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LKB1 signalling attenuates early events of adipogenesis and responds to adipogenic cues.

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ABSTRACT

cAMP-response element-binding protein (CREB) is required for the induction of adipogenic transcription factors such as CCAAT/enhancer-binding proteins (C/EBPs). Interestingly, it is known from other tissues that LKB1 and its substrates AMP-activated protein kinase (AMPK) and salt-inducible kinases (SIKs), negatively regulate gene expression by phosphorylating the CREB co-activator CRTC2 and class IIa histone deacetylases (HDACs), which results in their exclusion from the nucleus where they co-activate or inhibit their targets. In this study, we show that AMPK/SIK signalling is acutely attenuated during adipogenic differentiation of 3T3-L1 preadipocytes, which coincides with dephosphorylation and nuclear translocation of CRTC2 and HDAC4. When subjected to differentiation, 3T3-L1 preadipocytes in which LKB1 expression was stably reduced using shRNA (LKB1-shRNA), as well as LKB1 knockout mouse embryonic fibroblasts (LKB1-/- MEFs), differentiated more readily into adipocyte-like cells and accumulated more triglycerides compared to scrambled-shRNA 3T3-L1 cells or Wt MEFs. In addition, the phosphorylation of CRTC2 and HDAC4 was reduced, and the mRNA expression of adipogenic transcription factors C/EBPα, peroxisome proliferator-activated receptor γ (PPARγ) and adipocyte-specific proteins such as hormone sensitive lipase (HSL), fatty acid synthase (FAS), aP2, Glut4 and adiponectin was increased in the absence of LKB1. The mRNA and protein expression of CHOP-10, a dominant negative member of the C/EBP family, was reduced in LKB1 shRNA expressing cells, providing a potential mechanism for the up-regulation of Pparg and Cebpa. These results support the hypothesis that LKB1 signalling keeps preadipocytes in their non-differentiated form.
INTRODUCTION

Adipogenesis is a multi-step process through which progenitor cells differentiate into functional adipocytes, and involves a cascade of adipogenic transcription factors and genes that define the adipocyte phenotype (Rosen 2005). In cultured preadipocytes, the CCAAT/enhancer-binding proteins β and δ (C/EBPβ and C/EBPδ) are induced within hours following induction of differentiation. C/EBPβ and C/EBPδ then induce the expression of peroxisome proliferator-activated receptor γ (PPARγ) and CCAAT/enhancer-binding protein α (C/EBPα) (Christy, et al. 1991; Clarke, et al. 1997; Wu, et al. 1995; Yeh, et al. 1995). Once expressed, PPARγ and C/EBPα cooperate to induce the majority of genes involved in the adipocyte phenotype such as adipocyte protein (Fabp4, aP2), hormone sensitive lipase (Lipe, HSL) and fatty acid synthase (Fasn, FAS) (Cornelius, et al. 1994).

Signalling pathways that induce the adipogenic transcriptional machinery remain poorly characterized. It is generally accepted that this occurs partly through the cAMP/protein kinase A (PKA) pathway via the activation of cAMP-response element-binding protein (CREB). CREB is required for adipocyte differentiation by inducing the expression of C/EBPβ and potentially C/EBPδ (Belmonte, et al. 2001; Reusch, et al. 2000; Zhang, et al. 2004). CREB activity is regulated by PKA-dependent phosphorylation, and also requires co-activators such as CREB-regulated transcription co-activators (CRTC) (Conkright, et al. 2003; Ravnskjaer, et al. 2007; Screaton, et al. 2004; Xu, et al. 2007). When phosphorylated, CRTC bind to 14-3-3 proteins and are sequestered in the cytoplasm. Upon increasing levels of cAMP, CRTC are dephosphorylated and translocate to the nucleus where they co-activate CREB (Screaton et al. 2004).

The tumour suppressor kinase LKB1 and its substrates in the AMP-activated protein kinase (AMPK) family inhibit CREB-mediated gene expression via phosphorylation of CRTC (Koo, et al. 2005; Muraoka, et al. 2009; Screaton et al. 2004). Another recently identified group of substrates for AMPK family members, is the class IIa histone deacetylases (HDAC 4, 5, 7). Like CRTC, these HDACs are regulated by phosphorylation and binding to 14-3-3 proteins, preventing their nuclear entry (Bassel-Duby...
and Olson 2006; Haberland, et al. 2009; McKinsey, et al. 2000). In the nucleus, class IIa HDACs have
been shown to regulate gene expression, such as Glut4 in adipocytes (Weems, et al. 2012).

AMPK and its related kinases the salt inducible kinases 1-3 (SIK1-3) are phosphorylated and activated by
the common and constitutively active upstream kinase LKB1 (Hawley, et al. 2003; Lizcano, et al. 2004;
Woods, et al. 2003). The net phosphorylation of the activation (T-) loop residue T172 and thus activity of
AMPK is greatly induced by the binding of AMP (Hardie 2008; Sanders, et al. 2007; Suter, et al. 2006).
In this way, AMPK functions as an energy sensor, restoring energy levels by phosphorylating a wide
array of substrates (Carling 2004; Carling, et al. 2008). Several reports demonstrate that activators of
AMPK, including AICAR and A-769662, inhibit adipogenesis (Giri, et al. 2006; Lee, et al. 2011; Zhou, et
al. 2009). Both PKA and PKB, which are activated during adipocyte differentiation, have been suggested
to inhibit AMPK activity by phosphorylating S485 on the catalytic subunit (Hurley, et al. 2006). We have
previously shown that PKA-activation in adipocytes results in phosphorylation and binding of SIK2 and
SIK3 to 14-3-3 proteins (Berggreen, et al. 2012; Henriksson, et al. 2012), and this is predicted to inhibit
their function.

Based on its potential ability to respond to adipogenic stimuli, as well as cellular energy levels, we
hypothesise that in the absence of appropriate signals, the LKB1/AMPK/SIK signalling pathway serves to
keep adipocyte progenitors in their non-differentiated form. This inhibitory effect of LKB1 signalling on
adipogenesis might be mediated through its action on CRTC2/CREB and HDACs.

MATERIALS AND METHODS

Materials

3T3-L1 cells were obtained from American Type Culture Collection and used within 15 passages of the
original source. LKB1-deficient immortalized mouse embryonic fibroblasts (MEFs) were generated by
Professor Tomi Mäkelä, University of Helsinki, and kindly provided by Professor Dario Alessi,
University of Dundee. DMEM, trypsin/EDTA, dexamethasone, rosiglitazone, IBMX, insulin, H89 and
Nile Red were purchased from Sigma. Collagenase Type I was obtained from Gibco™. Precast Novex
SDS polyacrylamide 4-12% Bis-Tris gels, lauryl dodecyl sulfate (LDS) sample buffer, fetal bovine serum, Hoechst 33258 nucleic acid stain, DNase I amplification grade and SuperScript™ II RNaseH reverse transcriptase were obtained from Invitrogen. Infinity Triglycerides reagent was purchased from Thermo Scientific. QIAzol™ lysis reagent, RNAeasy® - and miRNeasy® Mini Kits were purchased from Qiagen.

Taqman® Gene Expression Assays for Stk11 (LKB1), Ddit3 (CHOP-10), Ribosomal protein S29 (Rps29, RPS29) and TATA box binding protein (Tbp, TBP) were obtained from Applied Biosystems. The QuantiTect Primers Assays with SYBR® Green detection for Cebpd (C/EBPδ), Dlk1 (Pref1), Glut4 (GLUT4), AdipoQ (adiponectin), 18S ribosomal RNA (Rn18s, 18S) and Rps29 were obtained from Qiagen. The primers for Pparg (PPARγ, forward primer: 5′-CTG TTT TAT GCT GTT ATG GGT GAA A-3′ and reverse primer: 5′-GCA CCA TGC TCT GGG TCA A-3′), Cebpa (C/EBPα, forward primer: 5′-ATA GAC ATC AGC GCC TAC ATC GA-3′ and reverse primer: 5′-CTG TCG GCT GTG CTG GAA-3′), Cebp (C/EBPβ, forward primer: 5′-CAG CGC ACC GGG TTT C-3′ and reverse primer: 5′-CGC AGG AAC ATC TTT AAG GTG ATT-3′), Fabp4 (aP2, forward primer: 5′-TTC GAT GAA ATC ACC-3′ and reverse primer: 5′-GGG CTG CTT TCC ATC-3′) and Fasn (FAS, forward primer: 5′-TGG TGA ATT GTC TCC-3′ and reverse primer: 5′-CAG GTT CAT CAC GAG-3′) were obtained from DNA Technology.

Phosphocellulose (P81) paper was obtained from Whatman and protease inhibitor cocktail tablets were from Roche. Protein G-Sepharose was purchased from GE Healthcare, and 32Pγ-ATP from Perkin Elmer. AMARA-, LKB1tide-, HDAC5tide- and Sakamototide peptides were synthesized by GL Biochem, China.

The following antibodies were used for western blotting: anti-AMPK, anti-phospho-AMPK (Thr172), anti-phospho-AMPK (S485), anti-HDAC4, anti-phospho-HDAC4(S246)/HDAC5(S259)/HDAC7(S155), anti-CREB, anti-phospho-CREB (S133), anti-phospho-PKA substrate and anti-HSP90 were purchased from Cell Signaling Technology; the antibody against LKB1 was purchased from AbCam; the antibody against GAPDH was purchased for Sigma; the antibody against CRTC2 was purchased from Calbiochem; the antibody against H3 was purchased from Millipore, and the antibody against CHOP-10 was from Santa Cruz (GADD153). Anti-phospho-CRTC2 (S275) was a kind gift from Robert Screaton, University
of Ottawa, Canada. The antibodies anti-FAS and anti-HSL were kindly provided by Professor Eva
Degerman and Cecilia Holm respectively, Lund University, Sweden. Anti-AMPKα1 antibody used for
kinase assays was kindly provided by Professor Grahame Hardie, University of Dundee, UK. Anti-SIK2,
anti-phospho-SIK2 (S358) and anti-SIK3 antibodies were raised in rabbit and affinity-purified by
Innovagen against peptides corresponding to residues 906-926 of human SIK2, residues 351-365 of
human SIK2 and residues 1349-1369 of human SIK3 respectively.
Horseradish peroxidase conjugated secondary antibodies were obtained from Biosource (anti-rabbit),
Pierce (anti-sheep), Santa Cruz (anti-goat) and GE Healthcare (anti-mouse).

**Cell culture and adipogenic differentiation**

3T3L1 fibroblasts were cultured and differentiated as previously described (Gormand, et al. 2011). MEFs
were cultured to subconfluence in growth medium (DMEM containing 10 % (v/v) FCS, 1 % (v/v)
penicillin/streptomycin) at 37 °C and 95 % air/5 % CO₂. Differentiation was induced on two-day post-
confluent cells by incubating them in growth medium supplemented with 0.5 mM IBMX, 5 µg/ml insulin,
1 µM dexamethasone and 0.1 µM rosiglitazone for 6 days, with one change of medium on day 3.
Hereafter the cells were cultured in growth medium. Cells were harvested in lysis buffer (50 mM
Tris/HCl pH 7.5, 1 mM EGTA, 1 mM EDTA, 1 mM sodium orthovanadate, 10 mM sodium-β-
glycerophosphate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 0.27 M sucrose, 1% w/v NP40,
1 mM dithiothreitol (DTT) and complete protease inhibitor cocktail (one tablet/50 ml)) or QIAzol™ after
washing with PBS.

**Generation of lentivirus**

The shRNA plasmid expression vectors were designed as described by Desclaux et al (Desclaux, et al.
2009). Briefly, the shRNA sequence to silence the expression of the mouse LKB1 gene was ordered from
MWG (Germany). The DNA fragment encoding LKB1-shRNA was generated by annealing two
complementary oligonucleotides and the resulting double-stranded DNA fragments were inserted in
pcDNA-ΔU6min (Amar, et al. 2006) between *BbsI* and *BamHI* restriction sites, generating the plasmid pcDNA-ΔU6-shLKB1.

The expression cassette allowing effective LKB1-shRNA under the control of the U6 promoter was excised from pcDNA-ΔU6min-shLKB1 and inserted into the lentivector precursor plasmid pFlap-PGK-GFP-WPRE (Desclaux et al. 2009) using *MluI* and *BamHI* sites. As control, a lentiviral vector expressing a "scrambled" shRNA (Scrbl-shRNA) sequence was produced. Lentiviral vector particles were produced as previously described (Philippe, et al. 2006). The amount of lentiviral vectors applied to cells is expressed as multiplicity of infection (MOI), corresponding to the number of transducing particles per cell.

**Generation of LKB1-deficient 3T3-L1 cells.**

3T3-L1 preadipocytes were seeded at a density of 5 500 cells per well in 12-well plates. Cells were transduced 24 h later at 1.5 MOI with lentivector encoding either Scrbl-shRNA or LKB1-shRNA. Contact with the vector was allowed for 16 h, after which the medium was replaced. 24 h later, cells were reseeded onto 10 cm dishes and allowed to grow to 60% confluence. Cells were then frozen in DMEM supplemented with 10% DMSO.

**Lipid staining and cell imaging.**

Intracellular lipids were stained with 10 μg/ml Nile Red in the medium for 15 min and nuclei were stained with 10 μg/ml of Hoescht 33258 for 30 min. Cells were washed twice with PBS and fresh medium was added. Stained cells were imaged under a fluorescent microscope (Olympus IX71) using FITC, TRITC and DAPI filters. Ten pictures per plate were randomly taken and each plate corresponded to one condition per experiment. Images were processed and analysed using cellSens software from Olympus. The percentage of differentiated 3T3-L1 cells was calculated as the number of cells stained with Nile Red over the total number of cells (number of nuclei stained with Hoechst).

**Quantification of intracellular triglyceride content**
To determine the triglyceride (TG) content, cells seeded onto 12-well plates were washed twice with PBS before being lysed in 500 µl 0.25 M NaOH. TGs were extracted in 1 ml hexane:isopropanol (3:2) mixture, dried using a SpeedVac and resuspended in isopropanol containing 1 % triton. The TG content was measured by chemiluminescence at 510 nm using the Infinity Triglycerides reagent, compared to a known concentration of TG solution and a calibrator.

**Immunoprecipitation and kinase activity assay**

Immunoprecipitations from lysates for western blotting and kinase activity assays were performed as described previously (Gormand et al. 2011). Phosphotransferase activity towards the AMARA peptide (AMARAASAAALARRR) for AMPK (Dale, et al. 1995), the LKB1tide (SNLYHQGFLQTFGSPLYRR) for LKB1 (Lizcano et al. 2004), the HDAC5tide (PLRKTASEPNKRRR) for SIK2 (Berdieux, et al. 2007; Henriksson et al. 2012) and the Sakamototide (ALNRTSSDSALHRRR) for SIK3 (Zagorska, et al. 2010), was measured with 200 µM of the peptide substrates. One Unit (U) of activity was defined as that which catalysed the incorporation of 1 nmol of $^{32}$P/min into the substrate.

**Western Blot analysis**

Western blot analysis was performed on equal amount of total protein as described previously (Gormand et al. 2011). Quantification of total amount of protein was normalised to either GAPDH to compare the amount of proteins in lysates from undifferentiated cells, or HSP90 to compare the amount of proteins in lysates from undifferentiated and differentiated cells (since the amount of GAPDH protein varies between preadipocytes and mature adipocytes). Images were acquired and analysed using ChemiDoc™ XRS+ and the Image Lab™ software from Bio-Rad.

**RNA and miRNA preparation and quantitative real-time PCR**

Total RNA and miRNAs were isolated using RNeasy® - and miRNeasy® Mini Kits according to the manufacturer's recommendations. Total RNA (1 µg) was treated with DNase I and reversely transcribed as described previously (Gormand et al. 2011). The cDNA was used in quantitative PCR reactions using
Taqman or Sybrgreen chemistry in an ABI 7900 Sequence Detection System. Relative abundance of mRNA was calculated after normalisation to the geometric mean of two internal control genes (Rps29 and Rn18s) (Ferguson, et al. 2010; Vandesompele, et al. 2002). MicroRNAs were analysed using Qiagen miScript kits, reagents and primers (Turczynska, et al. 2012). Each sample was analysed in duplicates.

Cytoplasmic and nuclear fractionation

Subcellular fractionation was performed using the NE-PER® Nuclear and Cytoplasmic Extraction kit (Thermo Scientific) according to the manufacturer’s recommendations with minor changes. Cells were washed in ice-cold PBS and scraped in 200 µl CERI containing complete protease inhibitors/10 cm dish. Homogenates were vortexed and incubated for 15 min on ice. 11 µl of ice-cold CERII was added, and homogenates were incubated for 1 min on ice, vortexed and centrifuged for 5 min at 16 000 g. The supernatant containing the cytoplasmic fraction was collected. Pellets were washed twice in ice-cold PBS and resuspended in 100 µl of ice-cold NER containing complete protease inhibitors. Samples were vortexed for 15 s and incubated on ice for 10 min. This step was repeated 4 times before centrifugation for 10 min at 16 000 g. The supernatant containing the nuclear fraction was recovered.

Confocal fluorescence microscopy

3T3-L1 preadipocytes were cultured on cover slips in 6-well plates. 2 days post confluent cells were stimulated for 1 h with differentiation medium, washed, fixed and probed with antibodies, as described (Henriksson et al. 2012). Confocal images were acquired with a Zeiss LSM 510 META microscope using excitation wavelengths 405 (Hoechts nuclear stain), 488 (BODIPY) or 561 (Alexa 594) nm. A Plan-Apochromat 63x/1.4 oil DIC objective was used and a frame size of 1024x1024. Z-stacks with 5-9 layers within the nucleus were used and maximum intensity projection applied.

Statistical analysis

Results in this study are presented as mean + SEM of the indicated number of independent experiments. Statistical analysis were performed using Student’s t test (two-tailed, unpaired) or two-way ANOVA with multiple comparisons, and differences were considered statistically significant when * p < 0.05, ** p <
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10

0.01, *** p < 0.001 and **** p < 0.0001. To enable comparison of qPCR and western blot data from several independent experiments, a control sample in each experiment was set to 1 or 100%. A representative blot displaying technical duplicates or triplicates from one experiment is generally shown.

RESULTS

LKB1 depletion facilitates 3T3-L1 adipocyte differentiation.

Based on the fact that the LKB1 signalling is known to regulate CREB activity, we hypothesised that the silencing of LKB1 may result in an altered adipogenic program in the 3T3-L1 preadipocyte cell line. We generated a 3T3-L1 cell line with stable expression of shRNA targeted against LKB1 mRNA. As a complement to this model system, we also employed wild type (Wt) and LKB1-deficient (LKB1-/-) mouse embryonic fibroblasts (MEFs), in which the activity of AMPK and its related kinases has been shown to be dramatically reduced (Lizcano et al. 2004). The silencing of LKB1 in the 3T3-L1 cells was confirmed by quantitative real-time PCR, LKB1 kinase activity assay, and by western blot (Fig. 2a).

Staining of cellular lipids showed that ≈ 75 % of LKB1-shRNA expressing 3T3-L1 cells were able to differentiate into mature and lipid-storing adipocytes, while only ≈ 41 % of Scrbl-shRNA cells had differentiated (Fig. 1a). Moreover, LKB1-deficient MEFs accumulated twice as much lipids as did Wt MEFs, as judged by quantification of the Nile Red staining (Fig. 1a and S1). As a complement to lipid staining, the triglyceride content of the whole 3T3-L1 cell population at day 9 was quantified biochemically (Fig. 1b), showing that the LKB1-shRNA cells contained significantly more triglycerides than Scrbl-shRNA cells. Furthermore, Pref1, a marker for preadipocytes, was significantly more expressed in Scrbl-shRNA cells at day 6, which indicates that less cells had differentiated compared to LKB1-shRNA cells (Fig. 1c). The adipocyte-specific proteins and/or mRNAs for HSL, FAS, aP2, Glut4 and adiponectin were up-regulated in LKB1-shRNA 3T3-L1 cells and in LKB1-/- MEFs (Fig. 1d, S1, S2).

In addition, Pparg and Cebpa were induced earlier and were significantly up-regulated in differentiating 3T3-L1 cells expressing LKB1-shRNA (Fig. 1e and S2). In LKB1-/- MEFs, Pparg and Cebpa were up-regulated already in the fibroblast stage, and their expression remained up-regulated throughout differentiation (S1). As shown in supplementary figure 2 (S2), the PPARγ targets miR-103 and miR-107,
which have been previously demonstrated to be induced during differentiation, were up-regulated in LKB1-shRNA cells, particularly in the late stages of differentiation (day 6) (Esau, et al. 2004; Li, et al. 2011). Interestingly, *Cebpd* was significantly higher in the LKB1-shRNA cells 30 min after induction of differentiation (Fig. 1f). However, there was no consistent difference in the expression of *Cebpb* mRNA (Fig. 1f) or protein expression (data not shown) between the Scrbl- and LKB1-shRNA expressing 3T3-L1 cells or in LKB1-/- MEFs (S1). We also analysed the mRNA- and protein expression of CHOP-10, a dominant-negative member of the C/EBP family, which has been shown to inhibit adipocyte differentiation by sequestering/inactivating C/EBPβ (Tang and Lane 2000). As previously reported (Huang, et al. 2005), CHOP-10 mRNA- and protein levels decline upon adipocyte differentiation (Fig. 1g). Notably, there was a reduction in the expression of CHOP-10 both at the mRNA and protein level in the LKB1-shRNA expressing cells at several time points, including before the initiation of differentiation. This provides a potential mechanism whereby C/EBPβ activity, and thus PPARγ and C/EBPα expression, may be increased in the LKB1-shRNA expressing cells, without an induction of C/EBPβ expression levels. To rule out that the lack of LKB1 might facilitate the adipogenesis by potentially enhancing clonal expansion, we counted cells at different time points before and after the initiation of differentiation, and found that LKB1- and Scr-shRNA expressing cells did not differ significantly in this regard (S2).

Taken together, these data show that 3T3-L1 preadipocytes and MEFs expressing lower levels of LKB1 displayed an improved ability to differentiate into adipocyte-like cells, expressed higher levels of early adipogenic genes, and lower levels of the anti-adipogenic gene *Ddit3* (CHOP-10). These data are in line with the hypothesis that LKB1 signalling attenuates the induction of differentiation in preadipocytes.

**LKB1 regulates CRTC2 and class IIa HDACs in 3T3-L1 fibroblasts.**

To gain more insight into the molecular mechanisms downstream of LKB1, which may mediate effects on adipogenic gene expression, we investigated whether the phosphorylation of CRTC2 and the class IIa HDAC4, 5, 7 is regulated by LKB1 in 3T3-L1 preadipocytes, before and 1h after the induction of differentiation. As shown in Fig. 2b and 2c, the phosphorylation of CRTC2 and class IIa HDACs on sites previously reported to control their activity (Mihaylova, et al. 2011; Screaton et al. 2004), was lower in
LKB1-shRNA cells, as was the phosphorylation of these proteins in cells treated with the differentiation medium for 1h. There was no difference in the phosphorylation of CREB on S133, between the Scrbl- and LKB1-shRNA expressing cells (data not shown).

We next monitored how the reduced LKB1 expression affected the phosphorylation and/or activity status of AMPK, SIK2 and SIK3 – potential substrates downstream of LKB1 reported to directly regulate CRTC2 and class II HDACs (Mihaylova et al. 2011; Muraoka et al. 2009; Screaton et al. 2004). In the absence of LKB1, the activity and specific phosphorylation of AMPK on the activity controlling T172 site was reduced (∼50%), as was the total kinase activity of SIK2 and SIK3 in the basal state and after 1 h of induction of differentiation (Fig. 2d-f). There was however no significant reduction of the specific activity of SIK2 and SIK3 in LKB1-shRNA cells (Fig. 2e and 2f).

**Acute regulation of AMPK, SIKs, CRTC2 and HDAC4 following adipocyte differentiation.**

In the experiments described in Fig. 2, we noted that treatment of Scrbl-shRNA expressing 3T3-L1 preadipocytes with differentiation medium for 1h, resulted in a dephosphorylation of CRTC2 and class IIa HDACs, in particular HDAC4 (Fig. 2b and 2c). This suggests that the activity of these proteins, and potentially their upstream regulators, may be subject to acute regulation by adipogenic stimuli. To test if this is the case, we treated 3T3-L1 preadipocytes with differentiation medium for different time points, and analysed the phosphorylation/activity and localisation status of AMPK, SIK2, SIK3, CRTC2 and HDAC4. Within an hour, AMPKα1 T172 phosphorylation and activity was significantly reduced by 35%, while the phosphorylation on S485 was increased (Fig. 3a). The phosphorylation of SIK2 on S358 was significantly induced within 10 min and was increased 25-fold within 1 h (Fig. 3b). This phosphorylation coincided with a translocation of SIK2 from the nuclear to the cytosolic fraction, where it may not be able to act on its downstream substrates (Fig. 3f). Notably, the kinase activity of SIK3 was significantly reduced within 30 min, and this coincided with increased phosphorylation of SIK3 on pPKA consensus sites (Fig. 3c). These data demonstrate that AMPK, SIK2 and SIK3 were rapidly inhibited following the induction of adipocyte differentiation.
The inactivation of AMPK/SIK signalling coincided with dephosphorylation of CRTC2 on S275 and HDAC4 on S246 (Fig. 3d and 3e), and a translocation of these proteins to the nucleus, as shown by fractionation (Fig. 3f) and fluorescence confocal imaging (CRTC2, Fig. 3g). We also analysed the expression and phosphorylation status of LKB1, AMPK, CRTC2 and class IIA HDACs at time points throughout the whole differentiation process. As shown in Supplementary figure 3 (S3), LKB1 protein expression was reduced by 50% at day 3 after differentiation, but returned to original levels at day 6. Notably, the T172 phosphorylation of AMPK was markedly reduced at day 1 and day 3, as was the phosphorylation of CRTC2 and class IIA HDACs (S3). These changes were mirrored by an increase in S485 phosphorylation of AMPK and phosphorylation of PKA substrates, as monitored using a PKA consensus motif antibody. This data demonstrate that the acute inhibition of AMPK signalling persisted at least until day 3 after inducing differentiation.

Mechanisms underlying the activation of CRTC2 and the inhibition of AMPK/SIK signalling by adipogenic stimuli.

Treatment with either insulin or dexamethasone had no significant effect on AMPKα1 activity, AMPK T172 phosphorylation, SIK2 S358 phosphorylation, phosphorylation of SIK3 on PKA consensus sites, or on the phosphorylation state of CRTC2 (Fig. 4a-d). However, upon stimulation with the cAMP-inducing agent IBMX, AMPKα1 activity and T172 phosphorylation (Fig. 4a) were significantly reduced, and the phosphorylation of SIK2 on S358 (Fig. 4b) was increased. Moreover, following IBMX treatment, SIK3 was phosphorylated on PKA consensus sites (Fig. 4c) and its activity was reduced (data not shown). This coincided with a significantly reduced phosphorylation of CRTC2 on S275 (Fig. 4d). IBMX was the only stimulation that mimicked the effects on AMPK, SIK2, SIK3 and CRTC2 observed when the cells were treated with the complete differentiation medium. The phosphorylation of AMPK on S485 was induced both by insulin and IBMX, however insulin stimulation alone did not result in inhibition of T172 phosphorylation or AMPKα1 activity (Fig. 4a). To confirm that the differentiation medium inhibits AMPK/SIK signalling, and consequently the phosphorylation of CRTC2, through the cAMP/PKA pathway, we employed the PKA inhibitor H89 (Fig. 4e-h). In the presence of H89, the phosphorylation of
CREB on S133, a known PKA site, was prevented, confirming the efficacy of the inhibitor (Fig. 4e). The effects of the differentiation medium on the phosphorylation of AMPK (T172, S485), SIK2 (S358), SIK3 (PKA sites) and CRTC2 were all reversed in the presence of H89 in the stimulated cells (Fig. 4e-h). We conclude that inhibition of AMPK/SIK signalling, and subsequent dephosphorylation of CRTC2, following adipogenic differentiation of 3T3-L1 cells is most likely mediated by activation of the cAMP/PKA pathway in response to IBMX.

DISCUSSION

In this paper we tested the hypothesis that LKB1 signalling might keep adipocyte precursors in their non-mature form, and that adipogenic stimuli attenuate LKB1 signalling in order for differentiation to occur. In summary, we demonstrate that three substrates downstream of LKB1 are acutely inhibited following the initiation of differentiation in a PKA dependent manner, and that this coincides with dephosphorylation and nuclear translocation of CRTC2 and class IIa HDACs. Moreover, silencing of LKB1 resulted in a marked reduction in the mRNA and protein level of CHOP-10, increase in Pparg expression and a facilitated differentiation into adipocytes. Our working model for the regulation of adipogenesis by LKB1 is summarised in Fig. 5.

AMPK, SIK2 and SIK3 were all inhibited within one hour after inducing adipogenic differentiation, and this inhibition coincided with dephosphorylation and nuclear translocation of CRTC2 and class IIa HDACs. Results from IBMX treatment and PKA inhibition suggest that cAMP/PKA signalling mediated the inhibition of AMPK/SIK signalling that we observed. Studies have shown that phosphorylation of AMPK on S485 by PKA and/or PKB inversely correlates with AMPK T172 phosphorylation and kinase activity (Berggreen, et al. 2009; Hurley et al. 2006), which could provide a mechanistic explanation to the inhibition of AMPK during differentiation. In our experiments, we did observe an induction of S485 phosphorylation in response to the adipogenic cocktail. However, while treatment of cells with insulin alone resulted in the phosphorylation of S485, it did not affect AMPK T172 phosphorylation or activity, arguing against the hypothesis that S485 mediates AMPK inhibition during differentiation. The
phosphorylation of SIK2 and SIK3, as well as the cytosolic translocation of SIK2 that we observed in response to IBMX and/or complete differentiation medium, is in line with our previous studies in mature adipocytes, demonstrating the regulation of these kinases by cAMP/PKA on many levels, including re-localisation of SIK2 to the cytosol and inhibition of SIK3 kinase activity respectively (Berggreen et al. 2012; Henriksson et al. 2012). Based on our previous results and the fact that PKA-phosphorylation has been shown to inhibit SIK1 and SIK2 cellular function in other systems (Katoh, et al. 2004; Screaton et al. 2004), we believe that the phosphorylation of SIK2 by PKA (on S358) and its cytosolic translocation inhibit its action on CRTC2 and class IIa HDACs.

To directly address if LKB1 inhibits CRTC2 and class IIa HDACs in 3T3-L1 cells, and the ability of these cells to differentiate, we generated 3T3-L1 preadipocytes with stable expression of LKB1-shRNA. We confirmed that these cells displayed markedly reduced activities of LKB1 and its substrates AMPK, SIK2 and SIK3, although the latter appeared to be partly due to reduced expression of these proteins. The fact that a ≈ 90 % reduction in LKB1 activity did not result in a larger attenuation of the specific activities of AMPK, SIK2 and SIK3 was not entirely unexpected. We have previously shown that in adipocytes isolated from mice expressing only 10 % of LKB1 activity compared to wild type, AMPK activity was reduced by 40 % and the activities of SIK2 and SIK3 were only reduced by approximately 25 % (Gormand et al. 2011). This either suggests the existence of alternative upstream kinases or a large spare capacity in the LKB1 signalling pathway – the latter being supported by the almost complete lack of activity of SIK2 and SIK3 in LKB1 deficient cells and tissues (Al-Hakim, et al. 2005; Lizcano et al. 2004). The effect of LKB1 silencing on CRTC2 and HDAC4 in 3T3-L1 preadipocytes was greater than any of the individual effects on AMPK, SIK2 or SIK3, suggesting that it may be the combined reduction of the AMPK/SIK signalling, or inhibition of other AMPK-related kinases, that leads to reduced CRTC2 and HDAC4 phosphorylation. LKB1 regulates 14 kinases of the AMPK family, many of which have similar substrate specificity and are likely to be expressed in 3T3-L1 fibroblasts. This complicates any attempt to pinpoint if a specific substrate of LKB1 might be chiefly responsible for downstream effects, or if they play redundant roles.
Employing two different cellular models, we show that a reduction in LKB1 expression results in an increased ability of the cells to differentiate. A key underlying mechanism appears to be that LKB1 loss results in higher levels of the master adipogenic transcription factor PPARγ as well as C/EBPα quite early in the differentiation process. The phenotype was stronger in the LKB1−/− MEFs, which is in line with the complete absence of LKB1, and dramatic reduction of AMPK, SIK2 and SIK3 activities (data not shown and (Lizcano et al. 2004)), and a barely detectable level of CRTC2 phosphorylation in these cells (data not shown). The fact that Pparg and Cebpα were upregulated early during differentiation and that the number of adipocytes was increased, suggest that the phenotype we observed is due to LKB1-regulation of transcriptional events controlling the actual differentiation program, rather than directly affecting lipid accumulation and/or the expression of adipocyte specific proteins. Zhang et al demonstrated that deletion of LKB1 in mouse adipose tissue, employing Fabp4-mediated Cre recombinase expression, resulted in reduced amount of white adipose tissue and expression of adipogenic genes (Zhang, et al. 2013). Since Fabp4/aP2 is only expressed in the later stages of adipocyte differentiation, this experimental model in fact did not address the role of LKB1 in differentiating preadipocytes (like ours). In support of our present findings, another upstream kinase of AMPK, CaMKK2, has also been shown to inhibit adipocyte differentiation and adipogenic gene transcription, as shown in CaMKK2-null MEFs and 3T3-L1 preadipocytes treated with CaMKK shRNA and CaMKK inhibitors (Lin, et al. 2011).

Our original hypothesis was that C/EBPs might be up-regulated in LKB1-silenced cells, due to activation of CRTC2/CREB. The lack of induction of C/EBPβ mRNA or protein expression in LKB1-shRNA expressing cells does not support this notion. In LKB1−/− MEFs we observed that C/EBPβ mRNA levels tended to be increased before the initiation of adipogenesis, but the difference was not statistically significant in the 2-way Anova and did not persist after the addition of differentiation medium, speaking against altered C/EBPβ levels as a primary mechanism mediating the phenotype. However, we cannot rule out the possibility that the higher expression of C/EBPδ, another potential CREB target gene, that we observed at one time point contributes to the induction of PPARγ and C/EBPα in the LKB1-shRNA 3T3-L1 cells (Belmonte et al. 2001; Reusch et al. 2000; Rosen and MacDougald 2006). In search for
additional mechanisms underlying the up-regulation of \textit{Pparg} and \textit{Cebpa}, we analysed the dominant-negative C/EBP family member CHOP-10, which inhibits C/EBP\(\beta\) by sequestration/inactivation (Huang et al. 2005; Tang and Lane 2000). Indeed, the mRNA and protein level of CHOP-10 was markedly reduced in LKB1-shRNA expressing cells, potentially allowing for more C/EBP\(\beta\) to bind to DNA and stimulate gene expression. At present, we do not know how LKB1 regulates CHOP-10 expression, and this will be of future interest to address. We find it interesting that CRITC2 and HDAC4 were dephosphorylated as a result of LKB1 silencing as well as adipogenic stimulation, but our data do not exclude the involvement of other substrates of the AMPK family of kinases.

In summary, this study demonstrates that LKB1 silencing in 3T3-L1 preadipocytes promotes the dephosphorylation of HDAC4 and CRITC2, induces the expression of \textit{Pparg} and \textit{Cebpa}, and facilitates adipocyte differentiation. We also demonstrate that the AMPK/SIK signalling is inhibited following the initiation of differentiation, and hypothesise that in the absence of adipogenic stimuli, LKB1/AMPK/SIK signalling serves to keep preadipocytes in their non-differentiated form.

\textbf{DECLARATION OF INTEREST}

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

\textbf{FUNDING}

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**FIGURE LEGENDS**

**Figure 1**: LKB1-silenced 3T3-L1 preadipocytes and LKB1 null MEFs display an increased ability to differentiate into adipocytes.

(a) 3T3-L1 preadipocytes transduced with scrambled-shRNA (Scrbl-shRNA) or LKB1-shRNA (LKB1-shRNA), as well as wild-type (Wt) or LKB1-deficient (LKB1\(^{-/-}\)) MEFs were subjected to a differentiation protocol for up to 10 days. The ability of these cells to differentiate into adipocytes was evaluated by quantifying the number of differentiated cells (Nile red stained cells) over the total number of cells (Hoechst stained cells) or the total amount of Nile red staining (LKB1\(^{-/-}\) MEFs) in fluorescent microscopy images, and (b) by a biochemical measurement of triglyceride (TG) accumulation. (c) The mRNA level of the preadipocyte marker Pref1 was measured by qPCR in Scrbl-shRNA or LKB1-shRNA expressing...
3T3-L1 preadipocytes. (d) Cell lysates from Scrbl-shRNA or LKB1-shRNA expressing 3T3-L1 preadipocytes were analysed by western blot for protein expression of FAS and HSL at 10 days after induction of differentiation. (e-g) The mRNA level measured by qPCR of the adipogenic transcription factors PPARγ and C/EBPα (e), C/EBPδ and C/EBPβ (f), and the dominant negative C/EBP family member CHOP-10 (g) in the LKB1-shRNA cells was compared to those in the Scrbl-shRNA cells after induction of differentiation at the time points indicated on the figures. The protein amount of CHOP-10 was measured by western blot in cell lysates from Scrbl- or LKB1-shRNA expressing 3T3-L1 preadipocytes after induction of differentiation at the time points indicated on the figures. The blot, showing one experiment, is representative of three independent experiments. The data represent the mean ±SEM of three independent experiments, each in which the data was expressed as fold- or % of a control condition, and the means were considered significantly different when *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001 (unpaired t-test in 1a and two-way ANOVA with multiple comparisons in b-g). Time point 0 corresponds to the day when the differentiation medium was added (2 days post-confluent).

Figure 2: The silencing of LKB1 in 3T3-L1 preadipocytes results in a reduced phosphorylation of CRTC2 and class IIa HDACs.

(a) 3T3-L1 preadipocytes were transduced with scrambled- (Scrbl-shRNA) and LKB1-shRNA (LKB1-shRNA). The silencing of LKB1 was confirmed at the mRNA level by qPCR and at the protein level by kinase activity assay and western blot. (b-f) Adipogenic differentiation was induced for 1h on 2 day post-confluent preadipocytes and cell homogenates were analysed for phosphorylation and/or kinase activity of CRTC2 (b), class IIa HDACs (c), AMPK (d), SIK2 (e) and SIK3 (f). The data represent the mean ±SEM of three independent experiments, each in which the data was expressed as fold- or % of a control condition, and the means were considered significantly different when *p<0.05, **p<0.01 and ***p<0.001 (unpaired t-test in 2a and Two-way ANOVA with multiple comparisons in b-f). A representative blot displaying technical duplicates from one experiment is shown.

Figure 3: Acute regulation of AMPK/SIK/CRTC2/HDAC4 by adipogenic differentiation.
3T3-L1 preadipocytes were treated with differentiation medium for up to 1 h. Cell homogenates were collected at different time points as indicated in the figures and analysed for phosphorylation state and/or in vitro kinase activity of AMPK (a), SIK2 (b), SIK3 (c), CRTC2 (d) and HDAC4 (e). Results are presented as the mean ±SEM of three independent experiments, each in which the data was expressed as % of a control condition, and the means were considered significantly different from time = 0 when *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001 (unpaired student t test). A representative blot displaying technical duplicates from one experiment is shown. Subcellular localisation of SIK2, CRTC2 and HDAC 4, 5, 7 (f) was analysed by western blot after performing subcellular fractionation of cell homogenates. The purity of the fractions was monitored by the presence of GAPDH and histone 3 (H3). Results are presented as the mean ±SEM of three independent experiments (made in triplicates), each in which the data was expressed as % of a control condition, and the means were considered significantly different when *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001 (unpaired student t-test). A representative blot displaying technical triplicates from one experiment is shown. The re-localisation of CRTC2 to the nucleus was also analysed by immunocytochemistry and confocal fluorescence microscopy (g).

Figure 4: Effects of the differentiation medium on the AMPK/SIK/CRTC2 signalling pathway is mediated by cAMP/PKA.

3T3-L1 preadipocytes were treated with 10 µg/ml insulin, 0.5 mM IBMX, 1 µM dexamethasone (Dex) or complete differentiation medium (Diff Medium) for 30 min (SIK3) or 1 h (AMPK, SIK2 and CRTC2). Cell homogenates were analysed for phosphorylation state and/or in vitro kinase activity of AMPK (a), SIK2 (b), SIK3 (c) and CRTC2 (d). The data represent the mean ±SEM of three independent experiments (made in duplicates), each in which the data was expressed as % of a control condition, and the means were considered significantly different from the basal state when *p<0.05, **p<0.01 and ****p<0.0001 (unpaired student t-test). A representative blot displaying technical duplicates from one experiment is shown. 3T3-L1 preadipocytes were treated with (H89) or without (DMSO) 100 µM H89 for 30 min before adding the adipogenic cocktail (10 µg/ml insulin, 0.5 mM IBMX, 1 µM dexamethasone; +Diff...
Medium) or not (Normal Medium) for 1 h. Cell homogenates were analysed for phosphorylation state of CREB and AMPK (e), SIK2 (f), SIK3 (g) and CRTC2 (h). The figures represent the mean ±SEM of three independent experiments (performed in duplicates), each in which the data was expressed as % of a control condition. A representative blot displaying technical duplicates from one experiment is shown.

Figure 5: Model for the regulation of adipogenesis by the LKB1/AMPK/SIK signalling pathway.
(a) Before the addition of differentiation cocktail to preadipocytes, the activity of the LKB1 signalling pathway, through AMPK, SIKs and other putative kinases, maintains a certain level of phosphorylation of transcriptional co-regulators such as CRTC2 and class IIa HDACs, which prevents their translocation to the nucleus, where they would otherwise contribute to the regulation of certain genes. LKB1/AMPK/SIK may also prevent adipogenesis through other as yet unknown targets. (b) Upon addition of the differentiation cocktail, LKB1 substrates are inhibited in response to increased levels of cAMP, induced for example by the phosphodiesterase inhibitor IBMX. CRTC2 and the class IIa HDACs are dephosphorylated and can translocate to the nucleus, where they affect gene expression that regulates adipogenesis, for example CHOP-10, C/EBPδ, C/EBPα and PPARγ. CHOP-10 can regulate C/EBPβ activity without affecting C/EBPβ expression levels. Several of the effects induced by adipogenic medium is mimicked by LKB1 silencing.

Supplementary figure 1 (S1): Expression of adipogenic and lipogenic markers during the differentiation of wild type (Wt) and LKB1−/− MEFs.
The ability of LKB1-deficient MEFs to differentiate into adipocytes (day 9) compared to Wt MEFs, was evaluated by fluorescent microscopy imaging where lipids were stained with Nile Red, and by quantifying the amount of adipocyte proteins FAS and HSL by western blot. Western blot quantifications are presented as the mean ±SEM of three independent experiments, each in which the data was expressed as % of a control condition, and the means were considered significantly different when *p<0.05 (Two-way ANOVA with multiple comparisons). mRNA levels of PPARγ, C/EBPα, C/EBPβ, aP2, Glut4 and adiponectin during differentiation of wild type (Wt) and LKB1−/− MEFs were measured by qPCR. Time 0 corresponds to the day when the differentiation medium was added (2 days post-confluent). Results are
presented as the mean +SEM of three experiments, each in which the data was expressed as fold- of a
control condition, and the means were considered significantly different when \( *p<0.05 \) and \( ***p<0.01 \)
(Two-way ANOVA with multiple comparisons).

**Supplementary figure 2 (S2): Expression of adipogenic markers in Scrbl-shRNA and LKB1-shRNA**

*expressing 3T3L1 preadipocytes and adipocytes.*

mRNA levels of PPAR\( \gamma \), C/EBP\( \alpha \), Glut4, FAS, aP2, adiponectin, mir-103 and miR-107 during the whole
differentiation process of 3T3-L1 cells (expressing Scrbl- and LKB1-shRNA). Time 0 corresponds to the
day when the differentiation medium was added (2 days post-confluent). Results are presented as the
mean +SEM of one representative experiment (made in duplicate), in which the data was expressed as
fold- of a control condition, and the means were considered significantly different when \( *p<0.05 \),
\( **p<0.01 \), \( ***p<0.001 \) and \( ****p<0.0001 \) (Two-way ANOVA with multiple comparisons). Clonal
expansion before and after adding the differentiation cocktail was measured by counting cells in a Bürker
chamber. The data is expressed as the fold change compared to day -2, which is the day when the cells
reach 100% of confluency, and represent the mean +SEM of 3 independent experiments.

**Supplementary figure 3 (S3): Expression of the LKB1 signalling during the differentiation of 3T3-L1**

*Cells.*

The protein expression and phosphorylation state of LKB1, AMPK, PKA substrates, CRTC2 and class IIa
HDACs was measured in cell homogenates by western blot. The data represent the mean +SEM of three
independent experiments (made in duplicates), each in which the data was expressed as % of a control
condition. The means were considered significantly different from the basal state when \( **p<0.01 \) and
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3f.

3g.

135x200mm (300 x 300 DPI)
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143x238mm (300 x 300 DPI)
Model for the regulation of adipogenesis by the LKB1/AMPK/SIK signalling pathway.
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149x128mm (300 x 300 DPI)