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

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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/EBP α , peroxisome proliferator-activated receptor γ (PPAR γ) 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/EBP β and C/EBP δ) are induced within hours following induction of differentiation. C/EBP β and
38 C/EBP δ then induce the expression of peroxisome proliferator-activated receptor γ (PPAR γ) and
39 CCAAT/enhancer-binding protein α (C/EBP α) (Christy, et al. 1991; Clarke, et al. 1997; Wu, et al. 1995;
40 Yeh, et al. 1995). Once expressed, PPAR γ and C/EBP α 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/EBP β and potentially C/EBP δ (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 (CRTC) (Conkright,
49 et al. 2003; Ravnskjaer, et al. 2007; Sreaton, 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 (Sreaton 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; Sreaton 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 ³²Pγ-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 Sreaton, 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-AMPK α 1 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 % CO₂. Differentiation was induced on two-day post-
120 confluent cells by incubating them in growth medium supplemented with 0.5 mM IBMX, 5 μ g/ml insulin,
121 1 μ M dexamethasone and 0.1 μ M 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 QIAzolTM 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- Δ U6min (Amar, et al. 2006) between *BbsI* and *BamHI* restriction sites, generating the plasmid
133 pcDNA- Δ U6-shLKB1.
134 The expression cassette allowing effective LKB1-shRNA under the control of the U6 promoter was
135 excised from pcDNA- Δ U6min-shLKB1 and inserted into the lentivector precursor plasmid pFlap-PGK-
136 GFP-WPRE (Desclaux et al. 2009) using *MluI* and *BamHI* sites. As control, a lentiviral vector expressing
137 a "scrambled" shRNA (Scrb1-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 transducing 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 Scrb1-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 μ g/ml Nile Red in the medium for 15 min and nuclei were
149 stained with 10 μ g/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 μ l 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 μ M 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 ChemiDocTM XRS+ and
176 the Image LabTM 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 μ g) 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 CER I containing complete protease inhibitors/10 cm dish.
189 Homogenates were vortexed and incubated for 15 min on ice. 11 µl of ice-cold CER II 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 $\approx 75\%$ of LKB1-shRNA expressing 3T3-L1 cells were able to
219 differentiate into mature and lipid-storing adipocytes, while only $\approx 41\%$ 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 PPAR γ 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/EBP β (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/EBP β activity, and thus PPAR γ and C/EBP α expression,
244 may be increased in the LKB1-shRNA expressing cells, without an induction of C/EBP β 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 CRT2 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 CRT2 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 CRT2 and class IIa HDACs on sites
257 previously reported to control their activity (Mihaylova, et al. 2011; Sreaton 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 CRT2 and class II HDACs (Mihaylova et al. 2011; Muraoka et al. 2009; Srean 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).

268 **Acute regulation of AMPK, SIKs, CRT2 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 CRT2 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, CRT2 and
275 HDAC4. Within an hour, AMPK α 1 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 AMPK α 1 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, AMPK α 1 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 AMPK α 1 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

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; Sreaton 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 $\approx 90\%$ 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 PPAR γ as well as C/EBP α 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/EBP β mRNA or protein expression in LKB1-shRNA
379 expressing cells does not support this notion. In LKB1^{-/-} MEFs we observed that C/EBP β 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/EBP β levels as a primary mechanism mediating the phenotype. However, we cannot
383 rule out the possibility that the higher expression of C/EBP δ , another potential CREB target gene, that we
384 observed at one time point contributes to the induction of PPAR γ and C/EBP α 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/EBP β 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/EBP β 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**

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

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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 (Scrb1-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 Scrb1-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 PPAR γ and C/EBP α **(e)**, C/EBP δ and C/EBP β **(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 $\mu\text{g/ml}$ 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 $\mu\text{g/ml}$ 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/EBP δ , C/EBP α and PPAR γ . CHOP-10 can regulate C/EBP β
630 activity without affecting C/EBP β 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 PPAR γ , C/EBP α , C/EBP β , 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

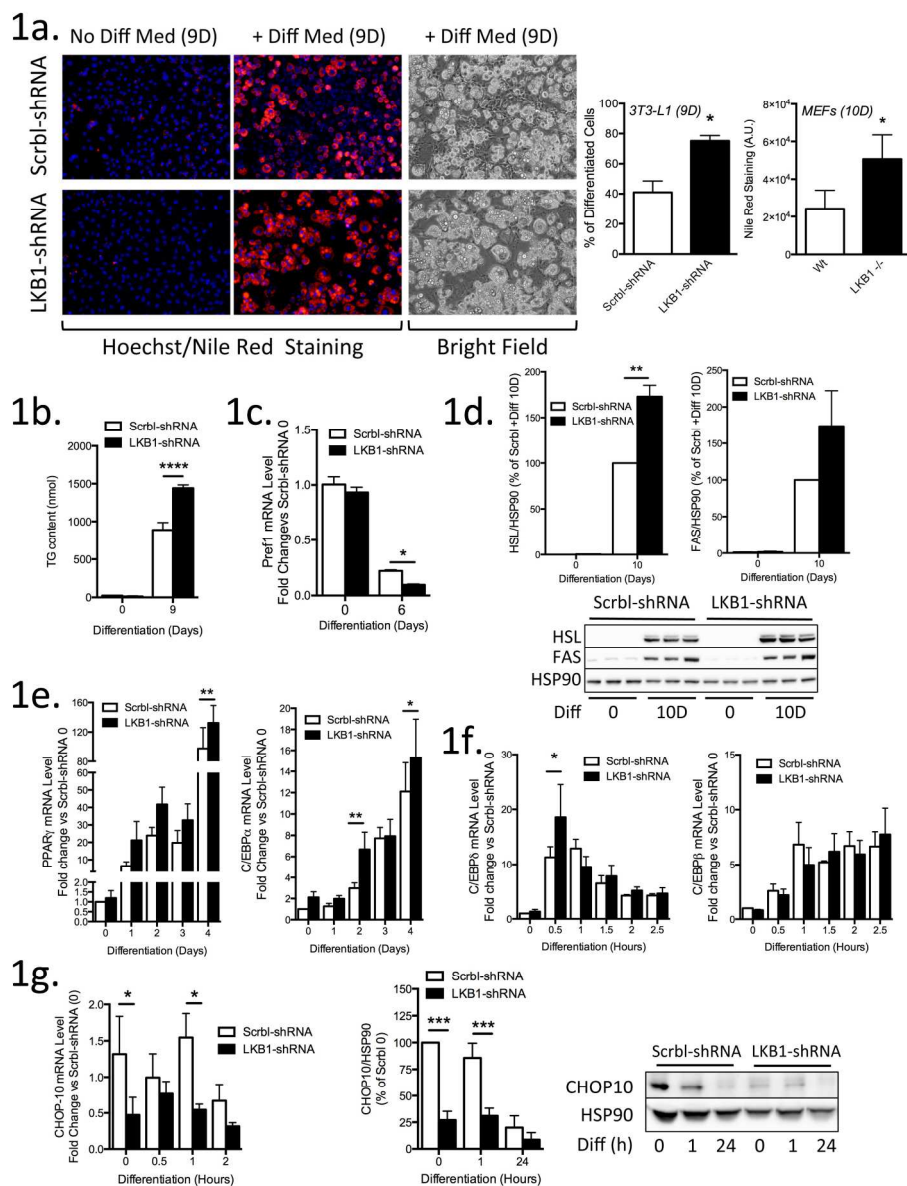
642 presented as the mean +SEM of three experiments, each in which the data was expressed as fold- of a
643 control condition, and the means were considered significantly different when * $p < 0.05$ and *** $p < 0.01$
644 (Two-way ANOVA with multiple comparisons).

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 PPAR γ , C/EBP α , 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 (Scrb1-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 Scrb1-shRNA or LKB1-shRNA expressing 3T3-L1 preadipocytes. (d) Cell lysates from Scrb1-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 PPAR γ and C/EBP α (e), C/EBP δ and C/EBP β (f), and the dominant negative C/EBP family member CHOP-10 (g) in the LKB1-shRNA cells was compared to

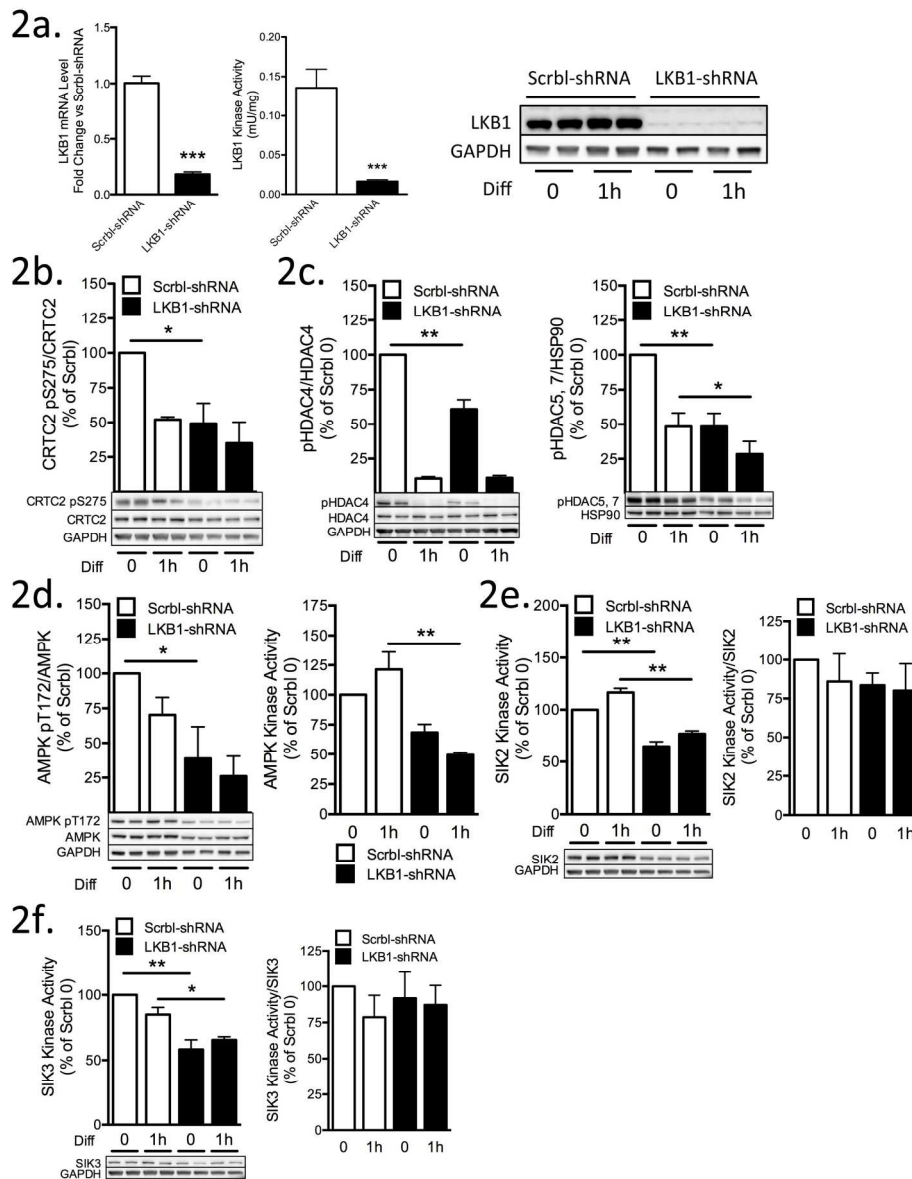
those in the Scrbl-shRNA cells after induction of differentiation at the time points indicated on the figures.

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 \pm 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).

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For Review Only

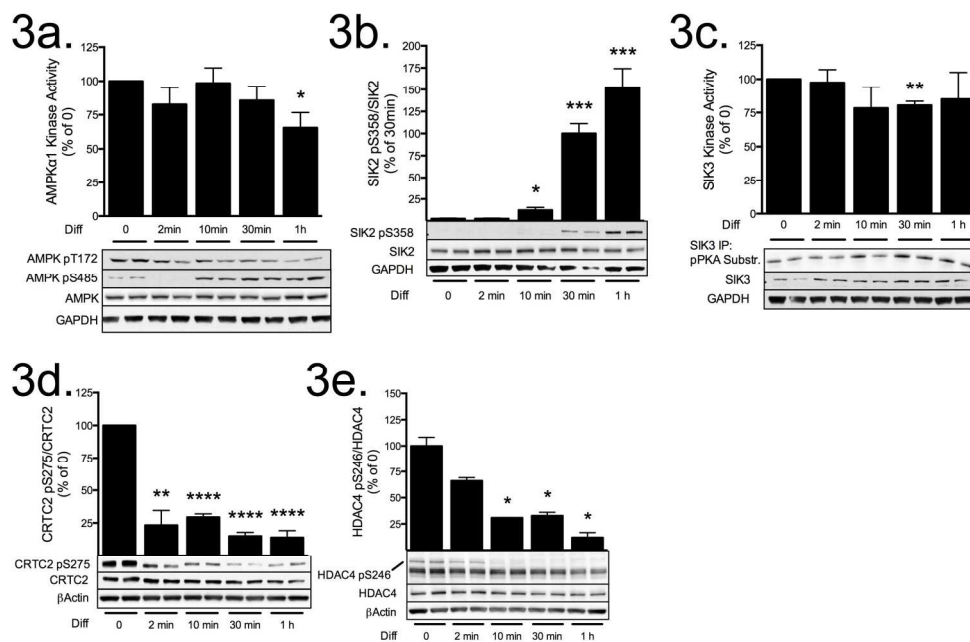


The silencing of LKB1 in 3T3-L1 preadipocytes results in a reduced phosphorylation of CRTC2 and class IIa HDACs.

(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.

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For Review Only

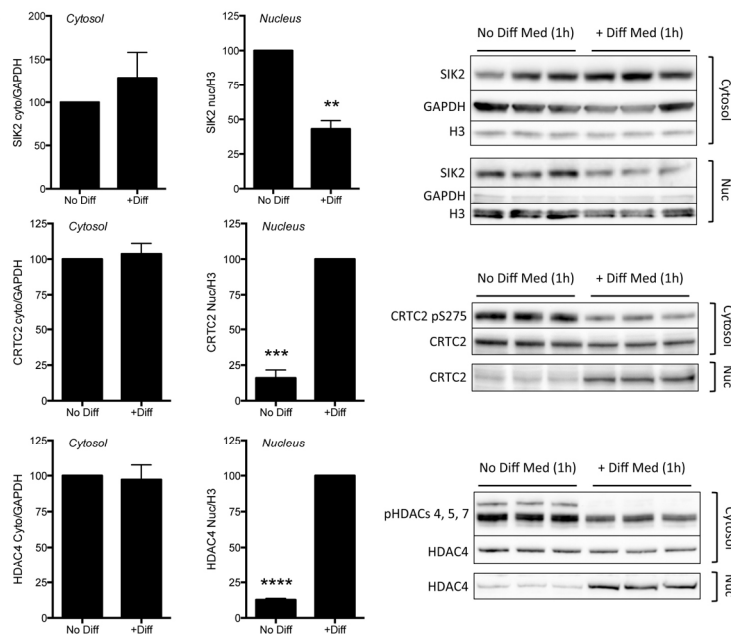


Acute regulation of AMPK/SIK/CRTC2/HDAC4 by adipogenic differentiation.

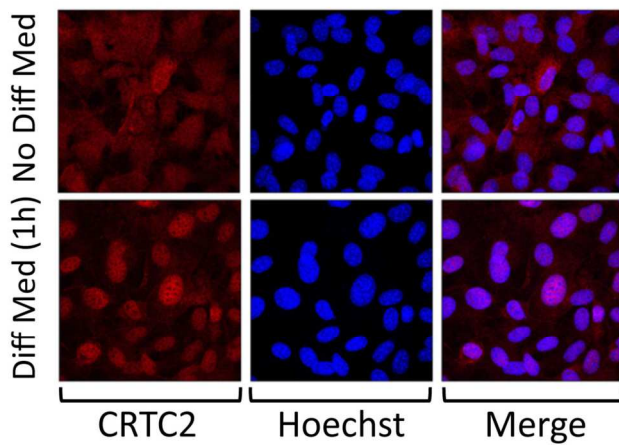
3T3-L1 preadipocytes were treated with differentiation medium for up to 1 h. Cell homogenates were collected at different time points as indicated in the figures and analysed for phosphorylation state and/or in vitro kinase activity of AMPK (a), SIK2 (b), SIK3 (c), CRTC2 (d) and HDAC4 (e). Results are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

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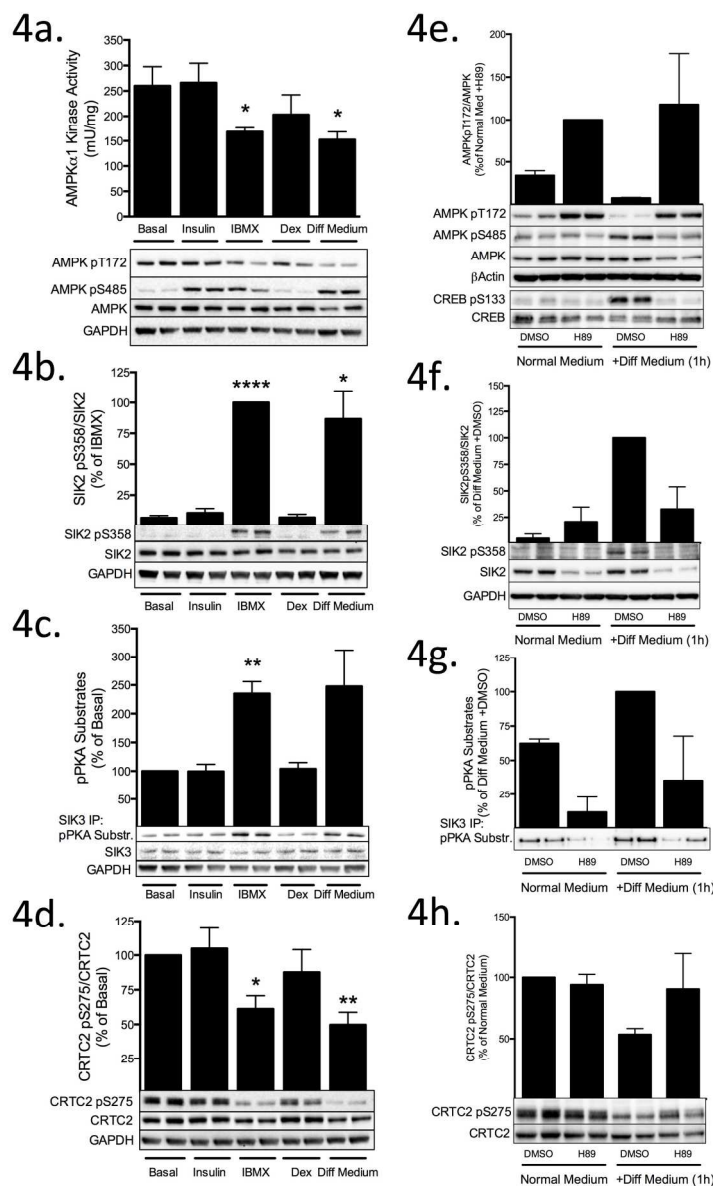
3f.



3g.



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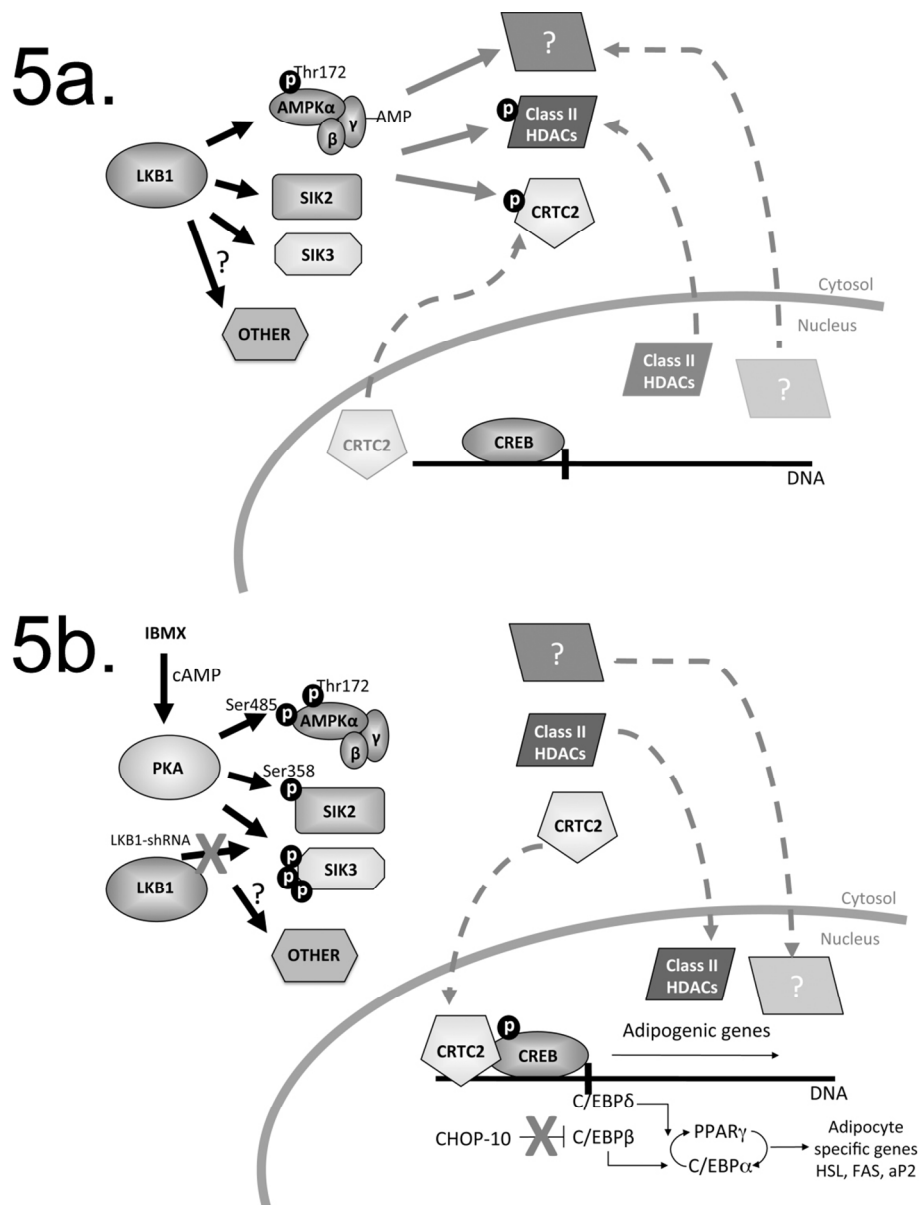


Effects of the differentiation medium on the AMPK/SIK/CRTC2 signalling pathway is mediated by cAMP/PKA. 3T3-L1 preadipocytes were treated with 10 μ g/ml insulin, 0.5 mM IBMX, 1 μ M dexamethasone (Dex) or complete differentiation medium (Diff Medium) for 30 min (SIK3) or 1 h (AMPK, SIK2 and CRTC2). Cell 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 \pm 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 μ M H89 for 30 min before adding the adipogenic cocktail (10 μ g/ml insulin, 0.5 mM IBMX, 1 μ M 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 \pm 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.

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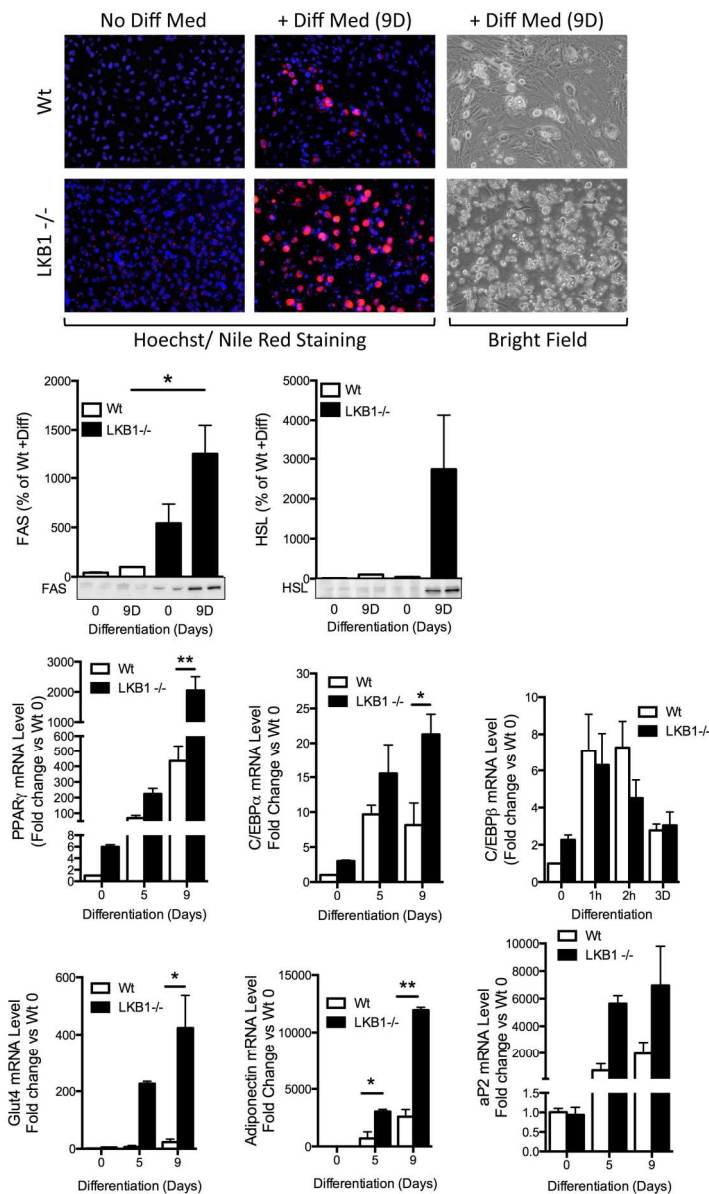


Model for the regulation of adipogenesis by the LKB1/AMPK/SIK signalling pathway.

(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/EBP δ , C/EBP α and PPAR γ . CHOP-10 can regulate C/EBP β activity without affecting C/EBP β expression levels. Several of the effects induced by adipogenic medium is mimicked by LKB1 silencing.

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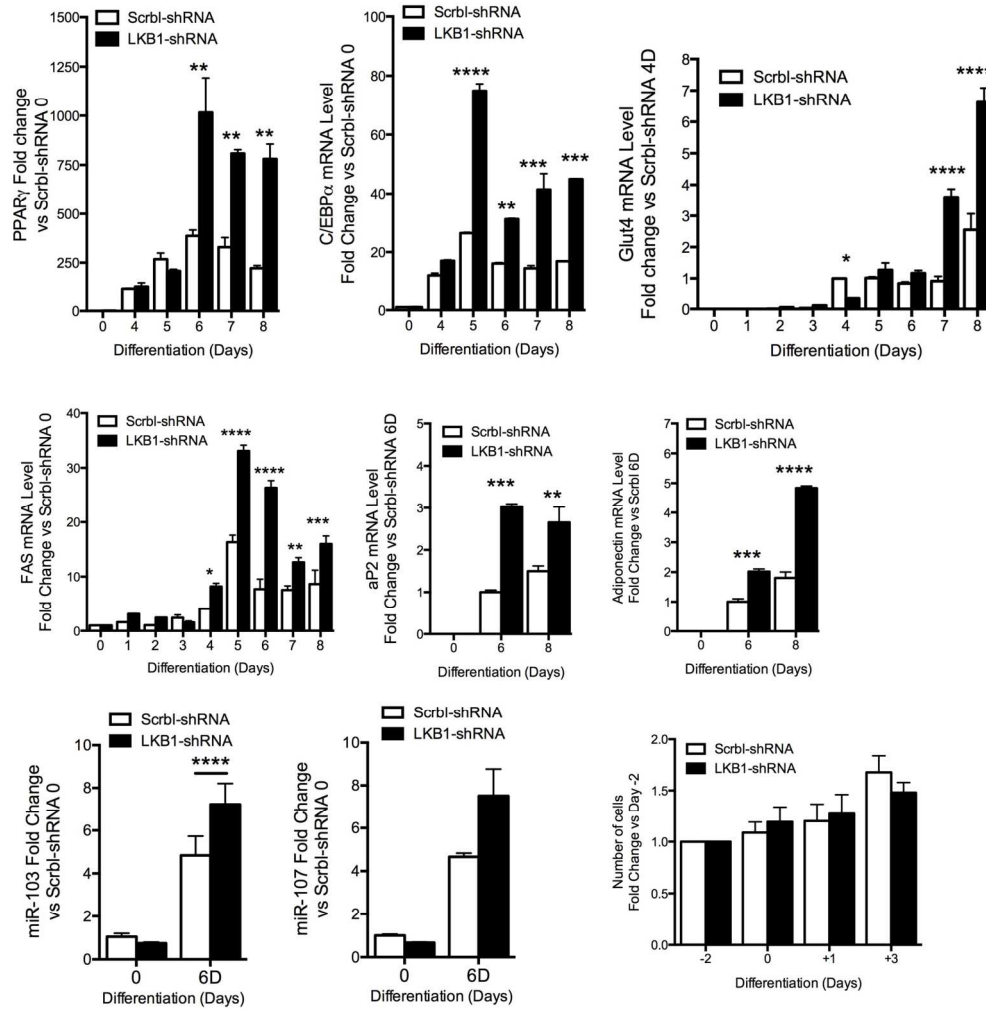


Expression of adipogenic and lipogenic markers during the differentiation of wild type (Wt) and LKB1^{-/-} MEFs.

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 \pm 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 PPAR γ , C/EBP α , C/EBP β , 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 \pm 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).

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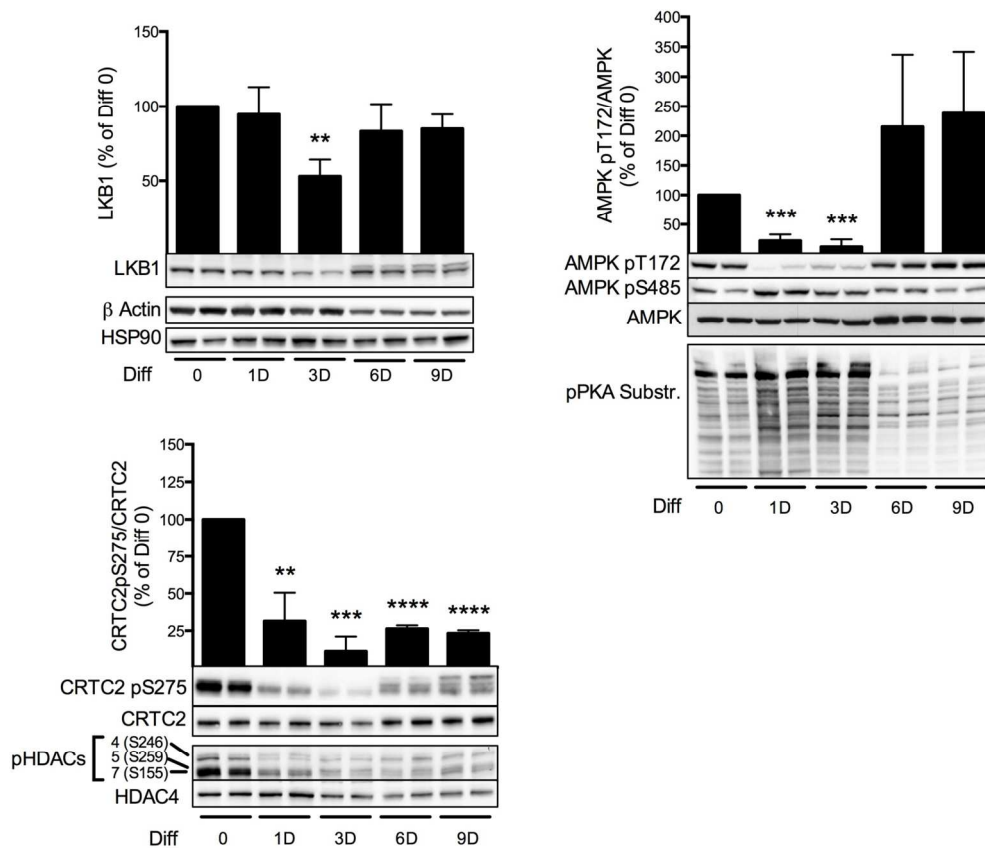
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Expression of adipogenic markers in Scrbl-shRNA and LKB1-shRNA expressing 3T3L1 preadipocytes and adipocytes.

mRNA levels of PPAR γ , C/EBP α , 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.

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