Insulin and cellular stress induced glucose uptake in 3T3-L1 adipocytes

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Chapter II

An introduction to 3T3-L1 adipocytes.

The adipocyte is a remarkable cell type in several aspects. For years the adipocyte has been viewed as a rather passive cell, simply a deposit site of excess energy in the form of lipids [1;2]. However, work with tissue-specific knock-out mice, the complex phenotype of patients with altered adipocyte function and the description of a range of proteins secreted by these cells, have established the adipocyte as a major regulator of whole body energy-homeostasis, influencing metabolic settings in key organs such as muscle, liver and brain [3-5]. Furthermore, the tight connection between adipocyte-mediated vascular remodelling and several types of cancer also identify adipose tissue as an important endocrine organ [6-8]. Aside from its endocrine role, the adipocyte serves to protect other organs from the deleterious effects of excessive intracellular triglyceride storage [9-11]. Thus, although adipose tissue accounts for only ~10% of whole body glucose uptake, an adipose tissue specific GLUT4 knock-out mouse displays glucose intolerance caused by a secondary insulin-resistance in muscle- and liver-cells [12].

Main effectors in this cross-talk are the "adipokines" TNFα , adiponectin and leptin [13-15]. Increases in levels of TNFα, as seen in the obese state, are associated with a deleterious impact on insulin-sensitivity in adipocytes, muscle and liver (Fig. 1). Conversely, adiponectin has a positive effect on insulin-sensitivity by stimulating fatty acid oxidation through the activation of AMPK and PPARγ [16-19]. The central role of adiponectin is illustrated by the adipocyte-specific insulin-receptor knock-out mouse. Although these adipocytes are no longer capable of insulin-induced glucose uptake, blood glucose levels are normal, due to an elevation in levels of adiponectin in these mice [20;21].

Another adipokine, acting in conjunction with adiponectin is the satiety hormone, leptin. This hormone regulates food intake through its effects on the hypothalamus [22], and mediates metabolic effects on peripheral tissues [23;24]. Adipocyte selective reduction of leptin receptors has profound effects on the regulation of metabolic genes, characterising an autoendocrine-loop in these cells [25]. Other functions of leptin involve regulation of AMPK, leading to fatty acid oxidation [26-28], the lipogenic transcription factor SREBP-1c [26] and PGC-1α, a powerful inducer of mitochondrial biogenesis (Fig. 1)[29;30]. The involvement of adipokines in metabolic homeostasis is further illustrated by the occurrence of insulin resistance associated with lipodystrophy.

Fig. 1

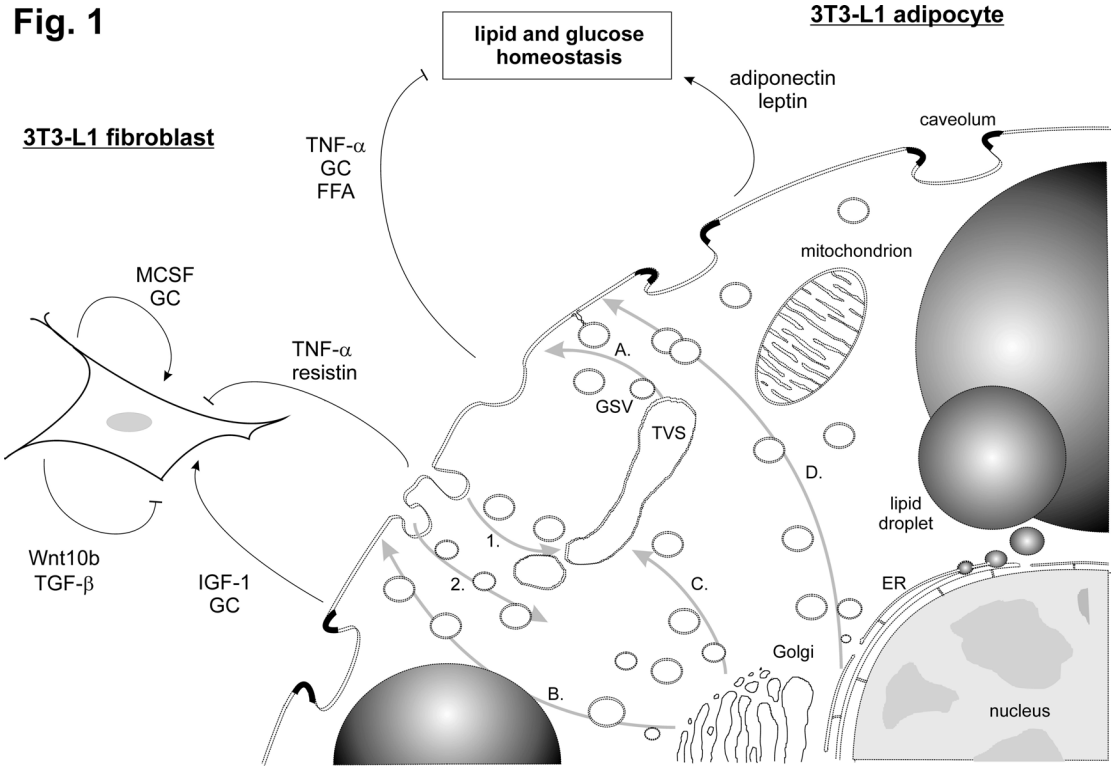

Fig. 1 A schematic overview of a 3T3-L1 adipocyte : cellular organelles, main vesicle pathways and adipokine signalling.
A fully mature adipocyte is an endocrine cell involved in regulating whole body lipid and glucose homeostasis through the secretion of both stimulatory (adiponectin, leptin) and inhibitory (TNF-α, glucocorticoids (GC) and Free Fatty Acids) adipokines. Aside from regulating metabolic settings in target tissues the adipocyte is also tightly involved in adipogenesis through TNF-α, resistin, IGF-1 and GC-signalling. Autocrine factors derived from the pre-adipocyte involved in regulating differentiation are MCSF, TGF-β and Wnt10b.
Characteristic cell-components of the fully mature adipocyte are the caveolae and the lipid droplets, which are derived from the Endoplasmic Reticulum (ER). The Low Density Microsomal fraction (LDM) consists of several vesicular components involved in cellular trafficking. Main trafficking routes contributing to the LDM are endocytotic : 1. recycling of GLUT4 from the plasma-membrane, 2. clathrin-coated pits involved in recycling of receptors, and exocytotic : A. translocation of insulin-responsive GLUT4 Storage Vesicles (GSV) towards the plasma membrane, B. direct endosomal shuttling of GLUT4 containing vesicles (either from Golgi stacks or from the endosomal Tubulo-Vesicular Sorting (TVS) compartment), C. shuttling of GLUT4 containing vesicles towards the TVS and translocation of synthesised adipokines from either the ER or Golgi.

In mice models of lipodystrophy, injections with adiponectin and leptin ameliorate insulin-resistance accompanied by clearance of triglycerides in muscle and liver [26;31;32].
The 3T3-L1 adipogenic cell-line was established thirty years ago when Green and Meuth noted a high tendency in clones of Swiss 3T3 fibroblasts to undergo spontaneous adipogenic conversion [33-35]. Though immortalised 3T3-L1 cells are not transformed as is evidenced by their contact-inhibition (Fig. 2). At this stage cellular changes in the postmitotic adipoblasts are readily apparent with the cell flattening out, the nucleoli becoming visible (see Fig. 2B) and at a molecular level, the upregulation of the growth-arrest associated gene 2 [36]. Overriding contact-inhibition results in a fully transformed phenotype and loss of the ability to differentiate [37]. When fully arrested, cells are challenged with a potent adipogenic cocktail consisting of insulin, IBMX and dexamethasone (Table I)[38;39]. Whereby the phosphodiesterase–inhibitor IBMX can be replaced by PPARγ agonists [40]. At this stage a number of crucial events take place : The medium becomes viscoelastic due to the excretion of highly crosslinked hyaluronic acid and the induction of metalloproteinases indicating an important outside-in signalling contribution [41-44]. The cells round up without losing the filipoda-connections with which they are linked to one-another (Fig. 2C and D). At this stage profound cell-morphological differences between lots of FCS become readily apparent, initiating the discrepancy in adipocytes differentiated under different batches of FCS (see Fig. 3A). Subsequently the cells undergo 2-4 rounds of clonal expansion and arrest in G1, whereas many other cells simply round up and enter apoptosis. Components of the p53-signalling pathway : Mdm-2, p21 and its family member p27 are tightly regulated at this stage [45-48]. The pocket-proteins, pRb, p130 and p107 are involved in regulating adipogenesis too : After a distinct switch to p107 during the clonal expansion stage the re-emergence of p130 as the main E2F-binding protein marks the final commitment of the cell to enter the G0 state (see Fig. 4)[49-52]. Remarkably, when these contact-inhibited cells are passaged into a new culture p130 will not re-emerge again which prevents 3T3-L1 cells from entering the differentiation programme a second time. The pRb protein meanwhile, regulates C/EBPβ activity and drives differentiation towards white over brown adipose tissue [53-56]. Important components of the mitogenic response are the MAPK family members. Illustrating this in PC12-cells, studies of MAPK activation in response to either EGF or NGF demonstrated that the determination between mitogenesis or differentiation is highly dependent on the kinetics of MAPK signalling [57;58].

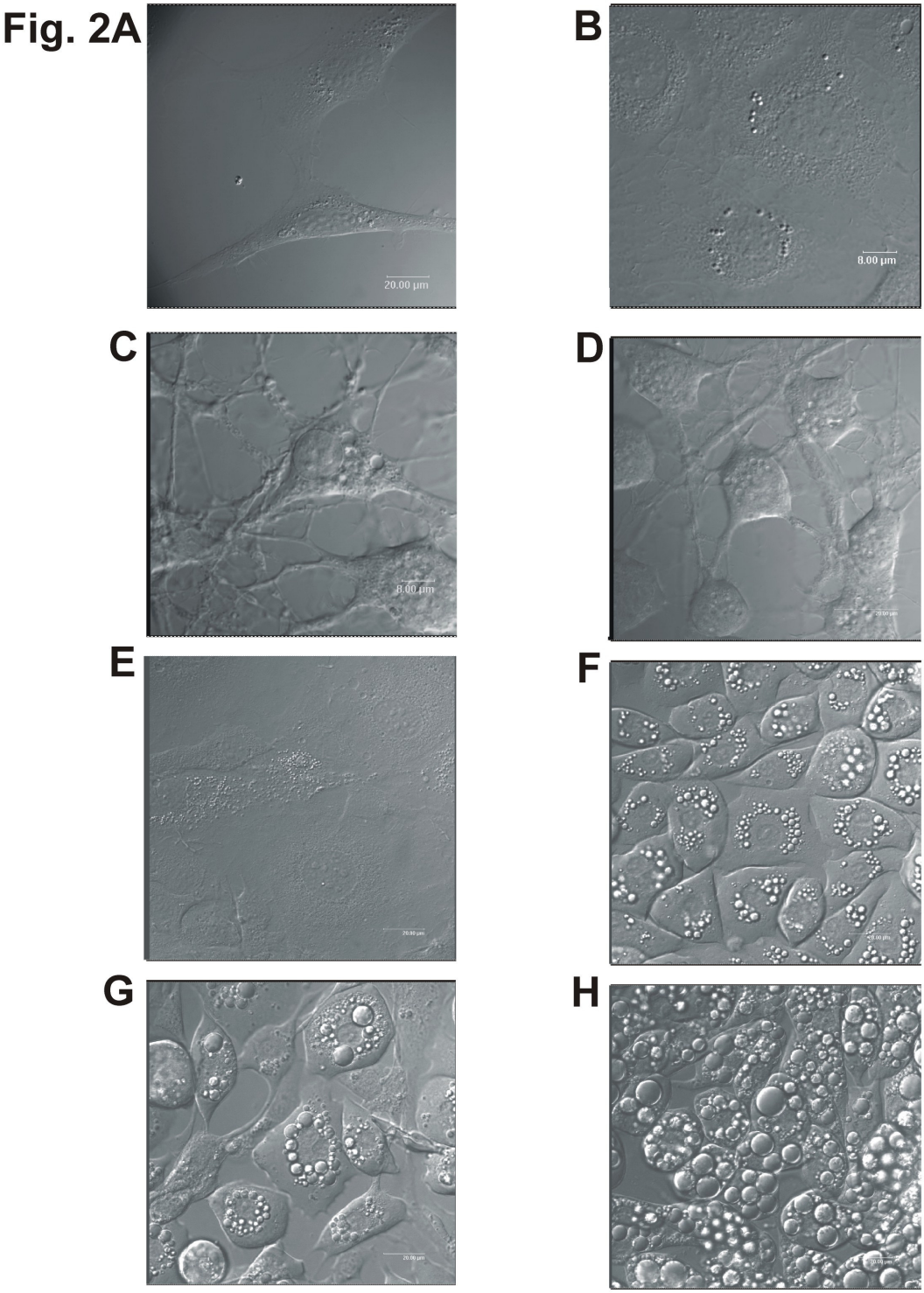

Fig. 2 Nomarski-photographs of differentiating adipocytes.
Panel A. growing 3T3-L1 fibroblasts, B. fibroblasts, flattened out at the growth arrested stage, C. and D. pre-adipocytes in Diff. I with the extended filipodic connections and their cytoplasmic components shrunk to barely more than the nucleus. E. early in Diff. II, flattened out cells and the start of lipid droplet formation. F. and G. maturation of the lipid droplets. H. fully mature 3T3-L1 adipocytes. Size (in micrometer) is indicated by a white bar in the photographs.

Fig. 3A

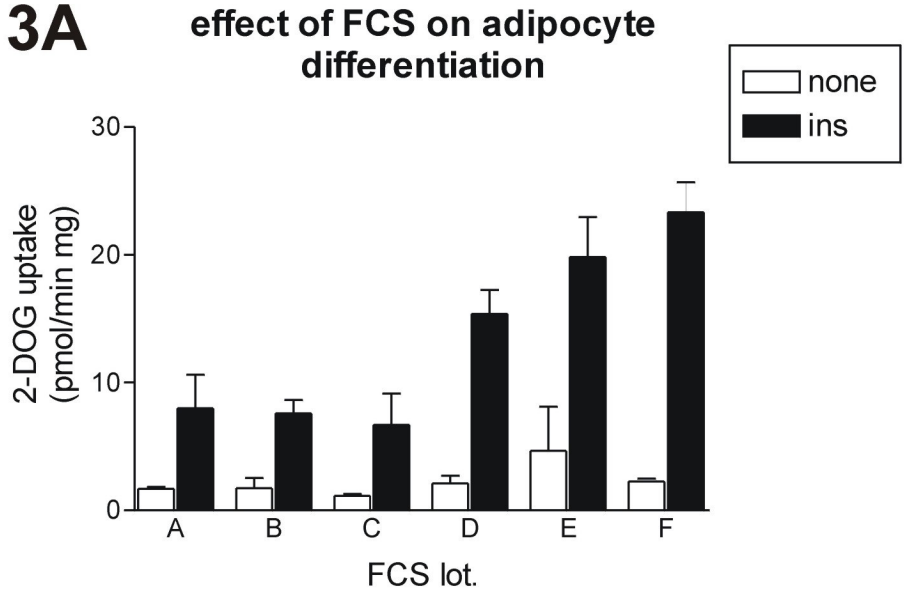

Fig. 3B

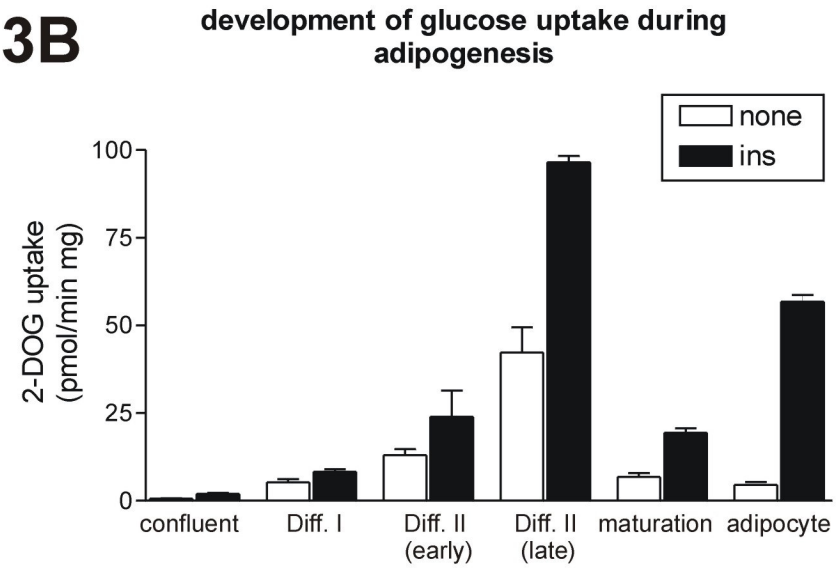

Fig. 3 Analysis of insulin-induced glucose uptake.
Panel A. 3T3-L1 adipocytes differentiated using several batches of Foetal Calf Serum (FCS) demonstrating profound differences in basal levels of glucose uptake (white bars) and insulin-stimulated glucose uptake (black bars). Lot A and F, and lot D and E were obtained from the same supplier. Panel B. Development of insulin-responsiveness during adipogenesis. 3T3-L1 fibroblasts (confluent stage) have a slight response to insulin. The increase in the Dif. I response is mediated by a stress-induced increase in GLUT1 synthesis. During Diff. II the insulin responsive GLUT4 and associated vesicular compartments generate a profound increase in insulin-induced glucose uptake capacity, though at this stage the differentiating adipocytes are insulin-resistant as can be seen in the maturation stage. After completion of the maturation stage, the now fully mature adipocyte has overcome its initial insulin-resistance and downmodulated GLUT1-mediated glucose uptake leading to the profound response in insulin-induced glucose uptake over characteristically low basal levels observed.

During the initial stages of adipogenesis the induction of MAPK family members ERK-1/2 leads to the induction of PPARγ and C/EBPα [59-63]. However, after this initial stage ERK signalling is terminated. Prolonged activation, such as induced by EGF-signalling, inhibits adipocyte differentiation through the inhibition of crucial adipogenic transcription factors (Fig. 4)[64-66]. Meanwhile p38 MAPK induces activation of C/EBPβ [67-69], though similar to ERK-1/2, prolonged activation inhibits adipogenesis through the activity of CHOP [70-72].
Another key transcription factor in adipogenesis is CREB [73], which is crucial in preventing apoptosis through its inhibition of several pro-apoptotic genes such as ICE and by stimulating PKB expression [74]. Subsequently, the downregulation of pre-adipocyte factor-1 and the induction of C/EBPβ and –δ induces the upregulation of PPARγ and C/EBPα (Fig. 4)[40;75-79]. These latter two regulate the late-stage genes in adipogenesis, such as GLUT4, aP2 and adiponectin. Simultaneously the characteristic insulin-responsive microsomal-vesicular GLUT4 storage compartment is formed (Fig. 3B)[80]. To be precise, C/EBPα is not required for the generation of an "adipocyte" as such, but is crucial for conferring proper insulin-responsiveness on the cell. Thus in a C/EBPα knock-out mouse adipocytes are incapable of lipid accumulation [81-85]. On the other hand, an adipose-specific PPARγ knock-out mice displays adipocyte hypocellularity and loss of leptin and adiponectin [86-88]. The insulin present in the cocktail induces the activation of PI-3' kinase through the IGF I Receptor [89-91], regulating the FKHR-transcription factors, C/EBPα and SREBP1 (Fig. 4)[92-95]. The lipid- and cholesterol-metabolism genes regulated by SREBP1 mediate the synthesis of endogenous ligands for PPARγ [96;97], illustrating autocrine signalling loops involved in adipogenesis (Fig. 1). Potent adipogenesis stimulating factors are Macrophage Colony-Stimulating Factor (MCSF), Insulin-like Growth Factor-1 (IGF-I) and Glucocorticoids (GC)[98-101]. The latter are not generated by the adipocyte as such. Rather, both primary pre-adipocytes and fully mature adipocytes express 11β-hydroxysteroid dehydrogenase 1, which catalyses the conversion of inactive corticosterone to active cortisol (a glucocorticoid)[102-104]. Conversely, the aforementioned TNFα, resistin, Transforming Growth Factor-β (TGFβ) and Wnt10b-signalling maintains adipocytes in an undifferentiated form [105-109]. Matter of factly, the Wnt-signalling components β-catenin and GSK-3β are extensively downregulated during the first days of differentiation [75;110;111].
With the onset of C/EBPα and PPARγ the pre-adipocyte matures as is visible by the formation of lipid droplets in the perinuclear region (see

Fig. 2F and 3B). These droplets are derived from the endoplasmic reticulum and covered by the adipocyte-specific perilipins (Fig. 1)[112;113]. PKA-mediated perilipin phosphorylation induces a conformational change of the perilipins allowing access to Hormone Sensitive Lipase and induces translocation of HSL towards the lipid-droplet [114-116]. PKA is acutely stimulated by lipolytic-hormones explaining the large cellular effects of these hormones on adipocytes [117;118]. Conversely, insulin inhibits lipolysis by activating phosphodiesterase-3, which leads to a loss of PKA activity [119]. Furthermore, insulin also induces the formation of an inhibitory complex between HSL and lipotransin [120]. Consequently, the presence of insulin in the Diff. II medium allows the lipid droplets to coalesce and expand until only a small number of large droplets is left, taking up roughly 70% of the cell-volume (Fig. 2G and H). Recent analysis of the protein profile found associated with these lipid droplets suggests that it is an important signalling compartment [121]. This is illustrated by the observation that when perilipins are ablated in knock-out mice, the mice become resistant to diet-induced obesity. Microarray analysis of these mice demonstrates a coordinated upregulation of genes involved in beta-oxidation, the Krebs cycle and the electron transport chain concomitant with a downregulation of genes involved in lipogenesis [122]. During adipogenesis cellular levels of mitochondria also increase, accompanied by qualitative changes in the mitochondrial composition (Fig. 1)[123;124]. In contrast to many continuous cell-lines, the 3T3-L1 adipocyte employs oxidative phosphorylation as a source of ATP [125]. Intriguingly, in response to insulin adipocytes also activate fatty acid oxidation in the mitochondria, even though the net effect of insulin is lipogenesis. Though this ‘futile cycle’ may seem a waste of energy, this cycle generates body heat and intermediates needed for the synthesis of other biochemical compounds [126].

Another cell-morphological feature of adipocytes is the presence of caveolae in the plasma-membrane (Fig. 1). According to the lipid-ordering hypothesis, membranes co-exist in two predominant forms : the liquid-disordered state, composed of phospholipids with relatively rapid lateral diffusion and the lipid-rafts, which are high in cholesterol- and sphingolipid-content resulting in a more rigid and confining environment [127;128].

Fig. 4

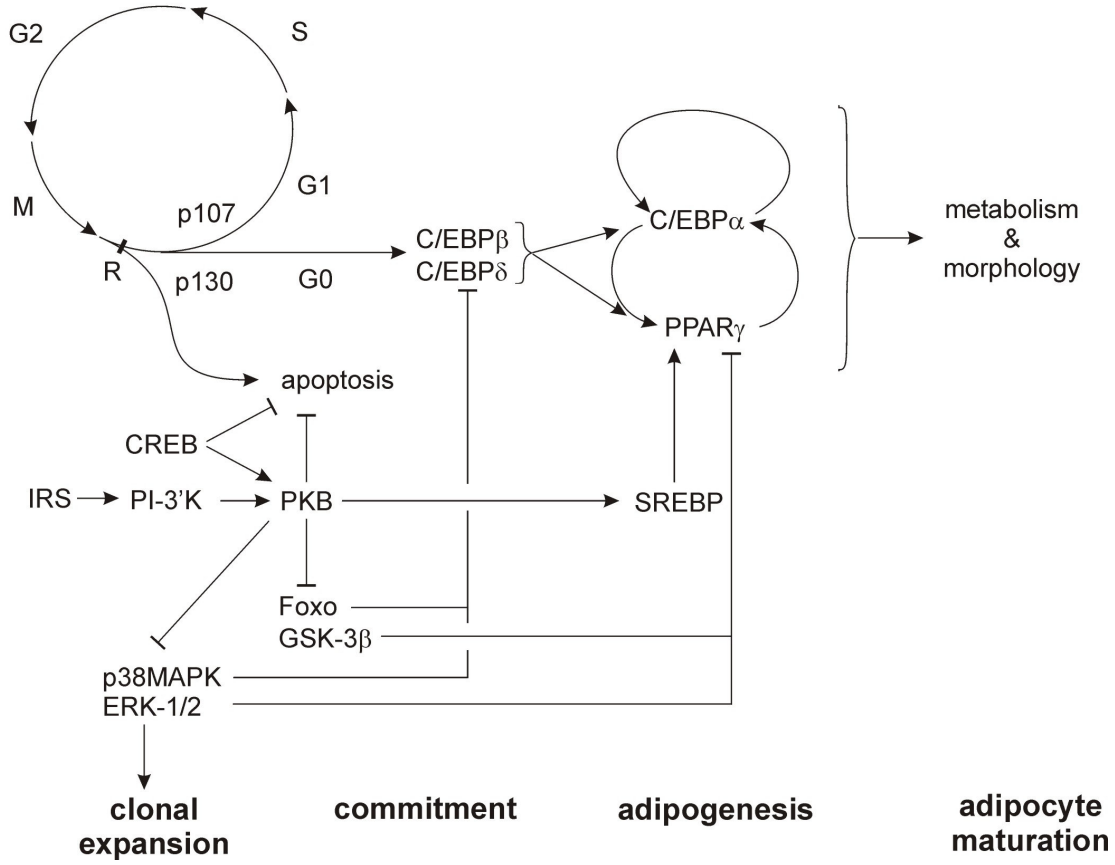

Fig. 4 Signalling pathways and stages involved in 3T3-L1 adipogenesis.
Mitotic cell stages involved in clonal expansion are indicated by their respective phases (Gap_1, Synthesis, Gap_2 and Mitosis), with the Restriction point involved in growth arrest and switch to the Gap_0 differentiation pathway. This stage is under control of the pocket proteins p107 and p130, IGF-I signalling (PI-3'kinase and PKB) and MAPK-signalling (ERK-1,-2 and p38). Apoptosis-induced cell loss occurs throughout the differentiation process, but is indicated in this picture as an alternative side-route of the cell-cycle.
Entry of the G_0 marks the entry of the commitment-stage dominated by C/EBPβ and –δ. From this stage onwards, continued MAPK- or GSK-3β signalling at this stage inhibits adipogenesis. C/EBPβ and –δ induce the main adipogenic transcription factors C/EBPα and PPARγ in conjunction with autocrine signalling. C/EBPα and PPARγ are interlocked in positive autoregulatory loops and mediate transcription of adipocyte-specific genes leading to the formation of a fully mature insulin-responsive 3T3-L1 adipocyte.

Caveolae are a specialised lipid raft characterised by the structural protein caveolin-1 forming the neck of these invaginations, thereby restricting random diffusion of the caveolar constituents [129-131]. At the plasma-membrane they form 50-100 nm omega-shaped invaginations morphologically distinct from clathrin coated pits [132;133]. In adipocytes a higher order organisation of the caveolae in "rosetta"-structures exists, though the precise reason for this clustering of caveolae remains unclear [134;135]. The two other members of the caveolin-family, Cav-2 and –3, also target exclusively to caveolae [136;137]. Whereas Cav-1 and –2 are coexpressed [138;139], Cav-3 expression is limited to muscle cells [136]. During adipogenesis, caveolae increase dramatically in number concomitant with an increase in caveolin-expression [140;141]. However, Cav-1 knock-out mice display a mild phenotype, such as exercise intolerance and decreased vascular tone, but no overt diabetes [142;143]. And treatment of adipocytes with the cholesterol chelating compounds nystatin and filipin has no effect on insulin-stimulated glucose uptake [144]. Although treatment with the more potent agent methyl-β-cyclodextrin inhibits IRS-1 activation, a total depletion of membrane-cholesterol also affects the organisation of the actin-cytoskeleton [145;146]. Yet, a direct interaction between the insulin receptor and caveolin is required for stabilisation of the insulin receptor [147-149]. And indeed, Cav-1 knock-out mice display a pronounced loss of the number of insulin receptors [150]. Furthermore, Cav-1 knock-out mice are lean, resistant to diet-induced obesity and display adipocyte abnormalities with attenuated serum leptin and adiponectin levels and loss of lipid homeostasis [151]. At face value, these mice resemble an adipocyte specific insulin-receptor knock-out (FIRKO) mouse [21]. There are some substantial differences though, such as a decrease in brown fat mass, an increase in plasma leptin and adiponectin and consequently a reduction in serum triglyceride levels in FIRKO mice with the opposite occurring in Cav-1 null mice. This is due to additional functions of the caveolae, such as its involvement in lipid homeostasis and signalling [152-154].

One of the hallmarks of a fully differentiated 3T3-L1 adipocyte is its marked insulin-induced glucose uptake, mediated by GLUT4 (Fig. 3B)[80;155]. In unstimulated cells GLUT4 is mainly localised in several intracellular vesicular compartments distinct from those employed by adipokines, demonstrating adipocytes maintain several insulin-responsive membrane compartments [156-159]. Among the intracellular structures harbouring GLUT4 are : a tubulo-vesicular endosomal recycling compartment [160-163], AP1/clathrin coated vesicles budding from either the TGN or endosomes, AP2 coated vesicles budding from the plasma-membrane, and a distinct population of GLUT4 Storage Vesicles (GSV)

harbouring a preponderance of GLUT4 and excluding general endosomal markers (Fig. 1)[161;164-167]. Of these LDM-vesicles (as they are collectively known) especially the GSV translocate rapidly towards the plasma-membrane in a PI-3'kinase dependent manner. However, endosomal ablation also causes a partial block of insulin-stimulated GLUT4 translocation, illustrating an direct involvement of the endosomal compartment as well [164;165;168]. This endosomal pathway is involved in GLUT4 translocation induced by cellular stress, exercise and GTPγS [168-170].
With respect to the cytoskeleton in support of these structures, during adipogenesis the fibroblastic "stress-like" F-actin filaments disappear and are replaced by a cortical F-actin structure accompanied by a rearrangement of the cytoskeleton structures involved in GLUT4 translocation [171-176]. Furthermore, a novel type of actin filament, the so-called cav-actin (caveolae associated F-actin) originates in the cell, associated with the aforementioned rosetta-structures [177]. Recent data show either actin stabilising, or actin disrupting pharmacological agents severely inhibit insulin-induced glucose uptake suggesting the cytoskeleton is actively involved in regulating GLUT4 translocation, rather than acting passively as a barrier or a molecular railroad [178-187].
In conclusion, the process of 3T3-L1 adipogenesis highlights the complex molecular rearrangements implemented in a terminally differentiating cell. The re-routing of MAPK-signalling pathways, closing down of Wnt-signalling and enabling CAP-signalling occurs in intimate association with cell-morphological alterations such as the formation of caveolae, the cortical actin structure and insulin-responsive GLUT4 storage vesicles. In the fully differentiated adipocyte, a complex signalling interplay exists between these cellular structures embedding the insulin-signalling pathway and the secreted adipokines.

Table I Experimentel set-up of 3T3-L1 adipogenesis

day	medium	comments
1	normal	Normal adipocyte-culturing medium consists of DMEM with 10% FCS. The FCS serum deployed throughout the procedure must have been tested for its adipogenic potential (see also Fig. 3A). Routinely cells are set up 1:20, though as high as 1:100 can be maintained.
4	normal	Usually cells are now roughly 70% confluent and have to be passaged into a new culture to prevent contact-inhibition. Up till passage 8 can be used, thereafter the cells rapidly lose adipogenic potential through the consequent "selection" of the fastest growing (transformed) cells with each passage.
7	normal	
10	normal	Usually the cells are now fully confluent and growth arrested (Fig. 2B). The cells are left in their contact-inhibited state for at least two days.
12	Diff. I	Differentiation I medium consists of 1.6 μM insulin, 0.5 mM IBMX, 0.25 μM dexamethasone and 10% FCS. The following day cells show their characteristically "stressed" appearance as depicted in Fig. 2C and D.
15	Diff. II	Differentiation II medium consists of 1.6 μM insulin and 10% FCS. Addition of this medium should be applied with care as the stressed cells are but loosely attached at this stage. The following day cells show their "relaxed" appearance as depicted in Fig. 2E.
18	Diff. II	A second treatment with insulin. At these stages the medium becomes highly viscous and acidified, making it sometimes prudent to refresh the medium an additional time in between. Cells are as depicted in Fig. 2F, by eye the plate looks clustered-opaque due to the presence of lipid droplets in the cells.
21	normal	The cells need time to recover from their initial insulin-resistance, as can be observed in Fig. 3B.
23	normal	Due to the fact that adipocytes are metabolically more active, leading to medium acidification, and excrete (amongst others) TNFα, the medium has to be replenished more regularly than in their fibroblastic stage.
25	normal	
27	normal	At this moment the cells are fully mature (see Fig. 2G and H) and highly insulin-responsive (see Fig. 3B). Roughly 95% of the cells will have been converted into mature, lipid laden adipocytes.
30	normal	
33	end culture	From the start adipocytes are lost due to apoptosis and cell death. As the adipocytes are terminally differentiated, lost cells are not replenished. Non-converted (fibroblastic) cells however keep on dividing and will occupy any open place available. Furthermore, at this stage adipocytes are rapidly becoming insulin-resistance as a consequence of their secreted adipokines.

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