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DLK2 Is a Transcriptional Target of KLF4 in the Early Stages of Adipogenesis

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The epidermal growth factor-like protein DLK2, highly homologous to DLK1, has been identified as a modulator of adipogenesis *in vitro*. Knocking down *Dlk2* expression prevents adipogenesis of 3T3-L1 cells but enhances that of the mesenchymal cell line C3H10T1/2. The expression of *Dlk2* shows two peaks along this differentiation process: the first one, in response to 3-isobutyl-1-methylxanthine (IBMX) and dexamethasone (Dex), and the second, shortly after exposure to insulin. Nothing is known about the transcriptional regulation of *Dlk2* during adipogenesis. Here, we report that, during early adipogenesis of 3T3-L1 cells, *Dlk2* expression is controlled independently by IBMX and Dex. We also show that KLF4, a transcription factor critical for the control of early adipogenesis, binds directly to the *Dlk2* promoter and increases *Dlk2* expression in response to IBMX. Overexpression of KLF4 leads to an increase in DLK2 expression levels, whereas KLF4 knockdown downregulates the transcriptional activity of the *Dlk2* promoter. Finally, we demonstrate that KLF4 regulates the basal expression of *Dlk2* in C3H10T1/2 cells, and it is required for the adipogenic differentiation of those cells. These results indicate that KLF4 mediates the transcriptional regulation of *Dlk2* in response to IBMX during the early stages of adipogenesis.

Introduction

DLK2 is a transmembrane protein belonging to the epidermal growth factor-like family.[1] DLK2 shares structural and functional features with DLK1,[1] a protein that participates in several differentiation processes, including adipogenesis,[2] hematopoiesis,[3–5] osteogenesis[6] and adrenal gland and neuroendocrine cell differentiation.[7–9] DLK1 has also been involved in peripheral and central nervous system differentiation,[10] as well as in growth arrest and increased malignancy of undifferentiated tumors.[9,11,12] DLK1 has also been reported to participate in the process of wound healing.[13]

DLK2 modulates the adipogenesis of 3T3-L1 cells and of the mesenchymal cell line C3H10T1/2, but in an opposite way as DLK1 does.[1] Both cell lines are well-established models for the analysis of adipogenesis *in vitro*: C3H10T1/2 is as a mesenchymal cell

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Abbreviations used: IBMX, 3-isobutyl-1-methylxanthine; KLF, Krüppel-like factor; Dex, dexamethasone; Ins, insulin; shRNA, short hairpin RNA; RT-qPCR, reverse-transcription real-time PCR; ChIP, chromatin immunoprecipitation; EMSA, electrophoretic mobility shift assay.

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line able to differentiate to adipocytes, osteocytes, chondrocytes and myocytes, whereas 3T3-L1 is a more committed cell line that can only differentiate to adipocytes.[1] 3T3-L1 cells with decreased *Dlk2* expression levels show diminished levels of adipogenesis. This is in marked contrast with *Dlk1*, whose overexpression is inhibitory and whose decreased expression enhances the differentiation response of 3T3-L1 cells. On the other hand, as it happened with *Dlk1*,[14] C3H10T1/2 cells also respond differently than 3T3-L1 cells when the expression of *Dlk2* is modified; in this case, the reduction of *Dlk2* expression in those cells leads to an increase in adipogenesis.[1] Both cell lines also show differences in the pattern of *Dlk2* expression during adipogenesis. In 3T3-L1 cells, *Dlk2* shows a sharp increase in its expression within the first 24 h, followed by a decrease at 48 h, a second peak of expression between 2 and 8 h after insulin (Ins) treatment and a final decrease that is maintained until the end of the process. However, in C3H10T1/2 cells, *Dlk2* shows an initial decrease in expression, followed by a marked increase in its expression immediately after Ins addition.[1] The differences in the adipogenic process between these two murine cell lines are due, in part, to different cell contexts, regarding the level of expression and activation of NOTCH1, DLK1 and DLK2.[1,15]

Dlk2 expression has been detected in several adult mouse tissues, showing a different pattern of expression than *Dlk1*. *Dlk2* is highly expressed in lung, brain, adipose tissue, testes, adult liver, placenta, ovaries and thymus. Interestingly, fetal and adult liver display an opposite expression pattern for *Dlk1* and *Dlk2*. *Dlk2* expression is absent from liver during the first days of life, when *Dlk1* expression is elevated, but increases around the 16th day after birth, when *Dlk1* expression starts to decline. These data suggest that the expression of *Dlk1* and *Dlk2* is inversely regulated along mouse liver development.[1] Our group has recently described the mapping of *Dlk2* core promoter and the location of a proximal Sp1 response element, which controls *Dlk2* basal transcription.[16]

Krüppel-like factors (KLFs) are zinc finger proteins that constitute an important class of transcriptional regulators. The KLF family plays a crucial role in numerous cellular processes, including the control of cell proliferation, cell differentiation and development.[17,18] The 17 members of the KLF family identified to date bind to CACCC elements and/or GC-rich DNA sequences in the regulatory regions of target genes.[19] Different studies have demonstrated that several members of the Krüppel-like family play a role in adipocyte biology. Thus, KLF2 and KLF3 negatively regulate adipogenesis,[20,21] whereas KLF5 and KLF15 are necessary for adipocyte differentiation.[22,23] KLF6 has been identified as an inductor of adipogenesis through repression of *Dlk1*.[24] All of these KLFs are thought to function through different transcriptional coactivators or repressors. KLF4, which has been involved in important cellular and physiological processes, such as cell cycle control, transcriptional regulation, DNA repair, apoptosis, differentiation and cell fate determination,[25–32] has also been reported as an early regulator of adipogenesis.[26] Expression of KLF4 is augmented in white adipose tissue as compared to *in vitro* fully differentiated 3T3-L1 adipocytes.[33] During 3T3-L1 cell differentiation, induced using the standard 3-isobutyl-1-methylxanthine (IBMX), dexamethasone (Dex) and Ins cocktail, *Klf4* mRNA appears 30 min after the beginning of treatment and peaks at 2 h post-induction. The expression pattern of *Klf4* along the early stages of 3T3-L1 adipogenesis is similar to that of *Cebp/β* and appears to be induced in response to cAMP. Knockdown of *Klf4* inhibits adipogenesis and downregulates CEBP/β levels. KLF4 and KROX20, another transcription factor that modulates adipogenesis, bind together to the *Cebp/β* promoter and transactivate it in the early stages of 3T3-L1 adipogenesis. In turn, CEBP/β regulates the expression of *Klf4* and *Krox20* through a negative feedback loop.[26]

In this report, we analyze the transcriptional regulation of *Dlk2* during the early stages of adipogenesis. We first show that *Dlk2* responds to both IBMX and Dex independently. Focusing on the response to IBMX, we show that *Dlk2* and *Klf4* show a similar expression pattern, with an increase in their expression in the first hours after treatment. We next show that KLF4 binds to the promoter of *Dlk2* and upregulates its transcriptional activity and that knockdown of KLF4 decreases *Dlk2* expression during 3T3-L1 differentiation and inhibits the adipogenesis of these cells. Finally, we show that, in the mesenchymal cell line C3H10T1/2, KLF4 shows the same expression pattern and modulates the extent of the adipogenic differentiation in a similar way as in 3T3-L1 cells. We conclude that the early activation of *Dlk2* transcription in response to IMBX is in part mediated by KLF4.

Results

The early expression of *Dlk2* during adipogenesis of 3T3-L1 cells is induced independently by IBMX and Dex

The adipogenic differentiation protocol consists of a 2-day treatment with IBMX and Dex, followed by a 5-day treatment with Ins.[2] In a previous report, we demonstrated that, in 3T3-L1 cells, *Dlk2* shows two peaks of expression: the first one, 2–4 h after the exposure to IBMX and Dex, and the second one, soon after the exposure to Ins.[1] In an attempt to dissect the transcriptional regulation of *Dlk2* during

adipogenesis, we first tested which components of the standard differentiation cocktail, IBMX, Dex and Ins, were able to activate *Dlk2* expression during the first hours of the differentiation process. Confluent 3T3-L1 cells were exposed to the three components of the differentiation cocktail, alone or in combination, and *Dlk2* mRNA levels were measured 2 h after treatment. As shown in Fig. 1a, *Dlk2* expression was induced by IBMX and Dex and, to a lesser extent, by Ins as expected. As a control, we analyzed the expression pattern of KLF4, a transcription factor that modulates early stages of 3T3-L1 adipogenesis by regulating the expression levels of *C/ebpβ*. It is known that, in 3T3-L1 cells, out of the three components of the standard differentiation cocktail, KLF4 responds solely to IBMX, showing a peak of expression 2 h post-treatment.[26] We observed the expected increase in *Klf4* expression when the cells were induced with IBMX, as well as a slight increase with Ins (Fig. 1a). Focusing on the effects of IBMX, we next observed that *Dlk2* and *Klf4* gene expression increased proportionally to IBMX concentration after 2 h of treatment (Fig. 1b). Although *Dlk2* also responded to Dex and Ins, the fact that its response to IBMX was similar to that of *Klf4* suggested us that KLF4 might participate in the IBMX-induced *Dlk2* transactivation. Therefore, we decided to further analyze whether KLF4 played a role in controlling the expression of *Dlk2* induced by IBMX.

Using IBMX as the only stimulus, we next analyzed the expression pattern of *Dlk2* and *Klf4* in confluent 3T3-L1 cells during the first 24 h of treatment. We also included *Dlk1* as a negative control because its expression is downregulated during the adipogenesis process.[1] As expected, *Klf4* showed a peak of expression 1–2 h after induction. Interestingly, *Dlk2* showed a similar peak of expression 2 h after treatment, but with a slight delay in respect to *Klf4*. *Dlk1* did not respond to IBMX treatment (Fig. 1c). Similar results were obtained when the protein levels were analyzed: although both DLK2 and KLF4 showed a peak in their expression levels 4 h after treatment with IBMX, KLF4 increased more markedly than DLK2 (Fig. 1d). However, the increase in DLK2 protein did not match the increase in *Dlk2* mRNA, thus suggesting the possibility of additional levels of regulation, such as mRNA or protein stability or translational or posttranslational control, which would be interesting to analyze in future studies. Taken together, these results suggested that the transcriptional activation of *Dlk2* in 3T3-L1 cells in response to the adipogenic signal IBMX might be mediated by KLF4.

The promoter region of *Dlk2* contains KLF4 response elements

To analyze whether KLF4 played a role in the transcriptional regulation of *Dlk2*, we first screened a proximal 2-kb fragment of the *Dlk2* promoter for the presence of putative KLF4 consensus binding sites, using the application MatInspector®‡. This application found three potential binding sites for KLF4 in that region, two of which were located upstream of the transcription start site, at positions −313/−326 (KLF4-A) and −261/−273 (KLF4-B), and a third one located within the first intron, at position +551/+563 (KLF4-C) (Fig. 2a). This latter potential binding site is conserved among several species, including *Homo sapiens*, *Mus musculus*, *Rattus norvegicus*, *Canis familiaris* and *Bos taurus* (ElDorado®; data not shown). To test whether KLF4 regulates *Dlk2* transcription, we first did a transfection-based promoter analysis using the luciferase system. 3T3-L1 and NIH3T3 cells were co-transfected with *Dlk2* promoter–luciferase reporter constructs, containing the potential KLF4 binding sites, along with a plasmid expressing KLF4 (pCMVSport-KLF4) or a control plasmid (pCMVSport). The pattern of response to KLF4 was similar in both cell lines; here, we show the luciferase values obtained with 3T3-L1 cells. Our results showed that overexpression of KLF4 resulted in a 2.5- to 3-fold induction in the luciferase activity displayed by cells transfected with pGL3Basic(−375/+1), which contains the KLF4-A and KLF4-B binding sites, and of cells transfected with pGL3Basic(−212/+641) and pGL3Basic(+1/+641), both of which contain the KLF4-C binding site (Fig. 2a). The highest activity was obtained with pGL3Basic(−375/+641), which contains the three KLF4 putative binding sites. We also analyzed the transcriptional response to KLF4 of other irrelevant promoters such as *Dlk1* (Fig. 2a), *Nos2* minimal promoter and *Hey1* (data not shown). None of them showed any activation in the presence of the mentioned transcription factor. To analyze the responsiveness of each of the three KLF4 binding sites to KLF4, we next mutated each one of them, separately. Mutations of KLF4-A and KLF4-B were performed in the −375/+1 fragment, whereas the mutation of the KLF4-C site was performed in the +1/+641 fragment. The three mutations separately led to a 60% reduction in the KLF4-induced transcriptional activity of the fragments −375/+1 and +1/+641, respectively (Fig. 2b), confirming that all three sites are necessary for a correct KLF4-mediated *Dlk2* expression. Similar results were obtained with NIH3T3 cells (data not shown). Thus, our results show the existence of functional KLF4 response elements in the proximal *Dlk2* promoter and within the first intron of the gene and indicate that *Dlk2* appears to be transcriptionally regulated by KLF4.

To further analyze the transcriptional regulation of *Dlk2* by KLF4, we knocked down the expression

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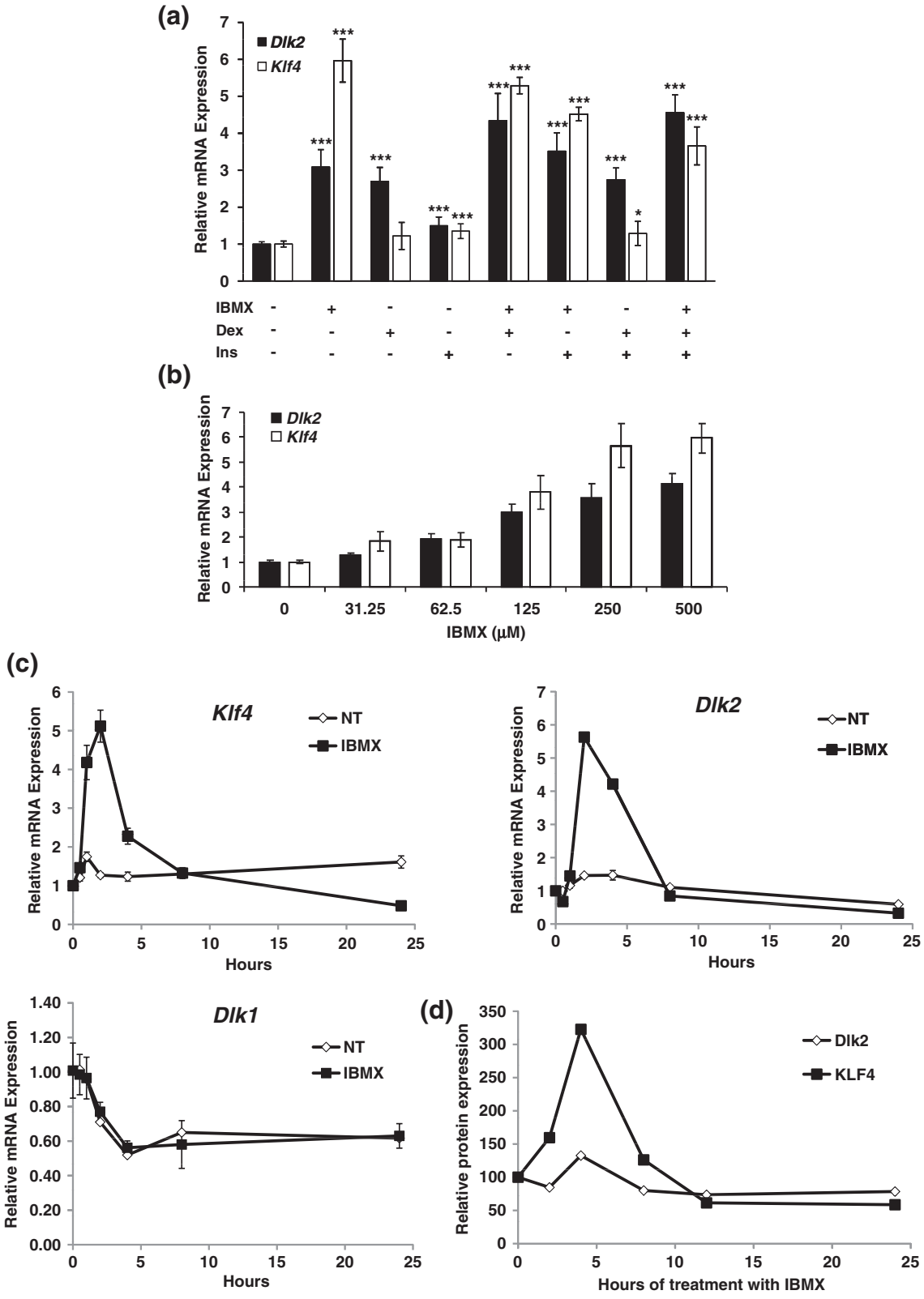

Fig. 1 (*legend on next page*)

of *Klf4* in NIH3T3 cells, using short hairpin RNA (shRNA) technology, and examined the transcriptional activity of the *Dlk2* promoter. We used two different shKLF4 plasmids, both of which were able to decrease the expression level of *Klf4* mRNA in NIH3T3 cells. As shown in Fig. 2c, when KLF4 expression was knocked down by around 50%, the transcriptional activity of the fragments +1/+641 and −212/+641 (containing the KLF4-C site) showed a strong and highly significant decreased that averaged a 60%; however, the transcriptional activity of the fragment −375/+1 (containing the KLF4-A and KLF4-B sites) showed a moderate, but still significant, decrease only when shKLF4#5 was used (Fig. 2c). These data indicate that endogenous KLF4 is also actively regulating *Dlk2* expression.

KLF4 upregulates *Dlk2* mRNA and protein expression

We next sought to determine whether the transactivation of the *Dlk2* promoter by KLF4 resulted in an increment in the amount of *Dlk2* mRNA and protein. For that, NIH3T3 and 3T3-L1 cells were transfected with a *Klf4* expression plasmid (pLPCX-KLF4) or a control plasmid (pLPCX), and the mRNA and protein levels of both *Dlk2* and *Klf4* were measured. The overexpression of *Klf4* led to moderate and constant increments of *Dlk2* mRNA levels of around 1.8-fold in NIH3T3 cells and 1.5-fold in 3T3-L1 cells (Fig. 3a). On the other hand, KLF4 overexpression in NIH3T3 and 3T3-L1 cells resulted in an increment in DLK2 protein levels over the control cells, 2.8-fold and 1.4-fold, respectively (Fig. 3b). No increase in *Dlk1* mRNA expression levels in response to KLF4 overexpression was detected (Fig. 3a). Thus, our data so far indicate that KLF4 is able to transactivate two separate regions in the promoter and first intron of *Dlk2* and that the increase in the transcriptional activity induced by KLF4 is associated with an upregulation of *Dlk2*, both at mRNA and protein levels.

To further investigate whether *Dlk2* is a transcriptional target of KLF4, we generated 3T3-L1 cells in which *Klf4* expression was stably knocked down by means of two different shRNAs (shKLF4#4 and shKLF4#5). We observed a clear decrease in the number of adipocytes when confluent cultures of those transfectants were differentiated and stained with oil red O, as compared to cells transfected with the control shRNA (shControl), as previously reported.[26] The control shRNA cell line showed a slight, nonsignificant decrease in the number of adipocytes when compared to the non-transfected cells (data not shown). This result was confirmed by the clear decrease in the expression of *C/ebpα*, *aP2* and *Pparγ2* at 2, 4 and 7 days of the differentiation process in cells transfected with both shRNAs, as determined by reverse-transcriptase real-time PCR (RT-qPCR) (Fig. 4a).

We next used those two sh*Klf4*-transfected cell lines to test whether the IBMX-induced expression of *Dlk2* during the early stages of adipogenesis was affected by the downregulation of KLF4. We analyzed the expression levels of *Dlk2* and *Klf4* in response to IBMX at different times, up to 24 h. As expected, control cells (shControl) showed maximal *Klf4* mRNA expression 1 h after the treatment with IBMX and maximal *Dlk2* mRNA expression around 2 h after the treatment (Fig. 4b). Moreover, cells transfected with the *Klf4* shRNAs (shKLF4#4 and shKLF4#5) showed a substantial and sustained reduction in the expression of *Klf4* mRNA when compared to control cells (Fig. 4b). As *C/ebpβ* expression is regulated by KLF4 during 3T3-L1 adipogenesis,[26] we analyzed its expression in cells transfected with shKLF4 and observed a significant reduction in the peak of activation (Fig. 4b), demonstrating that, in our cells, reduced *Klf4* expression levels also resulted in the expected reduction in *C/ebpβ* expression levels. Interestingly, *Dlk2* mRNA expression levels in response to 2- and 4-h treatments with IBMX were equally reduced in both shKLF4 transfectants, as compared to control cells. Taken together, these results indicate that *Dlk2*, as *C/ebpβ*, is one of the KLF4 transcriptional targets during the early events of adipogenesis and that the sharp increase in the expression of *Dlk2* is due, at least in part, to the action of KLF4.

KLF4 binds to the *Dlk2* promoter

To assess whether KLF4 directly binds to the *Dlk2* promoter and is responsible of its transactivation, we performed chromatin immunoprecipitation (ChIP) analysis. We first used 3T3-L1 cells, either

Fig. 1. *Dlk2* is independently induced by IBMX and Dex and shows a similar expression pattern in response to IBMX than *Klf4*. (a) Confluent 3T3-L1 cells were exposed to the three components of the adipogenic cocktail, IBMX, Dex and Ins, either individually or in combination, and *Dlk2* and *Klf4* mRNA levels were measured by RT-qPCR 2 h after treatment. The average values of three independent experiments are shown. (b) Confluent 3T3-L1 cells were treated with increasing amounts of IBMX (0–500 μM) and *Dlk1*, *Dlk2* and *Klf4* mRNA levels were analyzed by RT-qPCR 2 h after induction. The average values of three independent experiments are shown. (c and d) Confluent 3T3-L1 cells were treated with IBMX for 24 h, and the mRNA levels for *Dlk1*, *Dlk2* and *KLF4* (c) or protein levels for DLK2 and KLF4 (d) were analyzed at the indicated times. Phosphoriboprotein P0 (mRNA) and tubulin (protein) were used as internal controls. A representative experiment out of three independent experiments is shown. (*, ** and ***, statistical significance *versus* non-treated controls in Student's *t*-test, with p-value<0.05, p-value<0.01 and p-value<0.001, respectively.)

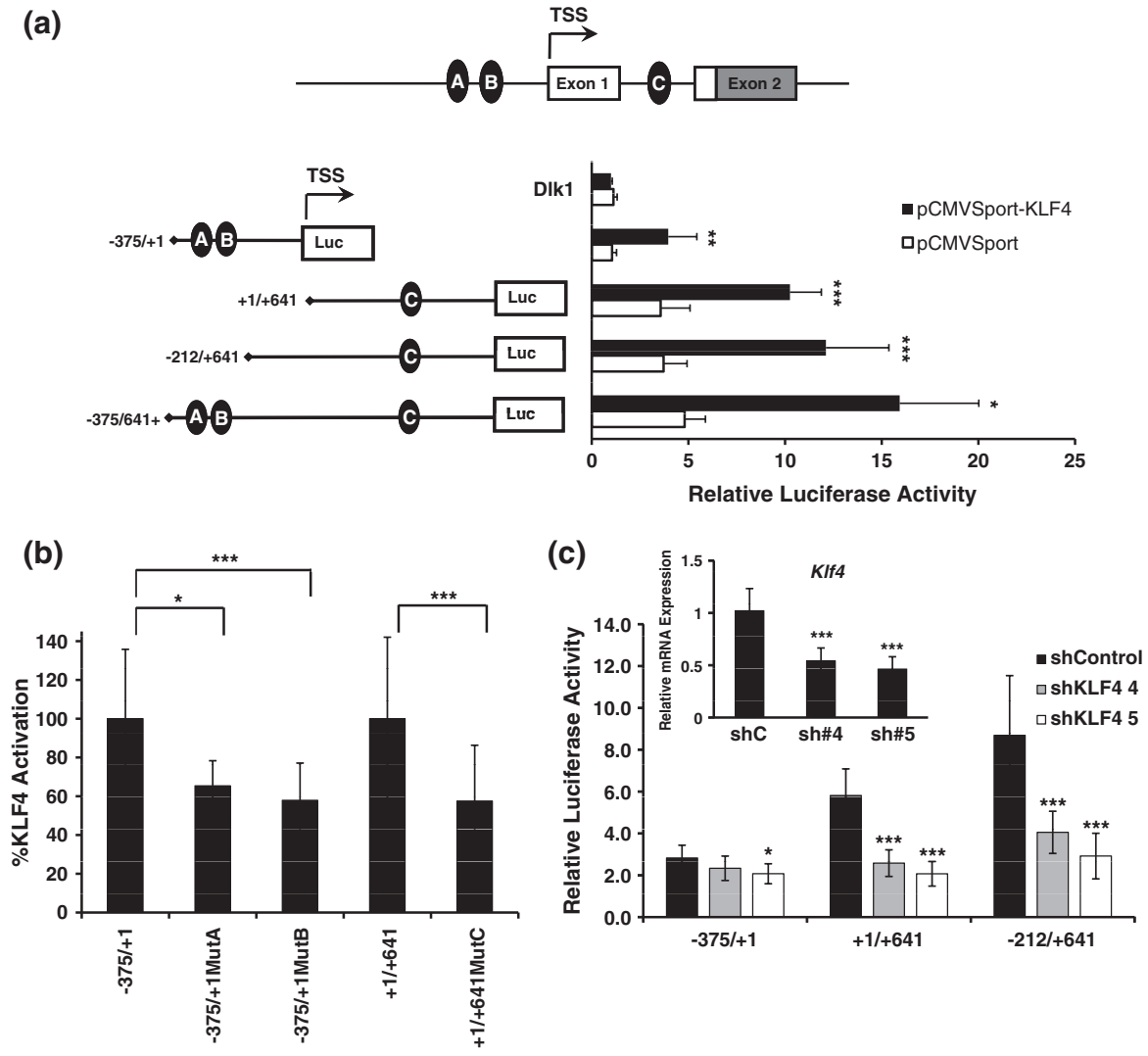

Fig. 2. KLF4 transactivates the *Dlk2* promoter. (a) Schematic representation of the three putative KLF4 binding sites (named KLF4-A, KLF4-B or KLF4-C) found on the *Dlk2* promoter by the bioinformatics application MatInspector. The three potential KLF4 binding sites are located at positions −313/−326 (KLF4-A), −261/−273 (KLF4-B) and +551/+563 (KLF4-C). 3T3-L1 cells were transiently transfected with pGL3Dlk1, pGL3Basic(−375/+1), pGL3Basic(−212/+641), pGL3Basic(+1/+641) or pGL3Basic(−375/+641), along with pCMVSport (control) or pCMVSport-KLF4 to drive the overexpression of KLF4. Luciferase activities were measured 24 h after transfections. The plasmid pRL-TK was co-transfected in all cases, and each luciferase relative light unit value was normalized with its corresponding value of *Renilla* activity. The average of at least three independent experiments is shown. (b) 3T3-L1 cells were transiently transfected with pGL3Basic(−375/+1), pGL3Basic(−375/+1MutA), pGL3Basic(−375/+1MutB), pGL3Basic(+1/+641) or pGL3Basic(+1/+641MutC), along with the plasmid pRL-TK, and with pCMVSport-KLF4. The normalized average values from three independent experiments are shown. (c) NIH3T3 cells were transfected with pGL3Basic(−375/+1), pGL3Basic(−212/+641) or pGL3Basic(+1/+641) plasmids, together with either an shKLF4 plasmid (shKLF4#4, sh#4, or shKLF4#5, sh#5) or an shControl plasmid (shC). *Klf4* mRNA expression levels were determined by RT-qPCR 24 h after transfection. Normalized luciferase values corresponding to three independent experiments are show. (*, ** and ***, significant *versus* control in Student's *t*-test with p-value<0.05, p-value<0.01 and p-value<0.001, respectively.)

treated or not with IBMX (see Materials and Methods), to prepare native chromatin, and then we performed the ChIP analyses with the ChIP-IT Express Kit (Active Motif, Carlsbad, CA, USA). Normal rabbit IgG was used as a negative control, whereas antibodies against RNA-polymerase II were used as positive controls of immunoprecipitation. When an antibody against KLF4 H-180 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) was used, the IBMX-induced cells showed a clear increase in the amount of immunoprecipitated DNA specific to *Dlk2*. The normalized increase in

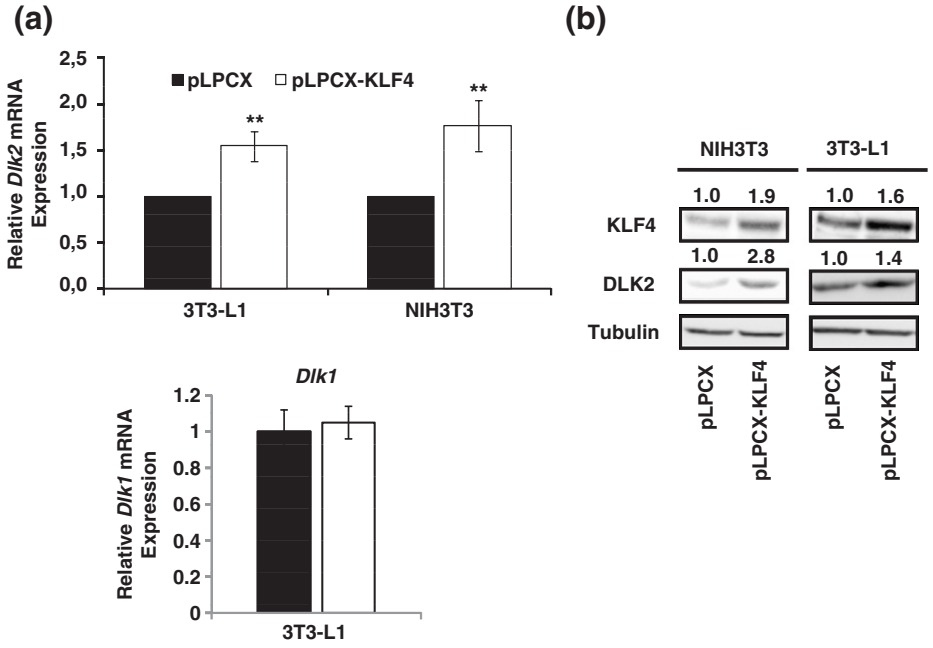

Fig. 3. KLF4 induces the expression of *Dlk2*. 3T3-L1 and NIH3T3 cells were transiently transfected with a *Klf4* expression plasmid (pLPCX-KLF4) or with a control plasmid (pLPCX). (a) Twenty-four hours after transfection, the *Dlk1*, *Dlk2* and *Klf4* mRNA levels were determined by RT-qPCR, using the phosphoriboprotein P0 mRNA as an internal control. The average values corresponding to three independent experiments are shown. A control analysis of *Dlk1* expression in response to KLF4 is included (b). DLK2 and KLF4 protein levels were determined 24 h after transfections by Western blot analysis. Image acquisition was performed with a LAS/3000 Mini Fujifilm. Quantitation was performed with the MultiGauge software (Fujifilm). A representative experiment is shown. All densitometric values were first normalized to α-tubulin, and then referred to their corresponding pLPCX values.

the amount of immunoprecipitated DNA was around 9-fold, as visualized by performing a PCR reaction with specific oligonucleotides and quantifying the density of the amplified DNA fragments (Fig. 5a). Our results indicate first that KLF4 is able to specifically bind to the *Dlk2* promoter region, suggesting that *Dlk2* is a real transcriptional target of KLF4. Secondly, the fact that the amount of immunoprecipitated *Dlk2*-specific DNA was increased in IBMX-treated cells, in which KLF4 expression is induced, indicates that the peak of *Dlk2* expression detected in 3T3-L1 cells in response to IBMX is due, at least in part, to the binding and the subsequent transactivation of *Dlk2* by KLF4.

Considering the limited resolution of the ChIP technology, we next explored, by electrophoretic mobility shift assay (EMSA), which of the three KLF4 potential consensus binding sites, located on the *Dlk2* promoter and its first intron, could be occupied preferentially by this transcription factor. Three double-stranded oligonucleotides, each one corresponding to one of the KLF4 potential binding sites, were radiolabeled and incubated with KLF4 full-length recombinant protein (Abnova). As shown in Fig. 5b, we detected that DNA–protein complexes were formed with the three oligonucleotides, although some of them did not seem to be specific to KLF4. The labeled oligonucleotides were exposed to the recombinant KLF4 protein (lanes 2 and 2′) and additionally were competed out by a specific KLF4 oligonucleotide (G3) (lane 3), by the corresponding cold oligonucleotide (lane 4) or by the corresponding KLF4-mutated cold oligonucleotide (lane 5). We also included a sample in which we added a specific antibody against KLF4 (lane 6). As shown in Fig. 5b, only oligonucleotides KLF4-B and KLF4-C produced a specific DNA–protein complexes (white arrow) that were competed out by the consensus and the cold, but not by the mutated, oligonucleotides; additionally, both oligonucleotides showed a supershift band when exposed to the specific KLF4 antibody (black arrows, SS), accompanied by the disappearance of the specific DNA–protein complex, which can be consider itself as a competition.[34,35] Judging by the intensity of the retarded bands, it seems that the affinity of KLF4 for the oligonucleotide KLF4-C is lower than the affinity for KLF-B. The retarded bands appearing with oligonucleotide KLF4-A are probably nonspecific because they were not competed with the corresponding oligonucleotides nor were they retarded by the addition of KLF4 antibody. Taken

together, our results indicate that KLF4 is able to bind to the *Dlk2* promoter, to both the −273/−261 and the +551/+563 binding sites, but most likely not to the −326/−313 site, and that way is able to control its transcription during the early events of adipogenesis.

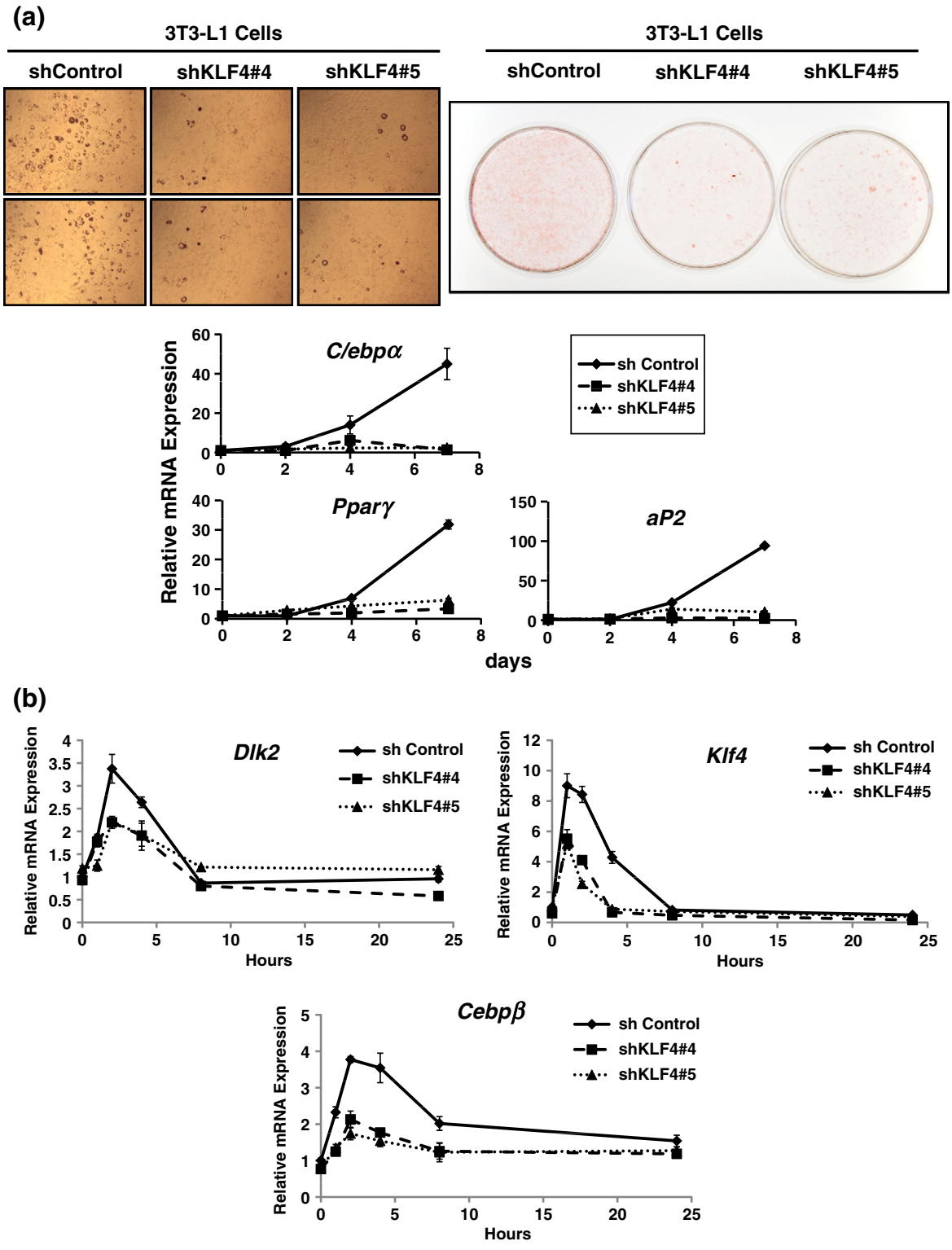

Fig. 4 (*legend on next page*)

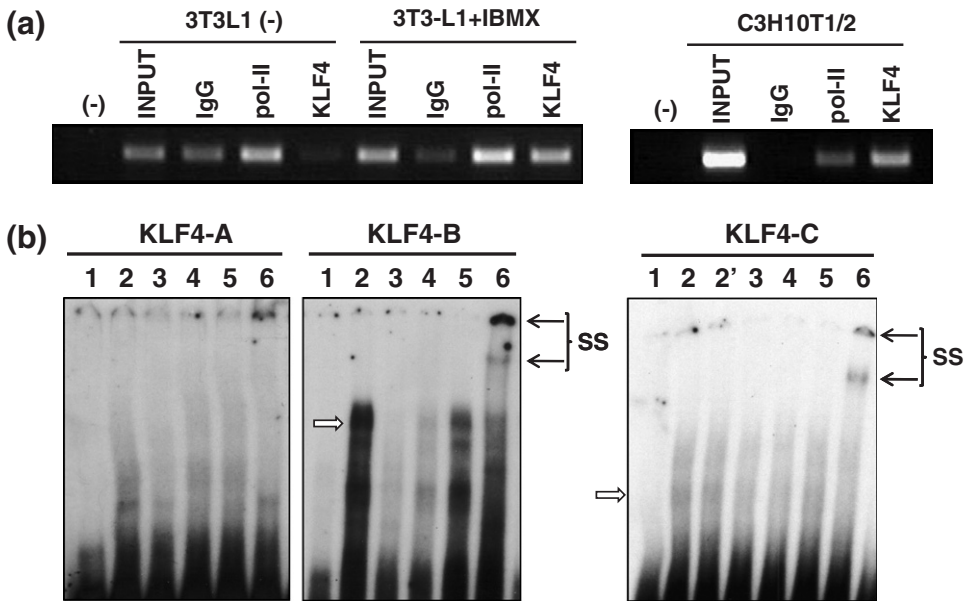

Fig. 5. KLF4 binds to the *Dlk2* promoter. (a) ChIP analyses were performed using native chromatin from either untreated or IBMX-treated 3T3-L1 cells [named 3T3L1 (–) or 3T3L1 + IBMX, respectively] or C3H10T1/2 cells, using the ChIP-IT Express Kit. Chromatin was incubated with normal rabbit IgG (IgG), antibodies against RNA-polymerase II (pol-II) or antibodies against KLF4 H180 (KLF4) and immunoprecipitated with protein G magnetic beads (Active Motif). Purified DNA was analyzed by PCR, with the oligonucleotides Dlk2MluI − 212U and Dlk2HindIII + 177L (see Materials and Methods). The electrophoretic analysis in a 1% agarose gel of a representative experiment is shown. (b) EMSAs were performed with 0.5 μg of KLF4 full-length recombinant protein. ^{32}P-labeled oligonucleotides KLF-A, KLF-B or KLF-C, containing the potential KLF4 binding sites located at positions −313/−326, −261/−273 and +551/+563, were incubated with buffer (lane 1) or with the purified protein (lanes 2 and 2′). For competition assays, the reaction was preincubated with a 100-fold excess of cold oligonucleotide before the addition of the labeled oligonucleotide, either G3, a consensus KLF4 sequence (lane 3), the corresponding unlabeled nucleotide (lane 4) or the corresponding unlabeled KLF4-mutated nucleotide (lane 5). For supershift assays (lane 6), the samples were incubated with 2 μg of KLF4 antibody (H-180; Santa Cruz Biotechnology, Inc.). White arrows indicate the specific KLF4–oligo complexes, and black arrows indicate the antibody supershifted bands (SS).

Dlk2 expression during early adipogenesis of C3H10T1/2 cells is not exclusively controlled by KLF4

Whereas *Dlk2* expression knockdown reduces the adipogenic potential of 3T3-L1 cells, the decrease in *Dlk2* expression enhances adipogenesis of the mesenchymal cell line C3H10T1/2. This effect is the opposite of the one exerted by *Dlk1* in both cell lines.[1] As mentioned in Introduction, *Dlk2* expression pattern along adipogenesis is also different in both cell lines: C3H10T1/2 cells show a decrease in its expression during the first 48 h after the induction of adipogenesis, followed by a marked induction in response to Ins, which is different from the bimodal stimulation of *Dlk2* expression shown by 3T3-L1 cells. Since *Dlk2* is a transcriptional target for KLF4 in 3T3-L1 cells, we investigated whether that transcription factor was also involved in the transcriptional control of *Dlk2* during the adipogenic differentiation of C3H10T1/2 cells. We first examined the potential binding of KLF4

Fig. 4. KLF4 induces *Dlk2* expression in response to IBMX in 3T3-L1 cells. 3T3-L1 cells were stably transfected with two KLF4-specific shRNA clones, shKLF4#4 and shKLF4#5. Stable transfection with shControl was used as a control. (a) Stably transfected cells were induced to differentiate under the standard protocol. The differentiated cells were stained with Red Oil O to reveal intracellular lipid accumulation in newly generated adipocytes. The 5× magnification micrographs (left) and whole plate pictures (right) are shown. mRNA samples were taken at 2, 4 and 7 days of the differentiation process, and RT-qPCR analysis of *C/ebpα*, *aP2* and *Pparγ2* expression was performed (lower part). All values were referred to the shControl values. (b) RT-qPCR analysis of *Klf4*, *Dlk2* and *C/ebpβ* expression patterns of 3T3-L1 cells stably transfected with shControl, shKLF4#4 or shKLF4#5. Confluent cells were treated with IBMX for 24 h, and the mRNA levels were analyzed by RT-qPCR at the indicated times. In all cases, the phosphoriboprotein P0 mRNA level was used as an internal control for quantitative PCR analysis. All values are referred to the zero-time value of the shControl. A representative experiment is shown.

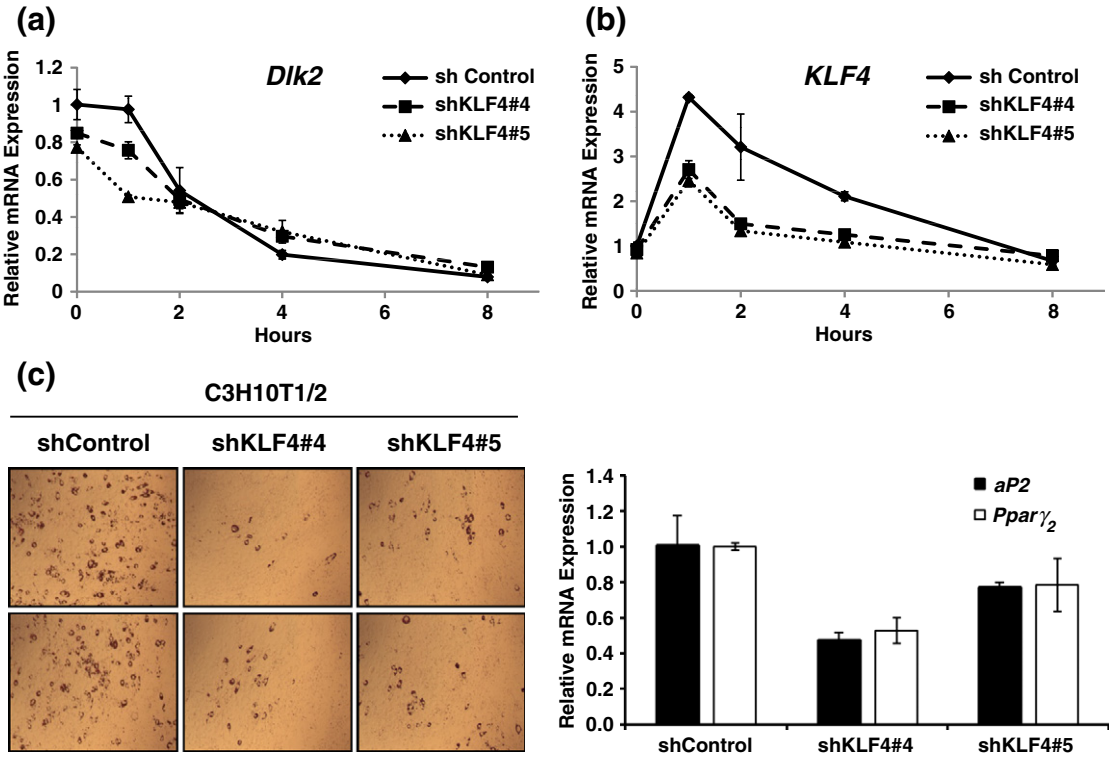

Fig. 6. Analysis of *Dlk2* expression in response to IBMX in C3H10T1/2 cells. C3H10T1/2 cells were transfected with two KLF4-specific shRNA clones (shKLF4#4 and shKLF4#5) or with shControl. (a and b) C3H10T1/2 confluent cells transfected with shControl, shKLF4#4 or shKLF4#5 were treated with IBMX for 8 h, and the *Klf4* (a) and *Dlk2* (b) mRNA levels were measured at the indicated times by RT-qPCR. In all samples, the mRNA of the phosphoriboprotein P0 was used as an internal control for quantitative PCR analysis. All values are referred to the zero-time value of the shControl. A representative experiment is shown. (c) Transfected cells were induced to differentiate under the standard protocol. The differentiated cells were stained with Red Oil O to reveal lipid accumulation in newly generated adipocytes (left panel). RT-qPCR analysis of *aP2* and *Pparγ2* expression was performed at the end of the differentiation process. The average values of three independent experiments are shown (right panel).

to the *Dlk2* promoter in those cells. As shown in Fig. 5a, chromatin corresponding to the *Dlk2* promoter immunoprecipitated when an antibody specific to KLF4 was used. This result indicated that KLF4 binds to and is implicated in the transcriptional control of *Dlk2* also in C3H10T1/2 cells. We next explored whether *Klf4* expression responded similarly to IBMX during the early stages of adipogenesis of those cells. As shown in Fig. 6b, *Klf4* expression increased in the first hour after treatment with IBMX, and its expression decreased to baseline levels after 8 h. Thus, our results suggest that the control of *Klf4* expression in C3H10T1/2 cells during the early stages of adipocyte differentiation could be similar to that observed in 3T3-L1 cells.

The fact that the expression pattern of *Klf4* and *Dlk2* in C3H10T1/2 cells was so different made us question whether KLF4 could be responsible for the initial decrease in *Dlk2* expression detected in the early adipogenesis of these mesenchymal cells. To investigate that, we treated C3H10T1/2 cells, transfected or not with the two shKLF4 constructs, with IBMX for up to 8 h, and we analyzed *Dlk2* and *Klf4* expression by RT-qPCR at different time points. As expected, the peak-of-*Klf4* expression in response to IBMX was significantly decreased in the shRNA-transfected cell lines (Fig. 6a). Interestingly, the forced reduction of *Klf4* expression led to a decrease in the basal level of expression of *Dlk2*, which was sustained only during the first 2 h of treatment with IBMX (Fig. 6a). *Dlk2* expression levels were undistinguishable from shControl cells at longer time points after induction with IBMX. These results indicate again that KLF4 activates the expression of *Dlk2* in C3H10T1/2 cells, but its activation may not contribute as much as in 3T3-L1 cells to the *Dlk2* expression pattern during the first hours after induction with IBMX. Thus, our results suggest that additional factors other than KLF4 must be responsible for the gradual decline of *Dlk2* expression during early adipogenesis of C3H10T1/2 cells.

To the best of our knowledge, nothing is known about the role of KLF4 in the onset of adipogenesis of C3H10T1/2 cells. Thus, we decided to explore whether the adipogenic potential of C3H10T1/2 cells could be modulated by *Klf4*. As shown in Fig. 6c, forced reduction of *Klf4* expression by transfection with both shKLF4 constructs led to a clear decrease in the adipogenic capacity of these cells, as measured by the accumulation of intracellular lipids or by analyzing the expression levels of the adipogenic markers *aP2* and *Pparγ2* by RT-qPCR. Interestingly, our results with C3H10T1/2 cells indicate that, similar to 3T3-L1 cells, an increase in KLF4 expression at the beginning of the differentiation process is necessary for the correct onset of adipogenesis. Taken together, our data indicate that both *Dlk2* and KLF4 seem to play an important role in the early steps of the adipogenic differentiation of C3H10T1/2 cells, but their actions appear not to be directly related.

Discussion

Adipogenesis is a well-characterized process, controlled by a complex transcriptional cascade leading to the expression of genes required for the acquisition and maintenance of the adipocyte phenotype. Our research group previously identified DLK2 as a modulator of adipogenesis in 3T3-L1 and C3H10T1/2 cells. This study is the first to analyze the transcriptional regulation of *Dlk2* in adipogenesis.

Interestingly, knocking down *Dlk2* expression leads to different effects in both cell lines: whereas the differentiation levels are reduced in 3T3-L1 cells, C3H10T1/2 cells show a greater adipogenic potential.[1] Recently, KLF4 has been described as a crucial factor for adipogenesis, acting as a modulator of *C/ebpβ* expression.[26] KLF4 is a transcription factor that has been implicated in many other physiological processes, such as cell cycle control, cell reprogramming, DNA repair, apoptosis, cancer and so on. It can function as a transcription activator or repressor, depending on the cell context (for a review, see Refs. 36 and 37). In this paper, we have analyzed the transcriptional regulation of *Dlk2* during the early events of adipogenesis and have identified KLF4 as an important transactivator of *Dlk2* expression.

Our data and those of other investigators[26] demonstrate that *Klf4* expression during 3T3-L1 adipogenesis is induced by agents that elevate intracellular cAMP, such as IBMX, and reaches a maximum of expression 2 h after treatment. Previous data from our laboratory indicated that, in 3T3-L1 cells, *Dlk2* shows an expression pattern similar to that of *Klf4* during the first 8 h of the differentiation process in response to adipogenic stimuli.[1] In this study, we show that the expression pattern of *Dlk2* in the early stages of 3T3-L1 adipogenesis closely follows in time that of *Klf4* and that its expression is regulated by both IBMX and Dex. Using bioinformatics tools, we found three potential KLF4 binding sites on the *Dlk2* promoter that we named KLF4-A, KLF4-B or KLF4-C respectively (Fig. 2). Our ChIP and EMSA analyses showed that KLF4 binds to the *Dlk2* promoter and is responsible of its transactivation in response to IBMX. Using luciferase assays, and either by overexpressing KLF4 or by knocking down its expression, we have shown that the activity of the *Dlk2* promoter is modulated by KLF4. The mutation of the KLF4-A and KLF4-B binding sites strongly reduced KLF4-mediated transactivation. Moreover, the mutation of the KLF4-C binding site, located within the first *Dlk2* intron and the only one conserved among species, also blocked the transcription of *Dlk2* mediated by KLF4. All these results demonstrate that *Dlk2* is a transcriptional target for KLF4.

In agreement with Birsoy *et al.*, using shRNA technology, we have observed that 3T3-L1 cells with reduced *Klf4* expression display lower adipogenic differentiation.[26] Our data show that decreasing *Klf4* expression leads to diminished *Dlk2* expression. These results are in agreement with the effects caused by the forced reduction of *Dlk2* expression on 3T3-L1 adipogenesis and suggest that the enhancing effects of KLF4 on the adipogenesis of these cells may be mediated, in part, by the increase in *Dlk2* expression triggered by this transcription factor. However, the fact that the reduction of *Dlk2* expression in cells with lower expression levels of *Klf4* during the first hours of treatment with IBMX was only partial suggests the involvement of other factors in the temporal expression of *Dlk2* along adipogenesis.

Considering that C3H10T1/2 cells respond differently than 3T3-L1 cells to changes in *Dlk2* expression in regard to their adipogenic potential, it was therefore relevant to analyze both the role of KLF4 in the adipogenic differentiation of C3H10T1/2 cells and its involvement on the regulation of *Dlk2* expression in this cell line. Our results indicated, first, that KLF4 regulates C3H10T1/2 adipogenesis in a way similar to that of 3T3-L1, with a decrease in the adipocyte differentiation level in response to a forced reduction in the expression of *Klf4*. Second, the expression pattern of *Klf4* in response to IBMX is very similar in both cell lines, reaching a maximum of expression 2 h after the treatment is initiated. Third, we observed that a decrease in *Klf4* expression levels results in a decrease in *Dlk2* expression during the first 2 h of exposure to IBMX in both cell lines (Figs. 4b and 6a). However, the expression pattern of *Dlk2* in response to IBMX shows striking differences between both cell lines: whereas, in 3T3-L1 cells, *Dlk2* expression peaks 2 h after IBMX

stimulation, following an expression pattern similar to that of *Klf4*, in C3H10T1/2 cells, *Dlk2* expression slowly and constantly decreases during the first 8 h of treatment and, as previously described, continues to decline during the first 2 days of treatment.[1] These data suggest, again, that despite the similar role played by KLF4 on the control of *Dlk2* expression in both cell lines, some other factors must contribute to the overall transcriptional regulation of *Dlk2* expression during adipocyte differentiation of those cells, particularly in C3H10T1/2 cells, where, unlike 3T3-L1 cells, KLF4 appears not to be the principal regulator of *Dlk2* expression during the adipogenic process.

Since *Dlk1* shares structural and functional features with *Dlk2* and since the regulation of the expression of both genes seems to be reciprocal in some instances,[1] it would be interesting to investigate whether KLF4 is a key regulator of *Dlk1* expression, as it happens with *Dlk2*. In this regard, it has been recently reported that *Dlk1* is directly regulated by IBMX during the adipogenic differentiation of human mesenchymal cells, in a cAMP-independent manner.[38] This regulation is probably not mediated by KLF4, considering that the increase in KLF4 expression in response to IBMX treatment is cAMP dependent.[26] Besides, our data show that *Dlk1* is not regulated by IBMX in our cell models, at least during the first 24 h of treatment. In addition, the *Dlk1* promoter does not respond to the overexpression of KLF4, and the expression of *Dlk1* mRNA is not upregulated by KLF4.

The identification of the factors responsible for the effects of Dex on *Dlk2* expression, as well as the analysis of the downregulation of *Dlk2* during the adipogenesis of C3H10T1/2 cells, although beyond the scope of this paper, would be of great value to acquire a complete view of the regulation of *Dlk2* during the adipogenesis.

Materials and Methods

Cell culture and differentiation assays

Mammalian cells were cultured at 37 °C in a 5% (v/v) CO_2 humidified atmosphere, in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal bovine serum, 2 mM L-glutamine, 1 unit/ml penicillin and 1 μg/ml streptomycin, all of them from Lonza (Verviers, Belgium). The cell lines used were 3T3-L1 (ATCC CCL-92.1), C3H10T1/2 (clone 8, ATCC CCL-226) and NIH3T3 (ATCC CRL-1658). Adipocyte differentiation was induced according to standard procedures.[2] The level of adipogenesis was determined by staining with Red Oil O, microscopic examination and image acquisition, performed as described previously.[2] In addition, the expression of *Pparγ2*, *C/ebpα* and *aP2* was monitored by RT-qPCR as a way of measuring the degree of adipogenesis.

DNA constructs

pCMVSport-KLF4 expression plasmid was purchased from American Type Culture Collection (IMAGE Clone ID 3156339). pLPCX-KLF4 expression plasmid derivates from pCMVSport-KLF4 digested with EcoRI–NotI. The 2150-kb *Klf4* cDNA fragment thus released was isolated by standard procedures and cloned into the EcoRI–NotI sites of pLPCX. Plasmids for shRNA assays pLKO.1-shControl, pLKO.1-shKLF4#4 (Clone TRCN0000095372) and pLKO.1-shKLF4#5 (Clone TRCN0000095373) were purchased from Sigma-Aldrich (Saint Louis, MO, USA). To clone different DNA fragments from the *Dlk2* promoter region, we used the BAC RP23-135A16 clone as a DNA template for PCR (BACPAC Resources, EE.UU.), which contains the complete sequence of the *Dlk2* promoter. To locate the promoter region and to name and locate the different oligonucleotides used in this work, we used sequence information from clone FM180474 (European Molecular Biology Laboratory Nucleotide Sequence Database). The DNA fragments from the *Dlk2* promoter cloned into vector pGL3Basic (Promega, Madison, WI, USA) were amplified by PCR using the following oligonucleotides: 5′-TAT ACG CGT TGG GTG AGG GGC AGA GTG G-3′ and 5′-TAT AAG CTT GCT GAG GCG ACC CCG AGC G-3′ for DNA fragment −375/+1, 5′-ATT CTC GAG CCC AGA TTC CCG AGT GCT CGG C-3′ and 5′-TAG AAG CTT CAC CTG TGG GAT GGC ACG GGC-3′ for DNA fragment +1/+641 and, finally, 5′-TAT CTC GAG GAA GGG AGG GGC GAA GAG C-3′ and 5′-TAG AAG CTT CAC CTG TGG GAT GGC ACG GGC-3′ for DNA fragment −212/+641. PCR amplifications were performed under standard conditions, after which DNAs were digested with HindIII–MluI for fragment −375/+1 or with HindIII–XhoI for fragments +1/+641 and −212/+641. The digested fragments were then cloned into the vector pGL3Basic. The mutant luciferase constructs pGL3Basic(−375/+1MutA), pGL3Basic(−375/+1MutB) and pGL3Basic(+1/+641MutC) were generated with the QuikChange site-directed mutagenesis kit (Stratagene, San Diego, CA, USA), using the pGL3Basic(−375/+1) and pGL3Basic(+1/+641) constructs as templates. The primers used for mutagenesis were 5′-GAA AAT GGG AAG ACA GC***C TTT*** **CGC** GCA CCT TCG GCC CGC G-3′ and 5′-CGC GGG CCG AAG GTG CGC G***AA A***GG CTG TCT TCC CAT TTT C-3′ for pGL3Basic(−375/+1MutA), 5′-GTG TAT GCC CAG TTC AGC TCC ***GGG*** GAT TTT CTA ATT TCG GAG-3′ and 5′-GTC CGA AAT TAG AAA ATC ***CCC*** GGA GCT GAA CTG GGC ATA CAC-3′ for pGL3Basic(−375/+1MutB) and 5′-CTC CCC ACC CGG GGA GT***A AA***T CTC CTT CTC CTG CTC C-3′ and 5′-GGA GCA GGA GAA GGA GA***T TT***A CTC CCC GGG TGG GGA G-3′ for pGL3Basic(+1/+641MutC). All the constructs were sequence verified, using 0.5–1 μg of each plasmid for sequencing with the ABI PRISM dRhodamine Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Carlsbad, CA, USA).

Transient and stable transfections

All transfections were performed using FuGene HD reagent (Roche Applied Science, Indianapolis, IN, USA). For gene expression analysis by RT-qPCR and Western blot, NIH3T3 and 3T3-L1 cells were transfected with

pCLPCX or pLPCX-KLF4 in 6-well or 100-mm dishes. For stable transfections, 3T3-L1 cells were transfected with the control vector or with any of the two clones of shRNA (Sigma-Aldrich) for mouse KLF4 described above. The transfected cells were selected for 7 days in media containing 4.0 μg/ml puromycin.

RT-qPCR analysis

Total RNA was isolated from cells using an RNeasy kit (Qiagen Inc., Valencia, CA, USA), including the optional DNase treatment step to remove potential genomic DNA contamination. The RNA was then reverse transcribed using "RevertAid H Minus First Strand cDNA Synthesis Kit" (Fermentas, Madrid, Spain), according to the manufacturer's recommendations. This cDNA was used as a template in PCR reactions. RT-qPCR was performed using FastStart SYBR Green Master Mix and a 7500 Fast RT-qPCR System (Applied Biosystems). The PCR conditions were the following: an initial denaturation step at 95 °C for 20 s and 40 cycles of 3 s at 95 °C, followed by 30 s at 60 °C. The P0 riboprotein gene was used as a control to compare the C_T of the different samples.[39] The sequences of the primers used were as follows: 5′-ACA GCC ACC CAC ACT TGT GAC TAT-3′ and 5′-AGT GTA AGG TTT CTC GCC TGT GT-3′ for *Klf4*, 5′-AGT CAC ATG GTC CAG GGC ATC TT-3′ and 5′-AAC CTT CGA GGA GGA GCT GTC T-3′ for *aP2*, 5′-TGA AGC CCA TCG AGG ACA TC-3′ and 5′-TGA CGA TCT GCC TGA GGT CTG-3′ for *Pparγ2*, 5′-GCT GAG TTG ACC AGT GAC A-3′ and AAA CCA TCC TCT GGG TCT CC-3′ for *C/ebpα* and 5′-TGA TGC AAT CCG GAT CAA ACG TGG-3′ and 5′-TTT AAG TGA TTA CTC AGG GCC CGG C-3′ for *C/ebpβ*. Finally, the P0 primers used were 5′-AAG CGC GTC CTG GCA TTG TCT-3′ and 5′-CCG CAG GGG CAG CAG TGG T-3′. For *Dlk1* and *Dlk2*, previously described primers were used.[1]

Luciferase reporter assays

The luciferase reporter plasmids along with the effector plasmids were transfected into NIH3T3 or 3T3-L1 cells at 80–90% confluence in 24-well plates using FuGene HD reagent (Roche Applied Science). The pRL-TK (Promega), carrying the *Renilla* luciferase under the control of the thymidine kinase promoter, was also co-transfected as an internal control for transfection efficiency. Cells were harvested 24 h after transfection, and luciferase activities were then analyzed using the Dual-Luciferase Assay Kit (Promega) and the MLX Microtiter Plate Luminometer (Dynex Technologies, Chantilly, VA, USA), as recommended by the manufacturer. Transfection efficiency was normalized to the *Renilla* luciferase activity measured in the same lysate.

Chromatin immunoprecipitation

ChIP analyses of 3T3-L1 and C3H10T1/2 cells were performed using the ChIP-IT Express Kit (Active Motif), following the manufacturer's recommendations. We first treated 2×10^7 confluent 3T3-L1 cells with 500 μM IBMX for 6 h and then formaldehyde-cross-linked them; DNA was sheared by sonication using a Bioruptor Sonication System (Diagenode, Belgium). C3H10T1/2 cells were formaldehyde-cross-linked, and DNA was sheared by enzymatic digestion following the ChIP-IT Express Kit instructions. The sheared chromatin was incubated with 3 μg of normal rabbit IgG (sc-2027), or the corresponding antibodies against RNA-polymerase II (sc-899), and KLF4 (H-180, sc-20691) (Santa Cruz Biotechnology, Inc.). Immunoprecipitation was performed with the magnetic beads included in the ChIP-IT Express Kit, following the manufacturer's recommendations. For PCR, 5 μl of the 100-μl total immunoprecipitated DNA was analyzed using GC-Rich PCR System (Roche Applied Science), with the oligonucleotides Dlk2MluI−212U, 5′-TAT ACG CGT GAA GGG AGG GGC GAA GAG C-3′ and Dlk2HindIII+177L, 5′-ATG AAG CTT AGG GAC CGC GTC CTC CTA GCT TC-3′. To calculate the fold increase in KLF4 binding to *Dlk2* promoter, we quantified the electrophoresed PCR reactions using Quantity One (Bio-Rad), and the corresponding KLF4 values were normalized against INPUT values.

Electrophoretic mobility shift assay

EMSA was performed by incubating at room temperature 0.5 μg of KLF4 full-length recombinant protein (Abnova) for 20 min in a 20-μl binding reaction mixture containing 10 mM Hepes (pH 8.0), 80 mM KCl, 1 mM DTT, 5% glycerol, 0.15 μg/μl bovine serum albumin, 0.4 mM $MgCl_2$, 2 μM $ZnSO_4$, 0.02% IGEPAL, 1 μg poly(dI-dC) and 40,000 cpm of ^{32}P-labeled double-stranded DNA probe. Following incubation, reaction mixtures were loaded and electrophoresed on a 6% polyacrylamide gel and subjected to autoradiography. A 100-fold molar excess of unlabeled probe was added for competition, as indicated in the corresponding figures. For competition and supershift experiments, proteins were preincubated with unlabeled probe or with the anti-KLF4 antibody [GKLF (H-180); Santa Cruz Biotechnology, Inc.] for 1 h at 4 °C. Labeled probe was then added and incubated for 20 min at room temperature. Gel shift assay oligonucleotide sequences used were as follows: 5′-GGT GTA TGC CCA GTT CAG CTC CTT TGA TTT TCT AAT TTC GGA G-3′ and 5′-CTC CGA AAT TAG AAA ATC AAA GGA GCT GAA CTG GGC ATA CAC C-3′ for KLF4-A site, 5′-TGG GCA AGA AAA TGG GAA GAC AGC AGG GCG CGC ACC TTC GGC C-3′ and 5′-GGC CGA AGG TGC GCG CCC TGC TGT CTT CCC ATT TTC TTG CCC A-3′ for KLF4-B site, 5′-GAG TCC CTC TCC TTC TCC TGC TCC TGT CAC TTG GAA CCA G-3′ and 5′-CTG GTT CCA AGT GAC AGG AGC AGG AGA AGG AGA GGG ACT C-3′ for KLF4-C site, 5′-GGT GTA TGC CCA GTT CAG CTC CGG GGA TTT TCT AAT TTC GGA G-3′ and 5′-CTC CGA AAT TAG AAA ATC CCC GGA GCT GAA CTG GGC ATA CAC C-3′ for KLF4-Amut, 5′-TGG GCA AGA AAA TGG GAA GAC AGC CTT TCG CGC ACC TTC GGC C-3′ and 5′-GGC CGA AGG TGC GCG AAA GGC TGT CTT CCC ATT TTC TTG CCC A-3′ for KLF4-Bmut, 5′-GAG TGG GTC TCC TTC TCCT G-3′ and 5′-CAG GAG AAG GAG ACC CAC TC-3′ for KLF4-Cmut and, finally, 5′-AGC CAG GCC TGG CTG GGT GGC CCT GGG GAT GTC ACC ACG CCT CTC TG-3′ and 5′-GCC CAG AGA GGC GTG GTG ACA TCC CCA GGG CCA CCC AGC CAG GCC TG-3′ for specific KLF4 G3.[26]

Preparation of cell extracts and Western blot analysis

For the preparation of total protein extracts, cells were homogenized and vortexed for 30 min on ice with hypertonic lysis buffer [50 mM Tris–HCl (pH 7.4), 300 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 2 mM DTT and 1 μg/μl of the protease inhibitor cocktail]. The samples were centrifuged for 10 min, and the supernatants containing the total protein extracts were collected. Protein concentrations were determined by using the detergent-compatible protein reagent from Bio-Rad Laboratories.

For Western blot analysis, 100 μg of total protein extract was separated by SDS-PAGE using 10% polyacrylamide-SDS gels and was transferred to nitrocellulose membranes. Western blots were performed as described previously.[40] Rabbit anti-KLF4 (H-180) was purchased from Santa Cruz Biotechnology, Inc., and mouse anti-tubulin was purchased from Sigma-Aldrich. Rabbit anti-DLK2 antibody was generated by immunization with two peptides from the DLK2 intracellular region (QDQECQVSMLPAGK and DLPPEPGKTTALC) conjugated with KLH. The immune serum was purified by affinity chromatography against the peptide DLPPEPGKTTALC. Horseradish-peroxidase-conjugated anti-rabbit and anti-mouse secondary antibodies were purchased from Santa Cruz Biotechnology, Inc.

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References

1. Nueda, M. L., Baladron, V., Garcia-Ramirez, J. J., Sanchez-Solana, B., Ruvira, M. D., Rivero, S. *et al.* (2007). The novel gene *EGFL9/Dlk2*, highly homologous to *Dlk1*, functions as a modulator of adipogenesis. *J. Mol. Biol.* **367**, 1270–1280.
2. Garcés, C., Ruiz-Hidalgo, M. J., Bonvini, E., Goldstein, J. & Laborda, J. (1999). Adipocyte differentiation is modulated by secreted delta-like (dlk) variants and requires the expression of membrane-associated dlk. *Differentiation*, **64**, 103–114.
3. Bauer, S. R., Ruiz-Hidalgo, M. J., Rudikoff, E. K., Goldstein, J. & Laborda, J. (1998). Modulated expression of the epidermal growth factor-like homeotic protein dlk influences stromal-cell–pre-B-cell interactions, stromal cell adipogenesis, and pre-B-cell interleukin-7 requirements. *Mol. Cell. Biol.* **18**, 5247–5255.
4. Kaneta, M., Osawa, M., Sudo, K., Nakauchi, H., Farr, A. G. & Takahama, Y. (2000). A role for Pref-1 and HES-1 in thymocyte development. *J. Immunol.* **164**, 256–264.
5. Raghunandan, R., Ruiz-Hidalgo, M., Jia, Y., Ettinger, R., Rudikoff, E., Riggins, P. *et al.* (2008). Dlk1 influences differentiation and function of B lymphocytes. *Stem Cells Dev.* **17**, 495–507.
6. Abdallah, B. M., Jensen, C. H., Gutierrez, G., Leslie, R. G., Jensen, T. G. & Kassem, M. (2004). Regulation of human skeletal stem cells differentiation by Dlk1/Pref-1. *J. Bone Miner. Res.* **19**, 841–852.
7. Floridon, C., Jensen, C. H., Thorsen, P., Nielsen, O., Sunde, L., Westergaard, J. G. *et al.* (2000). Does fetal antigen 1 (FA1) identify cells with regenerative, endocrine and neuroendocrine potentials? A study of FA1 in embryonic, fetal, and placental tissue and in maternal circulation. *Differentiation*, **66**, 49–59.
8. Okamoto, M., Takemori, H., Halder, S. K., Nonaka, Y. & Hatano, O. (1998). Implication of ZOG protein (zona glomerulosa-specific protein) in zone development of the adrenal cortex. *Endocr. Res.* **24**, 515–520.
9. Van Limpt, V. A., Chan, A. J., Van Sluis, P. G., Caron, H. N., Van Noesel, C. J. & Versteeg, R. (2003). High delta-like 1 expression in a subset of neuroblastoma cell lines corresponds to a differentiated chromaffin cell type. *Int. J. Cancer*, **105**, 61–69.
10. Costaglioli, P., Come, C., Knoll-Gellida, A., Salles, J., Cassagne, C. & Garbay, B. (2001). The homeotic protein dlk is expressed during peripheral nerve development. *FEBS Lett.* **509**, 413–416.
11. Laborda, J., Sausville, E. A., Hoffman, T. & Notario, V. (1993). *dlk*, a putative mammalian homeotic gene differentially expressed in small cell lung carcinoma and neuroendocrine tumor cell line. *J. Biol. Chem.* **268**, 3817–3820.
12. van Limpt, V., Chan, A., Caron, H., Sluis, P. V., Boon, K., Hermus, M. C. & Versteeg, R. (2000). SAGE analysis of neuroblastoma reveals a high expression of the human homologue of the *Drosophila Delta* gene. *Med. Pediatr. Oncol.* **35**, 554–558.
13. Samulewicz, S. J., Seitz, A., Clark, L. & Heber-Katz, E. (2002). Expression of preadipocyte factor-1 (Pref-1), a delta-like protein, in healing mouse ears. *Wound Repair Regen.* **10**, 215–221.
14. Nueda, M. L., Baladrón, V., Sánchez-Solana, B., Ballesteros, M. A. & Laborda, J. (2007). The EGF-like protein dlk1 inhibits notch signaling and potentiates adipogenesis of mesenchymal cells. *J. Mol. Biol.* **367**, 1281–1293.
15. Sánchez-Solana, B., Nueda, M. L., Ruvira, M. D., Ruiz-Hidalgo, M. J., Monsalve, E. M., Rivero, S. *et al.* (2011). The EGF-like proteins DLK1 and DLK2 function as inhibitory non-canonical ligands of NOTCH1 receptor that modulate each other's activities. *Biochim. Biophys. Acta*, **1813**, 1153–1164.
16. Rivero, S., Ruiz-García, A., Díaz-Guerra, M. J. M. & Laborda, J. (2011). Characterization of a proximal Sp1 response element in the mouse Dlk2 gene promoter. *BMC Mol. Biol.* **12**, 52.
17. Kaczynski, J., Cook, T. & Urrutia, R. (2003). Sp1- and Krüppel-like transcription factors. *Genome Biol.* **4**, 206.
18. Suske, G., Bruford, E. & Philipsen, S. (2005). Mammalian SP/KLF transcription factors: bring in the family. *Genomics*, **85**, 551–556.

19. Pearson, R., Fleetwood, J., Eaton, S., Crossley, M. & Bao, S. (2008). Krüppel-like transcription factors: a functional family. *Int. J. Biochem. Cell Biol.* **40**, 1996–2001.
20. Banerjee, S. S., Feinberg, M. W., Watanabe, M., Gray, S., Haspel, R. L., Denkinger, D. J. *et al.* (2003). The Krüppel-like factor KLF2 inhibits peroxisome proliferator-activated receptor-γ expression and adipogenesis. *J. Biol. Chem.* **278**, 2581–2584.
21. Sue, N., Jack, B. H., Eaton, S. A., Pearson, R. C., Funnell, A. P., Turner, J. *et al.* (2008). Targeted disruption of the basic Krüppel-like factor gene (*Klf3*) reveals a role in adipogenesis. *Mol. Cell. Biol.* **28**, 3967–3978.
22. Mori, T., Sakaue, H., Iguchi, H., Gomi, H., Okada, Y., Takashima, Y. *et al.* (2005). Role of Krüppel-like factor 15 (KLF15) in transcriptional regulation of adipogenesis. *J. Biol. Chem.* **280**, 12867–12875.
23. Oishi, Y., Manabe, I., Tobe, K., Tsushima, K., Shindo, T., Fujiu, K. *et al.* (2005). Krüppel-like transcription factor KLF5 is a key regulator of adipocyte differentiation. *Cell Metab.* **1**, 27–39.
24. Li, D., Yea, S., Dolios, G., Martignetti, J. A., Narla, G., Wang, R. *et al.* (2005). Regulation of Krüppel-like factor 6 tumor suppressor activity by acetylation. *Cancer Res.* **65**, 9216–9225.
25. Alder, J. K., Georgantas, R. W., III, Hildreth, R. L., Kaplan, I. M., Morisot, S., Yu, X. *et al.* (2008). Kruppel-like factor 4 is essential for inflammatory monocyte differentiation *in vivo*. *J. Immunol.* **180**, 5645–5652.
26. Birsoy, K., Chen, Z. & Friedman, J. (2008). Transcriptional regulation of adipogenesis by KLF4. *Cell Metab.* **7**, 339–347.
27. Chen, X., Johns, D. C., Geiman, D. E., Marban, E., Dang, D. T., Hamlin, G. *et al.* (2001). Krüppel-like factor 4 (gut-enriched Krüppel-like factor) inhibits cell proliferation by blocking G1/S progression of the cell cycle. *J. Biol. Chem.* **276**, 30423–30428.
28. Feinberg, M. W., Wara, A. K., Cao, Z., Lebedeva, M. A., Rosenbauer, F., Iwasaki, H. *et al.* (2007). The Kruppel-like factor KLF4 is a critical regulator of monocyte differentiation. *EMBO J.* **26**, 4138–4148.
29. Ghaleb, A. M., Aggarwal, G., Bialkowska, A. B., Nandan, M. O. & Yang, V. W. (2008). Notch inhibits expression of the Krüppel-like factor 4 tumor suppressor in the intestinal epithelium. *Mol. Cancer Res.* **6**, 1920–1927.
30. Yoon, H. S., Chen, X. & Yang, V. W. (2003). Krüppel-like factor 4 mediates p53-dependent G1/S cell cycle arrest in response to DNA damage. *J. Biol. Chem.* **278**, 2101–2105.
31. Yoon, H. S. & Yang, V. W. (2004). Requirement of Krüppel-like factor 4 in preventing entry into mitosis following DNA damage. *J. Biol. Chem.* **279**, 5035–5041.
32. Yusuf, I., Kharas, M. G., Chen, J., Peralta, R. Q., Maruniak, A., Sareen, P. *et al.* (2008). KLF4 is a FOXO target gene that suppresses B cell proliferation. *Int. Immunol.* **20**, 671–681.
33. Soukas, A., Socci, N. D., Saatkamp, B. D., Novelli, S. & Friedman, J. M. (2001). Distinct transcriptional profiles of adipogenesis *in vivo* and *in vitro*. *J. Biol. Chem.* **276**, 34167–34174.
34. Lee, H. J., Kang, Y. M., Moon, C. S., Joe, M. K., Lim, J. H., Suh, Y. H. *et al.* (2009). KLF4 positively regulates human ghrelin expression. *Biochem. J.* **420**, 403–411.
35. Yang, T., Chen, C., Zhang, B., Huang, H., Wu, G., Wen, J. & Liu, J. (2010). Induction of Krüppel-like factor 4 by high-density lipoproteins promotes the expression of scavenger receptor class B type I. *FEBS J.* **277**, 3780–3788.
36. Nandan, M. O. & Yang, V. W. (2009). The role of Krüppel-like factors in the reprogramming of somatic cells to induced pluripotent stem cells. *Histol. Histopathol.* **24**, 1343–1355.
37. Simmen, R. C., Pabona, J. M., Velarde, M. C., Simmons, C., Rahal, O. & Simmen, F. A. (2010). The emerging role of Krüppel-like factors in endocrine-responsive cancers of female reproductive tissues. *J. Endocrinol.* **204**, 223–231.
38. Jing, K., Heo, J. Y., Song, K. S., Seo, K. S., Park, J. H., Kim, J. S. *et al.* (2009). Expression regulation and function of Pref-1 during adipogenesis of human mesenchymal stem cells (MSCs). *Biochim. Biophys. Acta*, **1791**, 816–826.
39. Laborda, J. (1991). 36B4 cDNA used as an estradiol-independent mRNA control is the cDNA for human acidic ribosomal phosphoprotein PO. *Nucleic Acids Res.* **19**, 3998.
40. Baladrón, V., Ruiz-Hidalgo, M. J., Nueda, M. L., Díaz-Guerra, M. J., García-Ramírez, J. J., Bonvini, E. *et al.* (2005). dlk acts as a negative regulator of Notch1 activation through interactions with specific EGF-like repeats. *Exp. Cell Res.* **303**, 343–359.