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

2   Amélie Gormand[1], Christine Berggreen[1], Lahouari Amar[1], Emma Henriksson[1,3], Ingrid Lund[2], Sebastian

3   Albinsson[1], Olga Göransson[1]

4   [1]The Department of Experimental Medical Science, Lund University, Lund, Sweden

5   [2]The Department of Biomedicine, Karolinska Institute, Stockholm, Sweden

6   [3]Current affiliation: The Department of Chemical Physiology, The Scripps Research Institute, La Jolla,

7   US.

8   **Corresponding author:** Olga Göransson, Department of Experimental Medical Science, Protein

9   Phosphorylation Research Group BMC C11, 221 84 Lund, Sweden, Olga.Goransson@med.lu.se

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13

14   **ABSTRACT**

15   cAMP-response element-binding protein (CREB) is required for the induction of adipogenic transcription

16   factors such as CCAAT/enhancer-binding proteins (C/EBPs). Interestingly, it is known from other tissues

17   that LKB1 and its substrates AMP-activated protein kinase (AMPK) and salt-inducible kinases (SIKs),

18   negatively regulate gene expression by phosphorylating the CREB co-activator CRTC2 and class IIa

19   histone deacetylases (HDACs), which results in their exclusion from the nucleus where they co-activate

20   or inhibit their targets. In this study, we show that AMPK/SIK signalling is acutely attenuated during

21   adipogenic differentiation of 3T3-L1 preadipocytes, which coincides with dephosphorylation and nuclear

22   translocation of CRTC2 and HDAC4. When subjected to differentiation, 3T3-L1 preadipocytes in which

23   LKB1 expression was stably reduced using shRNA (LKB1-shRNA), as well as LKB1 knockout mouse

24   embryonic fibroblasts (LKB1$^{-/-}$ MEFs), differentiated more readily into adipocyte-like cells and

25   accumulated more triglycerides compared to scrambled-shRNA 3T3-L1 cells or Wt MEFs. In addition,

26   the phosphorylation of CRTC2 and HDAC4 was reduced, and the mRNA expression of adipogenic

27   transcription factors C/EBPα, peroxisome proliferator-activated receptor γ (PPARγ) and adipocyte-

28   specific proteins such as hormone sensitive lipase (HSL), fatty acid synthase (FAS), aP2, Glut4 and

29   adiponectin was increased in the absence of LKB1. The mRNA and protein expression of CHOP-10, a

30   dominant negative member of the C/EBP family, was reduced in LKB1 shRNA expressing cells,

31   providing a potential mechanism for the up-regulation of *Pparg* and *Cebpa*. These results support the

32   hypothesis that LKB1 signalling keeps preadipocytes in their non-differentiated form.

33   **INTRODUCTION**

34   Adipogenesis is a multi-step process through which progenitor cells differentiate into functional

35   adipocytes, and involves a cascade of adipogenic transcription factors and genes that define the adipocyte

36   phenotype (Rosen 2005). In cultured preadipocytes, the CCAAT/enhancer-binding proteins β and δ

37   (C/EBPβ and C/EBPδ) are induced within hours following induction of differentiation. C/EBPβ and

38   C/EBPδ then induce the expression of peroxisome proliferator-activated receptor γ (PPARγ) and

39   CCAAT/enhancer-binding protein α (C/EBPα) (Christy, et al. 1991; Clarke, et al. 1997; Wu, et al. 1995;

40   Yeh, et al. 1995). Once expressed, PPARγ and C/EBPα cooperate to induce the majority of genes

41   involved in the adipocyte phenotype such as adipocyte protein (*Fabp4*, aP2), hormone sensitive lipase

42   (*Lipe*, HSL) and fatty acid synthase (*Fasn*, FAS) (Cornelius, et al. 1994).

43   Signalling pathways that induce the adipogenic transcriptional machinery remain poorly characterized. It

44   is generally accepted that this occurs partly through the cAMP/protein kinase A (PKA) pathway via the

45   activation of cAMP-response element-binding protein (CREB). CREB is required for adipocyte

46   differentiation by inducing the expression of C/EBPβ and potentially C/EBPδ (Belmonte, et al. 2001;

47   Reusch, et al. 2000; Zhang, et al. 2004). CREB activity is regulated by PKA-dependent phosphorylation,

48   and also requires co-activators such as CREB-regulated transcription co-activators (CRTCs) (Conkright,

49   et al. 2003; Ravnskjaer, et al. 2007; Screaton, et al. 2004; Xu, et al. 2007). When phosphorylated, CRTCs

50   bind to 14-3-3 proteins and are sequestered in the cytoplasm. Upon increasing levels of cAMP, CRTCs

51   are dephosphorylated and translocate to the nucleus where they co-activate CREB (Screaton et al. 2004).

52   The tumour suppressor kinase LKB1 and its substrates in the AMP-activated protein kinase (AMPK)

53   family inhibit CREB-mediated gene expression via phosphorylation of CRTCs (Koo, et al. 2005;

54   Muraoka, et al. 2009; Screaton et al. 2004). Another recently identified group of substrates for AMPK

55   family members, is the class IIa histone deacetylases (HDAC 4, 5, 7). Like CRTCs, these HDACs are

56   regulated by phosphorylation and binding to 14-3-3 proteins, preventing their nuclear entry (Bassel-Duby

57   and Olson 2006; Haberland, et al. 2009; McKinsey, et al. 2000). In the nucleus, class IIa HDACs have

58   been shown to regulate gene expression, such as Glut4 in adipocytes (Weems, et al. 2012).

59   AMPK and its related kinases the salt inducible kinases 1-3 (SIK1-3) are phosphorylated and activated by

60   the common and constitutively active upstream kinase LKB1 (Hawley, et al. 2003; Lizcano, et al. 2004;

61   Woods, et al. 2003). The net phosphorylation of the activation (T-) loop residue T172 and thus activity of

62   AMPK is greatly induced by the binding of AMP (Hardie 2008; Sanders, et al. 2007; Suter, et al. 2006).

63   In this way, AMPK functions as an energy sensor, restoring energy levels by phosphorylating a wide

64   array of substrates (Carling 2004; Carling, et al. 2008). Several reports demonstrate that activators of

65   AMPK, including AICAR and A-769662, inhibit adipogenesis (Giri, et al. 2006; Lee, et al. 2011; Zhou, et

66   al. 2009). Both PKA and PKB, which are activated during adipocyte differentiation, have been suggested

67   to inhibit AMPK activity by phosphorylating S485 on the catalytic subunit (Hurley, et al. 2006). We have

68   previously shown that PKA-activation in adipocytes results in phosphorylation and binding of SIK2 and

69   SIK3 to 14-3-3 proteins (Berggreen, et al. 2012; Henriksson, et al. 2012), and this is predicted to inhibit

70   their function.

71   Based on its potential ability to respond to adipogenic stimuli, as well as cellular energy levels, we

72   hypothesise that in the absence of appropriate signals, the LKB1/AMPK/SIK signalling pathway serves to

73   keep adipocyte progenitors in their non-differentiated form. This inhibitory effect of LKB1 signalling on

74   adipogenesis might be mediated through its action on CRTC2/CREB and HDACs.

75   **MATERIALS AND METHODS**

76   **Materials**

77   3T3-L1 cells were obtained from American Type Culture Collection and used within 15 passages of the

78   original source. LKB1-deficient immortalized mouse embryonic fibroblasts (MEFs) were generated by

79   Professor Tomi Mäkelä, University of Helsinki, and kindly provided by Professor Dario Alessi,

80   University of Dundee. DMEM, trypsin/EDTA, dexamethasone, rosiglitazone, IBMX, insulin, H89 and

81   Nile Red were purchased from Sigma. Collagenase Type I was obtained from Gibco™. Precast Novex

82 SDS polyacrylamide 4-12% Bis-Tris gels, lauryl dodecyl sulfate (LDS) sample buffer, fetal bovine serum,

83 Hoechst 33258 nucleic acid stain, DNAse I amplification grade and SuperScript™ II RNaseH reverse

84 transcriptase were obtained from Invitrogen. Infinity Triglycerides reagent was purchased from Thermo

85 Scientific. QIAzol™ lysis reagent, RNeasy®- and miRNeasy® Mini Kits were purchased from Qiagen.

86 Taqman® Gene Expression Assays for *Stk11* (LKB1), *Ddit3* (CHOP-10), Ribosomal protein S29 (*Rps29*,

87 RPS29) and TATA box binding protein (*Tbp*, TBP) were obtained from Applied Biosystems. The

88 QuantiTect Primers Assays with SYBR® Green detection for *Cebpd* (C/EBPδ), *Dlk1* (Pref1), *Glut4*

89 (GLUT4), *AdipoQ* (adiponectin), 18S ribosomal RNA (*Rn18s*, 18S) and *Rps29* were obtained from

90 Qiagen. The primers for *Pparg* (PPARγ, forward primer: 5′-CTG TTT TAT GCT GTT ATG GGT GAA

91 A-3′ and reverse primer: 5′-GCA CCA TGC TCT GGG TCA A-3′), *Cebpa* (C/EBPα, forward primer: 5′-

92 ATA GAC ATC AGC GCC TAC ATC GA-3′ and reverse primer: 5′-CTG TCG GCT GTG CTG GAA-

93 3′), *Cebpb* (C/EBPβ, forward primer: 5'-CAG CGC ACC GGG TTT C-3' and reverse primer: 5'-CGC

94 AGG AAC ATC TTT AAG GTG ATT-3'), *Fabp4* (aP2, forward primer: 5'-TTC GAT GAA ATC ACC-

95 3' and reverse primer: 5'-GGT CGA CTT TCC ATC-3') and *Fasn* (FAS, forward primer: 5'-TGG TGA

96 ATT GTC TCC-3' and reverse primer: 5'-CAG GTT CAT CAC GAG-3') were obtained from DNA

97 Technology.

98 Phosphocellulose (P81) paper was obtained from Whatman and protease inhibitor cocktail tablets were

99 from Roche. Protein G-Sepharose was purchased from GE Healthcare, and ^{32}Pγ-ATP from Perkin Elmer.

100 AMARA-, LKB1tide-, HDAC5tide- and Sakamototide peptides were synthesized by GL Biochem, China.

101 The following antibodies were used for western blotting: anti-AMPK, anti-phospho-AMPK (Thr172),

102 anti-phospho-AMPK (S485), anti-HDAC4, anti-phospho-HDAC4(S246)/HDAC5(S259)/HDAC7(S155),

103 anti-CREB, anti-phospho-CREB (S133), anti-phospho-PKA substrate and anti-HSP90 were purchased

104 from Cell Signaling Technology; the antibody against LKB1 was purchased from AbCam; the antibody

105 against GAPDH was purchased for Sigma; the antibody against CRTC2 was purchased from Calbiochem;

106 the antibody against H3 was purchased from Millipore, and the antibody against CHOP-10 was from

107 Santa Cruz (GADD153). Anti-phospho-CRTC2 (S275) was a kind gift from Robert Screaton, University

108   of Ottawa, Canada. The antibodies anti-FAS and anti-HSL were kindly provided by Professor Eva

109   Degerman and Cecilia Holm respectively, Lund University, Sweden. Anti-AMPKα1 antibody used for

110   kinase assays was kindly provided by Professor Grahame Hardie, University of Dundee, UK. Anti-SIK2,

111   anti-phospho-SIK2 (S358) and anti-SIK3 antibodies were raised in rabbit and affinity-purified by

112   Innovagen against peptides corresponding to residues 906-926 of human SIK2, residues 351-365 of

113   human SIK2 and residues 1349-1369 of human SIK3 respectively.

114   Horseradish peroxidase conjugated secondary antibodies were obtained from Biosource (anti-rabbit),

115   Pierce (anti-sheep), Santa Cruz (anti-goat) and GE Healthcare (anti-mouse).

116   **Cell culture and adipogenic differentiation**

117   3T3L1 fibroblasts were cultured and differentiated as previously described (Gormand, et al. 2011). MEFs

118   were cultured to subconfluence in growth medium (DMEM containing 10 % (v/v) FCS, 1 % (v/v)

119   penicillin/streptomycin) at 37 °C and 95 % air/5 % CO_2. Differentiation was induced on two-day post-

120   confluent cells by incubating them in growth medium supplemented with 0.5 mM IBMX, 5 µg/ml insulin,

121   1 µM dexamethasone and 0.1 µM rosiglitazone for 6 days, with one change of medium on day 3.

122   Hereafter the cells were cultured in growth medium. Cells were harvested in lysis buffer (50 mM

123   Tris/HCl pH 7.5, 1 mM EGTA, 1 mM EDTA, 1 mM sodium orthovanadate, 10 mM sodium-β-

124   glycerophosphate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 0.27 M sucrose, 1% w/v NP40,

125   1 mM dithiothreitol (DTT) and complete protease inhibitor cocktail (one tablet/50 ml)) or QIAzol^{TM} after

126   rinsing with PBS.

127   **Generation of lentivirus**

128   The shRNA plasmid expression vectors were designed as described by Desclaux et al (Desclaux, et al.

129   2009). Briefly, the shRNA sequence to silence the expression of the mouse LKB1 gene was ordered from

130   MWG (Germany). The DNA fragment encoding LKB1-shRNA was generated by annealing two

131   complementary oligonucleotides and the resulting double-stranded DNA fragments were inserted in

132   pcDNA-ΔU6min (Amar, et al. 2006) between *Bbs*I and *Bam*HI restriction sites, generating the plasmid

133   pcDNA-ΔU6-shLKB1.

134   The expression cassette allowing effective LKB1-shRNA under the control of the U6 promoter was

135   excised from pcDNA-ΔU6min-shLKB1 and inserted into the lentivector precursor plasmid pFlap-PGK-

136   GFP-WPRE (Desclaux et al. 2009) using *Mlu*I and *Bam*HI sites. As control, a lentiviral vector expressing

137   a "scrambled" shRNA (Scrbl-shRNA) sequence was produced. Lentiviral vector particles were produced

138   as previously described (Philippe, et al. 2006). The amount of lentiviral vectors applied to cells is

139   expressed as multiplicity of infection (MOI), corresponding to the number of transducting particles per

140   cell.

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

142   3T3-L1 preadipocytes were seeded at a density of 5 500 cells per well in 12-well plates. Cells were

143   transduced 24 h later at 1.5 MOI with lentivector encoding either Scrbl-shRNA or LKB1-shRNA. Contact

144   with the vector was allowed for 16 h, after which the medium was replaced. 24 h later, cells were

145   reseeded onto 10 cm dishes and allowed to grow to 60 % confluence. Cells were then frozen in DMEM

146   supplemented with 10 % DMSO.

147   **Lipid staining and cell imaging.**

148   Intracellular lipids were stained with 10 µg/ml Nile Red in the medium for 15 min and nuclei were

149   stained with 10 µg/ml of Hoescht 33258 for 30 min. Cells were washed twice with PBS and fresh medium

150   was added. Stained cells were imaged under a fluorescent microscope (Olympus IX71) using FITC,

151   TRITC and DAPI filters. Ten pictures per plate were randomly taken and each plate corresponded to one

152   condition per experiment. Images were processed and analysed using cellSens software from Olympus.

153   The percentage of differentiated 3T3-L1 cells was calculated as the number of cells stained with Nile Red

154   over the total number of cells (number of nuclei stained with Hoechst).

155   **Quantification of intracellular triglyceride content**

156 To determine the triglyceride (TG) content, cells seeded onto 12-well plates were washed twice with PBS

157 before being lysed in 500 µl 0.25 M NaOH. TGs were extracted in 1 ml hexane:isopropanol (3:2)

158 mixture, dried using a SpeedVac and resuspended in isopropanol containing 1 % triton. The TG content

159 was measured by chemiluminescence at 510 nm using the Infinity Triglycerides reagent, compared to a

160 known concentration of TG solution and a calibrator.

161 **Immunoprecipitation and kinase activity assay**

162 Immunoprecipitations from lysates for western blotting and kinase activity assays were performed as

163 described previously (Gormand et al. 2011). Phosphotransferase activity towards the AMARA peptide

164 (AMARAASAAALARRR) for AMPK (Dale, et al. 1995), the LKB1tide

165 (SNLYHQGKFLQTFCGSPLYRR) for LKB1 (Lizcano et al. 2004), the HDAC5tide

166 (PLRKTASEPNLKRRR) for SIK2 (Berdeaux, et al. 2007; Henriksson et al. 2012) and the Sakamototide

167 (ALNRTSSDSALHRRR) for SIK3 (Zagorska, et al. 2010), was measured with 200 µM of the peptide

168 substrates. One Unit (U) of activity was defined as that which catalysed the incorporation of 1 nmol of

169 ^{32}P/min into the substrate.

170 **Western Blot analysis**

171 Western blot analysis was performed on equal amount of total protein as described previously (Gormand

172 et al. 2011). Quantification of total amount of protein was normalised to either GAPDH to compare the

173 amount of proteins in lysates from undifferentiated cells, or HSP90 to compare the amount of proteins in

174 lysates from undifferentiated and differentiated cells (since the amount of GAPDH protein varies between

175 preadipocytes and mature adipocytes). Images were acquired and analysed using ChemiDoc^{TM} XRS+ and

176 the Image Lab^{TM} software from Bio-Rad.

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

178 Total RNA and miRNAs were isolated using RNeasy®- and miRNeasy® Mini Kits according to the

179 manufacturer's recommendations. Total RNA (1 µg) was treated with DNase I and reversely transcribed

180 as described previously (Gormand et al. 2011). The cDNA was used in quantitative PCR reactions using

181   Taqman or Sybrgreen chemistry in an ABI 7900 Sequence Detection System. Relative abundance of

182   mRNA was calculated after normalisation to the geometric mean of two internal control genes (*Rps29* and

183   *Rn18s*) (Ferguson, et al. 2010; Vandesompele, et al. 2002). MicroRNAs were analysed using Qiagen

184   miScript kits, reagents and primers (Turczynska, et al. 2012). Each sample was analysed in duplicates.

185   **Cytoplasmic and nuclear fractionation**

186   Subcellular fractionation was performed using the NE-PER® Nuclear and Cytoplasmic Extraction kit

187   (Thermo Scientific) according to the manufacturer's recommendations with minor changes. Cells were

188   washed in ice-cold PBS and scraped in 200 µl CERI containing complete protease inhibitors/10 cm dish.

189   Homogenates were vortexed and incubated for 15 min on ice. 11 µl of ice-cold CERII was added, and

190   homogenates were incubated for 1 min on ice, vortexed and centrifuged for 5 min at 16 000 g. The

191   supernatant containing the cytoplasmic fraction was collected. Pellets were washed twice in ice-cold PBS

192   and resuspended in 100 µl of ice-cold NER containing complete protease inhibitors. Samples were

193   vortexed for 15 s and incubated on ice for 10 min. This step was repeated 4 times before centrifugation

194   for 10 min at 16 000 g. The supernatant containing the nuclear fraction was recovered.

195   **Confocal fluorescence microscopy**

196   3T3-L1 preadipocytes were cultured on cover slips in 6-well plates. 2 days post confluent cells were

197   stimulated for 1 h with differentiation medium, washed, fixed and probed with antibodies, as described

198   (Henriksson et al. 2012). Confocal images were acquired with a Zeiss LSM 510 META microscope using

199   excitation wavelengths 405 (Hoechts nuclear stain), 488 (BODIPY) or 561 (Alexa 594) nm. A Plan-

200   Apochromat 63x/1.4 oil DIC objective was used and a frame size of 1024x1024. Z-stacks with 5-9 layers

201   within the nucleus were used and maximum intensity projection applied.

202   **Statistical analysis**

203   Results in this study are presented as mean + SEM of the indicated number of independent experiments.

204   Statistical analysis were performed using Student's t test (two-tailed, unpaired) or two-way ANOVA with

205   multiple comparisons, and differences were considered statistically significant when * $p < 0.05$, ** $p <$

206   0.01, *** p < 0.001 and **** p < 0.0001. To enable comparison of qPCR and western blot data from

207   several independent experiments, a control sample in each experiment was set to 1 or 100%. A

208   representative blot displaying technical duplicates or triplicates from one experiment is generally shown.

209   **RESULTS**

210   **LKB1 depletion facilitates 3T3-L1 adipocyte differentiation.**

211   Based on the fact that the LKB1 signalling is known to regulate CREB activity, we hypothesised that the

212   silencing of LKB1 may result in an altered adipogenic program in the 3T3-L1 preadipocyte cell line. We

213   generated a 3T3-L1 cell line with stable expression of shRNA targeted against LKB1 mRNA. As a

214   complement to this model system, we also employed wild type (Wt) and LKB1-deficient (LKB1$^{-/-}$) mouse

215   embryonic fibroblasts (MEFs), in which the activity of AMPK and its related kinases has been shown to

216   be dramatically reduced (Lizcano et al. 2004). The silencing of LKB1 in the 3T3-L1 cells was confirmed

217   by quantitative real-time PCR, LKB1 kinase activity assay, and by western blot (Fig. 2a).

218   Staining of cellular lipids showed that ≈ 75 % of LKB1-shRNA expressing 3T3-L1 cells were able to

219   differentiate into mature and lipid-storing adipocytes, while only ≈ 41 % of Scrbl-shRNA cells had

220   differentiated (Fig. 1a). Moreover, LKB1-deficient MEFs accumulated twice as much lipids as did Wt

221   MEFs, as judged by quantification of the Nile Red staining (Fig. 1a and S1). As a complement to lipid

222   staining, the triglyceride content of the whole 3T3-L1 cell population at day 9 was quantified

223   biochemically (Fig. 1b), showing that the LKB1-shRNA cells contained significantly more triglycerides

224   than Scrbl-shRNA cells. Furthermore, *Pref1*, a marker for preadipocytes, was significantly more

225   expressed in Scrbl-shRNA cells at day 6, which indicates that less cells had differentiated compared to

226   LKB1-shRNA cells (Fig. 1c). The adipocyte-specific proteins and/or mRNAs for HSL, FAS, aP2, Glut4

227   and adiponectin were up-regulated in LKB1-shRNA 3T3-L1 cells and in LKB1$^{-/-}$ MEFs (Fig. 1d, S1, S2).

228   In addition, *Pparg* and *Cebpa* were induced earlier and were significantly up-regulated in differentiating

229   3T3-L1 cells expressing LKB1-shRNA (Fig. 1e and S2). In LKB1$^{-/-}$ MEFs, *Pparg* and *Cebpa* were up-

230   regulated already in the fibroblast stage, and their expression remained up-regulated throughout

231   differentiation (S1). As shown in supplementary figure 2 (S2), the PPARγ targets miR-103 and miR-107,

232   which have been previously demonstrated to be induced during differentiation, were up-regulated in

233   LKB1-shRNA cells, particularly in the late stages of differentiation (day 6) (Esau, et al. 2004; Li, et al.

234   2011). Interestingly, *Cebpd* was significantly higher in the LKB1-shRNA cells 30 min after induction of

235   differentiation (Fig. 1f). However, there was no consistent difference in the expression of *Cebpb* mRNA

236   (Fig. 1f) or protein expression (data not shown) between the Scrbl- and LKB1-shRNA expressing 3T3-L1

237   cells or in LKB1$^{-/-}$ MEFs (S1). We also analysed the mRNA- and protein expression of CHOP-10, a

238   dominant-negative member of the C/EBP family, which has been shown to inhibit adipocyte

239   differentiation by sequestering/inactivating C/EBPβ (Tang and Lane 2000). As previously reported

240   (Huang, et al. 2005), CHOP-10 mRNA- and protein levels decline upon adipocyte differentiation (Fig.

241   1g). Notably, there was a reduction in the expression of CHOP-10 both at the mRNA and protein level in

242   the LKB1-shRNA expressing cells at several time points, including before the initiation of differentiation.

243   This provides a potential mechanism whereby C/EBPβ activity, and thus PPARγ and C/EBPα expression,

244   may be increased in the LKB1-shRNA expressing cells, without an induction of C/EBPβ expression

245   levels. To rule out that the lack of LKB1 might facilitate the adipogenesis by potentially enhancing clonal

246   expansion, we counted cells at different time points before and after the initiation of differentiation, and

247   found that LKB1- and Scr-shRNA expressing cells did not differ significantly in this regard (S2).

248   Taken together, these data show that 3T3-L1 preadipocytes and MEFs expressing lower levels of LKB1

249   displayed an improved ability to differentiate into adipocyte-like cells, expressed higher levels of early

250   adipogenic genes, and lower levels of the anti-adipogenic gene *Ddit3* (CHOP-10). These data are in line

251   with the hypothesis that LKB1 signalling attenuates the induction of differentiation in preadipocytes.

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

253   To gain more insight into the molecular mechanisms downstream of LKB1, which may mediate effects on

254   adipogenic gene expression, we investigated whether the phosphorylation of CRTC2 and the class IIa

255   HDAC4, 5, 7 is regulated by LKB1 in 3T3-L1 preadipocytes, before and 1h after the induction of

256   differentiation. As shown in Fig. 2b and 2c, the phosphorylation of CRTC2 and class IIa HDACs on sites

257   previously reported to control their activity (Mihaylova, et al. 2011; Screaton et al. 2004), was lower in

258 LKB1-shRNA cells, as was the phosphorylation of these proteins in cells treated with the differentiation

259 medium for 1h. There was no difference in the phosphorylation of CREB on S133, between the Scrbl-

260 and LKB1-shRNA expressing cells (data not shown).

261 We next monitored how the reduced LKB1 expression affected the phosphorylation and/or activity status

262 of AMPK, SIK2 and SIK3 – potential substrates downstream of LKB1 reported to directly regulate

263 CRTC2 and class II HDACs (Mihaylova et al. 2011; Muraoka et al. 2009; Screaton et al. 2004). In the

264 absence of LKB1, the activity and specific phosphorylation of AMPK on the activity controlling T172

265 site was reduced ($\approx$ 50 %), as was the total kinase activity of SIK2 and SIK3 in the basal state and after 1

266 h of induction of differentiation (Fig. 2d-f). There was however no significant reduction of the *specific*

267 activity of SIK2 and SIK3 in LKB1-shRNA cells (Fig. 2e and 2f).

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

269 In the experiments described in Fig. 2, we noted that treatment of Scrbl-shRNA expressing 3T3-L1

270 preadipocytes with differentiation medium for 1h, resulted in a dephosphorylation of CRTC2 and class IIa

271 HDACs, in particular HDAC4 (Fig. 2b and 2c). This suggests that the activity of these proteins, and

272 potentially their upstream regulators, may be subject to acute regulation by adipogenic stimuli. To test if

273 this is the case, we treated 3T3-L1 preadipocytes with differentiation medium for different time points,

274 and analysed the phosphorylation/activity and localisation status of AMPK, SIK2, SIK3, CRTC2 and

275 HDAC4. Within an hour, AMPKα1 T172 phosphorylation and activity was significantly reduced by 35

276 %, while the phosphorylation on S485 was increased (Fig. 3a). The phosphorylation of SIK2 on S358 was

277 significantly induced within 10 min and was increased 25-fold within 1 h (Fig. 3b). This phosphorylation

278 coincided with a translocation of SIK2 from the nuclear to the cytosolic fraction, where it may not be able

279 to act on its downstream substrates (Fig. 3f). Notably, the kinase activity of SIK3 was significantly

280 reduced within 30 min, and this coincided with increased phosphorylation of SIK3 on pPKA consensus

281 sites (Fig. 3c). These data demonstrate that AMPK, SIK2 and SIK3 were rapidly inhibited following the

282 induction of adipocyte differentiation.

283   The inactivation of AMPK/SIK signalling coincided with dephosphorylation of CRTC2 on S275 and

284   HDAC4 on S246 (Fig. 3d and 3e), and a translocation of these proteins to the nucleus, as shown by

285   fractionation (Fig. 3f) and fluorescence confocal imaging (CRTC2, Fig. 3g). We also analysed the

286   expression and phosphorylation status of LKB1, AMPK, CRTC2 and class IIa HDACs at time points

287   through-out the whole differentiation process. As shown in Supplementary figure 3 (S3), LKB1 protein

288   expression was reduced by 50% at day 3 after differentiation, but returned to original levels at day 6.

289   Notably, the T172 phosphorylation of AMPK was markedly reduced at day 1 and day 3, as was the

290   phosphorylation of CRTC2 and class IIa HDACs (S3). These changes were mirrored by an increase in

291   S485 phosphorylation of AMPK and phosphorylation of PKA substrates, as monitored using a PKA

292   consensus motif antibody. This data demonstrate that the acute inhibition of AMPK signalling persisted at

293   least until day 3 after inducing differentiation.

294   **Mechanisms underlying the activation of CRTC2 and the inhibition of AMPK/SIK signalling by**

295   **adipogenic stimuli.**

296   Treatment with either insulin or dexamethasone had no significant effect on AMPKα1 activity, AMPK

297   T172 phosphorylation, SIK2 S358 phosphorylation, phosphorylation of SIK3 on PKA consensus sites, or

298   on the phosphorylation state of CRTC2 (Fig. 4a-d). However, upon stimulation with the cAMP-inducing

299   agent IBMX, AMPKα1 activity and T172 phosphorylation (Fig. 4a) were significantly reduced, and the

300   phosphorylation of SIK2 on S358 (Fig. 4b) was increased. Moreover, following IBMX treatment, SIK3

301   was phosphorylated on PKA consensus sites (Fig. 4c) and its activity was reduced (data not shown). This

302   coincided with a significantly reduced phosphorylation of CRTC2 on S275 (Fig. 4d). IBMX was the only

303   stimulation that mimicked the effects on AMPK, SIK2, SIK3 and CRTC2 observed when the cells were

304   treated with the complete differentiation medium. The phosphorylation of AMPK on S485 was induced

305   both by insulin and IBMX, however insulin stimulation alone did not result in inhibition of T172

306   phosphorylation or AMPKα1 activity (Fig. 4a). To confirm that the differentiation medium inhibits

307   AMPK/SIK signalling, and consequently the phosphorylation of CRTC2, through the cAMP/PKA

308   pathway, we employed the PKA inhibitor H89 (Fig. 4e-h). In the presence of H89, the phosphorylation of

13

309   CREB on S133, a known PKA site, was prevented, confirming the efficacy of the inhibitor (Fig. 4e). The

310   effects of the differentiation medium on the phosphorylation of AMPK (T172, S485), SIK2 (S358), SIK3

311   (PKA sites) and CRTC2 were all reversed in the presence of H89 in the stimulated cells (Fig. 4e-h). We

312   conclude that inhibition of AMPK/SIK signalling, and subsequent dephosphorylation of CRTC2,

313   following adipogenic differentiation of 3T3-L1 cells is most likely mediated by activation of the

314   cAMP/PKA pathway in response to IBMX.

315   **DISCUSSION**

316   In this paper we tested the hypothesis that LKB1 signalling might keep adipocyte precursors in their non-

317   mature form, and that adipogenic stimuli attenuate LKB1 signalling in order for differentiation to occur.

318   In summary, we demonstrate that three substrates downstream of LKB1 are acutely inhibited following

319   the initiation of differentiation in a PKA dependent manner, and that this coincides with

320   dephosphorylation and nuclear translocation of CRTC2 and class IIa HDACs. Moreover, silencing of

321   LKB1 resulted in a marked reduction in the mRNA and protein level of CHOP-10, increase in *Pparg*

322   expression and a facilitated differentiation into adipocytes. Our working model for the regulation of

323   adipogenesis by LKB1 is summarised in Fig. 5.

324   AMPK, SIK2 and SIK3 were all inhibited within one hour after inducing adipogenic differentiation, and

325   this inhibition coincided with dephosphorylation and nuclear translocation of CRTC2 and class IIa

326   HDACs. Results from IBMX treatment and PKA inhibition suggest that cAMP/PKA signalling mediated

327   the inhibition of AMPK/SIK signalling that we observed. Studies have shown that phosphorylation of

328   AMPK on S485 by PKA and/or PKB inversely correlates with AMPK T172 phosphorylation and kinase

329   activity (Berggreen, et al. 2009; Hurley et al. 2006), which could provide a mechanistic explanation to the

330   inhibition of AMPK during differentiation. In our experiments, we did observe an induction of S485

331   phosphorylation in response to the adipogenic cocktail. However, while treatment of cells with insulin

332   alone resulted in the phosphorylation of S485, it did not affect AMPK T172 phosphorylation or activity,

333   arguing against the hypothesis that S485 mediates AMPK inhibition during differentiation. The

334   phosphorylation of SIK2 and SIK3, as well as the cytosolic translocation of SIK2 that we observed in

335   response to IBMX and/or complete differentiation medium, is in line with our previous studies in mature

336   adipocytes, demonstrating the regulation of these kinases by cAMP/PKA on many levels, including re-

337   localisation of SIK2 to the cytosol and inhibition of SIK3 kinase activity respectively (Berggreen et al.

338   2012; Henriksson et al. 2012). Based on our previous results and the fact that PKA-phosphorylation has

339   been shown to inhibit SIK1 and SIK2 cellular function in other systems (Katoh, et al. 2004; Screaton et

340   al. 2004), we believe that the phosphorylation of SIK2 by PKA (on S358) and its cytosolic translocation

341   inhibit its action on CRTC2 and class IIa HDACs.

342   To directly address if LKB1 inhibits CRTC2 and class IIa HDACs in 3T3-L1 cells, and the ability of

343   these cells to differentiate, we generated 3T3-L1 preadipocytes with stable expression of LKB1-shRNA.

344   We confirmed that these cells displayed markedly reduced activities of LKB1 and its substrates AMPK,

345   SIK2 and SIK3, although the latter appeared to be partly due to reduced expression of these proteins. The

346   fact that a ≈ 90 % reduction in LKB1 activity did not result in a larger attenuation of the *specific* activities

347   of AMPK, SIK2 and SIK3 was not entirely unexpected. We have previously shown that in adipocytes

348   isolated from mice expressing only 10 % of LKB1 activity compared to wild type, AMPK activity was

349   reduced by 40 % and the activities of SIK2 and SIK3 were only reduced by approximately 25 %

350   (Gormand et al. 2011). This either suggests the existence of alternative upstream kinases or a large spare

351   capacity in the LKB1 signalling pathway – the latter being supported by the almost complete lack of

352   activity of SIK2 and SIK3 in LKB1 deficient cells and tissues (Al-Hakim, et al. 2005; Lizcano et al.

353   2004). The effect of LKB1 silencing on CRTC2 and HDAC4 in 3T3-L1 preadipocytes was greater than

354   any of the individual effects on AMPK, SIK2 or SIK3, suggesting that it may be the combined reduction

355   of the AMPK/SIK signalling, or inhibition of other AMPK-related kinases, that leads to reduced CRTC2

356   and HDAC4 phosphorylation. LKB1 regulates 14 kinases of the AMPK family, many of which have

357   similar substrate specificity and are likely to be expressed in 3T3-L1 fibroblasts. This complicates any

358   attempt to pinpoint if a specific substrate of LKB1 might be chiefly responsible for downstream effects,

359   or if they play redundant roles.

360   Employing two different cellular models, we show that a reduction in LKB1 expression results in an

361   increased ability of the cells to differentiate. A key underlying mechanism appears to be that LKB1 loss

362   results in higher levels of the master adipogenic transcription factor PPARγ as well as *C*/EBPα quite early

363   in the differentiation process. The phenotype was stronger in the LKB1$^{-/-}$ MEFs, which is in line with the

364   complete absence of LKB1, and dramatic reduction of AMPK, SIK2 and SIK3 activities (data not shown

365   and (Lizcano et al. 2004)), and a barely detectable level of CRTC2 phosphorylation in these cells (data

366   not shown). The fact that *Pparg* and *Cebpa* were upregulated early during differentiation and that the

367   number of adipocytes was increased, suggest that the phenotype we observed is due to LKB1-regulation

368   of transcriptional events controlling the actual differentiation program, rather than directly affecting lipid

369   accumulation and/or the expression of adipocyte specific proteins. Zhang et al demonstrated that deletion

370   of LKB1 in mouse adipose tissue, employing *Fabp4*-mediated Cre recombinase expression, resulted in

371   *reduced* amount of white adipose tissue and expression of adipogenic genes (Zhang, et al. 2013). Since

372   *Fabp4*/aP2 is only expressed in the later stages of adipocyte differentiation, this experimental model in

373   fact did not address the role of LKB1 in differentiating preadipocytes (like ours). In support of our present

374   findings, another upstream kinase of AMPK, CaMKK2, has also been shown to inhibit adipocyte

375   differentiation and adipogenic gene transcription, as shown in CaMKK2-null MEFs and 3T3-L1

376   preadipocytes treated with CaMKK shRNA and CaMKK inhibitors (Lin, et al. 2011).

377   Our original hypothesis was that C/EBPs might be up-regulated in LKB1-silenced cells, due to activation

378   of CRTC2/CREB. The lack of induction of C/EBPβ mRNA or protein expression in LKB1-shRNA

379   expressing cells does not support this notion. In LKB1$^{-/-}$ MEFs we observed that C/EBPβ mRNA levels

380   tended to be increased before the initiation of adipogenesis, but the difference was not statistically

381   significant in the 2-way Anova and did not persist after the addition of differentiation medium, speaking

382   against altered C/EBPβ levels as a primary mechanism mediating the phenotype. However, we cannot

383   rule out the possibility that the higher expression of C/EBPδ, another potential CREB target gene, that we

384   observed at one time point contributes to the induction of PPARγ and C/EBPα in the LKB1-shRNA 3T3-

385   L1 cells (Belmonte et al. 2001; Reusch et al. 2000; Rosen and MacDougald 2006). In search for

386 additional mechanisms underlying the up-regulation of *Pparg* and *Cebpa*, we analysed the dominant-

387 negative C/EBP family member CHOP-10, which inhibits C/EBPβ by sequestration/inactivation (Huang

388 et al. 2005; Tang and Lane 2000). Indeed, the mRNA and protein level of CHOP-10 was markedly

389 reduced in LKB1-shRNA expressing cells, potentially allowing for more C/EBPβ to bind to DNA and

390 stimulate gene expression. At present, we do not know how LKB1 regulates CHOP-10 expression, and

391 this will be of future interest to address. We find it interesting that CRTC2 and HDAC4 were

392 dephosphorylated as a result of LKB1 silencing as well as adipogenic stimulation, but our data do not

393 exclude the involvement of other substrates of the AMPK family of kinases.

394 In summary, this study demonstrates that LKB1 silencing in 3T3-L1 preadipocytes promotes the

395 dephosphorylation of HDAC4 and CRTC2, induces the expression of *Pparg* and *Cebpa*, and facilitates

396 adipocyte differentiation. We also demonstrate that the AMPK/SIK signalling is inhibited following the

397 initiation of differentiation, and hypothesise that in the absence of adipogenic stimuli, LKB1/AMPK/SIK

398 signalling serves to keep preadipocytes in their non-differentiated form.

399 **DECLARATION OF INTEREST**

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555   **FIGURE LEGENDS**

556   *Figure 1: LKB1-silenced 3T3-L1 preadipocytes and LKB1 null MEFs display an increased ability to*

557   *differentiate into adipocytes.*

558   (**a**) 3T3-L1 preadipocytes transduced with scrambled-shRNA (Scrbl-shRNA) or LKB1-shRNA (LKB1-

559   shRNA), as well as wild-type (Wt) or LKB1-deficient (LKB1$^{-/-}$) MEFs were subjected to a differentiation

560   protocol for up to 10 days. The ability of these cells to differentiate into adipocytes was evaluated by

561   quantifying the number of differentiated cells (Nile red stained cells) over the total number of cells

562   (Hoechst stained cells) or the total amount of Nile red staining (LKB1$^{-/-}$ MEFs) in fluorescent microscopy

563   images, and (**b**) by a biochemical measurement of triglyceride (TG) accumulation. (**c**) The mRNA level

564   of the preadipocyte marker Pref1 was measured by qPCR in Scrbl-shRNA or LKB1-shRNA expressing

565   3T3-L1 preadipocytes. (**d**) Cell lysates from Scrbl-shRNA or LKB1-shRNA expressing 3T3-L1

566   preadipocytes were analysed by western blot for protein expression of FAS and HSL at 10 days after

567   induction of differentiation. (**e-g**) The mRNA level measured by qPCR of the adipogenic transcription

568   factors PPARγ and C/EBPα (**e**), C/EBPδ and C/EBPβ (**f**), and the dominant negative C/EBP family

569   member CHOP-10 (**g**) in the LKB1-shRNA cells was compared to those in the Scrbl-shRNA cells after

570   induction of differentiation at the time points indicated on the figures. The protein amount of CHOP-10

571   was measured by western blot in cell lysates from Scrbl- or LKB1-shRNA expressing 3T3-L1

572   preadipocytes after induction of differentiation at the time points indicated on the figures. The blot,

573   showing one experiment, is representative of three independent experiments. The data represent the mean

574   +SEM of three independent experiments, each in which the data was expressed as fold- or % of a control

575   condition, and the means were considered significantly different when *p<0.05, **p<0.01, ***p<0.001

576   and ****p<0.0001 (unpaired t-test in 1a and two-way ANOVA with multiple comparisons in b-g). Time

577   point 0 corresponds to the day when the differentiation medium was added (2 days post-confluent).

578   *Figure 2: The silencing of LKB1 in 3T3-L1 preadipocytes results in a reduced phosphorylation of*

579   *CRTC2 and class IIa HDACs.*

580   (**a**) 3T3-L1 preadipocytes were transduced with scrambled- (Scrbl-shRNA) and LKB1-shRNA (LKB1-

581   shRNA). The silencing of LKB1 was confirmed at the mRNA level by qPCR and at the protein level by

582   kinase activity assay and western blot. (**b-f**) Adipogenic differentiation was induced for 1h on 2 day post-

583   confluent preadipocytes and cell homogenates were analysed for phosphorylation and/or kinase activity of

584   CRTC2 (**b**), class IIa HDACs (**c**), AMPK (**d**), SIK2 (**e**) and SIK3 (**f**). The data represent the mean +SEM

585   of three independent experiments, each in which the data was expressed as fold- or % of a control

586   condition, and the means were considered significantly different when *p<0.05, **p<0.01 and

587   ***p<0.001 (unpaired t-test in 2a and Two-way ANOVA with multiple comparisons in b-f). A

588   representative blot displaying technical duplicates from one experiment is shown.

589   *Figure 3: Acute regulation of AMPK/SIK/CRTC2/HDAC4 by adipogenic differentiation.*

590   3T3-L1 preadipocytes were treated with differentiation medium for up to 1 h. Cell homogenates were

591   collected at different time points as indicated in the figures and analysed for phosphorylation state and/or

592   in vitro kinase activity of AMPK (**a**), SIK2 (**b**), SIK3 (**c**), CRTC2 (**d**) and HDAC4 (**e**). Results are

593   presented as the mean +SEM of three independent experiments, each in which the data was expressed as

594   % of a control condition, and the means were considered significantly different from time = 0 when

595   *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001 (unpaired student t test). A representative blot

596   displaying technical duplicates from one experiment is shown. Subcellular localisation of SIK2, CRTC2

597   and HDAC 4, 5, 7 (**f**) was analysed by western blot after performing subcellular fractionation of cell

598   homogenates. The purity of the fractions was monitored by the presence of GAPDH and histone 3 (H3).

599   Results are presented as the mean +SEM of three independent experiments (made in triplicates), each in

600   which the data was expressed as % of a control condition, and the means were considered significantly

601   different when *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001 (unpaired student t-test). A

602   representative blot displaying technical triplicates from one experiment is shown. The re-localisation of

603   CRTC2 to the nucleus was also analysed by immunocytochemistry and confocal fluorescence microscopy

604   (**g**).

605   *Figure 4: Effects of the differentiation medium on the AMPK/SIK/CRTC2 signalling pathway is*

606   *mediated by cAMP/PKA.*

607   3T3-L1 preadipocytes were treated with 10 μg/ml insulin, 0.5 mM IBMX, 1 μM dexamethasone (Dex) or

608   complete differentiation medium (Diff Medium) for 30 min (SIK3) or 1 h (AMPK, SIK2 and CRTC2).

609   Cell homogenates were analysed for phosphorylation state and/or in vitro kinase activity of AMPK (**a**),

610   SIK2 (**b**), SIK3 (**c**) and CRTC2 (**d**). The data represent the mean +SEM of three independent experiments

611   (made in duplicates), each in which the data was expressed as % of a control condition, and the means

612   were considered significantly different from the basal state when *p<0.05, **p<0.01 and ****p<0.0001

613   (unpaired student t-test). A representative blot displaying technical duplicates from one experiment is

614   shown. 3T3-L1 preadipocytes were treated with (H89) or without (DMSO) 100 μM H89 for 30 min

615   before adding the adipogenic cocktail (10 μg/ml insulin, 0.5 mM IBMX, 1 μM dexamethasone; +Diff

616   Medium) or not (Normal Medium) for 1 h. Cell homogenates were analysed for phosphorylation state of

617   CREB and AMPK (**e**), SIK2 (**f**), SIK3 (**g**) and CRTC2 (**h**). The figures represent the mean +SEM of three

618   independent experiments (performed in duplicates), each in which the data was expressed as % of a

619   control condition. A representative blot displaying technical duplicates from one experiment is shown.

620   *Figure 5: Model for the regulation of adipogenesis by the LKB1/AMPK/SIK signalling pathway.*

621   (**a**) Before the addition of differentiation cocktail to preadipocytes, the activity of the LKB1 signalling

622   pathway, through AMPK, SIKs and other putative kinases, maintains a certain level of phosphorylation of

623   transcriptional co-regulators such as CRTC2 and class IIa HDACs, which prevents their translocation to

624   the nucleus, where they would otherwise contribute to the regulation of certain genes. LKB1/AMPK/SIK

625   may also prevent adipogenesis through other as yet unknown targets. (**b**) Upon addition of the

626   differentiation cocktail, LKB1 substrates are inhibited in response to increased levels of cAMP, induced

627   for example by the phosphodiesterase inhibitor IBMX. CRTC2 and the class IIa HDACs are

628   dephosphorylated and can translocate to the nucleus, where they affect gene expression that regulates

629   adipogenesis, for example CHOP-10, C/EBPδ, C/EBPα and PPARγ. CHOP-10 can regulate C/EBPβ

630   activity without affecting C/EBPβ expression levels. Several of the effects induced by adipogenic

631   medium is mimicked by LKB1 silencing.

632   *Supplementary figure 1 (S1): Expression of adipogenic and lipogenic markers during the*

633   *differentiation of wild type (Wt) and LKB1^{-/-} MEFs.*

634   The ability of LKB1-deficient MEFs to differentiate into adipocytes (day 9) compared to Wt MEFs, was

635   evaluated by fluorescent microscopy imaging where lipids were stained with Nile Red, and by

636   quantifying the amount of adipocyte proteins FAS and HSL by western blot. Western blot quantifications

637   are presented as the mean +SEM of three independent experiments, each in which the data was expressed

638   as % of a control condition, and the means were considered significantly different when *p<0.05 (Two-

639   way ANOVA with multiple comparisons). mRNA levels of PPARγ, C/EBPα, C/EBPβ, aP2, Glut4 and

640   adiponectin during differentiation of wild type (Wt) and LKB1^{-/-} MEFs were measured by qPCR. Time 0

641   corresponds to the day when the differentiation medium was added (2 days post-confluent). Results are

642   presented as the mean +SEM of three experiments, each in which the data was expressed as fold- of a

643   control condition, and the means were considered significantly different when *p<0.05 and ***p<0.01

644   (Two-way ANOVA with multiple comparisons).

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

646   *expressing 3T3L1 preadipocytes and adipocytes.*

647   mRNA levels of PPARγ, C/EBPα, Glut4, FAS, aP2, adiponectin, mir-103 and miR-107 during the whole

648   differentiation process of 3T3-L1 cells (expressing Scrbl- and LKB1-shRNA). Time 0 corresponds to the

649   day when the differentiation medium was added (2 days post-confluent). Results are presented as the

650   mean +SEM of one representative experiment (made in duplicate), in which the data was expressed as

651   fold- of a control condition, and the means were considered significantly different when *p<0.05,

652   **p<0.01, ***p<0.001 and ****p<0.0001 (Two-way ANOVA with multiple comparisons). Clonal

653   expansion before and after adding the differentiation cocktail was measured by counting cells in a Bürker

654   chamber. The data is expressed as the fold change compared to day -2, which is the day when the cells

655   reach 100% of confluency, and represent the mean +SEM of 3 independent experiments.

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

657   *Cells.*

658   The protein expression and phosphorylation state of LKB1, AMPK, PKA substrates, CRTC2 and class IIa

659   HDACs was measured in cell homogenates by western blot. The data represent the mean +SEM of three

660   independent experiments (made in duplicates), each in which the data was expressed as % of a control

661   condition. The means were considered significantly different from the basal state when **p<0.01 and

662   ***p<0.001 (unpaired student t-test). A representative blot displaying technical duplicates from one

663   experiment is shown.
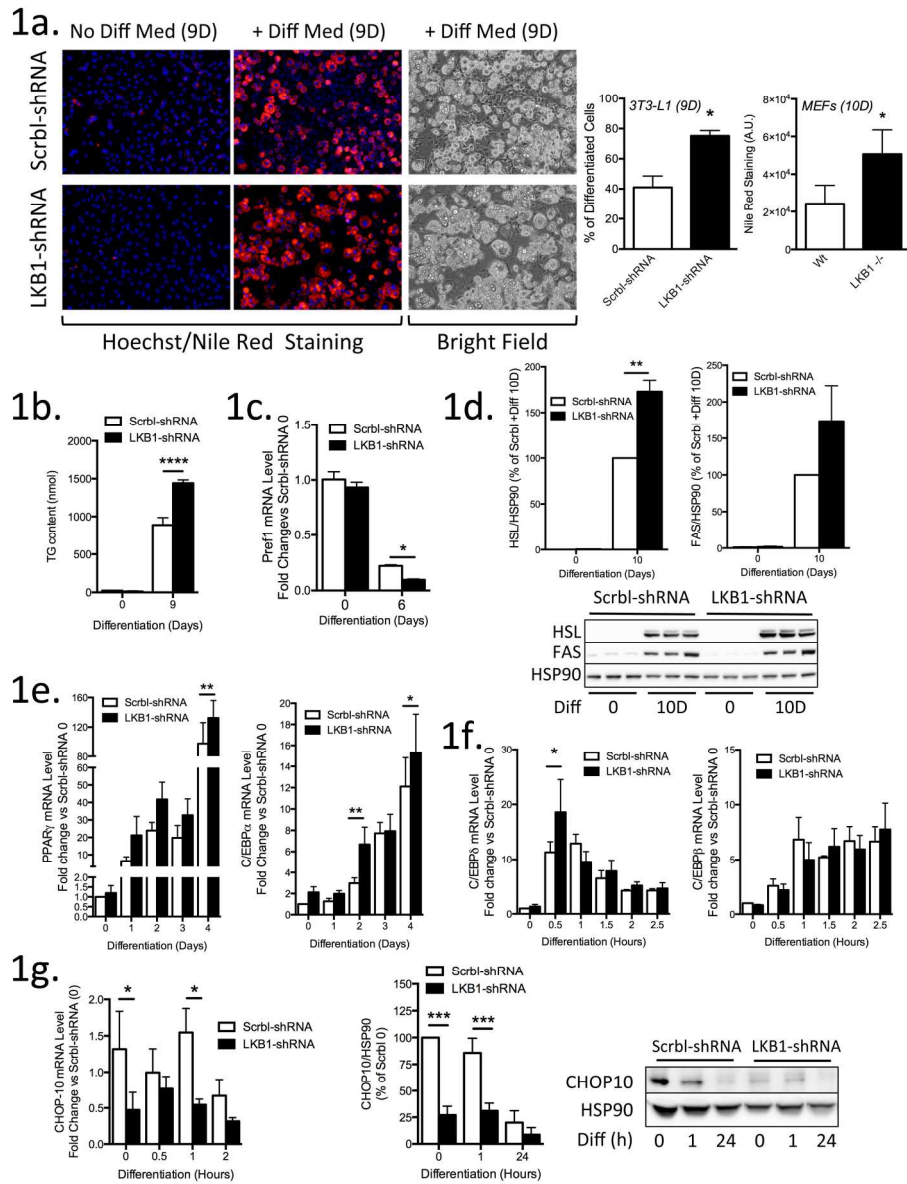
LKB1-silenced 3T3-L1 preadipocytes and LKB1 null MEFs display an increased ability to differentiate into adipocytes.
(a) 3T3-L1 preadipocytes transduced with scrambled-shRNA (Scrbl-shRNA) or LKB1-shRNA (LKB1-shRNA), as well as wild-type (Wt) or LKB1-deficient (LKB1-/-) MEFs were subjected to a differentiation 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).

188x245mm (300 x 300 DPI)
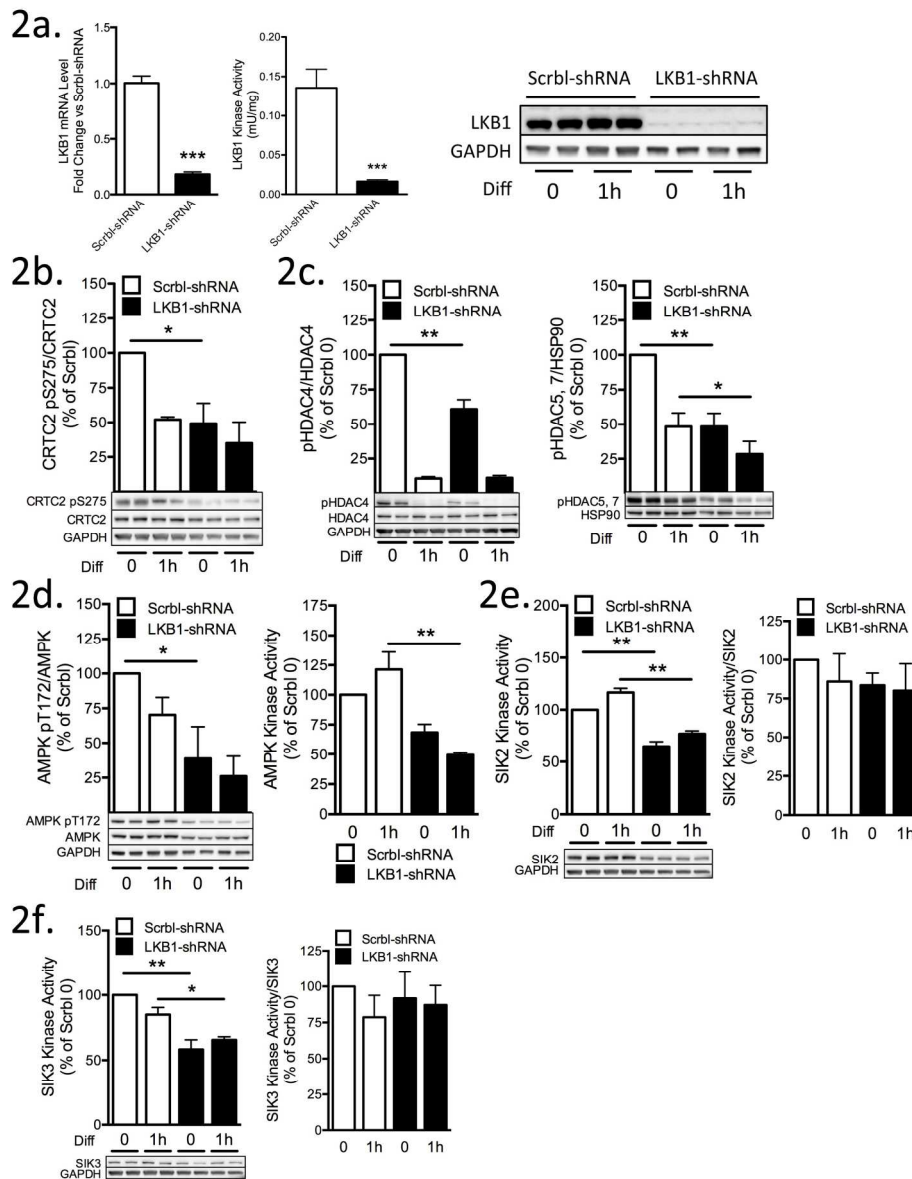
The silencing of LKB1 in 3T3-L1 preadipocytes results in a reduced phosphorylation of CRTC2 and class IIa HDACs.
(a) 3T3-L1 preadipocytes were transduced with scrambled- (Scrbl-shRNA) and LKB1-shRNA (LKB1-shRNA). The silencing of LKB1 was confirmed at the mRNA level by qPCR and at the protein level by 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.

185x239mm (300 x 300 DPI)
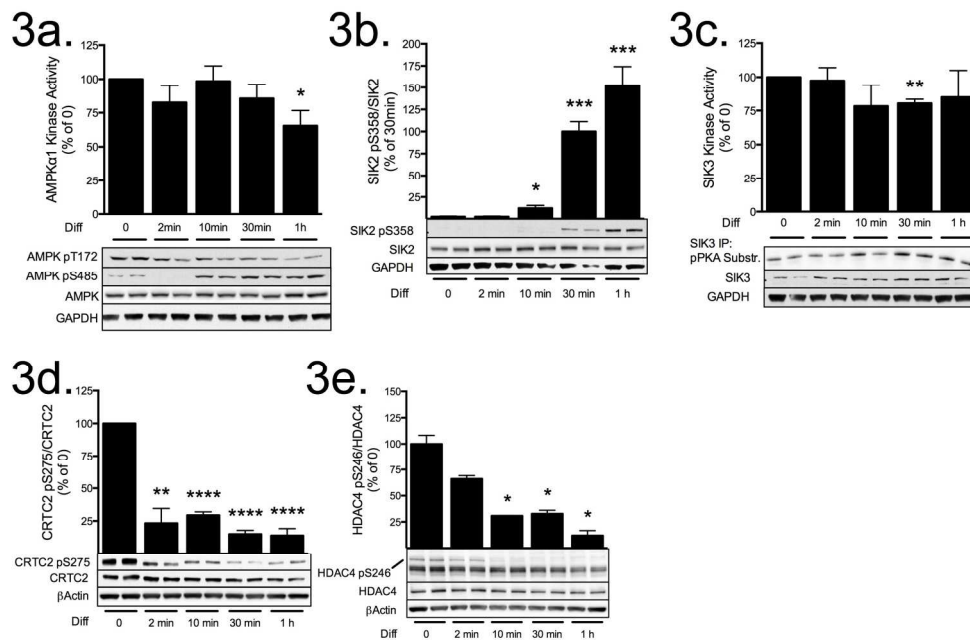
Acute regulation of AMPK/SIK/CRTC2/HDAC4 by adipogenic differentiation.
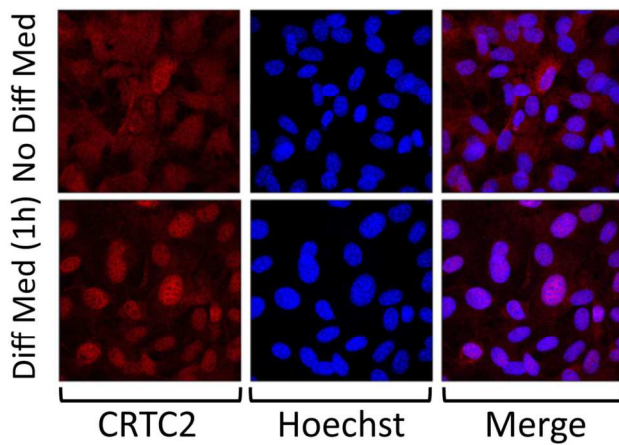3T3-L1 preadipocytes were treated with differentiation medium for up to 1 h. Cell homogenates were collected at different time points as indicated in the figures and analysed for phosphorylation state and/or in vitro kinase activity of AMPK (a), SIK2 (b), SIK3 (c), CRTC2 (d) and HDAC4 (e). Results are 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).
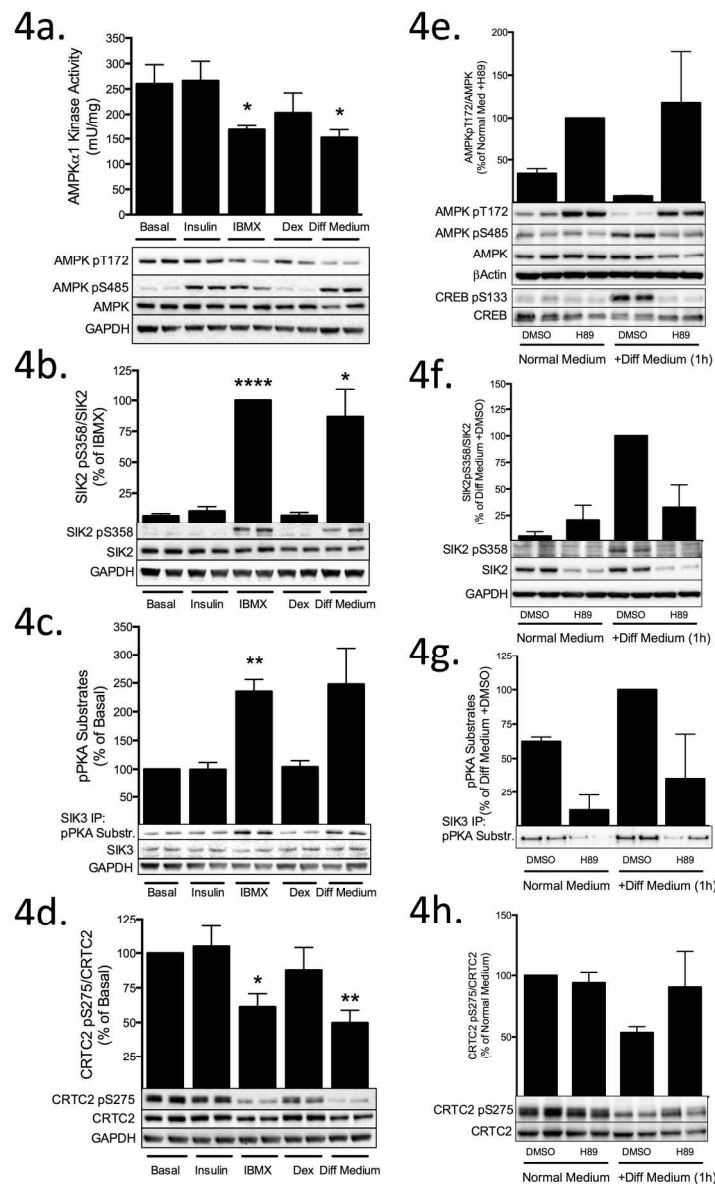
182x119mm (300 x 300 DPI)

3f.
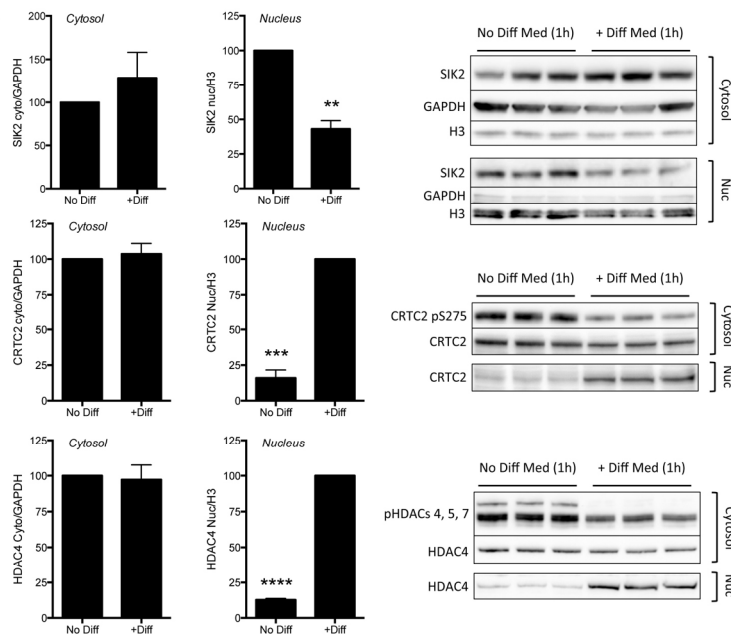
3g.

135x200mm (300 x 300 DPI)

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.

143x238mm (300 x 300 DPI)
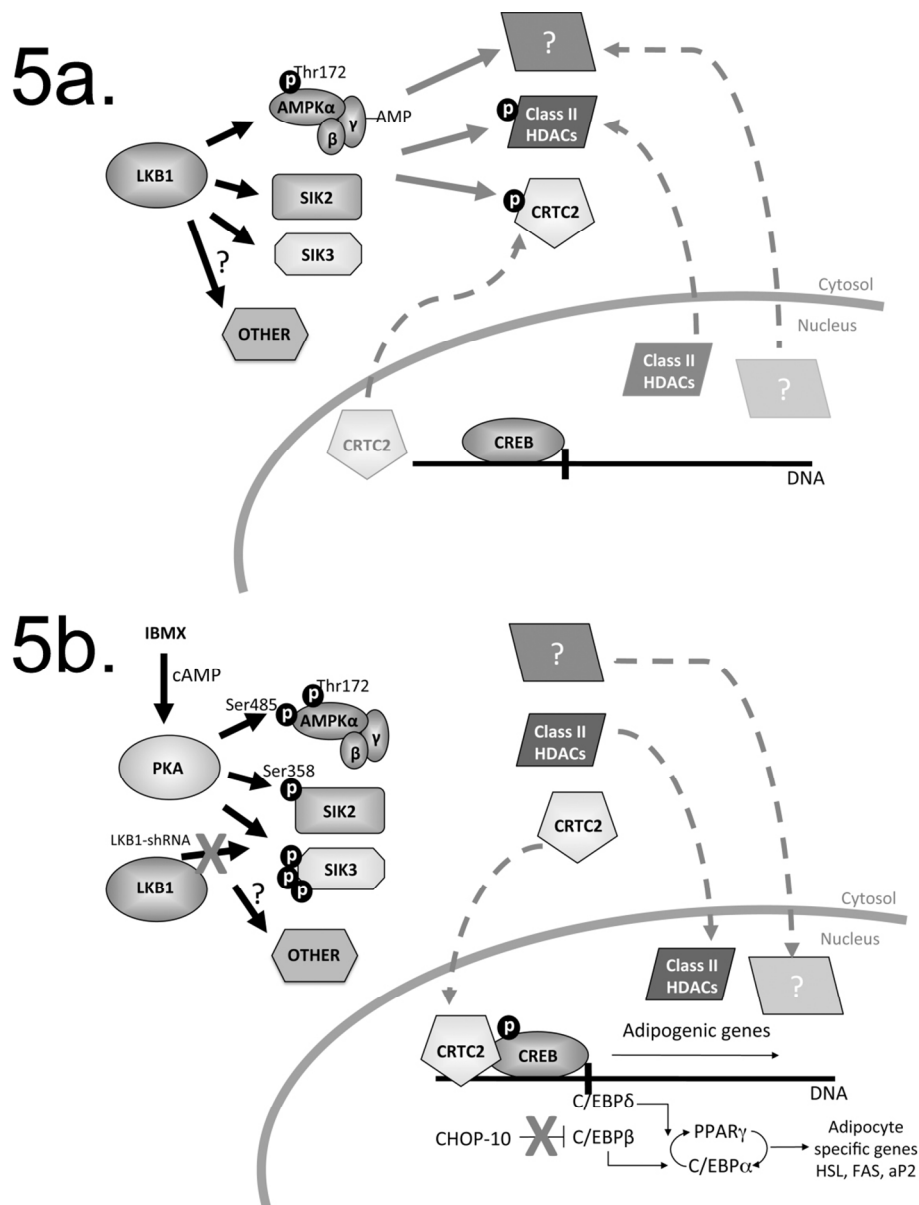
Model for the regulation of adipogenesis by the LKB1/AMPK/SIK signalling pathway.
(a) Before the addition of differentiation cocktail to preadipocytes, the activity of the LKB1 signalling pathway, through AMPK, SIKs and other putative kinases, maintains a certain level of phosphorylation of transcriptional 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.

100x128mm (300 x 300 DPI)
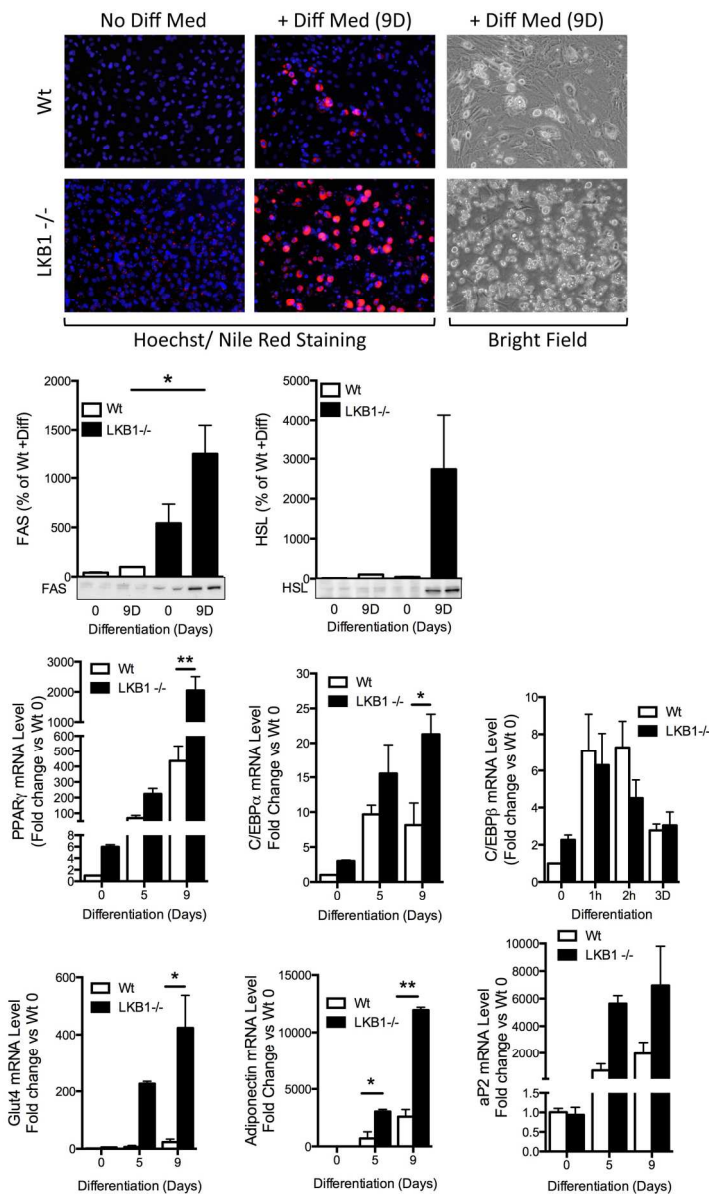
Expression of adipogenic and lipogenic markers during the differentiation of wild type (Wt) and LKB1-/- MEFs.

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).

135x227mm (300 x 300 DPI)
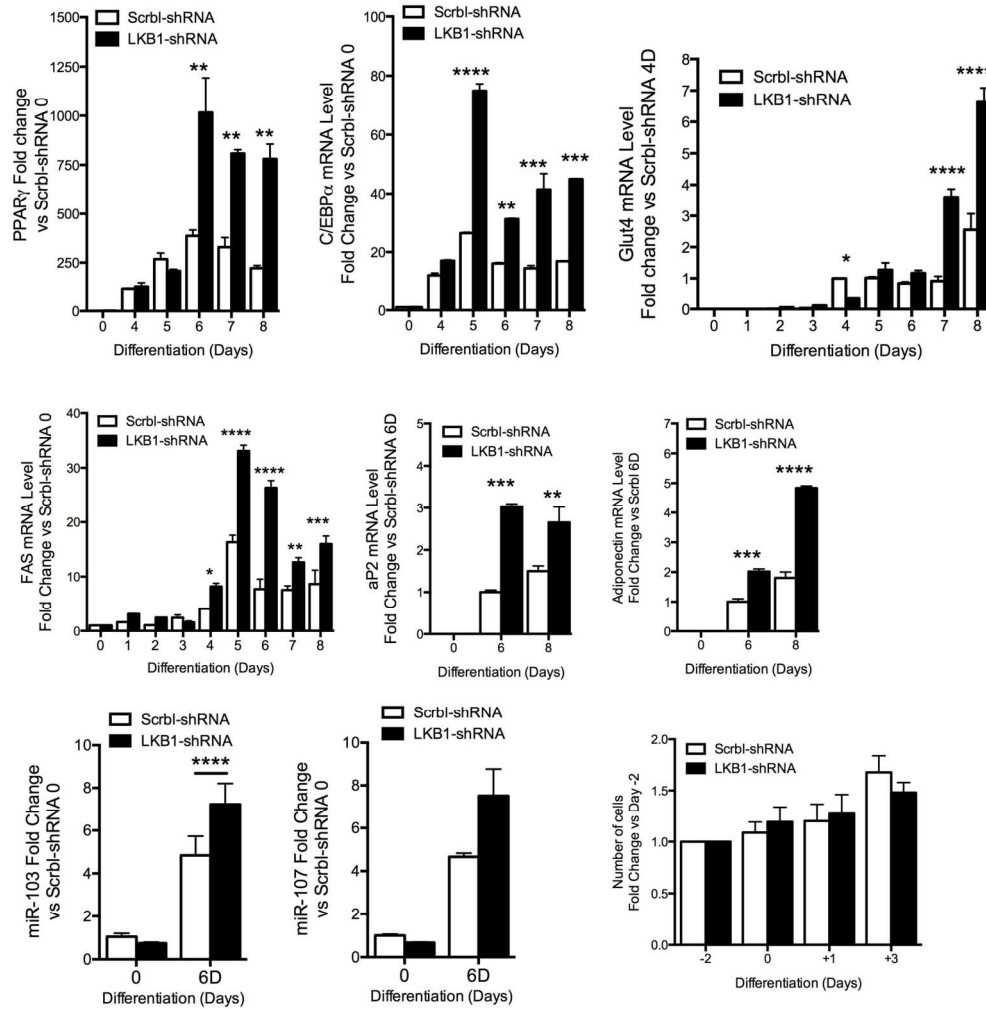
Expression of adipogenic markers in Scrbl-shRNA and LKB1-shRNA expressing 3T3L1 preadipocytes and adipocytes.

mRNA levels of PPARγ, C/EBPα, 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.

154x157mm (300 x 300 DPI)
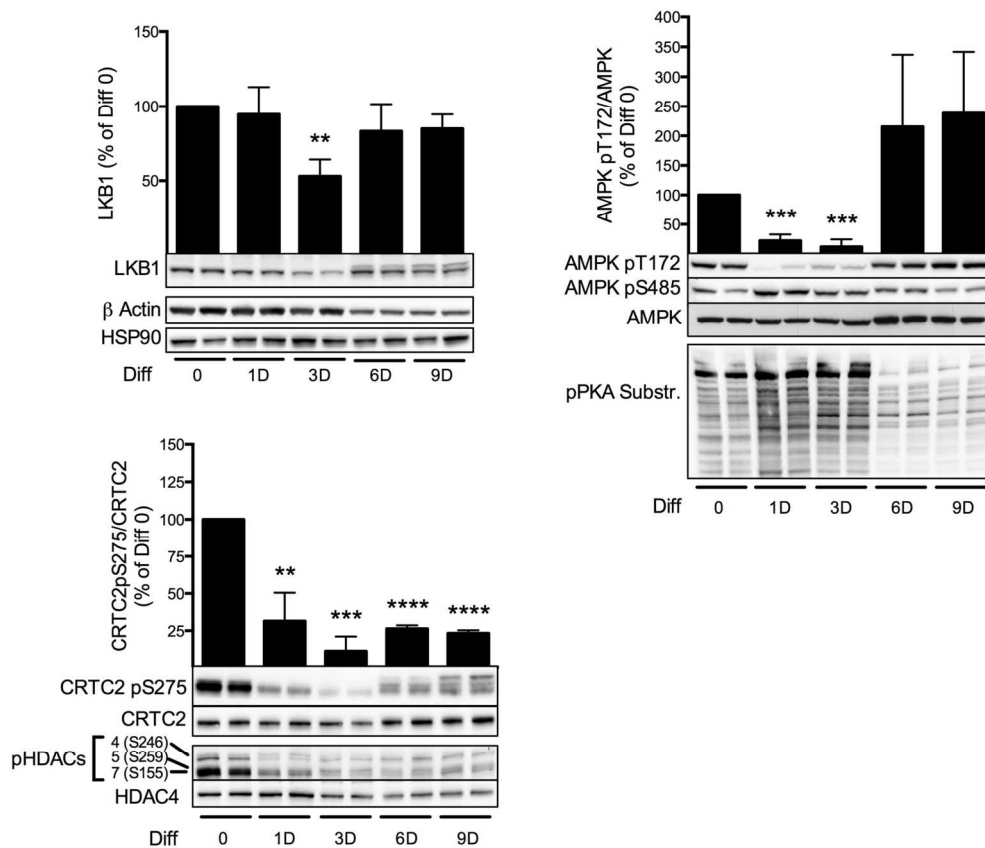
Expression of the LKB1 signalling during the differentiation of 3T3-L1 Cells.
The protein expression and phosphorylation state of LKB1, AMPK, PKA substrates, CRTC2 and class IIa HDACs was measured in cell homogenates by western blot. The data represent the mean +SEM of three independent experiments (made 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 ***p<0.001 (unpaired student t-test). A representative blot displaying technical duplicates from one experiment is shown.

149x128mm (300 x 300 DPI)