



# Function of pref-1 as an inhibitor of adipocyte differentiation

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During conversion of preadipocytes to adipocytes, growth arrest and subsequent activation of adipocyte genes by the transcription factors, C/EBP $\alpha$  and PPAR $\gamma$ , lead to adipogenesis. During differentiation, these cells not only start expressing those genes necessary for adipocyte function, but also undergo changes in morphology to become rounded lipid filled adipocytes. Various factors in cell–cell communication or cell–matrix interaction may govern whether preadipocytes are kept in an undifferentiated state or undergo differentiation. In an attempt to identify molecules that play critical roles in the conversion of preadipocytes to adipocytes, we cloned by differential screening several regulatory molecules, including pref-1. Pref-1 is an inhibitor of adipocyte differentiation and is synthesized as a plasma membrane protein containing 6 EGF-repeats in the extracellular domain. Pref-1 is highly expressed in 3T3-L1 preadipocytes, but is not detectable in mature fat cells. Dexamethasone, a component of standard differentiation agents, inhibits pref-1 transcription and thereby promotes adipogenesis. Downregulation of pref-1 is required for adipose conversion and constitutive expression of pref-1 inhibits adipogenesis. Conversely, decreasing pref-1 levels by antisense pref-1 transfection greatly enhances adipogenesis. The ectodomain of pref-1 is cleaved to generate a biologically active 50 kDa soluble form. There are four major forms of membrane pref-1 resulting from alternate splicing. Two of these forms which have a deletion that includes the putative processing site proximal to the membrane do not produce a biologically active soluble form. This indicates that alternate splicing may determine the range of action, juxtacrine or paracrine, of pref-1.

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## Introduction

3T3-L1 cells, which have often been used as a model system for adipogenesis, exhibit the properties of fibroblasts during growth.<sup>1</sup> When confluent cells are treated with dexamethasone (DEX) and methylisobutylxanthine (MIX), after several rounds of cell division, the cells withdraw from the cell cycle and undergo adipocyte differentiation over a period of about 7 days. The cells become round, *de novo* synthesis of long-chain fatty acids and esterification to triacylglycerol increase, and the cells accumulate large lipid droplets.<sup>2</sup> Many of the genes involved in fatty acid and lipid metabolism, including adipocyte fatty acid binding protein (aFABP, aP2), stearoyl CoA desaturase 1 (SCD1) and fatty acid synthase (FAS), are induced during adipocyte differentiation. When ectopically expressed, C/EBP $\alpha$  and PPAR $\gamma$  activate adipocyte genes and induce adipogenesis of fibroblasts when they are maintained in a medium contain-

ing serum and DEX that are permissive to differentiation. Growth arrest, which must precede the differentiation process, may also be a mode of action for C/EBP $\alpha$  and PPAR $\gamma$  in promoting adipogenesis. In addition to the transcription factors C/EBP $\alpha$  and PPAR $\gamma$ , various factors in cell–cell communication or cell–matrix interaction may govern whether preadipocytes are kept in an undifferentiated state or undergo differentiation. Rubin and coworkers originally demonstrated that DEX and MIX in the presence of fetal calf serum or IGF-1 (or high concentrations of insulin) trigger adipogenesis in 3T3-L1 cells.<sup>3</sup> Cell adhesion molecules and extracellular matrix (ECM) components also modulate the interaction of cells with their environment in a manner that influences cell differentiation. Maintaining 3T3 preadipocytes on fibronectin-coated dish interferes with requisite morphological changes and the expression of adipocyte-specific genes.<sup>4</sup> The process of adipocyte differentiation therefore requires not only cell cycle withdrawal and expression of specific transcription factors, but also a proper extracellular environment which transduces external signals to the nucleus via a cascade of intracellular signaling. In this review, we describe expression and function of one such molecule, preadipocyte factor-1 (pref-1), that we originally cloned by differential screening in an attempt to identify regulatory molecules for adipogenesis.<sup>5–7</sup>

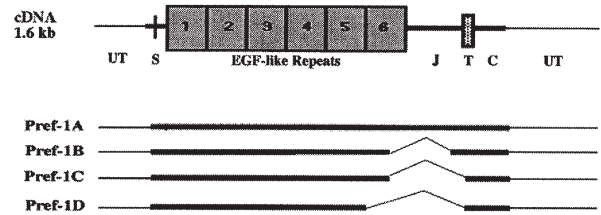
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## Pref-1 and its processing to generate soluble form

Pref-1 encodes a protein of 385 amino acids that contains two hydrophobic stretches: one located within the first 20 N-terminal residues that has the characteristics of a signal sequence and the other, spanning amino acids 300–322, presumably functioning as a single membrane-spanning domain. The most striking structural feature of pref-1 is the presence of six tandem EGF-repeats in the putative extracellular domain. The EGF-repeats of pref-1 maintain both the conserved spacing of six cysteines for the formation of three disulfide bonds, and other amino acids characteristic of the EGF-repeat motif which appears to be for protein–protein interaction and is found in proteins that affect growth and differentiation. This motif was initially described for EGF and is present in TGF $\alpha$  and heparin-binding (HB)-EGF which, by binding to the cell surface EGF receptor, act as signals for proliferation, growth inhibition or differentiation.<sup>8</sup> Several invertebrate cell-fate determining proteins, notably Drosophila Notch and Notch ligands, Delta and Serrate, also belong to the EGF-repeat family of proteins.<sup>9</sup> Notch is often regarded as keeping cells undifferentiated or antagonizing cell-fate determining signals. Those growth factors that function via EGF-receptor undergo proteolytic processing of membrane-bound precursors. Notch ligand Delta was recently shown to be cleaved to also generate a soluble form. Binding of Delta to Notch has been suggested to trigger processing of the intracellular domain of Notch which can then localize to the nucleus and involve in the transcriptional activities.

Pref-1 is found in the preadipocyte cell membrane with molecular weights of 45–60 kDa with N-linked oligosaccharides and sialic acid contributing to the heterogeneity of pref-1 protein.<sup>5</sup> In addition to the full-length form, three major shorter forms of pref-1, each containing in-frame deletions in the extracellular juxtamembrane region, are generated by alternate splicing<sup>10</sup> (Figure 1). The potential functional differences among the alternatively spliced products of the pref-1 gene are discussed below.

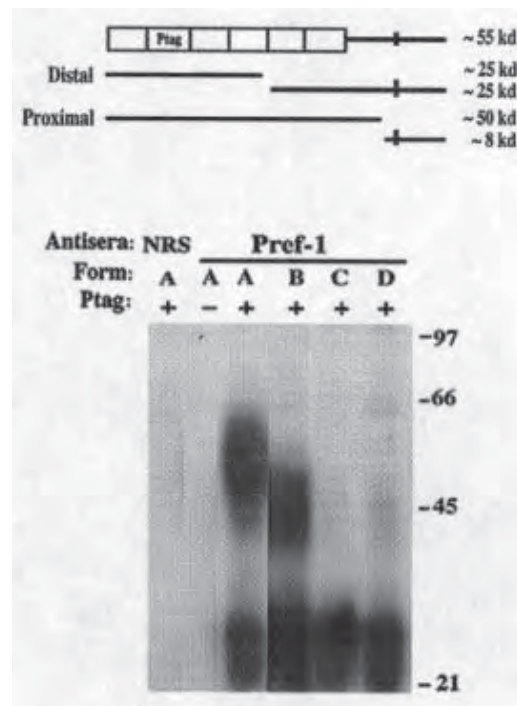
Membrane-associated pref-1 is cleaved at two sites in the extracellular domain. The processing of pref-1 was first evidenced by detection of the smaller membrane-associated residual pref-1 and release of regions of the ectodomain of pref-1.<sup>11</sup> In addition, pulse-chase analysis of media revealed that the major soluble form of pref-1 was a 50 kDa protein, with additional diffuse bands of 24–31 kDa. The increase in soluble pref-1 in the media with a concomitant decrease in the membrane-associated form was consistent with a precursor-product relationship; distinct pref-1 proteins of 31 kDa and a 24–25 kDa doublet, in addition to the 50 kDa major soluble form, were also found in the media. The diffuse nature of the 24–25 kDa doublet suggests glycosylation of the polypeptide backbone. Furthermore, expression of a pref-1 tagged with a



**Figure 1** The structure of pref-1 and the four major forms of pref-1 generated by alternate splicing.

phosphorylation site for A-kinase in the near N-terminal domain in COS cells resulted in the media a phosphorylated protein of 50 kDa, the same soluble product noted by metabolic labeling. The 24–25 kDa was also detected by *in vitro* phosphorylation. These proteins thus probably contain the N-terminal region of pref-1. The 31 kDa form of soluble pref-1 observed by metabolic labeling, on the other hand, was not detected by phosphorylation, indicating that it does not contain the phosphorylation tag. A percentage of the 50 kDa soluble pref-1 may be further processed at a site C-terminal to the tagged phosphorylation site to generate the N-terminal 25 kDa and the more C-terminal 31 kDa. This predicts that there are two processing events. A cleavage proximal to the membrane domain generates 50 kDa soluble pref-1 and a distal cleavage occurs between the phosphorylation tag and the transmembrane domain (Figure 2).

The major 50 kDa soluble form of pref-1 corresponds to the full ectodomain and suggests a



**Figure 2** The two cleavage sites for pref-1 and the soluble forms detected in the conditioned media when pref-1A, pref-1B, pref-1C and pref-1D were transfected into COS cells.

membrane-proximal processing event. The four alternately spliced forms of pref-1, with various extracellular juxtamembrane deletions, provided a system in which to determine if an alternate splicing mechanism controls the generation of soluble vs membrane-anchored pref-1. Strikingly, whereas pref-1A, pref-1B, pref-1C and pref-1D all expressed the 25 kDa in the media, only pref-1A and pref-1B expressed the larger soluble form; pref-1C and pref-1D with larger juxtamembrane deletions did not express the larger soluble form (Figure 2). The simplest interpretation of the absence of the 50 kDa processed product by pref-1C and pref-1D is that the spliced out sequence removes a cleavage site. This indicates that the sequence present in pref-1B but not in pref-1C contains a proximal processing site for the generation of the 50 kDa soluble pref-1. This localizes the membrane proximal cleavage event to within a 22 amino acid juxtamembrane sequence PEQHILKVSMLKELNKSTPLLTE.

### Pref-1 expression during adipogenesis

Pref-1 mRNA is highly expressed in 3T3-L1 preadipocytes at confluence and its expression is abolished in fully differentiated adipocytes (Figure 3). The downregulation of pref-1 during adipocyte differentiation is primarily at the transcriptional level (Figure 3).<sup>12</sup> Standard *in vitro* differentiation of 3T3-L1 preadipocytes involves induction of differentiation by treatment with a combination of DEX and MIX for 48 h, which results in fully differentiated adipocytes 3–5 days later. However, the exact mechanism by which DEX/MIX promotes adipocyte differentiation is not known. Cells treated with DEX/MIX for 48 h had a drastic reduction in all forms of the pref-1 protein and the degree of reduction in pref-1 protein

by DEX was comparable to that of DEX/MIX, indicating that decreased pref-1 protein content was due largely to the DEX effect.<sup>13</sup> All forms of the pref-1 transcript generated by alternate splicing decreased in the same fashion by DEX treatment and the negative regulation of the pref-1 by glucocorticoids is at the transcriptional level. We also found that the efficacy of DEX on pref-1 downregulation and on adipocyte differentiation were similar. Given that DEX may have multiple effects in adipocyte differentiation, it would be premature to conclude that the effects of DEX in adipocyte differentiation are mediated solely through its ability to dramatically reduce pref-1 levels. However, dramatically lowering pref-1 levels, via prolonged exposure to DEX/MIX, greatly optimized differentiation and indicate a function of glucocorticoids in promoting adipogenesis.

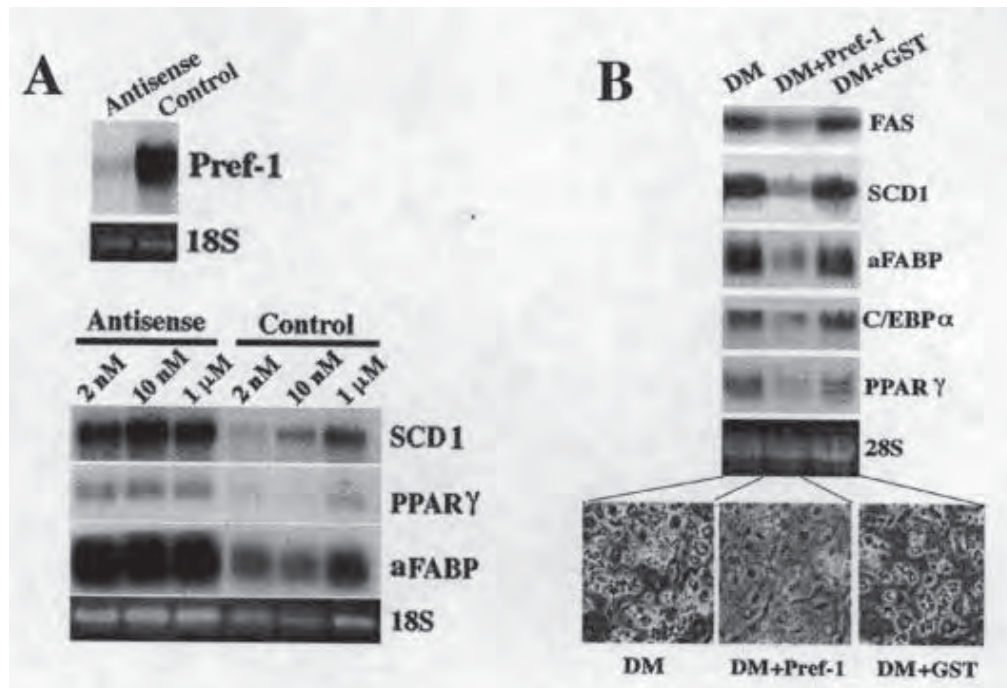
The decrease in pref-1 mRNA is one of the earliest responses of 3T3-L1 preadipocytes known to date upon treatment with a component of the adipogenic differentiation cocktail. If a major functional role of DEX in promoting adipocyte differentiation was via its reduction of pref-1 levels, then reducing pref-1 levels independent of DEX treatment would substitute for DEX treatment in the promotion of adipocyte differentiation. To test this, we attempted to eliminate endogenous pref-1 expression by stable transfection of pref-1 antisense construct into 3T3-L1 cells.<sup>13</sup> Upon confluence antisense and control cells were treated with 0.5 mM MIX alone (no DEX) or in combination with 2 nM, 10 nM or 1 μM DEX. For control cells, 10 nM DEX was highly effective in supporting adipocyte differentiation, while 2 nM was not. While the antisense cells showed a similar level of expression of the adipocyte markers at either 2 nM, 10 nM or 1 μM DEX, control cells exhibited the same DEX dose-responsiveness in their conversion to adipocytes in that marker expression was 70% at 10 nM and 33% at 2 nM compared to mRNA levels at 1 μM DEX, (Figure 4A). In the absence of DEX, while control cultures showed no expression of adipocyte marker mRNAs such as SCD1 and aFABP, the antisense cells showed low but significant SCD1 and aFABP mRNA levels. Similar differences in differentiation were observed when judged by rounded morphology and lipid accumulation. The antisense cells showed nearly complete conversion at all concentrations of DEX and even without added DEX 5–10% of cells differentiated. These data support a model whereby a major function of DEX in the adipocyte conversion process is through its ability to effectively reduce pref-1 transcription.



**Figure 3** Decrease in pref-1 mRNA levels during 3T3-L1 adipocyte differentiation (upper panel). Transcription run-on assay shows decrease in pref-1 transcription and increase in adipocyte marker SCD1 transcription in adipocytes when compared to that in preadipocytes.

### Pref-1 as an inhibitor of adipogenesis

The fact that pref-1 RNA and protein expression is abolished during adipocyte differentiation indicates



**Figure 4** Effect of pref-1 on adipogenesis. Transfection of antisense pref-1 to decrease pref-1 enhances adipocyte differentiation even at lower concentrations of dexamethasone as compared to control 3T3-L1 cells (panel A). Pref-1 (in the form of GST-fusion protein) when added to the 3T3-L1 cell culture media inhibits adipose conversion as judged by adipocyte marker expression and cell morphology (panel B).

that pref-1 is regulated during this process. The correlation of DEX suppression of pref-1 with adipogenesis and the enhancement of adipogenesis by antisense pref-1 expression are consistent with an inhibitory role for pref-1 in adipocyte differentiation. To further demonstrate the role of pref-1, we stably transfected pref-1 in 3T3-L1 cells. Since preadipocytes express high endogenous levels of pref-1 mRNA, the constitutively expressed form only increased total pref-1 RNA by at most 50%. However, it was not the absolute level of pref-1 that we were addressing here, but rather the inability to down-regulate its expression. Oil Red O staining of lipids, the expression of two adipocyte markers, SCD1 and aFABP and microscopic examination of the cultures revealed inhibition of adipocyte differentiation by pref-1.<sup>5</sup> The blockage of adipocyte differentiation in cells unable to down-regulate pref-1 levels due to its constitutive expression, argues that pref-1 downregulation is required for the conversion process, either in a permissive or instructive manner. The molecular basis of the pref-1 function in adipocyte differentiation remains unknown. However, the presence of EGF-like repeats in the protein and the demonstrated role of this motif in other molecules, form the basis for the following hypothesis. During 3T3-L1 differentiation, pref-1 may function via interaction of its EGF-like repeats with the EGF-like repeats of its putative receptor, thereby maintaining a preadipocyte morphology.

To test specifically the effect of soluble pref-1 on adipocyte differentiation, media of confluent 3T3-L1 preadipocytes were supplemented with *E. coli*-expressed pref-1 extracellular domain fused to glutathione-S-transferase (Figure 4B).<sup>11</sup> Addition of pref-1-glutathione-S-transferase fusion protein markedly inhibited differentiation, with only 10% of cells converting to adipocytes. Pref-1-treated cells had only 20% of the levels of the terminal adipocyte marker mRNAs FAS, SCD1 and aFABP. Moreover, the mRNA for C/EBP $\alpha$  and PPAR $\gamma$  decreased to a similar extent, indicating the inability of cells to express these transcription factors in the presence of soluble pref-1, confirming an early inhibitory effect of pref-1. The inhibition of adipogenesis by *E. coli*-expressed pref-1 also indicates that, although pref-1 is usually glycosylated, carbohydrate modification is not required for its function. Unlike pref-1A and pref-1B, which generate the inhibitory 50 kDa soluble pref-1, pref-1C and pref-1D do not produce biologically active soluble pref-1. We have confirmed the inhibitory effect of the larger soluble 50 kDa protein using conditioned media from COS cells transfected with pref-1A; pref-1D, which secretes only the smaller 25 kDa, did not affect adipose differentiation. Since the larger 50 kDa soluble form of pref-1 has inhibitory activity similar to that of the full-length form, release of the pref-1 ectodomain as a soluble factor allows switching between two active forms of pref-1, thereby regulating its range of action. Because all four alternately spliced

pref-1 cDNAs generate membrane-associated forms, while pref-1C and pref-1D generate only the 25 kDa soluble form and not the active larger form, we hypothesize that the mode of function, juxtacrine or paracrine depends on the alternate pref-1 transcript expressed.

## Conclusion

Given the abundant evidence that EGF-like proteins participate in cellular development and differentiation, the ability of forced expression to enhance 3T3-L1 adipocyte differentiation argues for a regulatory role of pref-1 in adipogenesis. It was shown that pref-1 also decreases during differentiation of rat primary preadipocytes in culture and soluble pref-1 inhibits their differentiation.<sup>14</sup> This indicates that pref-1 functions not only in immortalized preadipocyte cell lines but also in primary cells isolated from adipose tissue. Importantly, we found that the pref-1 ectodomain is cleaved to generate soluble pref-1 and that the soluble form is also antiadipogenic. We propose that pref-1 is a unique inhibitor of adipogenesis: pref-1, produced and secreted by the preadipocytes themselves, keeps the cells in an undifferentiated state and prevents differentiation, albeit by an unknown mechanism. In this regard, pref-1 is an excellent marker for preadipocytes. In transgenic mice overexpressing a truncated activated form of SREBP-1c, white fat failed to fully differentiate and showed increased pref-1 expression.<sup>15</sup> Unger and coworkers recently reported that adenovirus-induced hyperleptinemia transformed adipocytes into cells devoid of adipocyte marker expression but having increased pref-1, suggesting dedifferentiation of these cells.<sup>16</sup> Pref-1 may be a component of a feedback loop in maintaining an undifferentiated state. Pref-1 is not mitogenic and pref-1 does not affect c2c12 myoblast differentiation to myotubes (unpublished observations). This is in contrast to classic growth factors which are made by variety of cell types and inhibit differentiation of multiple types of cells generally by preventing the cell cycle withdrawal that must precede differentiation. There was no increase in pref-1 expression nor PPAR $\gamma$  expression changed in mitogenic growth factor-mediated inhibition of adipocyte differentiation.<sup>13</sup> Dedifferentiation of adipocytes by TNF $\alpha$  treatment decreased PPAR $\gamma$  but did not increase pref-1 expression.<sup>17</sup> On the other hand, inhibition of rat primary preadipocyte differentiation by growth hormone was reported to be by preventing a decrease in pref-1 levels.<sup>14</sup> We found that the decrease in pref-1 is an early event in that it precedes an increase in PPAR $\gamma$  expression and that constitutive expression of pref-1 inhibits adipogenesis and prevents expression of C/EBP $\alpha$  and PPAR $\gamma$ . This indicates that inhibition of PPAR $\gamma$  expression is a downstream event of pref-1

signaling and that attenuation of pref-1 signaling is required for C/EBP and PPAR $\gamma$  expression. Pref-1, as suggested by Ringold and coworkers, may be a non-reversible molecular checkpoint early in the adipocyte differentiation process.<sup>17</sup> How does pref-1 expression keep the cells in an undifferentiated state? Identification of the pref-1 receptor will provide us with key insights to pref-1 action and signaling.

## References

- Green H, Kehinde O. Spontaneous heritable changes leading to increased adipose conversion in 3T3 cells. *Cell* 1976; **7**: 105–113.
- Gregoire F, Smas CM, Sul HS. Understanding adipocyte differentiation. *Physiol Rev* 1998; **78**: 783–809.
- Smith PJ, Wise LS, Rubin CS. Insulin-like growth factor-I is an essential regulator of the differentiation of 3T3-L1 adipocytes. *J Biol Chem* 1988; **263**: 9402–9408.
- Spiegelman BS, Farmer SR. Decreases in tubulin and actin gene expression prior to morphological differentiation of 3T3 adipocytes. *Cell* 1982; **29**: 53–60.
- Smas CM, Sul HS. Pref-1, a protein containing EGF-like repeats, inhibits adipocyte differentiation. *Cell* 1993; **73**: 725–734.
- Smas CM, Sul HS. Molecular mechanisms of adipocyte differentiation and inhibitory action of pref-1. *Crit Rev Eukaryotic Gene Expression* 1997; **7**: 281–298.
- Sul HS, Smas CM, Mei B, Zhou L. Regulation of fat synthesis and adipose differentiation. *Prog Nucleic Acid Res Mol Biol* 1997; **60**: 317–345.
- Carpenter G, Cohen S. Epidermal growth factor. *J Biol Chem* 1990; **265**: 7709–7712.
- Artavanis-Tsakonas S, Rand MD, Lake RJ. Notch signaling: cell fate control and signal integration in development. *Science* 1999; **284**: 770–776.
- Smas CM, Green D, Sul HS. Structural characterization and alternate splicing of the gene encoding the preadipocyte EGF-like protein pref-1. *Biochemistry* 1994; **33**: 9257–9265.
- Smas CM, Chen L, Sul HS. Cleavage of membrane-associated pref-1 generates a soluble inhibitor of adipocyte differentiation. *Mol Cell Biol* 1997; **17**: 977–988.
- Smas CM, Kachinskas D, Liu C, Xie X, Dircks L, Sul HS. Transcriptional control of the pref-1 gene in 3T3-L1 adipocyte differentiation. Sequence requirement for differentiation-dependent suppression. *J Biol Chem* 1998; **273**: 31751–31758.
- Smas CM, Chen L, Zhou L, Latasa J-M, Sul HS. Transcriptional suppression of preadipocyte factor-1 by glucocorticoids promotes 3T3-L1 adipocyte differentiation. *J Biol Chem* 1999; **274**: 12632–12641.
- Hansen LH, Madsen B, Teisner B, Nielsen JH, Billestrup N. Characterization of the inhibitory effect of growth hormone on primary preadipocyte differentiation. *Mol Endocrinol* 1998; **8**: 1140–1149.
- Shimomura I, Hammer RE, Richardson JA, Ikemoto S, Bashmakov Y, Goldstein JL, Brown MS. Insulin resistance and diabetes mellitus in transgenic mice expression nuclear SREBP-1c in adipose tissue: model for congenital generalized lipodystrophy. *Genes Dev* 1998; **12**: 3182–3194.
- Zhou YT, Wang ZW, Higa M, Newgard CB, Unger RH. Reversing adipocyte differentiation: implications for treatment of obesity. *Proc Natl Acad Sci USA* 1999; **96**: 2391–2395.
- Xing H, Northrop JP, Grove JR, Kilpatrick KE, Su JL, Ringold GM. TNF $\alpha$ -mediated inhibition and reversal of adipocyte differentiation is accompanied by suppressed expression of PPAR $\gamma$  without effects on pref-1 expression. *Endocrinology* 1997; **138**: 2776–2783.