Krüppel-like Factor-6 Promotes Preadipocyte Differentiation through Histone Deacetylase 3-dependent Repression of DLK1*

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Preadipocyte differentiation occurs during distinct periods of human development and is a key determinant of body mass. Transcriptional events underlying adipogenesis are beginning to emerge, but the link between chromatin remodeling of specific target loci and preadipocyte differentiation remains elusive. We have identified Krüppel-like factor-6 (KLF6), a recently described tumor suppressor gene, as a repressor of the proto-oncogene Delta-like 1 (Dlk1), a gene encoding a transmembrane protein that inhibits adipocyte differentiation. Forced expression of KLF6 strongly inhibits Dlk1 expression in preadipocytes and NIH 3T3 cells in vivo, whereas down-regulation of KLF6 in 3T3-L1 cells by small interfering RNA prevents adipogenesis. Repression of Dlk1 requires HDAC3 deacetylase activity, which is recruited to the endogenous Dlk1 promoter where it interacts with KLF6. Our studies identify the interaction between HDAC3 and KLF6 as a potential mechanism underlying human adipogenesis, and highlight the role of KLF6 as a multifunctional transcriptional regulator capable of mediating adipocyte differentiation through gene repression.

Adipocyte differentiation requires coordinated expression of general and tissue-specific regulatory proteins in a defined sequence (1, 2). A critical regulator of adipogenesis is Dlk1, also called preadipocyte factor-1, whose sustained expression prevents differentiation of 3T3-L1 preadipocytes into adipocytes (3). Dlk1 encodes DLK1, a transmembrane epidermal growth factor repeat domain-containing protein highly expressed in preadipocytes and other cells (4). The abrupt down-regulation of Dlk1 following hormonal stimulation in preadipocytes is an early and necessary event in the phenotypic conversion to fat cells. Forced expression of Dlk1 prevents adipogenesis, whereas enforced down-regulation enhances adipocyte differentiation. Following the reduction in DLK1, rapid induction of adipogenic transcription factors, including SREBP1, C/EBPα/δ and C/EBPα, leads to terminal differentiation of adipocytes, and expression of adipocyte proteins, including leptin and adipin (1, 2).

Our previous work (5) has explored the activity of a transcription factor, KLF6 (also known as Zf9 or CPBP), a ubiquitously expressed 283-amino acid Krüppel-like zinc finger protein and a member of a growing family of related transcriptional regulators. KLF6 contains an 82-amino acid C-terminal DNA-binding domain identical to other Krüppel-like factors, and a 201-amino acid N-terminal activation domain, whose only homology is to KLF7 in its N-terminal 41 amino acids (6). KLF6 was originally identified as a rapidly induced mRNA following activation of hepatic stellate cells, a mesenchymal liver cell, during liver injury (7). Most interestingly, stellate cells harbor many features of adipocytes, including the storage of lipids as vitamin A esters (retinoids), the production of leptin, and phenotypic plasticity in defined biologic contexts (8). Transcriptional targets of KLF6 include transforming growth factor-β1 and its receptors (9), urokinase-type plasminogen activator (10), and the human immunodeficiency virus-long terminal repeat (11). Recently, we have established Krüppel-like factor 6 (KLF6) as a novel tumor suppressor gene frequently mutated in human prostate and colon cancers (12, 13). A key mechanism of tumor suppression by KLF6 is the transcriptional up-regulation of p21WAF1/CIP1, a cyclin-dependent kinase inhibitor whose induction also accounts for the growth suppressive activity of the tumor suppressor p53 (12).

Although many studies have described down-regulation of Dlk1 during adipocyte differentiation, the underlying mechanisms regulating this event have not been well characterized. The phenotype resemblance of hepatic stellate cells (the original source of KLF6) to adipocytes led us to explore a potential role of KLF6 in adipogenesis, particularly because stellate cell activation in liver injury is also accompanied by KLF6 induction (7, 8). In fact, previous studies have documented the induction of KLF6 during adipocyte differentiation, but its role in this process has been unclear (14).

Our preliminary experiments, using a commercial membrane array (Clontech), identified Dlk1 as a strongly repressed...
mRNA following induction of KLF6 in fibroblasts (data not shown). The aim of the present study was to investigate whether Dlk1 is a transcriptional target of KLF6 and, if so, to elucidate the mechanism of Dlk1 gene regulation by KLF6. Our data demonstrate that KLF6 promotes adipocyte differentiation by transcriptionally repressing Dlk1 expression, and this repression specifically requires the deacetylase activity of HDAC3.

MATERIALS AND METHODS

Expression Plasmids—pCIneo-KLF6 (human) expression plasmid was constructed as described previously (15, 20) in mammalian expression system were purchased and prepared for cloning (Invitrogen). The expression plasmid pVgRXR (Invitrogen) was constructed as described previously (7, 8). pCDNA3-HDAC3-FLAG (Invitrogen) and pIND-FLAG/HDAC3 construct. After 48 h, transfected 3T3-L1 cells were washed three times with serum-free Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic. Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Invitrogen) and 1% antibiotic-antimycotic. The resulting HDAC3-containing DNA fragment was then ligated into the EcoRI site of pBluescript SK(+) (Stratagene).

Stable Transfection and Tetacycline-inducible Expression of KLF6 in Cultured Cells—To establish cell lines stably expressing KLF6, NIH 3T3, 293, and 293T cells were transfected with 10 μg of pCIneo-KLF6, with or without pCDNA3-HDAC3, as indicated in the text, together with the expression plasmid pVgRXR (Invitrogen). The expression plasmid pIND for the ecdysone-inducible gene expression, and this repression specifically requires the deacetylase activity of HDAC3.

Expression plasmids pVgRXR and pIND for the ecdysone-inducible gene expression system constructs pTet-spliace and pTet-tTAk were purchased from Invitrogen. Expression plasmids pVgRXR and pIND for the ecdysone-inducible mammalian expression system were purchased and prepared for cloning (Invitrogen). The expression plasmid pVgRXR (Invitrogen) was used to establish 3T3-L1 cells under Zeocin (Invitrogen) selection (600 μg/ml) to produce cell lines, induced to differentiate 3T3-L1 cell line following the manufacturer's procedures (Invitrogen). Insertion of the FLAG-HDAC3 fragment into the pIND vector expression system was digested with pCDNA3-HDAC3-FLAG with EcoRI and NotI. The resulting HDAC3-containing DNA fragment was then ligated into the EcoRI site of pBluescript. The cDNA insert was confirmed by DNA sequence analysis. Cell culture and differentiation of adipocytes.

Stable Transfection—3T3-L1, NIH 3T3, HeLa, 293 and 293T cells lines were obtained from the American Tissue Culture Collection (ATCC). 3T3-L1 cells were grown in Dulbecco's modified Eagle's medium, supplemented with 10% calf serum (Invitrogen), 100 units/ml penicillin and 100 units/ml streptomycin, and 2 mM l-glutamine (Invitrogen). NIH 3T3, 293, and 293T cells were cultured in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum (Invitrogen) and with or without pCDNA3-HDAC3, as indicated in the text, together with the HDAC3-containing DNA fragment. The HDAC3-containing DNA fragment was then ligated into the EcoRI site of pBluescript SK(+) (Stratagene).

To establish cell lines stably expressing KLF6, NIH 3T3, 293, and 293T cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% calf serum, 1% antibiotic-antimycotic, and 0.5 mM isobutylmethylxanthine, 1 μM dexamethasone (Sigma), and 1 μM insulin (Sigma). Culture medium was changed every 3 days. For histology, cells were fixed with 2% formaldehyde, 0.2% glutaraldehyde in PBS for 15 min, rinsed in PBS, and stained with Oil Red O (0.6% in 60% isopropyl alcohol for 25 min).

Transfection Assays—Transient transfections were performed using Lipofectamine 2000 reagent (Invitrogen). For luciferase assay, 3T3-L1 cells or HeLa cells cultured in 10-cm plates (Corning Glass) were transfected with 10 μg of pCMV-EGFP empty vector or pCMV-KLF6, with or without pCDNA3-HDAC3, as indicated in the text, together with the HDAC3 fragment. The HDAC3-containing DNA fragment was then ligated into the EcoRI site of pBluescript SK(+) (Stratagene).

Stable Transfection and Tetracycline-inducible Expression of KLF6 in Cultured Cells—To establish cell lines stably expressing KLF6, NIH 3T3 cells line that expressed pTet-pTak, followed by selection with histidine-deficient Dulbecco's modified Eagle's medium (Invitrogen) and 2 μg/ml tetracycline (Sigma). For induction of KLF6, cells were washed three times with serum-free Dulbecco's modified Eagle's medium and changed to medium without tetracycline.

Stable Transfection and Ponerasterone A-inducible Expression of FLAG-tagged HDAC3 in 3T3-L1 Preadipocytes—Following the insertion of FLAG-tagged HDAC3 into expression vector pIND (see above), stable transfectants of 3T3-L1 cells carrying the pVgRXR transgene under Zeocin (Invitrogen) drug selection were used following transfection of the pIND-FLAG-HDAC3 construct. After 48 h, transfected 3T3-L1 cells were placed under growth selection with 400 μg/ml G418 (geneticin, Invitrogen) and 400 μg/ml Zeocin (Invitrogen) for 2 weeks to select for both transgene- and individual populations of stably transfected 3T3-L1 cells. Individual clones were then selected for the induction of the HDAC3-targeted gene using treatment with analogone A (5 μM) (Sigma) and analyzed by immunoblot studies.

Real Time Reverse-Transcription-PCR of Total RNA—3T3-L1 cells were cultured in 10-cm plates, induced to differentiate 3T3-L1 cell line following the manufacturer's procedures (Invitrogen). Insertion of the FLAG-HDAC3 fragment into the pIND vector expression system was digested with pCDNA3-HDAC3-FLAG with EcoRI and NotI. The resulting HDAC3-containing DNA fragment was then ligated into the EcoRI site of pBluescript. The cDNA insert was confirmed by DNA sequence analysis. Cell culture and differentiation of adipocytes.

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RNA was isolated using TRIzol reagent® and chloroform extraction. Total cellular RNA was extracted using Trizol reagent® and chloroform extraction. Total cellular RNA was extracted using Trizol reagent® and chloroform extraction. Total cellular RNA was extracted using Trizol reagent® and chloroform extraction. Total cellular RNA was extracted using Trizol reagent® and chloroform extraction. Total cellular RNA was extracted using Trizol reagent® and chloroform extraction. Total cellular RNA was extracted using Trizol reagent® and chloroform extraction. Total cellular RNA was extracted using Trizol reagent® and chloroform extraction. Total cellular RNA was extracted using Trizol reagent® and chloroform extraction.
formed using a commercial gel shift assay system (Promega). The oligonucleotide sequences from the Dlk1 promoter were as follows (with putative KLF6-binding site underlined, and mutated position(s) indicated): wild type, 5′-GCCGAGGCGTGGGCGTGCGGGC-3′; antisense, 5′-GCCCGCCGCCGCCAGCGCTCCTGGG-3′; mutant, sense, 5′-GGGAAAAATTGAAATGAAAGGGC-3′; antisense, 5′-GGGCGCCCTTTTATTCCCTGGG-3′.

Single-stranded oligonucleotides (Genelink) were labeled with 32P-ATP using T4 polynucleotide kinase. Double-stranded oligonucleotide probes were created by annealing complementary single-stranded oligonucleotides. Nuclear extracts from 3T3-L1 preadipocytes were prepared from cells induced at 18 °C for 12–15 h, and the hexahistidine-tagged components were purified by Talon cobalt metal affinity chromatography using P100 buffer with 100 mM imidazole to elute. Similar experiments were performed for untagged HDAC3 expressed on its own under identical conditions.

**In Vitro HDAC Assay—**Histone deacetylase activity was assayed essentially as described (18) with 50 μl of crude cell extract and then immunoprecipitated with rabbit polyclonal anti-KLF6 antibody for 2.5 h at 37 °C. Immunoprecipitates of human KLF6 were washed three times at 4 °C in a low stringency wash containing 0.1% Nonidet P-40 in IP wash buffer (0.5% Nonidet P-40, 10 mM Tris-HCl (pH 7.5), and 50 μl of poly(dI-dC); with or without unlabeled competitor oligonucleotides. A monoclonal antibody was generated for use in supershift assays. To do so, the N terminus of KLF6 (amino acids 1–200) was expressed as a recombinant glutathione S-transferase fusion protein in Escherichia coli, purified, and inoculated into BALB/c mice. Hybridomas were screened by enzyme-linked immunosorbent assay. The monoclonal antibody “2A2” was found to be suitable for KLF6 detection in Western blotting. The monoclonal antibody was incubated with the above nuclear extract in the presence of IP wash buffer (0.5% Nonidet P-40, 50 mM Tris-HCl (pH 7.4), and 500 mM KCl, 10 mM Tris-HCl (pH 7.5), and 50 μl of poly(dI-dC); with or without unlabeled competitor oligonucleotides.

**Chromatin Immunoprecipitation Assay—**Chromatin immunoprecipitation assays were performed using a commercial assay kit (Upstate Biotechnologies, Inc.). Briefly, 2 × 106 3T3-L1 cells at the 5th day of differentiation were cross-linked with 1% formaldehyde for 10 min at 37 °C, followed by cell lysis and sonication. The DNA fragments were cross-linked to DNA were immunoprecipitated with 10 μg of anti-Zif/ KLF6 antibody (R-173) (Santa Cruz Biotechnology) or two different anti-HDAC3 antibodies (a gift from Dr. Eric Verdin, and anti-HDAC3 antibody from Upstate Biotechnology, Inc., respectively) and 40 μl of salmon sperm DNA/protein-A-agarose beads. The protein-A-agarose-protein complexes were washed extensively and eluted, according to the manufacturer’s recommendations. The precipitated DNA was recovered by phenol/chloroform extraction and ethanol precipitation in the presence of 1 μl of the organic phase containing ethyl acetate and quantified by liquid scintillation counting. Samples were assayed in triplicate (in four separate experiments), and the nonenzymatic release of label was subtracted to obtain the final value.

**HDAC Pre-absorption Deacetylation Assays—** Cultures of 293T cells were transfected with FLAG-tagged KLF6 expression vector in 100-mm dishes. The cell lysate was precipitated for 30 min at 4 °C. The supernatant was immunoprecipitated at 4 °C for 1 h using 4 μl of the organic phase containing ethyl acetate and then centrifuged at 12,000 rpm for 1 min. 400 μl of the organic phase containing ethyl acetate was removed and mixed with 1 ml of scintillant (OCS, Amersham Biosciences). Release of [3H]acetic acid from the histone H4 peptide was measured in counts/min using a scintillation counter (model 1216 LKB-Wallace).

Inputs of each of the nuclear extracts used in the deacetylation assays were evaluated using immunoblot analysis to determine the presence or absence of specific HDAC protein in the nuclear extracts used in the HDAC assays. Equivalent amounts of nuclear extract were loaded onto 10% SDS-polyacrylamide gels and separated by electrophoresis. Separated proteins were then transferred onto polyvinylidene difluoride membranes and blotted with rabbit anti-human HDAC3 and HDAC2 antisera. Membranes were visualized using ECL by the manufacturer’s instructions (Amersham Biosciences).
KLF6 Represses Dlk1 Expression—We examined KLF6 and Dlk1 expression during differentiation of 3T3-L1 preadipocyte differentiation. 3T3-L1 cells were differentiated into adipocytes following incubation with a differentiation mixture (see "Materials and Methods"). KLF6 protein levels were determined by SDS-PAGE/Western analysis using cells lysates from days 0 to 8 during differentiation. Dlk1 mRNA expression was determined by real time quantitative reverse transcription-PCR. KLF6 represses Dlk1 gene expression in tetracycline-regulated NIH 3T3 cells. Northern blot demonstrates that upon KLF6 induction through tetracycline withdrawal, Dlk1 mRNA level is markedly diminished. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. C, KLF6 represses Dlk1 gene expression in 3T3-L1 preadipocytes. Western analysis demonstrates that expression of KLF6 via stable transfection in 3T3-L1 cells reduces the level of endogenous Dlk protein.

RESULTS

KLF6 Represses Dlk1 Expression—We examined KLF6 and Dlk1 expression during differentiation of 3T3-L1 cells from preadipocytes into adipocytes following incubation with a defined hormonal mixture that includes dexamethasone, insulin, and isobutylmethylxanthine (1, 3, 22) (Fig. 1A). Following the onset of differentiation by hormonal stimulation, KLF6 was induced in a biphasic pattern, with a transient induction immediately following adipogenic stimulation on day 0, followed by a sustained expression accompanying terminal differentiation, between days 4 and 8. Each peak of KLF6 protein at days 0 and 4 was followed by an abrupt decrease of Dlk1 mRNA as assessed by real time PCR, first by ~40% and then by more than 80%. To examine whether KLF6 transcriptionally represses Dlk1, we studied Dlk1 mRNA levels in a 3T3 fibroblast line with tetracycline-regulated KLF6 expression (12), and we examined Dlk1 protein levels in 3T3-L1 cells with forced expression of KLF6 following stable transfection. As shown in Fig. 1, B and C, KLF6 markedly reduced Dlk1 expression in both KLF6 tetracycline-regulated NIH 3T3 cells and in 3T3-L1 preadipocytes stably transfected with KLF6.

Dlk1 Is a Direct Transcriptional Target of KLF6—To establish Dlk1 as a potential target of KLF6 transactivation/repression, electrophoretic mobility shift assays were performed using nuclear extracts from 3T3-L1 preadipocytes and an oligonucleotide targeting the partial sequence in exons 2 and 3 of the Dlk1 mRNA, spanning the transcriptional start site, a region containing a GC box to which KLF6 is known to bind (2). These data further confirm a role of KLF6 in promoting adipocyte differentiation through repression of Dlk1.

Silencing of KLF6 Results in Sustained Dlk1 Expression and Impaired Adipogenesis—If KLF6 promotes adipocyte differentiation through repression of Dlk1, then diminished expression of KLF6 should lead to a decrease of adipogenesis through sustained Dlk1 (or DLK) expression and concomitant reduced expression of downstream adipogenic markers. To test this prediction, we examined the effects of KLF6 silencing on adipocyte differentiation of 3T3-L1 cells using retroviral gene transduction (Fig. 3), as well as stable gene expression via liposome-mediated transfection (data not shown). KLF6-specific gene silencing was accomplished by RNA interference targeting the partial sequence in exons 2 and 3 of the KLF6 genomic sequence (Fig. 3A). Retroviral gene transduction of the pSuperRetro vector containing the target KLF6 sequence generated cells with reduced expression of endogenous KLF6 compared with the control cell line infected only with the pSuperRetro control vector (Fig. 3B). We then evaluated the impact of retroviral siRNA-mediated down-regulation of KLF6 on expression of Dlk1 and other downstream adipogenic markers, including C/EBPα, PPARγ, adipin, aP2, and steroyl-CoA desaturase-1 (SCD1) following induction of adipocyte differentiation. As shown in Fig. 3, B and C, the level of Dlk1 failed to decrease in cells in which KLF6 had been silenced compared with vector-infected control cells, whose Dlk1 level markedly diminished during adipocyte differentiation. As predicted, the expression of key adipogenic mRNAs was significantly attenuated in siRNA-KLF6 cell lines but not in control cells (Fig. 3C).

To evaluate the degree of differentiation toward terminal adipocytes, cells in the 7th day of differentiation were stained with Oil Red O to reveal fat droplet content. As shown in Fig. 3D, there were significantly fewer terminally differentiated adipocytes in cultures in which KLF6 had been silenced than in control cultures. These data further confirm a role of KLF6 in promoting adipocyte differentiation through repression of Dlk1.

The Repression of Dlk1 by KLF6 Requires HDAC Activity—Because gene repression is often associated with the activity of histone deacetylases (HDACs), we examined whether the repression of Dlk1 by KLF6 was affected by the presence of the HDAC inhibitor TSA (23) (Fig. 4A). TSA completely abolished KLF6-dependent repression of Dlk1, indicating that HDAC activity is required for Dlk1 repression by KLF6.
present at the KLF6-containing transcriptional complex. To explore this possibility, we directly assayed HDAC activity in this transcriptional complex (Fig. 4B). KLF6 was immunoprecipitated under nondenaturing conditions from extracts of the 293T cell line and incubated with 3H-labeled and -acetylated histone H4 peptides. The KLF6-immunoprecipitate harbored HDAC activity, which was reduced by 80% by TSA. These data indicate that KLF6 represses \(\text{Dlk1} \) expression through recruitment of TSA-sensitive HDAC activity.

To determine whether an interaction could exist between KLF6 and HDACs, 3T3-L1 cells were subjected to induced differentiation for 8 days, and cells were recovered and prepared as nuclear lysate. Nuclear lysates were then immunoprecipitated using a number of antisera directed specifically at HDAC1–4, respectively. Results shown (Fig. 4C) demonstrate the association of endogenous HDAC3 with KLF6 in the post-induced 3T3-L1 cells, whereas antisera against HDAC1, -2, or -4 failed to detect a signal (data not shown). This finding suggested that HDAC3, which has already been shown to play a role in adipogenesis (24), is a candidate for interaction with KLF6.

**HDAC3 Acts as a Co-repressor of KLF6**—Among the HDACs identified and characterized to date, class I HDACs (HDAC1–3) and class II HDACs (HDAC4, -5, and -7 but not -6) are TSA-sensitive (25). Moreover, class II HDAC activity is dependent on a multiprotein complex containing HDAC3 (15). To determine which HDAC regulates \(\text{Dlk1} \) repression by KLF6, we explored their interaction with KLF6 by reciprocal co-immunoprecipitation following their transient transfection into 293T cells. By using this approach, KLF6 interacted with HDAC3 (Fig. 5, A and B) but not with HDAC1 or -2, or class II HDACs (data not shown). To localize further the region(s) of KLF6 responsible for HDAC3 interaction, truncation/deletion mutants of KLF6 were co-transfected with HDAC3, which localized an HDAC3 interaction domain between amino acids 28 and 128 (Fig. 5C).

To determine whether HDAC3 and KLF6 interact directly under native conditions, KLF6 protein containing an N-terminal combination hexahistidine-thioredoxin-TEV protease site fusion tag was co-expressed with HDAC3 in \(E. \) coli using a bicistronic expression plasmid (27). Talon cobalt metal affinity chromatography was used to purify the hexahistidine-tagged KLF6 fusion protein together with associated proteins, and the fractions were analyzed on Western blots using antisera against human KLF6 or HDAC3. Whereas HDAC3 alone bound minimally to the Talon column (Fig. 5D, top panel, lanes 1–3), when co-expressed with tagged KLF6, HDAC3 bound to and eluted from the metal affinity column (lanes 4–7), indicating a likely association between HDAC3 and KLF6. As shown Fig. 5D, lower panel, tagged KLF6 bound to and eluted from the Talon column as expected, although a significant proportion of expressed KLF6 appeared to be degraded at the C terminus. The identity of the bands in Fig. 5D, lower panel, lane 7, was corroborated by removing the N-terminal tag with TEV protease, which increased the mobility of the KLF6 bands by amounts corresponding to the HisTrxN tag (Fig. 5D, bottom).
Because bacterial co-expression of HDAC3 and KLF6 is sufficient for complex formation, our results suggest that HDAC3 can interact directly with KLF6 without other accessory or intermediate eukaryotic factors.

**HDAC3 Is Essential for KLF6-associated HDAC Activity**—To determine whether HDAC3 is essential for the deacetylase activity associated with KLF6, we took advantage of the fact that HDAC3 is dispensable in bacteria. We first transfected 293 cells with a pCIneo vector containing the KLF6 cDNA and verified that the expression of KLF6 was induced at the mRNA level (Fig. 5). We then tested whether the HDAC activity associated with KLF6 complex was abolished by the addition of the HDAC inhibitor TSA. As expected, TSA completely blocked the HDAC activity associated with KLF6 complex, indicating that HDAC3 is essential for KLF6-associated HDAC activity. These findings provide strong evidence that HDAC3 is essential for the deacetylase activity associated with KLF6.
activity associated with KLF6, we immunodepleted nuclear extracts from 293T cells transfected with FLAG-tagged KLF6 of specific HDACs using rabbit polyclonal antisera against several of the HDAC species, including HDAC1, HDAC2, HDAC3, HDAC4, HDAC5, and HDAC7. Deacetylase assays were conducted with anti-FLAG immunoprecipitates on each of the nuclear extracts depleted of each HDAC by immunabsorption. By using this approach, we found that HDAC3 is required for the deacetylase activity associated with KLF6 (Fig. 6A). In contrast, immunodepletion of HDAC2 (Fig. 6A) and HDAC4–6 (data not shown) did not diminish deacetylase activity associated with KLF6. These data indicate that HDAC3 is responsible for the protein deacetylase activity associated with KLF6. To validate the use of HDAC3 antisera to remove HDAC3 from the nuclear lysate, an immunoblot of nuclear lysate before and after absorption of HDAC3 by immunoprecipitation (Fig. 6B) confirmed the removal of HDAC3 from the supernatant used for KLF6 immunoprecipitation. We also tested the presence of KLF6 following the immunodepletion of HDAC3 and found a 60% loss in KLF6 protein as measured by densitometry (Fig. 6B). These studies confirm the dependence of HDAC3 for deacetylase activity associated with KLF6.

To determine whether KLF6 also specifically associates with other endogenous HDACs in differentiated 3T3-L1 cells, we performed immunoprecipitation/immunoblot analysis from nuclear lysates. As demonstrated in Fig. 6C, KLF6 is predominantly associated with HDAC3; very minor interactions are seen in the presence of the HDAC1 and HDAC7 immunoprecipitates but not with HDAC2, -4, and -5. To ensure that levels of protein lysate used between each sample were relatively equivalent, immunoblots were also probed with anti-β-actin antisera (Fig. 6C). Furthermore, we examined the presence of each of the HDACs in the 3T3-L1 cell nuclear lysate as well, with only significant expression of HDAC3 detected (Fig. 6C).

HDAC Activity Associated with KLF6 Is Independent of the Rb Protein—Recent studies implicate pRb110 in the regulation of adipogenesis through its association with HDAC3 (24). To exclude the possibility that HDAC activity associated with KLF6 is dependent on HDAC(s) that also associate with pRb110, we further exploited the use of immunodepleted nuclear extracts to test whether depletion of pRb could eliminate or reduce HDAC activity associated with KLF6. Immunoprecipitation of FLAG-tagged KLF6 from transfected 293T cells was used to measure HDAC activity following the pre-absorption of pRb110 from nuclear extracts. This pre-absorption of anti-KLF6 antibody. Bottom panel, FLAG epitope-tagged KLF6 and HDAC3 constructs were co-transfected. Anti-FLAG antibody was used for immunoprecipitation, and anti-HDAC3 antibody was used for immunoblot. E, KLF6 co-immunoprecipitated with endogenous HDAC3 in HeLa cells. An experiment identical to that shown before was performed in HeLa cells. C, mapping of the HDAC3-interaction domain in KLF6. A series of FLAG epitope-tagged KLF6 deletion constructs were co-transfected with the HDAC3 expression construct into 293T cells, followed by immunoprecipitation using an anti-FLAG antibody, and blotted with anti-HDAC3 antibody. The results indicate that amino acids 28–128 of KLF6 are required for interaction with HDAC3. D, KLF6 and HDAC3 complex when co-expressed in E. coli. Top panel, Western blot probed with anti-HDAC3 antibodies for HDAC3 expressed alone and HDAC3 co-expressed with HisTrxN-tagged KLF6. Lanes 1–3 show soluble cell extract, Talon metal affinity flow-through, and Talon eluent, respectively, for HDAC3 expressed alone. Lanes 4–8 show soluble cell extract, Talon metal affinity flow-through, and Talon eluent, respectively, for HDAC3 co-expressed with HisTrxNKL6. A faint 50-kDa HDAC3 immunoreactive band can be detected in lane 3 on an overexposed Western blot (data not shown). Bottom panel, Western blot of the same samples from the experiment described in the top panel probed with anti-KLF6 antibodies for co-expression of the HisTrxNKL6 protein (lanes 4–8).
pRb110 did not reduce HDAC activity associated with KLF6 (Fig. 6D), indicating that the HDAC activity associated with KLF6 is independent of pRb110-associated HDAC activity.

HDAC3 Mediates the Repression of Endogenous DLK1 through KLF6—Our data indicate that KLF6 associates in vivo with HDAC3 to form a stable complex repressing Dlk1 expression. However, they do not establish the relative contribution of this complex to occupancy of the native Dlk1 promoter during adipocyte differentiation. To address this issue, we performed chromatin immunoprecipitation assays to verify that KLF6 recruits HDAC3 to the endogenous Dlk1 promoter (Fig. 7A). Immunoprecipitation of chromatin using polyclonal antisera against either KLF6 or HDAC3 was performed on nuclear extracts from 3T3-L1 cells undergoing differentiation cross-linked with formaldehyde. Following reversal of the formaldehyde cross-links and proteinase K digestion, a fragment corresponding to −481 through −283 of the mouse Dlk1 promoter was amplified by PCR. Our results confirmed that the endogenous Dlk1 promoter was occupied by full-length but not mutant (Δ128) KLF6, together with HDAC3, following differentiation of 3T3-L1 cells (Fig. 7, A and B).

To determine the relative contribution of HDAC3 to transcriptional repression of Dlk1, an ecdysone/ponasterone-regulated system was used to control the induction of HDAC3 in 3T3-L1 cells. In 3T3-L1 preadipocytes stably expressing both the pVgRXR transactivator and pIND-FLAG/HDAC3 target transgenes, stimulation of HDAC3 expression in vivo by ponasterone A led to down-regulation of endogenous DLK1 protein, which was partially reversed by silencing of HDAC3 expression using either trichostatin A or HDAC3 siRNA (Fig. 7C). Further experiments confirmed that HDAC3 catalytic activity is specifically associated with KLF6 and independent of pRb. Nuclear extracts immunodepleted of HDAC3 or HDAC2 proteins were used to identify specific HDAC protein activity associated with KLF6. A, anti-FLAG (M2) agarose-bound protein was assayed for deacetylase activity following pre-absorption of specific HDAC3 and HDAC2 proteins. Preimmune serum was used to determine background levels of nonspecific protein absorption. Release of tritiated acetate was measured by scintillation counting. B, immunoblot analysis of nuclear lysate precleared of HDAC3. C, the specific association of KLF6 with the various endogenous classes I and II HDACs were evaluated in 3T3-L1 cell nuclear lysates following the differentiation protocol that was described (“Materials and Methods”). Antibodies against the various HDACs from the class I and II families (αHDAC1, αHDAC2, αHDAC3, αHDAC4, αHDAC5, and αHDAC7) were used to react with specific HDAC proteins from 3T3-L1 cell nuclear lysates, followed by precipitation with protein-A-agarose. Samples were then blotted with anti-KLF6 antisera (αKLF6). To monitor the levels of endogenous class I and class II HDACs, immunoblots were performed for each of the same HDACs with specific antibodies, with significant expression only of HDAC3 (HDAC5 was not detected in nuclear lysates—not shown). D, HDAC activity was measured, as described above, following the pre-absorption of pRb by immunodepletion of nuclear extracts using rabbit polyclonal antibody against pRb (C15, Santa Cruz Biotechnology). IP, immunoprecipitation.
thermore, from these experiments we conclude that HDAC3 is a direct mediator of Dlk1 repression in vivo.

To determine whether recruitment of HDAC3 is mediated directly by KLF6 recruitment to the Dlk1 locus, we established a stably transfected NIH 3T3 cell line that constitutively overexpresses HDAC3. These cells were transiently transfected with either the wild-type KLF6 plasmid or the KLF6 deletion mutant (Δ128) expression construct (which lacks part of the activation domain and the entire DNA-binding domain) in order to examine their respective ability to recruit HDAC3 to the Dlk1 promoter. Based on chromatin immunoprecipitation analysis (Fig. 7B), there was a direct correlation between wild-type and HDAC3 occupancy of the DLK1 locus and correspond to the loss of DLK1 expression in vivo. A, upper panel, anti-KLF6 antibody and two different antibodies against HDAC3 (labeled as anti-HDAC3a and anti-HDAC3b) were used for immunoprecipitation. PCRs for ChIP assay were performed using primers positioned at -481 and -263 bp upstream of the mouse Dlk1 promoter transcriptional start site. Middle panel, prior to each immunoprecipitation, 1% volume of each cell lysate was analyzed by PCR to determine the input signals from the Dlk1 promoter. Lower panel, to demonstrate the presence of KLF6 and HDAC3 in the anti-KLF6 and anti-HDAC3 immunoprecipitates, respectively, one-half of each of anti-KLF6 and anti-HDAC3a immunoprecipitates was subjected to SDS-PAGE/Western analysis, blotted with anti-KLF6 antibody or anti-HDAC3a antibody, respectively. B, ChIP analysis was conducted with NIH 3T3 cells stably transfected with pcDNA3.1/HDAC3 under G418 selection. After three serial passages under G418 selection (600 μg/ml), both wild-type KLF6 and deletion mutant of KLF6 (Δ128) were transiently transfected into the HDAC3 expression system as indicated above the image. After 48 h, cells (~5×10^6 cells) were fixed with formaldehyde, and chromatin was prepared for chromatin immunoprecipitation assays. ChIPs were performed with anti-histone H3 (Upstate Biotechnology, Inc.), anti-acetyl histone H3 (Upstate Biotechnology, Inc.), anti-HDAC3, and normal rabbit serum as a negative control as indicated below the image. C, stably transfected 3T3-L1 cells were used to induce the expression of HDAC3 in vivo by an ecdysone-inducible mammalian system (Invitrogen) with ponasterone A. RNA interference was conducted with siRNA duplexes directed against HDAC3 (Dharmacon). Transfection of siRNA into stably transfected 3T3-L1 cells was performed under induced and uninduced states of HDAC3 expression. Stably transfected 3T3-L1 cells were then monitored for the expression of HDAC3 by immunoblot analysis against mammalian HDAC3, and input levels were monitored by parallel immunoblot analysis of cyclophilin A as a control. Levels of Dlk1/pref1 were analyzed by immunoblot studies conducted with rabbit anti-mouse Dlk1/pref1 antisera. D, chromatin immunoprecipitation studies were performed on chromatin from pre- and post-induced (as indicated + and −, respectively) state of differentiation in 3T3-L1 cells. Mouse preadipocyte 3T3-L1 cells were cultured under selective conditions (see “Materials and Methods”) to induce cell differentiation. On day 0 (+) of induction, 5×10^6 3T3-L1 cells were fixed with formaldehyde and used in the ChIP experiments as shown. Following 8 days of induction (+), the same number of cells was recovered from formaldehyde fixation and used in the ChIP experiments. Immunoprecipitation (IP) of sonicated chromatin lysates was performed with polyclonal rabbit antisera as indicated. An approximate amount of 10% of the total input was used to monitor equivalent levels of chromatin used in the PCR from the sheared DNA templates. The levels of pre (+) and post (+)-induced 3T3-L1 cells from the chromatin input were monitored for HDAC3 protein by immunoblot analysis.
KLF6 expression and HDAC3 recruitment to the endogenous Dlk1 promoter. In contrast, under identical conditions, the KLF6 (ΔΔ28) deletion mutant failed to recruit HDAC3 to the same Dlk1 promoter element, further indicating that full-length KLF6 mediates the interaction of HDAC3 within the Dlk1 promoter.

To confirm additionally the requirement for KLF6 and HDAC3 to occupy the Dlk1 locus during adipogenesis, chromatin immunoprecipitation analysis was performed using extracts of 3T3-L1 cells isolated before or after induction of adipogenesis. Although minimal HDAC3 occupied the endogenous Dlk1 locus prior to induction of adipogenesis, the HDAC3-Dlk1 promoter occupancy was clearly demonstrable following adipogenic induction (Fig. 7D). Moreover, although KLF6 was also detectable prior to induction, its association with the promoter was also increased upon adipogenesis. These data support that conclusion that both HDAC3 and KLF6 co-occupy the Dlk1 promoter, but only in mature adipocytes and not preadipocytes.

**DISCUSSION**

Our data support a role for KLF6 in adipocyte differentiation. HDAC3 activity is specifically recruited to the transactivation domain of KLF6, which together directly bind to the Dlk1 promoter to achieve repression, leading to induction of key regulators of adipocyte differentiation. Silencing of KLF6 mRNA using either retroviral infection or stable transfection of KLF6 siRNA blocks 3T3-L1 cell differentiation, indicating that KLF6 function is necessary, albeit not sufficient, to direct preadipocytes into mature adipocytes. In fact, forced expression of KLF6 in the absence of a full differentiation mixture does not induce adipogenesis in 3T3-L1 cells (data not shown). Although several factors and upstream pathways converge to promote differentiation of adipocytes, there are no previous examples where direct transcriptional repression of a regulatory protein (Dlk1) controls this process. A potential contribution of KLF6 was suggested by an earlier report (14) in which murine KLF6/Zf9 was associated with adipogenesis immediately following the induction of 3T3-L1 cells with adipogenic hormones, but neither its activity nor regulatory targets were clarified.

**KLF6 is a Novel Transcriptional Repressor of Dlk1 and Modulates Adipocyte Differentiation**—Our data identify a novel transcriptional repressor function of KLF6 in promoting adipocyte differentiation, whereas previous studies have focused on its role as a transcriptional activator in tumor suppression (12, 28, 29) and other biologic contexts (8, 30, 31). Transactivation by KLF6 may be coupled with co-activator activities (32); however, our findings indicate a more complex transcriptional model for KLF6-mediated repression. Specifically, transcriptional repression by KLF6 is associated with HDAC3 activity in both 3T3-L1 and 293T cells. Other Krüppel-like factors may function as either transcriptional activators or repressors (33, 34), but none has been linked to cell type-specific transcriptional co-factors in this manner. Most interestingly, two recent studies (35, 36) implicate other Krüppel-like factors in mediating adipocyte differentiation through multiple and discrete transcriptional mechanisms.

In support of the data shown in Fig. 2, we performed ChIP shown in Fig. 7. Although there is a discrepancy between the DNA elements bound by KLF6 in vitro (Fig. 2) and the choice of genomic sequences used to amplify by PCR following ChIP (Fig. 7), these results are due to the inability to identify optimal primer sequences for PCR overlapping the KLF6-binding site of the Dlk1 promoter. To address this issue, a region proximal to the KLF6-binding site was selected both for optimal conditions for PCR and the capacity to detect the occupation of KLF6 within a proximal region of Dlk1. We account for this discrepancy because a consequence of shearing chromatin within ~500 bp to 1 kb will allow retention of the target sequences following the immunoprecipitation step. Therefore, amplification of the genomic region overlapping the putative KLF6 binding is capable of generating a signal during the PCR procedure.

**HDAC3 as a Critical Co-repressor in Adipocyte Differentiation**—The architecture of chromatin has a fundamental role regulating gene transcription in vivo. The relationship between KLF6 and the remodeling of chromatin structure via the modification of nucleosomal histones has not been studied previously. We demonstrate that KLF6 incorporates histone-modifying activity through the recruitment of HDAC3 (Figs. 4 and 5). HDAC3 is a member of the class I Rd/p 3-like histone deacetylases (37–40) that has activities clearly distinct from those of HDAC1 and -2 (37, 41). A role for HDAC3 in cell differentiation has been suggested based on its functional relationship to signaling mechanisms directing a number of crucial cell fate decisions (37, 42, 43). The evidence that KLF6 is a true HDAC3-associated protein is supported by its co-purification using a bicistronic bacterial expression system able to identify direct protein-protein interactions under native conditions (Fig. 5D). Furthermore, immunodepletion experiments also demonstrate that HDAC3 is responsible for the majority of the deacetylase associated with KLF6 (Fig. 6). These results indicate that HDAC3 is a specific co-factor for KLF6 activity during 3T3-L1 adipogenic differentiation. Our findings also exclude participation of other HDACs, including HDAC2, in cooperating with KLF6, a surprising finding, considering that several proteins that bind HDAC1/2 also partner with HDAC3 (44).

Our results further indicate that KLF6 complexes directly with HDAC3 (Fig. 5D). KLF6 appears to recruit directly HDAC3 to the endogenous Dlk1 promoter, and the induction of HDAC3 expression correlates directly with a decrease of Dlk1 levels in 3T3-L1 (Fig. 7). These data demonstrate that one potential mechanism directing repression of Dlk1 involves recruitment of HDAC3 through interaction with KLF6. Several previous studies indicate that HDAC3 participates in transcriptional repression through either direct interactions with transcriptional co-repressor molecules NCoR and SMRT to form stable ternary complex(es) (15, 37, 42, 45–47) or through binding to pRb (44). However, our experiments demonstrate that KLF6 complexes with HDAC3 independently of these co-repressors or pRb (Figs. 5D and 6D, and data not shown). The lack of NCoR/SMRT participation in directing complex formation between KLF6 and HDAC3 (Fig. 5D) suggests that KLF6 utilizes mechanism(s) of transcriptional repression independent of those associated with nuclear hormone receptor signaling that require NCoR/SMRT, receptors that contribute important permissive signals during adipocyte differentiation (41). Thus, HDAC3 is a nexus for multiple converging mechanisms that drive terminal differentiation of adipocytes, among which is its interaction with KLF6. Moreover, KLF6 represents one of only a few transcription factors utilizing HDAC3 in repressing transcription. Because our studies exclude the participation of some other class I and class II HDACs in this repression (Fig. 6, A and B), HDAC3 may be the only deacetylase activity recruited by KLF6 during adipogenesis, further reinforcing the central importance of HDAC3.

The outcome of the interaction between HDAC3 and KLF6 is the repression of Dlk1, a gene encoding an epidermal growth factor-like homotypic transmembrane protein whose down-regulation is required for adipocyte differentiation (48). Similarly, HDAC3 has been assigned recently a role in repressing PPARγ function by interacting with hypophosphorylated Rb (24). Combined with previous studies by Fajas et al. (24), our results indicate that HDAC3 appears to function as a central mediator...
Repression of DLK1 by KLF6 Promotes Adipogenesis

通过突变可能作为生长依赖性激酶抑制剂，如p21

Dlk1/H9004128) 失败于招募 HDAC3 到位

contribute to the loss of differentiating function of HDAC3

activity through mutation in neoplastic disease might directly

addition to the transcriptional repression of

differentiation at the expense of

transcriptional activation of p21

Ronon environment for differentiation of preadipocytes (51–53). Given our

cellular proliferation (49, 50). However, recent studies suggest

directing adipocyte differentiation through interplay between

emphasizes the role of HDAC3 and indicates KLF6 as a

indirecting adipocyte differentiation through interplay between

either KLF6 and/or Rb (Fig. 8), Rb...
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