Recently, a new anti-diabetic compound (SR1664) has been described. SR1664 binds to PPARγ, blocks its phosphorylation by Cdk5, and thus improves the biological parameters of diabetic mice (Choi et al. 2010; Choi et al. 2011). This compound has a powerful anti-diabetic activity without modifying the bone mass and without weight gain, the two main secondary effects of thiazolidinediones (TZD). Altogether these findings suggest that PPARγ phosphorylation by Cdk5 might be a major event in the development of type 2 diabetes.

The members (pRb, p107, p130) of the «pocket protein» family are key regulators of the physiology of AT. Deletion of pRb specifically in the adult AT leads to resistance to weight gain in animals fed on a lipid-rich diet, thanks to an increase of energy expenditure (Dali-Youcef et al. 2007). In these mice, both white and brown adipocytes have higher mitochondrial activity and the white AT shows morphological characteristics that are typical of the brown AT. In vitro, pRb -/- MEFs differentiate into adipose cells that have the phenotype of brown adipocytes and are characterized by the expression of PPAR gamma coactivator-1 (PGC-1), a major regulator of mitochondrial biogenesis, and of enzymes of the mitochondrial respiratory chain (Hansen et al. 2004). pRb thus regulates differentiation into

the white or brown adipocyte lineage. A similar phenotype has been reported in p107-/- mice which shows a strong reduction of the adipose mass and an increased number of mitochondria (Scime et al. 2005).

P21 and p27 are important regulators of adipogenesis. Loss of expression of these Cdk inhibitors induces hyperplasia of the AT. The p21-/-, p27-/-, and p21-/-p27-/- mice develop obesity characterized by a larger number of adipocytes (Naaz et al. 2004). These observations are however controversial because two other studies have shown that p21 -/- mice are protected against obesity induced by lipid-rich diet (Inoue et al. 2008) and that p27-/- mice do not have an AT phenotype (Lin et al. 2003).

Altogether, these in vivo studies using mice in which cell cycle regulators have been genetically ablated demonstrate that these factors play a crucial role in the physiopathology of the AT.

Cancer

The activity of the Cdk-pRb-E2F signaling pathway is often altered in many human cancers, such as glioblastoma as well as lung, ovary, breast, and colon cancers (Chen et al. 2009). Few studies have investigated the involvement of this pathway in tumors of the AT.

Liposarcomas are tumors derived from the primitive cells that have differentiated into adipocytes. The term "liposarcoma" covers a huge variety of neoplastic processes, mainly benign lesions and also more aggressive, malignant lesions with a high rate of relapse and/or metastases. In comparison to other cancer types, these soft tissue sarcomas are relatively rare.

Only deregulation of the Cdk4 gene, among the many cell cycle regulators, has been described in these cancers (Helias-Rodzewicz et al. 2009; Chung et al. 2009). Differentiated and dedifferentiated liposarcomas are characterized by amplification of a region in chromosome 12 that carries the Cdk4 gene. This amplification can in some cases lead to overexpression of Cdk4 in tumor cells in comparison to mature adipocytes. Moreover, a study has demonstrated that loss of Cdk4 copies by chemical treatment in liposarcomas was correlated with an increase of adipocyte differentiation (Helias-Rodzewicz et al. 2009). These data, obtained in cancer cells, are reminiscent of those from studies on Cdk4 in the 3T3-L1 adipocyte cell line (Abella et al. 2005).

Conclusion

The role of the Cdk-cyclin-pRb-E2F signaling pathway in proliferation, cell cycle regulation, apoptosis, and cancer has been much studied. This review describes the involvement of this signaling pathway in the physiology and physiopathology of the AT as well. These cell cycle regulators act directly on key regulators of adipocytes, such as PPARγ and C/EBP.

However, their function is not limited to the AT. Indeed, activation of the Cdk-Cyclin-pRb-E2F signaling pathway has also been reported in other metabolic, non-proliferative tissues, such as pancreas and muscle. Moreover, an increasing number of studies have described the activity of these factors in glucose and energy homeostasis (Annicotte et al. 2009; Blanchet et al. 2011).

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