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 studies in 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 the dephosphorylation and nuclear translocation of CRTC2 and HDAC4. When subjected to differentiation, 3T3-L1 preadipocytes in which the expression of LKB1 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 with scrambled-shRNA-expressing 3T3-L1 cells or Wt MEFs. In addition, the phosphorylation of CRTC2 and HDAC4 was reduced, and the mRNA expression of adipogenic transcription factors Cebpα, peroxisome proliferator-activated receptor γ (Pparg) and adipocyte-specific proteins such as hormone-sensitive lipase (HSL), fatty acid synthase (FAS), αP2, GLUT4 and adiponectin was increased in the absence of LKB1. The mRNA and protein expression of Ddit3/CHOP10, 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 Cebpα expression. These results support the hypothesis that LKB1 signalling keeps preadipocytes in their non-differentiated form.

Key Words
- adipogenesis
- LKB1
- AMPK
- SIKs
- CRTC2
- HDACs

Introduction
Adipogenesis is a multistep 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 protein β and δ (C/EBPβ and C/EBPδ) are induced within hours of induction of differentiation. C/EBPβ and C/EBPδ then induce the expression of
peroxisome proliferator-activated receptor γ (PPARγ) and C/EBPα (Christy et al. 1991, Wu et al. 1995, Yeh et al. 1995, Clarke et al. 1997). Once expressed, PPARγ and C/EBPα cooperate to induce the expression of 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 characterised. 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 the differentiation of adipocytes through induction of the expression of C/EBPβ and potentially C/EBPα (Reusch et al. 2000, Belmonte et al. 2001, Zhang et al. 2004). The activity of CREB is regulated by PKA-dependent phosphorylation and also requires co-activators such as CREB-regulated transcription co-activators (CRTC2s; Conkright et al. 2003, Screaton et al. 2004, Ravnskjaer et al. 2007, Xu et al. 2007). When phosphorylated, CRTC2s bind to 14-3-3 proteins and are sequestered in the cytoplasm. Upon increasing levels of cAMP, CRTC2s are dephosphorylated and translocated 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 CRTC2s (Screaton et al. 2004, Koo et al. 2005, Muraoka et al. 2009). Another recently identified group of substrates for 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, Woods et al. 2003, Lizcano et al. 2004). The net phosphorylation of the activation (T-) loop residue T172 and thus the activity of AMPK are greatly induced by the binding of AMP (Suter et al. 2006, Sanders et al. 2007, Hardie 2008). 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 have demonstrated that some activators of AMPK, including AICAR and A-769662, inhibit adipogenesis (Giri et al. 2006, Zhou et al. 2009, Lee et al. 2011). Both PKA and PKB, which are activated during the differentiation of adipocytes, have been suggested to inhibit the activity of AMPK by phosphorylating S485 on the catalytic subunit (Hurley et al. 2006). We have previously shown that the activation of PKA in adipocytes results in the phosphorylation and binding of SIK2 and SIK3 to 14-3-3 proteins (Berggreen et al. 2012, Henriksson et al. 2012), and this has been predicted to inhibit their function.

Based on its potential ability to respond to adipogenic stimuli, as well as cellular energy levels, in this study, we tested the hypothesis 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 the American Type Culture Collection and used within 15 passages of the original source. Lkb1-deficient immortalised mouse embryonic fibroblasts (MEFs) were generated by Prof. Tomi Mäkelä, University of Helsinki, and kindly provided by Prof. Dario Alessi, University of Dundee. DMEM, trypsin/EDTA, dexamethasone, rosiglitazone, 3-isobutyl-1-methyloxanthine (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 sulphate 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 Fisher Scientific (Waltham, MA, USA). QIAzol lysis reagent and RNeasy and miRNeasy Mini Kits were obtained from Qiagen. TaqMan Gene Expression Assays for Lkb1 (LKB1), Ddit3 (CHOP10), ribosomal protein S29 (Rps29) and TATA box-binding protein (Tbp (TBP)) were obtained from Applied Biosystems. The QuantiTect Primer Assays with SYBR Green detection for Cebpδ (C/EBPδ), Dlk1 (Pref1), Slc2a4/Glut4 (GLUT4), AdipoQ (adiponectin), 18S rRNA (Rn18s (18S)) and Rps29 were obtained from Qiagen. Primers for Pparγ (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

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primer: 5′-ATA GAC ATC AGC TAC ATC GA-3′ and reverse primer: 5′-CTG TCG GCT GTG CTG GAA-3′), Cebpβ (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 ACC ACC-3′ and reverse primer: 5′-GGT CGA TTT C-3′)

Whatman (GE Healthcare, Little Chalfont, Bucks, UK), (Risskov, Denmark).

Phosphocellulose (P81) paper was obtained from Whatman (GE Healthcare, Little Chalfont, Bucks, UK), and protease inhibitor cocktail tablets were obtained from Roche. Protein G Sepharose was purchased from GE Healthcare (anti-mouse).

Described previously (Gormand et al. 2011). MEFs were cultured to subconfluence in growth medium (DMEM containing 10% (v/v) FCS and 1% (v/v) penicillin/streptomycin) at 37 °C and 95% air/5% CO2. Differentiation was induced in 2-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 the medium being changed on day 3. The cells were then cultured in growth medium. The 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 and complete protease inhibitor cocktail (one tablet/50 ml)) or QAzol after rinsing with PBS.

**Generation of lentivirus**

The shRNA plasmid expression vectors were designed as described by Desclaux et al. (2009). Briefly, the shRNA sequence required for silencing the expression of the mouse Lkb1 gene was ordered from MWG (Ebersberg, Germany). The DNA fragment encoding Lkb1-shRNA was generated by annealing two complementary oligonucleotides, and the resulting double-stranded DNA fragments were inserted into pcDNAΔU6min (Amar et al. 2006) between BbsI and BamHI restriction sites, generating the plasmid pcDNAΔU6-shLKB1.

The expression cassette allowing effective Lkb1-shRNA expression under the control of the U6 promoter was excised from pcDNAΔU6min-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 described previously (Philippe et al. 2006). The amount of lentiviral vectors applied to cells is expressed as multiplicity of infection (MOI), corresponding to the number of transduction particles per cell.

**Generation of Lkb1-deficient 3T3-L1 cells**

3T3-L1 preadipocytes were seeded at a density of 5500 cells/well in 12-well plates. The cells were transduced 24 h later at 1.5 MOI with a lentivector encoding either Scrbl-shRNA or Lkb1-shRNA. Contact with the vector was allowed for 16 h, after which the medium was replaced. The cells were reseeded into 10 cm dishes 24 h later and allowed to grow to 60% confluence. The cells were then frozen in DMEM supplemented with 10% DMSO.

**Cell culture and adipogenic differentiation**

3T3-L1 fibroblasts were cultured and differentiated as described previously (Gormand et al. 2011). MEFs were cultured to subconfluence in growth medium (DMEM containing 10% (v/v) FCS and 1% (v/v) penicillin/streptomycin) at 37 °C and 95% air/5% CO2. Differentiation was induced in 2-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 the medium being changed on day 3. The cells were then cultured in growth medium. The 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 and complete protease inhibitor cocktail (one tablet/50 ml)) or QAzol after rinsing with PBS.

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**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 Hoechst 33258 for 30 min. The cells were washed twice with PBS and grown in fresh medium. Stained cells were imaged under a fluorescence microscope (Olympus IX71) using FITC, TRITC and 4',6-diamidino-2-phenylindole (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 triglyceride (TG) content, cells seeded into 12-well plates were washed twice with PBS before being lysed in 500 μl of 0.25 M NaOH. TGs were extracted in 1 ml of a hexane/isopropanol (3:2) mixture, dried using a SpeedVac and resuspended in isopropanol containing 1% Triton X100. TG content was measured by chemiluminescence at 510 nm using the Infinity Triglycerides reagent and compared with a known concentration of TG solution and a calibrator.

**Immunoprecipitation and kinase activity assay**

Immunoprecipitation from lysates for western blot analysis and kinase activity assay was performed as described previously (Gormand et al. 2011). Phosphotransferase activity towards the AMARA peptide (AMAR-AASAAALARRR) for AMPK (Dale et al., 1995), the LKB1tide (SNLYHQQKFLQTFCGSPLYR) for LKB1 (Lizzano et al., 2004), the HDAC5tide (PLRKTASEPNLRRR) for SIK2 (Berdeaux et al., 2007, Henriksson et al., 2012) and the Sakamototide (ALNRITSSDALTRRR) for SIK3 (Zagorska et al., 2010) was measured at 200 μM of the peptide substrates. One unit (U) of activity was defined as that which catalysed the incorporation of 1 nmol of 32P/min into the substrate.

**Western blot analysis**

Western blot analysis was carried out on equal amounts of total protein as described previously (Gormand et al., 2011). Quantification of the 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 (as 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 reverse-transcribed as described previously (Gormand et al., 2011). The cDNA was used in quantitative PCRs using TaqMan or SYBR Green chemistry in an ABI 7900 Sequence Detection System. The relative abundance of miRNA was calculated after normalisation to the geometric mean of two internal control genes (Rps29 and Rn18s; Vandesenompele et al., 2002, Ferguson et al., 2010). Qiagen miScript kits, reagents and primers were used to analyse miRNAs (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 CERI (200 μl) containing complete protease inhibitors/10 cm dish. Homogenates were vortexed and incubated for 15 min on ice. Ice-cold CERII (11 μl) was added to the homogenates, and the 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 with ice-cold PBS and resuspended in ice-cold NER (100 μl) containing complete protease inhibitors. Samples were vortexed for 15 s and incubated on ice for 10 min. This step was repeated four 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 six-well plates. Two-day post-confluent cells were stimulated for 1 h with differentiation medium, washed, fixed...
and probed with antibodies, as described previously (Henriksson et al. 2012). Confocal images were acquired with a Zeiss LSM 510 META microscope using an excitation wavelength of 405 (Hoechst nuclear stain), 488 (BODIPY) or 561 (Alexa 594) nm. A Plan-Apochromat 63×/1.4 oil DIC objective was used and a frame size of 1024×1024. Z-stacks with five to nine layers within the nucleus were used and maximum-intensity projection was applied.

Statistical analysis

Results are presented as means±S.E.M. of the indicated number of independent experiments. Statistical analysis was carried out 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<0.01, ***P<0.001 and ****P<0.0001. To enable comparison of qPCR and western blot analysis 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 LKB1 signalling is known to regulate the activity of CREB, we hypothesised that the silencing of Lkb1 may result in an altered adipogenic programme 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 Wt and Lkb1-deficient (Lkb1−/−) MEFs, in which the activity of AMPK and its related kinases has been shown to be dramatically reduced (Lizcano et al. 2004).

Staining of cellular lipids revealed that ≈75% of the Lkb1-shRNA-expressing 3T3-L1 cells were able to differentiate into mature and lipid-storing adipocytes, while only ≈41% of the Scrbl-shRNA-expressing cells had differentiated (Fig. 1a). Moreover, Lkb1-deficient MEFs accumulated twice as much lipids as did Wt MEFs, as revealed by Nile red staining (Fig. 1a and Supplementary Fig. 1, see section on supplementary data given at the end of this article). As a complement to lipid staining, the TG content of the whole 3T3-L1 cell population was quantified biochemically on day 9 (Fig. 1b), and Lkb1-shRNA-expressing cells were found to contain significantly higher levels of TG compared to Scrbl-shRNA-expressing cells. Furthermore, Dlk1 (Pref1), a marker for preadipocytes, was significantly more expressed in Scrbl-shRNA-expressing cells on day 6, which indicates that a lower number of cells had differentiated compared with Lkb1-shRNA-expressing cells (Fig. 1c).

The expression of adipocyte-specific proteins and/or mRNAs for HSL/Lipe, FAS/Fasn, aP2/Fabp4, GLUT4/Scl2a4 (glut4) and adiponectin/AdipoQ was up-regulated in Lkb1-shRNA-expressing 3T3-L1 cells and in Lkb1−/− MEFs (Fig. 1d and Supplementary Figs 1 and 2). In addition, the expression of Pparg and Cebpa was induced earlier and was significantly up-regulated in differentiating 3T3-L1 cells expressing Lkb1-shRNA (Fig. 1e and Supplementary Fig. 2, see section on supplementary data given at the end of this article). In Lkb1−/− MEFs, the expression of Pparg and Cebpa was already up-regulated in the fibroblast stage and remained so throughout differentiation (Supplementary Fig. 1). As shown in Supplementary Fig. 2, the expression of the PPARγ targets miR-103 and miR-107, which have previously been demonstrated to be induced during differentiation, was up-regulated in Lkb1-shRNA-expressing cells, particularly in the late stages of differentiation (day 6; Esau et al. 2004, Li et al. 2011). Interestingly, the expression of Cebpd was significantly higher in Lkb1-shRNA-expressing cells 30 min after the induction of differentiation (Fig. 1f). However, there was no consistent difference in the mRNA (Fig. 1f) or protein expression (data not shown) of Cebpb/C/EBPβ between the Scrbl-shRNA- and Lkb1-shRNA-expressing 3T3-L1 cells or in Lkb1−/− MEFs (Supplementary Fig. 1).

We also analysed the mRNA and protein expression of Ddit3/CHOP10, a dominant-negative member of the C/EBP family, which has been shown to inhibit the differentiation of adipocytes by sequestering/inactivating C/EBPβ (Tang & Lane 2000). As previously reported (Huang et al. 2005), the levels of Ddit3/CHOP10 declined upon the differentiation of adipocytes (Fig. 1g). Notably, there was a reduction in the expression of Ddit3/CHOP10 at both the mRNA and protein levels in 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 Lkb1-shRNA-expressing cells, without an induction of C/EBPβ expression levels. To rule out that the lack of Lkb1 might facilitate adipogenesis by potentially enhancing clonal expansion, we counted cells at different time points before and after the initiation of differentiation and found that Lkb1-shRNA- and Scrbl-shRNA-expressing cells did not differ significantly in this regard (Supplementary Fig. 2).
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 as well as 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 Dlk1 (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 analysis for the protein expression of FAS and HSL 10 days after the induction of differentiation. (e, f and g) The mRNA levels of the adipogenic transcription factors Pparγ and C/EBPα (e), C/EBPβ and C/EBPδ (f) and the dominant-negative C/EBP family member Ddit3 (CHOP10) (g) measured by qPCR in Lkb1-shRNA-expressing cells were compared with those in the Scrbl-shRNA-expressing cells after the induction of differentiation at the time points indicated in the figure. The protein amount of CHOP10 was measured by western blot analysis in lysates from Scrbl-shRNA- or Lkb1-shRNA-expressing 3T3-L1 preadipocytes after the induction of differentiation at the time points indicated in the figure. The blot, from one experiment, is representative of three independent experiments.

The data represent the mean ± S.E.M. of three independent experiments, in each of which the data were expressed as fold or percentage 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 (a) and two-way ANOVA with multiple comparisons in (b, c, d, e, f and g)). Time point 0 corresponds to the day when the differentiation medium was added (2 days post-confluent).
Figure 2
Silencing of \(\text{Lkb}1\) in 3T3-L1 preadipocytes results in a reduced phosphorylation of CRTC2 and class IIa HDACs. (a) 3T3-L1 preadipocytes were transduced with scrambled-shRNA (Scrbl-shRNA) and \(\text{Lkb}1\)-shRNA. The silencing of \(\text{Lkb}1\) was confirmed at the mRNA level by qPCR and at the protein level by kinase activity assay and western blot analysis. (b, c, d, e and f) Adipogenic differentiation was induced for 1 h in 2-day post-confluent preadipocytes, and cell homogenates were analysed for the phosphorylation and/or kinase activity of CRTC2 (b), class IIa HDACs (c), AMPK (d), SIK2 (e) and SIK3 (f). The data represent the mean±s.e.m. of three independent experiments, in each of which the data were expressed as fold or percentage 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 (a) and two-way ANOVA with multiple comparisons in (b, c, d, e and f)). A representative blot displaying technical duplicates from one experiment is shown.
Taken together, these data show that 3T3-L1 pre-adipocytes and MEFs expressing lower levels of LKB1 display an improved ability to differentiate into adipocyte-like cells, express higher levels of early adipogenic genes, and lower levels of the anti-adipogenic gene Ddit3. 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**

The silencing of Lkb1 in 3T3-L1 cells was confirmed by quantitative real-time PCR, LKB1 kinase activity assay and western blot analysis (Fig. 2a). To gain more insights into the molecular mechanisms downstream of LKB1, which may mediate effects on adipogenic gene expression, we investigated whether the phosphorylation of CRTC2 and class IIA HDAC4, HDAC5 and HDAC7 is regulated by LKB1 in 3T3-L1 preadipocytes, before and 1 h after the induction of differentiation. As shown in Fig. 2b and c, the phosphorylation of CRTC2 and class IIA HDACs on sites previously reported to control their activities (Screaton et al. 2004, Mihaylova et al. 2011) was lower in Lkb1-shRNA-expressing cells, as was the phosphorylation of these proteins in cells treated with the differentiation medium for 1 h. There was no difference in the phosphorylation of CREB on S133 between the Scrbl-shRNA- and Lkb1-shRNA-expressing cells (data not shown).

We next determined 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 (Screaton et al. 2004, Muraoka et al. 2009, Mihaylova et al. 2011). In the absence of LKB1, the activity and specific phosphorylation of AMPK on the activity controlling T172 site were 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, e and f). However, there was no significant reduction in the specific activities of SIK2 and SIK3 in Lkb1-shRNA-expressing cells (Fig. 2e and f).

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

In the experiments described in Fig. 2, we observed that treatment of Scrbl-shRNA-expressing 3T3-L1 preadipocytes with the differentiation medium for 1 h resulted in the dephosphorylation of CRTC2 and class IIA HDACs, in particular, HDAC4 (Fig. 2b and c). This indicates that the activities of these proteins, and potentially their upstream regulators, may be subject to acute regulation by adipogenic stimuli. To determine whether this is the case, we treated 3T3-L1 preadipocytes with the differentiation medium for different time periods and analysed the phosphorylation/activity and localisation status of AMPK, SIK2, SIK3, CRTC2 and HDAC4. Within an hour, AMPKα1 T172 phosphorylation and activity were 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 an 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 the dephosphorylation of CRTC2 on S275 and HDAC4 on S246 (Fig. 3d and e), 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 different time points throughout the whole differentiation process. As shown in Supplementary Fig. 3, see section on supplementary data given at the end of this article, the protein expression of LKB1 was reduced by 50% on day 3 after differentiation, but returned to original levels on day 6. Notably, the T172 phosphorylation of AMPK was markedly reduced on days 1 and 3, as was the phosphorylation of CRTC2 and class IIA HDACs (Supplementary Fig. 3). These changes were mirrored by an increase in the S485 phosphorylation of AMPK and the phosphorylation of PKA substrates, as monitored using a PKA consensus motif antibody. These data demonstrate that the acute inhibition of AMPK signalling persisted at least until day 3 after the induction of 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, b, c and 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 stimuli 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 by both insulin and IBMX; however, insulin stimulation alone did not result in the 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

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 figure and analysed for the 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 ± S.E.M. of three independent experiments (carried out in triplicates), in each of which the data were expressed as percentage 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's t-test). A representative blot displaying technical triplicates from one experiment is shown. Subcellular localisation of SIK2, CRTC2 and HDAC4, HDAC5 and HDAC7 (f) was analysed by western blot analysis 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 ± S.E.M. of three independent experiments (carried out in triplicates), in each of which the data were expressed as percentage 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's 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).
the cAMP/PKA pathway, we employed the PKA inhibitor H89 (Fig. 4e, f, g and 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 reversed in the presence of H89 in the stimulated cells (Fig. 4e, f, g and h). We conclude that the inhibition of AMPK/SIK signalling, and subsequent dephosphorylation of CRTC2, following adipogenic differentiation of 3T3-L1 cells is most likely mediated by the activation of the cAMP/PKA pathway in response to IBMX.

**Discussion**

In this study, we tested the hypothesis that LKB1 signalling might keep adipocyte precursors in their non-mature form and that adipogenic stimuli attenuate LKB1 signalling for differentiation to occur. We demonstrated that three substrates downstream of LKB1 are acutely inhibited following the initiation of differentiation in a PKA-dependent manner and that this coincides with the dephosphorylation and nuclear translocation of CRTC2 and class IIa HDACs. Moreover, silencing of Lkb1 resulted in a marked reduction in the mRNA and protein levels of Ddit3/CHOP10, an increase in Pparγ 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 inhibited within 1 h of induction of adipogenic differentiation, and this inhibition coincided with the dephosphorylation and nuclear translocation of CRTC2 and class IIa HDACs. The results from IBMX treatment and PKA inhibition assays indicate that cAMP/PKA signalling mediated the inhibition of
AMPK/SIK signalling that we observed. Studies have shown that the phosphorylation of AMPK on S485 by PKA and/or PKB is inversely correlated with AMPK T172 phosphorylation and kinase activity (Hurley et al. 2006, Berggreen et al. 2009), which could provide a mechanistic explanation for the inhibition of AMPK during differentiation. In our experiments, we did observe an induction of S485 phosphorylation in response to the adipogenic cocktail. However, although 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 the inhibition of AMPK 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 the findings of 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 the results of our previous studies and the fact that the phosphorylation by PKA 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 determine whether 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 an ≈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 with the WT, the activity of AMPK was reduced by 40% and the activities of SIK2 and SIK3 were only reduced by ~25% (Gorman et al. 2011). This indicates either the existence of alternative upstream kinases or a large spare capacity in the LKB1 signalling pathway – with the latter being supported by the almost complete lack of SIK2 and SIK3 activities in Lkb1-deficient cells and tissues (Lizcano et al. 2004, Al-Hakim et al. 2005). 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, indicating that it may be the combined reduction of the AMPK/SIK signalling, or inhibition of other AMPK-related kinases, that leads to a reduced phosphorylation of CRTC2 and HDAC4. 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 whether a specific substrate of LKB1 is involved.
might be chiefly responsible for downstream effects or whether the substrates play redundant roles.

Employing two different cellular models, we showed 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 $Lkb1^{-/-}$ 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 the expression of $Pparg$ and $Cebpα$ was up-regulated early during differentiation and that the number of adipocytes was increased indicates that the phenotype we observed is due to LKB1 regulation of transcriptional events controlling the actual differentiation program, rather than directly affecting the accumulation of lipids and/or the expression of adipocyte-specific proteins. Zhang et al. (2013) demonstrated that the deletion of $Lkb1$ in mouse adipose tissue, employing Fabp4-mediated Cre recombinase expression, resulted in a reduced amount of white adipose tissue and expression of adipogenic genes. As 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, which is in contrast to our approach. In support of the findings of the present study, 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 the expression of C/EBPs might be up-regulated in $Lkb1$-silenced cells due to the activation of CRTC2/CREB. The lack of induction of LKB1, mRNA and protein expression of $Cebpb/C/EBPβ$ in $Lkb1$-shRNA-expressing cells does not support this notion. In $Lkb1^{-/-}$ MEFs, we observed that $Cebpb$ mRNA levels tended to be increased before the initiation of adipogenesis, but the difference was not statistically significant in the two-way ANOVA and did not persist after the addition of the 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 $Cebpδ$, 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-expressing 3T3-L1 cells (Reusch et al. 2000, Belmonte et al. 2001, Rosen & MacDougald 2006). In search for additional mechanisms underlying the up-regulation of $Pparg$ and $Cebpα$ expression, we analysed the dominant-negative C/EBP family member CHOP10, which inhibits C/EBPβ by sequestration/inactivation (Tang & Lane 2000, Huang et al. 2005). Indeed, the mRNA and protein levels of Ddit3/CHOP10 were markedly reduced in $Lkb1$-shRNA-expressing cells, potentially allowing for more C/EBPβ to bind to DNA and stimulate gene expression. At present, we do not know how LKB1 regulates the expression of CHOP10, and this will be of future interest to address. We find it interesting that CRTC2 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, we demonstrate that $Lkb1$ silencing in 3T3-L1 preadipocytes promotes the dephosphorylation of HDAC4 and CRTC2, induces the expression of $Pparg$ and $Cebpα$, and facilitates the differentiation of adipocytes. We also demonstrate that AMPK/SIK signalling is inhibited following the initiation of differentiation. Based on these findings, we hypothesise that in the absence of adipogenic stimuli, the LKB1/AMPK/SIK signalling serves to keep preadipocytes in their non-differentiated form.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/JME-13-0296.

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.

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