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The Krüppel-like factor KLF15 inhibits transcription of the adrenomedullin gene in adipocytes

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ABSTRACT

KLF15 (Krüppel-like factor 15) plays a key role in adipocyte differentiation and glucose transport in adipocytes through activation of its target genes. We have now identified six target genes regulated directly by KLF15 in 3T3-L1 mouse adipocytes with the use of a combination of microarray-based chromatin immunoprecipitation and gene expression analyses. We confirmed the direct regulation by KLF15 of one of these genes, that for adrenomedullin, with the use of a luciferase reporter assay in 3T3-L1 preadipocytes and adipocytes. Such analysis revealed that the most proximal CACCC element in the promoter of the human adrenomedullin gene (located in the region spanning nucleotides −70 and −29) is required for trans-inhibition by KLF15. Furthermore, chromatin immunoprecipitation showed that KLF15 binds to this region of the human adrenomedullin gene promoter in cultured human adipocytes. These results thus implicate KLF15 in the regulation of adrenomedullin expression in adipose tissue.

Krüppel-like factors (KLFs) are transcriptional regulators that contain the C2H2 zinc-finger motif and play diverse roles in the regulation of cell proliferation, cell differentiation, and development [1,2]. All 17 members of the KLF family identified to date bind to GC-rich sequences including GC boxes and GT boxes (also known as CACCC boxes) [2,3]. Certain KLF proteins have been implicated in adipocyte function or in the pathogenesis of obesity [4–9]. We have previously shown that KLF15 regulates adipogenesis through induction of the peroxisome proliferator-activated receptor γ (PPARγ) gene [10]. In addition, overexpression of KLF15 induces adipocyte maturation and expression of the insulin-sensitive glucose transporter GLUT4 [11], although the physiological relevance of KLF15 in the development of obesity has remained unclear. We have also previously shown that KLF15 regulates the expression of mitochondrial acetyl-CoA synthase (AceCS2) in skeletal muscle [12] as well as that of phosphoenolpyruvate carboxykinase in hepatocytes [13]. We have now attempted to identify novel targets of KLF15 in mouse 3T3-L1 adipocytes by a combination of chromatin immunoprecipitation with a promoter oligonucleotide microarray (ChIP-chip) and analysis of gene expression with an expression oligonucleotide microarray. Our results indicate that KLF15 inhibits transcription of the adrenomedullin gene in adipocytes.

Materials and methods

Antibodies, reagents, and cells. Goat polyclonal antibodies to KLF15 and normal goat immunoglobulin G (IgG) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Insulin, dexamethasone, and isobutylmethylxanthine were from Sigma (St. Louis, MO). Mouse 3T3-L1 cells were obtained from American Type Culture Collection (Manassas, VA). 3T3-L1 preadipocytes were maintained and induced to differentiate into adipocytes as previously described [14]. Cultured human subcutaneous adipocytes (F-SA-75) were obtained from DS Pharma Biomedical (Osaka, Japan).

Adenoviral vectors and infection. An adenoviral vector encoding rat KLF15 (AxKLF15) was generated as previously described [10]. An adenoviral vector encoding β-galactosidase (Axβgal) was kindly provided by I. Saito (University of Tokyo, Tokyo, Japan). An adenoviral vector encoding a short hairpin RNA (shRNA) specific for mouse KLF15 mRNA under the control of the mouse U6 promoter (AxshKLF15) and a vector containing the U6 promoter alone

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(AxU6) were generated described in Supplementary materials and methods. 3T3-L1 preadipocytes or adipocytes were infected with adenoviral vectors as previously described [15].

Expression microarray analysis. Fully differentiated 3T3-L1 adipocytes at 7 days after the onset of induction of differentiation were infected with adenoviral vectors encoding KLF15 or β-galactosidase for 48 h. Total RNA was then isolated from the infected cells with the use of an RNeasy Lipid Tissue Mini Kit (Qiagen, Hilden, Germany). Portions (20 μg) of the RNA were used for the synthesis of biotin-labeled cRNA, which in turn was used to probe Murine Genome U74v2 microarrays (Affymetrix, Santa Clara, CA). The arrays were scanned and analyzed as described previously [10].

Promoter array analysis. Fully differentiated 3T3-L1 adipocytes at 9 days after the onset of differentiation induction were detached from culture dishes by exposure to 0.5% trypsin, washed twice with phosphate-buffered saline, lysed, and subjected to chromatin immunoprecipitation (ChIP) with antibodies to KLF15 or normal goat IgG. The resulting precipitates were then subjected to mouse promoter array analysis with a ChIP-DSL system (version M8K 1.0; Aviva Systems Biology, San Diego, CA). The array was scanned with a GenePix4000B instrument (Molecular Devices, Sunnyvale, CA) and analyzed with Array-Pro Analyzer software (Media Cybernetics, Bethesda, MD).

Quantitative RT-PCR analysis. Total RNA was isolated from 3T3-L1 preadipocytes or adipocytes with the use of an RNeasy Lipid Tissue Mini Kit (Qiagen) and was subjected to reverse transcription (RT). Portions of the resulting cDNA were subjected to quantitative real-time polymerase chain reaction (PCR) analysis in a Sequence Detector (model 7900; Invitrogen (Carlsbad, CA), with the use of specific primers and SYBR Green PCR Master Mix (Perkin-Elmer Life Sciences, Waltham, MA). The relative abundance of mRNAs was calculated with 36B4 mRNA as the invariant control. The PCR primers were described in Supplementary materials and methods.

Expression plasmids, reporter plasmids, and site-directed mutagenesis. A mammalian expression vector for mouse KLF15 (pcDNA3.1/KLF15) was generated as previously described [10]. Mammalian expression vectors either encoding the mouse KLF15 shRNA under the control of the mouse U6 promoter or containing the U6 promoter alone were generated by subcloning the corresponding DNA fragments into pcDNA3.1, from which the cytomegalovirus promoter was subsequently deleted. A luciferase reporter plasmid based on pGL3-basic (Promega, Madison, WI) and containing the human adrenomedullin gene promoter was described previously [16,17]. The human adrenomedullin gene promoter sequence in pGL3-basic was subjected to site-directed mutagenesis with the use of a QuikChange II kit (Stratagene, La Jolla, CA). The resulting plasmids were sequenced to confirm the introduced mutations.

Transient transfection and luciferase reporter assays. 3T3-L1 preadipocytes were cultured in 24-well plates and transfected with the use of the Lipofectamine reagent (Invitrogen). Cells in each well were thus transfected with 0.2 μg of the expression plasmid, 0.1 μg of the reporter plasmid, and 0.1 μg of pSV-β-galactosidase control vector (Promega). 3T3-L1 adipocytes were transfected by electroporation as described previously [18]; the amounts of DNA used for transfection were 2 μg of the expression plasmid, 1 μg of the reporter plasmid, and 1 μg of pSV-β-galactosidase for every three wells of a 24-well plate.

All cells were lysed in 100 μl of "1 Time Passive Lysis Buffer" (Promega) after transfection for 48 h, and portions (20 μl) of the lysates were subjected to assays for firefly luciferase (Promega) and β-galactosidase (Clontech, Palo Alto, CA). Promoter activity was determined as the ratio of luciferase to β-galactosidase activities.

ChIP. ChIP analysis was performed with the use of a ChIP assay kit (Upstate Biotechnology, Lake Placid, NY). In brief, cultured human subcutaneous adipocytes were cross-linked for 30 min with 1% formaldehyde in culture medium, washed with ice-cold phosphate-buffered saline, and lysed and sonicated in SDS lysis buffer [1% SDS, 10 mM EDTA, 50 mM Tris–HCl (pH 8.1)] containing aprotinin (1 μg/ml) and leupeptin (1 μg/ml). Lysates were incubated overnight at 4 °C with antibodies to KLF15 or normal goat IgG, after which protein A–agarose was added and each mixture was incubated for an additional 1 h. The precipitated DNA–protein complexes were washed, eluted from the agarose beads, and reverse cross-linked. DNA was extracted with phenol-chloroform and used as a template for PCR analysis with the primers 5′-GTGGCTGAGGAAAGAAAG-3′(sense) and 5′-AAGAAACCACTGAGTGGC-3′ (antisense). The PCR protocol included an initial incubation at 94 °C for 5 min followed by 35 cycles of 94 °C for 30 s, 52 °C for 30 s, and 72 °C for 30 s. The PCR products were fractionated by electrophoresis on a 2% agarose gel and stained with ethidium bromide.

Results and discussion

Screening for novel KLF15 target genes by combined ChIP-chip and expression microarray analysis in 3T3-L1 adipocytes

To identify novel target genes of KLF15 in 3T3-L1 adipocytes, we first performed ChIP-chip analysis. The precipitates obtained by ChIP with antibodies to KLF15 or with normal goat IgG were thus analyzed with mouse promoter oligonucleotide microarrays containing oligonucleotides corresponding to ~6000 distinct genes. Promoter regions of 132 genes showed a reproducibly significant difference in hybridization signal (Fig. 1A). We next profiled genes whose level of expression changed in association with overexpression of KLF15 in 3T3-L1 adipocytes. Total RNA isolated from 3T3-L1 adipocytes infected with adenoviral vectors encoding either KLF15 or β-galactosidase was thus analyzed with mouse oligonucleotide microarrays, revealing that forced expression of KLF15 was accompanied by an increase in the expression of 337 genes and a decrease in that of 274 genes (Fig. 1A). Integration of the ChIP-chip data with the expression microarray data resulted in the identification of six genes whose promoters bound KLF15 and whose expression was either increased (*Slc16a9*, *Cdk9*, *P4ha2*, and *Klf3*) or decreased (*Aprt* and *Adm*), according to a $\log_2$ ratio of >1.0 or <0.5, respectively, in KLF15-overexpresing 3T3-L1 adipocytes (Fig. 1B). Among these genes directly regulated by KLF15, we further investigated the role of KLF15 in transcription of the adrenomedullin gene (*Adm*).

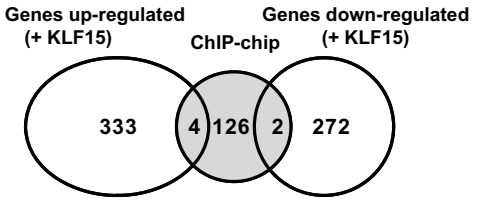
Effect of KLF15 on transcription of Adm in 3T3-L1 cells

Quantitative RT-PCR analysis confirmed that overexpression of KLF15 in 3T3-L1 adipocytes resulted in down-regulation of adrenomedullin mRNA (Fig. 2A and B). Consistent with previous observations, whereas the amount of KLF15 mRNA was increased during the induction of adipocyte differentiation in 3T3-L1 cells (Fig. 2C) [10], that of adrenomedullin mRNA was decreased (Fig. 2D) [16,19]. Furthermore, we found that overexpression of KLF15 in 3T3-L1 preadipocytes resulted in a significant decrease in the amount of adrenomedullin mRNA (Fig. 2E and F). Conversely, depletion of KLF15 in 3T3-L1 adipocytes by RNA interference resulted in an increase in the abundance of adrenomedullin mRNA (Fig. 2G and H). These findings thus suggested that KLF15 inhibits the transcription of *Adm* in 3T3-L1 adipocytes.

Effect of KLF15 overexpression on the activity of the ADM promoter in 3T3-L1 preadipocytes

To confirm that the KLF15-induced down-regulation of adrenomedullin mRNA was attributable to inhibition of *Adm*

A



B

Gene symbol	Transcript ID	ChIP-chip	Microarray
Slc16a9	NM_025807	9.6	3.7
Cdk9	NM_130860	8.7	2.3
P4ha2	NM_011031	9.7	2.0
KLF3	NM_008453	7.4	2.0
Aprt	NM_009698	9.6	0.48
Adm	NM_009627	14.8	0.50

Fig. 1. Identification of *Adm* as a target gene of KLF15 in 3T3-L1 adipocytes. (A) Venn diagram of the numbers of genes whose promoters were found to bind KLF15 by ChIP-chip analysis and whose expression was found to be increased or decreased in response to KLF15 overexpression by expression microarray analysis in 3T3-L1 adipocytes. (B) Data for the six genes identified by both types of analysis in (A). The ChIP-chip data are presented as the ratio of the signals obtained for immunoprecipitates prepared with antibodies to KLF15 and with normal goat IgG, whereas the expression microarray data are presented as the ratio of the signals obtained with cells overexpressing KLF15 and those expressing β-galactosidase.

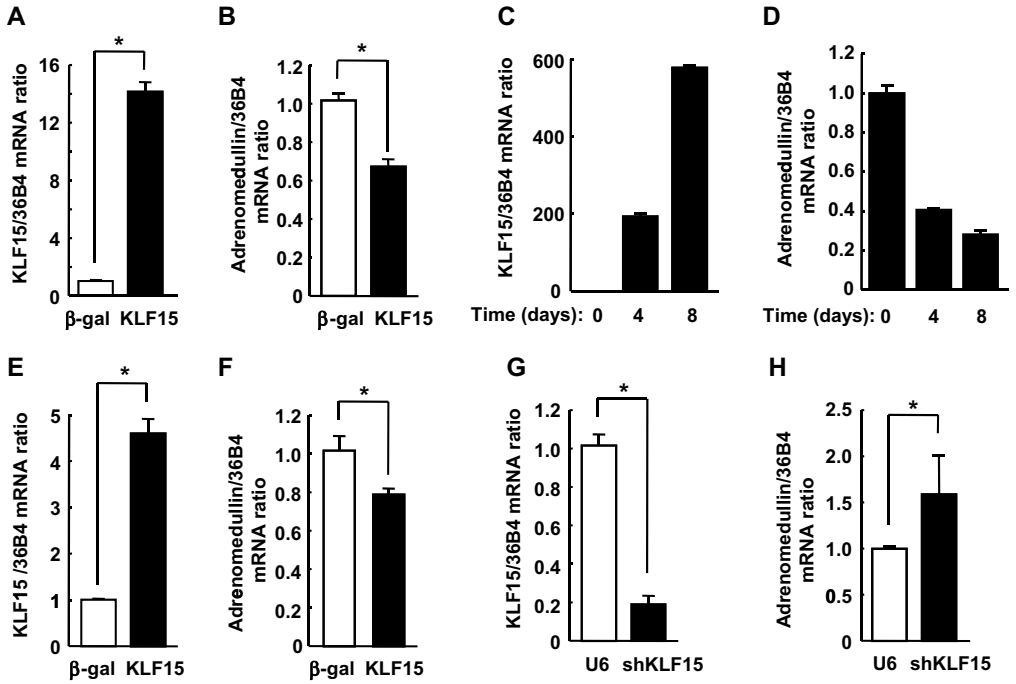


Fig. 2. Effect of KLF15 on transcription of *Adm* in 3T3-L1 cells. (A, B) Effect of overexpression of KLF15 on *Adm* expression in 3T3-L1 adipocytes. 3T3-L1 adipocytes were infected with adenoviral vectors encoding β-galactosidase (β-gal) or rat KLF15 for 48 h, after which the cells were subjected to quantitative RT-PCR analysis of mouse KLF15 (A) and adrenomedullin (B) mRNAs. Data are expressed relative to the corresponding value for cells infected with Axβgal. (C, D) Quantitative RT-PCR analysis of the abundance of KLF15 (C) and adrenomedullin (D) mRNAs at the indicated times after the onset of exposure to inducers of adipocyte differentiation in 3T3-L1 cells. Data are expressed relative to the corresponding value for time zero. (E, F) Quantitative RT-PCR analysis of mouse KLF15 (E) and adrenomedullin (F) mRNAs in 3T3-L1 preadipocytes infected with adenoviral vectors for β-galactosidase or rat KLF15. Data are expressed relative to the corresponding value for cells infected with Axβgal. (G, H) Quantitative RT-PCR analysis of KLF15 (G) and adrenomedullin (H) mRNAs in 3T3-L1 adipocytes infected with adenoviral vectors containing the U6 promoter alone or encoding KLF15 shRNA. Data are expressed relative to the corresponding value for cells infected with the control vector. All RT-PCR data are means ± SEM from three independent experiments. $^{*}P < 0.05$.

transcription, we examined the effect of overexpression of KLF15 in 3T3-L1 preadipocytes on the activity of the human adrenomedullin gene (*ADM*) promoter (nucleotides −4616 to +108 relative to the transcription start site) (Supplementary Fig. 1) ligated to the firefly luciferase gene in the pGL3-basic plasmid. Forced expression of KLF15 resulted in inhibition of *ADM* promoter activity (Fig. 3A). KLF15 was originally identified on the basis of its ability to bind to a GA element in the promoter of the CLC-K1 gene, which encodes a kidney-specific member of the CLC family of Cl^- channels [20]. The gene for GLUT4 in adipose or muscle tissue as well as that for AceCS2 in muscle are also targets of KLF15, which binds to CACCC elements in the promoters of these genes [11,12]. There are 10 CACCC sequences within the 4.6-kb proximal region of the human *ADM* promoter (Supplementary Fig. 1). To determine the relative importance of these sequences as functional KLF15 binding sites, we generated a series of 5′ deletion mutants of the promoter region. Deletion of the sequence from nucleotides −4616 to −71 did not affect transcriptional inhibition by KLF15 (Fig. 3A). However, further deletion to nucleotide −29 resulted in a marked loss of promoter activity as well as rendered such activity insensitive to KLF15 (Fig. 3A). We therefore next constructed two reporter plasmids, pGL3-AM(-70)m1 and pGL3-AM(-70)m2, that contain different mutations of the putative KLF15 binding site located in the region between nucleotides −70 and −29. The promoter activity of the two mutant constructs was unaffected by overexpression of KLF15 (Fig. 3B), indicating that the most proximal putative

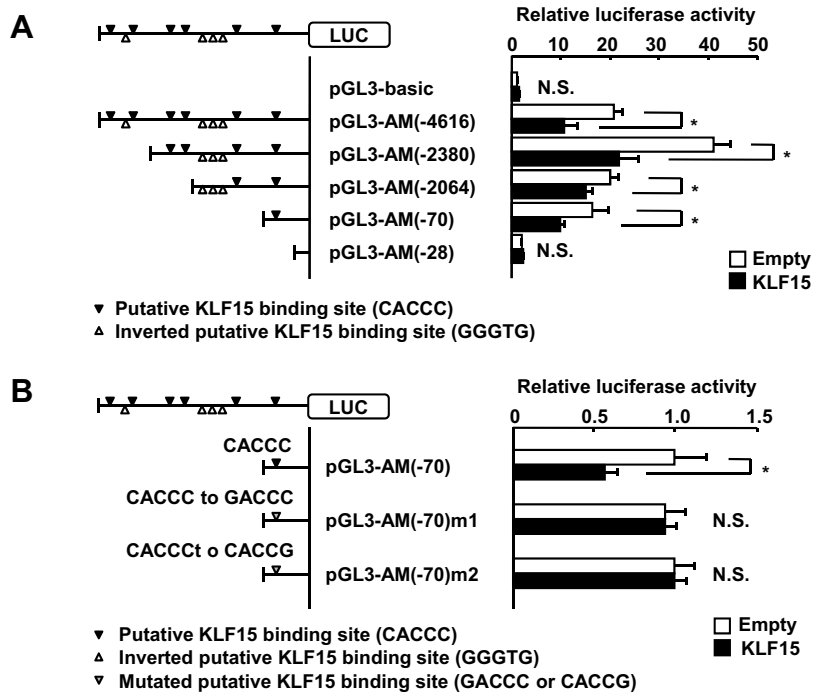


Fig. 3. Effect of KLF15 overexpression on *ADM* promoter activity in 3T3-L1 preadipocytes. 3T3-L1 preadipocytes were transiently transfected with pGL3-basic plasmids containing various deletion (A) or point-mutant (B) constructs of the *ADM* promoter as well as with an expression vector for KLF15 (pcDNA3.1/KLF15) or the corresponding empty vector (pcDNA3.1). Luciferase activity was normalized by β-galactosidase activity derived from pSV-β-galactosidase and is expressed relative to the normalized value for cells transfected with pGL3-basic (A) or pGL3-AM(-70) (B) as well as with the empty expression vector. Data are means ± SEM from three independent experiments. $^{*}P < 0.05$; N.S., not significant.

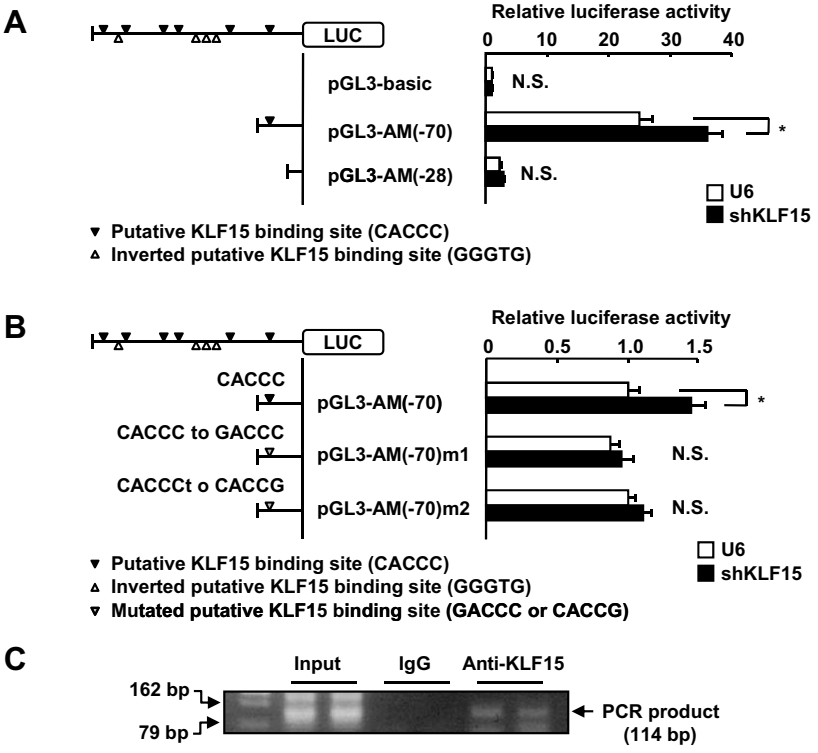


Fig. 4. Effect of depletion of KLF15 on *ADM* promoter activity in 3T3-L1 adipocytes as well as ChIP analysis of KLF15 binding to the *ADM* promoter in human adipocytes. (A, B) 3T3-L1 adipocytes were transiently transfected with the indicated pGL3-basic plasmids as well as with an expression vector for KLF15 shRNA or a control vector containing the U6 promoter alone and were assayed for luciferase activity as in Fig. 3. Data are means ± SEM from three independent experiments. $^{*}P < 0.05$; N.S., not significant. (C) ChIP analysis of the binding of endogenous KLF15 to the *ADM* promoter in cultured human adipocytes (F-SA-75). ChIP was performed with antibodies to KLF15 or with control IgG. The primers used to amplify the *ADM* promoter fragment are indicated in Supplementary Fig. 1.

KLF15 binding site plays the dominant role in trans-inhibition of *ADM* transcription by KLF15.

Effect of depletion of KLF15 on ADM promoter activity in 3T3-L1 adipocytes

Given that depletion of KLF15 induced the expression of *Adm* in 3T3-L1 adipocytes (Fig. 2H), we examined the effect of KLF15 depletion on *ADM* promoter activity in these cells. Transfection of 3T3-L1 adipocytes with a vector encoding KLF15 shRNA resulted in activation of the *ADM* promoter fragment containing the most proximal putative KLF15 binding site in the plasmid pGL3-AM(-70) (Fig. 4A). Furthermore, the m1 or m2 mutations of this putative KLF15 binding site rendered the *ADM* promoter fragment insensitive to KLF15 (Fig. 4B). Finally, a ChIP assay with antibodies to KLF15 showed that KLF15 bound to the region of the *ADM* promoter containing the most proximal putative KLF15 binding site in cultured human adipocytes (Fig. 4C). These results thus indicate that KLF15 binds to the *ADM* promoter and thereby regulates its activity.

Conclusions

We have identified new target genes of KLF15 in 3T3-L1 adipocytes with the combination of ChIP-chip and expression microarray analyses. The proteins encoded by three of these genes — *Slc16a9*, *P4ha2*, and *Aprt* — contribute to transport of monocarboxylic acids [21], collagen biosynthesis [22], and metabolism of purine nucleotides [23], respectively, but they have not been previously characterized in cultured adipocytes or adipose tissue of animals. The protein encoded by *Cdk9* is a member of the cyclin-dependent kinase (CDK) family and has been shown to participate in adipocyte differentiation through direct interaction with and phosphorylation of PPARγ [24]. The protein encoded by *Klf3* also regulates adipogenesis and binds to the promoter of the gene for CCAAT/enhancer-binding protein α (C/EBPα), a key adipogenic gene [5].

Our results suggest that expression of the adrenomedullin gene in cultured adipocytes is suppressed by KLF15 as a result of its interaction with the most proximal CACCC element located in the promoter region spanning nucleotides −70 to −29 of human *ADM*. Deletion of this promoter region resulted in almost a complete loss of transcriptional activity. The transcription factor Sp1 was previously shown to bind to this region of the *ADM* promoter and to induce *ADM* expression during adipocyte differentiation [17,25]. Thus, whereas KLF15 and Sp1 have been shown to induce synergistic activation of the *AceCS2* promoter in vitro [12], these proteins appear to regulate expression of *ADM* in a reciprocal manner.

Adrenomedullin is a potent vasodilatory peptide that was originally isolated from human pheochromocytoma cells [26]. Adipose tissue, especially mature adipocytes, was subsequently shown to be a major source of adrenomedullin in the body, and adipocyte-derived adrenomedullin was found to play a pathophysiologic role in adipogenesis or obesity as a member of the adipokine family of proteins [27,28]. Adipocyte-derived adrenomedullin is also thought to protect against the development of hypertension, insulin resistance, and the complications of these conditions in obese subjects [29]. We have found that expression of *Klf15* is decreased in adipose tissue of mice with high-fat diet–induced or genetic (*ob*/*ob*) obesity (T.N., H.S., M.K., unpublished observations), animals in which the expression of adrenomedullin in adipose tissue is increased [27]. The role of KLF15 in the regulation of adrenomedullin expression in adipose tissue and its contribution to the pathogenesis of obesity as well as to hypertension, insulin resistance, and their complications associated with obesity thus warrant further investigation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.12.020.

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