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JDP2 suppresses adipocyte differentiation by regulating histone acetylation

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Among the events that control cellular differentiation, the acetylation of histones plays a critical role in the regulation of transcription and the modification of chromatin. Jun dimerization protein 2 (JDP2), a member of the AP-1 family, is an inhibitor of such acetylation and contributes to the maintenance of chromatin structure. In an examination of *Jdp2* 'knock-out' (KO) mice, we observed elevated numbers of white adipocytes and significant accumulation of lipid in the adipose tissue in sections of scapulae. In addition, mouse embryo fibroblasts (MEFs) from *Jdp2* KO mice were more susceptible to adipocyte differentiation in response to hormonal induction and members of the CCAAT/enhancer-binding proteins (C/EBP) gene family were expressed at levels higher than MEFs from wild-type mice. Furthermore, JDP2 inhibited both the acetylation of histone H3 in the promoter of the gene for C/EBPδ and transcription from this promoter. Our data indicate that JDP2 plays a key role as a repressor of adipocyte differentiation by regulating the expression of the gene for C/EBPδ via inhibition of histone acetylation.
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In mammals, the strict control of adipocyte development, the mass of adipose tissue, the insulin sensitivity of adipocytes, and the appropriate metabolism of glucose and lipids is critical to the maintenance of energy homeostasis.[1,2] Adipogenesis, namely, the process whereby hormonal stimuli induce the differentiation of fibroblasts or mesenchymal cells to adipocytes, requires organized and controlled expression of a cascade of transcription factors and the modification of the chromatin within preadipocytes.[3–5] The factors involved in adipocyte differentiation include a nuclear receptor known as peroxisome proliferation-activated receptor gamma (PPARγ) and a group of CCAAT/enhancer-binding proteins (C/EBPs).[6,7] The rapid and transient induction of the expression of C/EBPβ and C/EBPδ is one of the earliest events in adipogenesis.[4] These transcription factors bind to specific sequences in the promoters of the *C/EBP*α gene and the *PPAR*γ*2* gene, inducing their expression, which, in turn, activates the full adipogenic program of gene expression.[8–10] Expression of PPARγ2 is also induced via an SREBP-1c-dependent pathway.[11] Once *PPAR*γ*2* and *C/EBP*α have been activated, 'cross-talk' between PPARγ2 and C/EBPα maintains the expression of each protein during adipocyte differentiation, even in the absence of C/EBPβ and C/EBPδ.[12,13]

Jun dimerization protein 2 (JDP2) is a DNA-binding protein that forms homodimers or heterodimers with c-Jun, ATF2 and C/EBPγ.[14–16] JDP2 can function not only as a transcriptional repressor but also as a coactivator in various types of cell,[15,17–20] and it is involved in a variety of biological phenomena such as proliferation and differentiation of cells and apoptosis.[14,15,17,18,20–22] For example, forced expression of JDP2 represses the retinoic acid-mediated (RA-mediated) transcription of the *c-jun* gene and the differentiation of F9 cells in response to RA.[18] By contrast, the expression of an exogenous gene for JDP2 promotes the formation of C2 myotube and strong expression of major myogenic markers in C2 myoblasts.[16] Moreover, ectopic expression of JDP2 in rhabdomyosarcoma cells induces myogenesis and the formation of incomplete myotubes.[16] Similar enhancement of cell differentiation by JDP2 was reported in the case of the induction of the formation of osteoblasts by RANKL.[23] These observations suggest that JDP2 might play multiple roles in the initial steps in the differentiation of cells. We demonstrated recently that JDP2 has histone-chaperone activity and participates in nucleosome assembly *in vitro*.[24] The molecular network of JDP2 between the chromatin modification and the regulation of proliferation, differentiation and apoptosis of cells still remains to be solved.

In the present study, we generated *Jdp2* 'knock-out' (KO) mice in order to study the activities of JDP2 *in vivo* and we found that JDP2 plays a role as a repressor of adipocyte differentiation. We also found that JDP2 targeted an

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Abbreviations: MEFs, mouse embryo fibroblasts; JDP2, Jun dimerization protein 2; KO, knock-out; PPARγ, peroxisome proliferation-activated receptor gamma; C/EBPs, CCAAT/enhancer-binding proteins; PGC-1α, PPARγ coactivator 1α; UCP-1, uncoupling protein 1; ChIP, chromatin immunoprecipitation; HDAC, histone deacetylase; HAT, histone acetyltransferase; RA, retinoic acid; DRE, differentiation response element; TG, triglyceride
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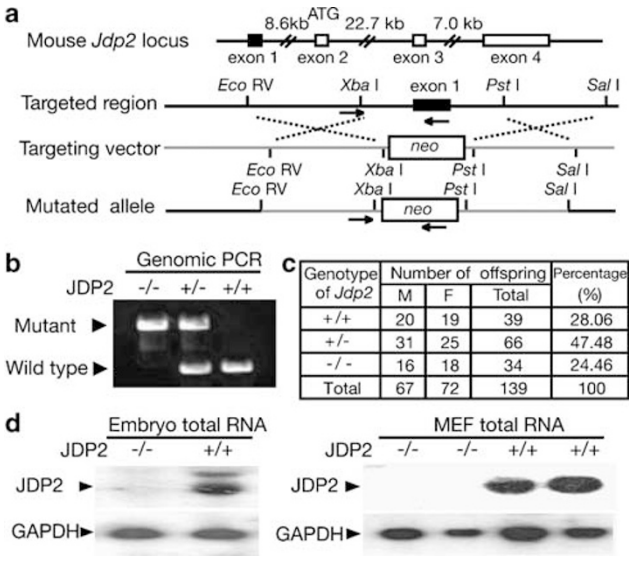
adipogenesis-related gene, *C/EBPδ*, and inhibited its expression via the regulation of histone acetylation.

Results

To generate JDP2 'knock-out' (KO) mice, we targeted exon 1 of the mouse *Jdp2* gene in an effort to disrupt the initiation of transcription of the gene and to generate JDP2 'knock-out' (KO) mice (Figure 1a). *Jdp2*$^{-/-}$, *Jdp2*$^{+/-}$ and *Jdp2*$^{+/+}$ mice were born normally, at appropriate frequencies, without any apparent physical abnormalities (Figure 1b and c). Northern blotting analysis of total RNA derived from embryos demonstrated that no JDP2 mRNA was transcribed from the mutated *Jdp2* allele (Figure 1d). We generated MEFs from embryos on day 11 and confirmed by Northern blotting that *Jdp2*$^{+/+}$ MEFs, but not *Jdp2*$^{-/-}$ MEFs, expressed JDP2 mRNA at significant levels (Figure 1d).

Since JDP2 promotes the spontaneous differentiation of C2C12 cells[16] and since ectopic JDP2 inhibits the RA-induced differentiation of F9 cells,[18] we examined the effects of endogenous JDP2 on the differentiation of MEFs to adipocytes. We exposed confluent MEFs to differentiation medium for 2, 3 or 4 days and then incubated them in growth medium that contained insulin (insulin-GM). After 7 days, significantly more adipocytes were evident in cultures of *Jdp2*$^{-/-}$ MEFs than in cultures of wild-type (WT) MEFs (Figure 2a). We analyzed the levels of aP2 mRNA, a common marker of adipocyte differentiation, and of PPGRγ2 and C/EBPα mRNA, key regulators of adipocyte differentiation, by real-time RT-PCR. We found that aP2, PPARγ2 and C/EBPα mRNAs were strongly expressed 5 days after the initiation of adipocyte differentiation and their levels of expression were higher in differentiated *Jdp2*$^{-/-}$ MEFs than in WT MEFs (Figure 2b). We also analyzed the expression of C/EBPβ and C/EBPδ, which are upstream regulators of the activity of C/EBPα at the early stage of adipogenesis but not at late stages. We found elevated levels of expression of C/EBPβ and C/EBPδ mRNAs even at late stage of adipocyte differentiation and levels were higher in differentiated *Jdp2*$^{-/-}$ MEFs than in WT MEFs. The level of expression of JDP2 mRNA did not change significantly before and after adipocyte differentiation in WT MEFs (Figure 2b). Moreover, when confluent *Jdp2*$^{-/-}$ MEFs were cultured for 20 days in Dulbecco's modified Eagle's medium (DMEM) that contained 10% fetal calf serum (FCS), the cells differentiated spontaneously and conspicuously to adipocytes (Figure 2c).

In order to confirm the hypothesis that JDP2 acts as a repressor of adipogenesis, we prepared a recombinant adenovirus vector that included JDP2 cDNA under control of the CAG promoter (Adeno-JDP2) and infected 3T3-L1 preadipocytes with this vector. As a negative control, we used a recombinant adenovirus vector that contained the gene for β-galactosidase from *Escherichia coli* (Adeno-lacZ). The 3T3-L1 cells that had been infected by these vectors expressed JDP2 and β-galactosidase, as revealed by immunoblotting analysis (Figure 3a). We exposed the infected cells to differentiation medium for 2 days and then cultured them in



Genotype of *Jdp2*	Number of offspring M	F	Total	Percentage (%)
+/+	20	19	39	28.06
+/-	31	25	66	47.48
-/-	16	18	34	24.46
Total	67	72	139	100

Figure 1 Targeted disruption of the *Jdp2* gene. (**a**) The wild-type (WT) *Jdp2* allele, the targeted allele, the targeting vector and the mutated allele. Exon 1 (black box) was replaced by a neomycin resistance gene (*neo*; white box) in the mutated allele. The arrows indicate the positions of primers used for the genomic PCR for which results are shown in (**b**). (**b**) Genotyping by genomic PCR. Mutant and WT alleles yielded amplified products of 788 and 593 bp, respectively. (**c**) The numbers of mice born with each genotype, as determined by genomic PCR. (**d**) The expression of mRNAs for JDP2 and GPDH in WT and *Jdp2*$^{-/-}$ total mouse embryos (left panel) and in MEFs (right panel), as analyzed by Northern blotting

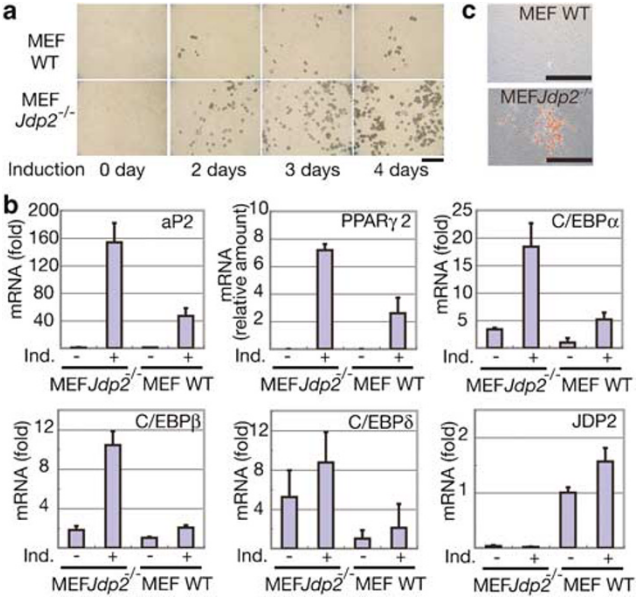


Figure 2 Inhibition by JDP2 of the differentiation of MEFs to adipocytes. (**a**) Differentiation of *Jdp2*$^{-/-}$ and wild-type (WT) MEFs. Confluent MEFs were exposed to differentiation medium for 0, 2, 3 and 4 days and then cultured for 1 week in the growth medium plus insulin (Insulin-GM). (**b**) Expression of adipocyte marker genes in differentiation-induced or uninduced *Jdp2*$^{-/-}$ and WT MEFs. After induction of adipocyte differentiation for two days, MEFs were cultured for a further 3 days in Insulin-GM. Total RNA was extracted and the levels of mRNAs for aP2, PPARγ2, C/EBPα, C/EBPβ, C/EBPδ and JDP2 were determined by real-time RT-PCR. Relative levels were normalized by reference to those of genomic DNA. (**c**) Confluent *Jdp2*$^{-/-}$ and WT MEFs after culture in DMEM medium that contained 10% FCS for 20 days. The cells were stained with Oil-Red. Scale bars, 0.5 mm

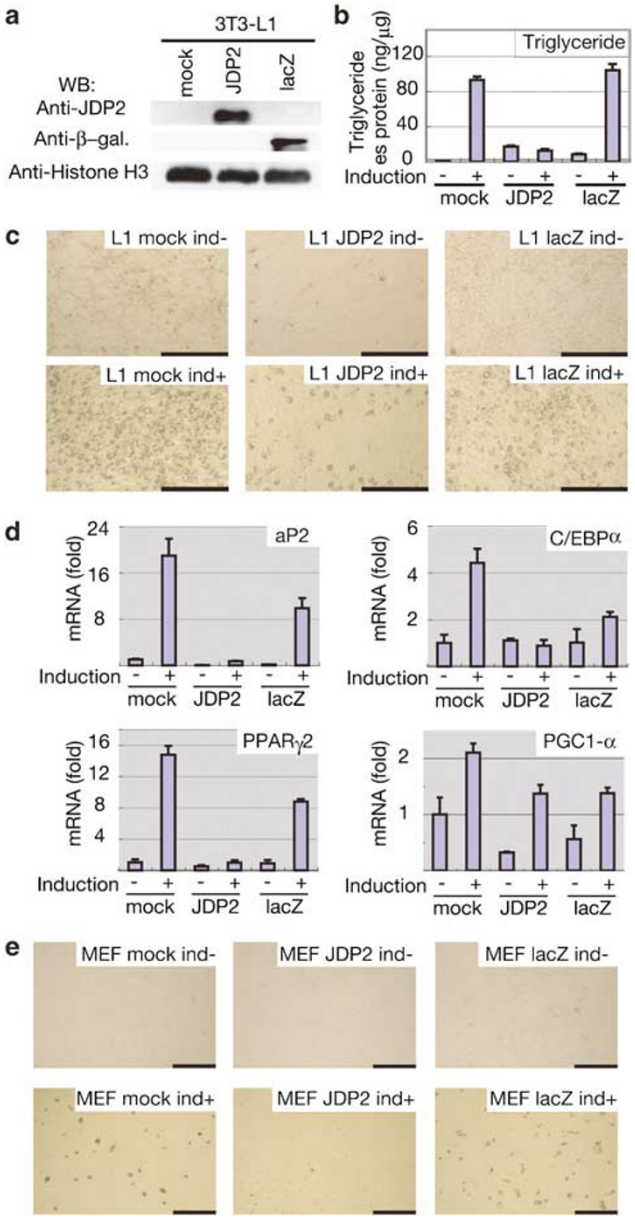


Figure 3 Inhibition of the differentiation to adipocyte of 3T3-L1 cells and MEFs by expression of ectopic JDP2. 3T3-L1 preadipocytes were infected with recombinant adenovirus that included cDNA that encoded JDP2 (Adeno-JDP2) or β-galactosidase (Adeno-LacZ), driven by the CAG promoter. The cells were exposed to differentiation medium for 2 days and then to Insulin-GM for 1 week. (**a**) Total cell lysates from Adeno-JDP2-, Adeno-LacZ- and mock-infected cells were subjected to Western blotting with antibodies against JDP2 and β-galactosidase. A loading control was blotted with antibodies specific for histone H3. (**b**) The same lysates were used to determine the levels of triglycerides in cells. Relative values were standardized by reference to amounts of total protein. (**c**) The morphology of cells was examined by light microscopy. Scale bars, 0.5 mm. (**d**) Total RNA was extracted and the levels of expressions of adipocyte marker genes (for aP2, C/EBPα, PPARγ2 and PGC1-α) were determined by real-time RT-PCR. Relative levels were normalized by reference of those of genomic DNAs. (**e**) *Jdp2*$^{-/-}$ MEFs were infected with Adeno-JDP2 or Adeno-LacZ and treated for the induction of adipocyte differentiation as indicated. The cells were examined by light microscopy. Scale bars, 0.5 mm

Insulin-GM for 7 days to induce adipocyte differentiation. We then lysed the cells and measured levels of triglycerides (TGs) as a marker of adipocytes. We found that the level of TGs was five- to six-fold lower in Adeno-JDP2-infected cells (17 ng/μg protein) than in Adeno-lacZ (104 ng/μg protein)- and mock (93 ng/μg protein)-infected cells (Figure 3b). Light microscopy revealed fewer adipocytes in cultures of Adeno-JDP2-infected cells than in cultures of Adeno-lacZ- and mock-infected cells (Figure 3c). When we compared the levels of mRNAs for adipocyte markers such as aP2, C/EBPα and PPARγ2, we found that the respective levels were significantly lower in Adeno-JDP2-infected cells (Figure 3d). We also infected *Jdp2*$^{-/-}$ MEFs with the Adeno-JDP2 vector in a 'rescue' experiment and then examined the induced differentiation of adipocytes. Light microscopy of cells revealed the almost complete absence of adipocytes in cultures of Adeno-JDP2-infected cells and increased numbers of adipocytes in Adeno-lacZ- and mock-infected cells after treatment to induce adipocyte differentiation (Figure 3e). These results strongly suggest that JDP2 is a negative regulator of adipocyte differentiation.

To study the effects of JDP2 on the formation of adipose tissue, we prepared sections of mouse adipose tissue from scapulae of *Jdp2*$^{-/-}$ and WT mice. Staining with hematoxylin and eosin (Figure 4a–d) revealed significant accumulation of lipid in the adipose tissue from 3-week-old *Jdp2*$^{-/-}$ mice. Moreover, the adipose tissue of 3-week-old Jdp2$^{-/-}$ mice consisted mostly of adipocytes that contained single large lipid droplets and resembled white adipocytes (Figure 4c), whereas many and significantly smaller lipid droplets, which are characteristic of brown adipocytes, were observed in the adipose tissue from 3-week-old WT mice (Figure 4d). When the adipose tissue was stained with antibodies against PGC-1α, a marker of brown adipocytes, we detected stronger signals due to expression of PGC-1α in the adipose tissue from WT mice than in the adipose tissue from *Jdp2*$^{-/-}$ mice (Figure 4e–g). Taken together, our results indicate that JDP2 plays a role in development of adipose tissue *in vivo* and that the preadipocytes in scapulae of JDP2-deficient mice preferentially differentiate to white adipocytes, while those of WT mice mostly differentiate to brown adipocytes.

In order to identify the genes that are targets of JDP2 in the inhibition of adipocyte differentiation, we analyzed the expression of adipogenesis-related genes during adipogenesis. We extracted total RNA from *Jdp2*$^{-/-}$ MEFs and WT MEFs 48 and 72 h after the start of the induction of adipogenesis and measured relative levels of expression of mRNAs for PPARγ2, C/EBPα, C/EBPβ and C/EBPδ by real-time RT-PCR (Figure 5a–d). We normalized levels by reference to levels of mRNA for glyceraldehyde-3-phosphate dehydrogenase (GPDH). We found that the expression of C/EBPδ mRNA was strongly suppressed 48 h after the start of induction in WT MEFs (Figure 5d), whereas that of C/EBPβ mRNA was suppressed to a more limited extent (Figure 5c). The expression of C/EBPα mRNA was also inhibited in the presence of JDP2 (Figure 5b). By contrast, we found no conspicuous difference in the level of expression of PPARγ2 mRNA between *Jdp2*$^{-/-}$ MEFs and WT MEFs (Figure 5a). The expression of JDP2 did not change during induction of adipocyte differentiation in MEF WT cells (Figure 5e). Our

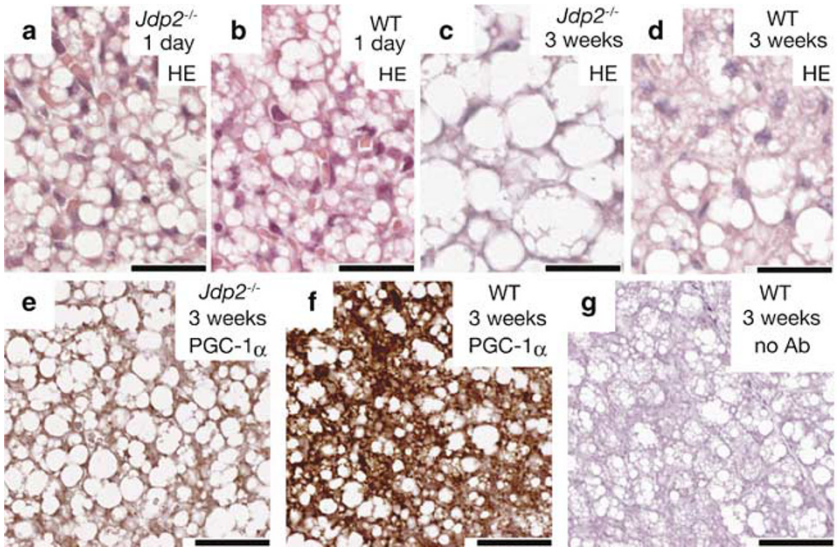


Figure 4 Accumulation of lipids in *Jdp2*$^{-/-}$ mice. Staining with hematoxylin and eosin (**a–d**) or immunostaining with (**e** and **f**) or without (**g**) antibodies against PGC-1α of sections of adipose tissue from scapulae of 1-day-old (**a** and **b**) and 3-week-old (**c–g**) *Jdp2*$^{-/-}$ (**a**, **c** and **e**) and WT (**b**, **d**, **f** and **g**) mice, as indicated. Scale bars, 0.05 mm

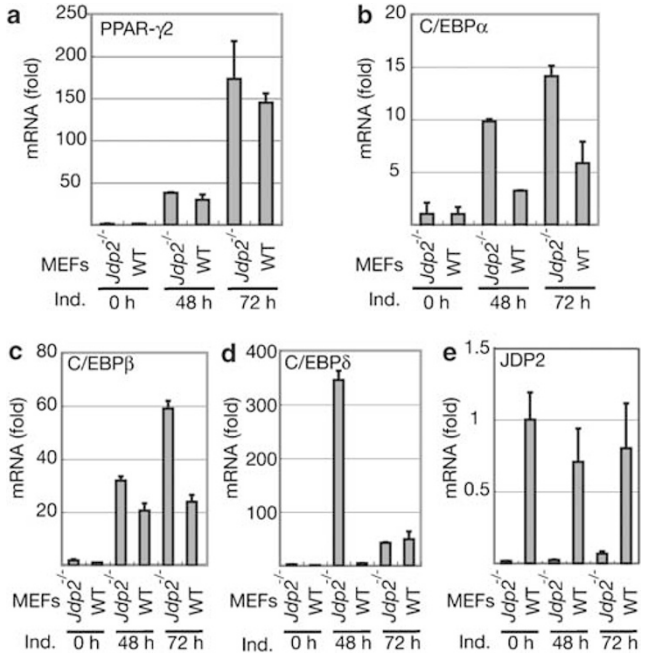


Figure 5 Induced expression of adipogenesis-related early genes in *Jdp2*$^{-/-}$ and wild-type MEFs. MEFs were stimulated to induce adipocyte differentiation for 0, 48 and 72 h. Total RNA was used for real-time RT-PCR to detect mRNA for (**a**) PPARγ2; (**b**) C/EBPα; (**c**) C/EBPβ, (**d**) C/EBPδ and (**e**) JDP2. Relative levels were normalized by reference to those of GPDH mRNA

data suggested that JDP2 targeted the genes for C/EBPs, but not for PPARγ2, and inhibited their expression, with resultant repression of the differentiation of MEFs to adipocytes.

To study the effects of JDP2 on the expression of C/EBPs in further detail, we performed chromatin immunoprecipitation (ChIP) assays using lysates of *Jdp2*$^{-/-}$ MEFs and WT MEFs, with or without induction of adipogenesis, and found that JDP2 was recruited to the *C/EBPδ* and *C/EBPβ* genes but not to the *C/EBPα* gene (Figure 6a). Since repression of expression of C/EBPδ was more significant than that of C/EBPβ (Figure 5c and d) in the presence of JDP2, it seemed likely that the *C/EBPδ* gene might be a target of JDP2. To confirm our results, we cloned a DNA fragment that corresponded to nucleotides (nts) -224 to $+35$ from the site of initiation of transcription of the mouse *C/EBPδ* gene by genomic PCR. The results of a transactivation assay with a promoter–luciferase construct revealed that the cloned fragment of the *C/EBPδ* promoter was sufficient for activation of transcription in response to inducers of adipogenesis, and that such activation was abolished in the presence of JDP2 (Figure 6b). Gel shift analysis using several oligodeoxynucleotide probes that corresponded to the *C/EBPδ* promoter and purified histidine-tagged recombinant JDP2 revealed that the cloned fragment of the *C/EBPδ* promoter included two AP-1-like JDP2-binding sites (data not shown). We engineered single- and double-substitution mutations at these AP-1-like sites in the *C/EBPδ* promoter. Subsequent gel shift assays demonstrated that JDP2 could bind to the promoter of the *C/EBPδ* gene with a single mutation at an AP-1-like site (either C/EBPδ-MW or C/EBPδ-WM), but it failed to bind to the doubly mutated site (C/EBPδ-MM) (Figure 6c). Moreover, when we used the WT promoter (C/EBPδ-WW), we detected two appropriately shifted bands on the gel.

To show that JDP2 was also unable to bind to the doubly mutated *C/EBPδ* promoter *in vivo*, we transfected *Jdp2*$^{-/-}$ MEFs with wild-type (WW), single-mutant (MW or WM) or double-mutant (MM) forms of the *C/EBPδ* promoter. Cells were stimulated in differentiation medium and then we performed ChIP assays using antibodies against JDP2. To distinguish the activity of the reporter plasmid from that of the endogenous promoter we used a DNA fragment that corresponded to part of the pGL3 plasmid in these assays. Our results showed that JDP2 was recruited to the WT and the singly mutated AP-1-like site in the *C/EBPδ* promoter but not to the doubly mutated promoter, as we had expected (Figure 6d).

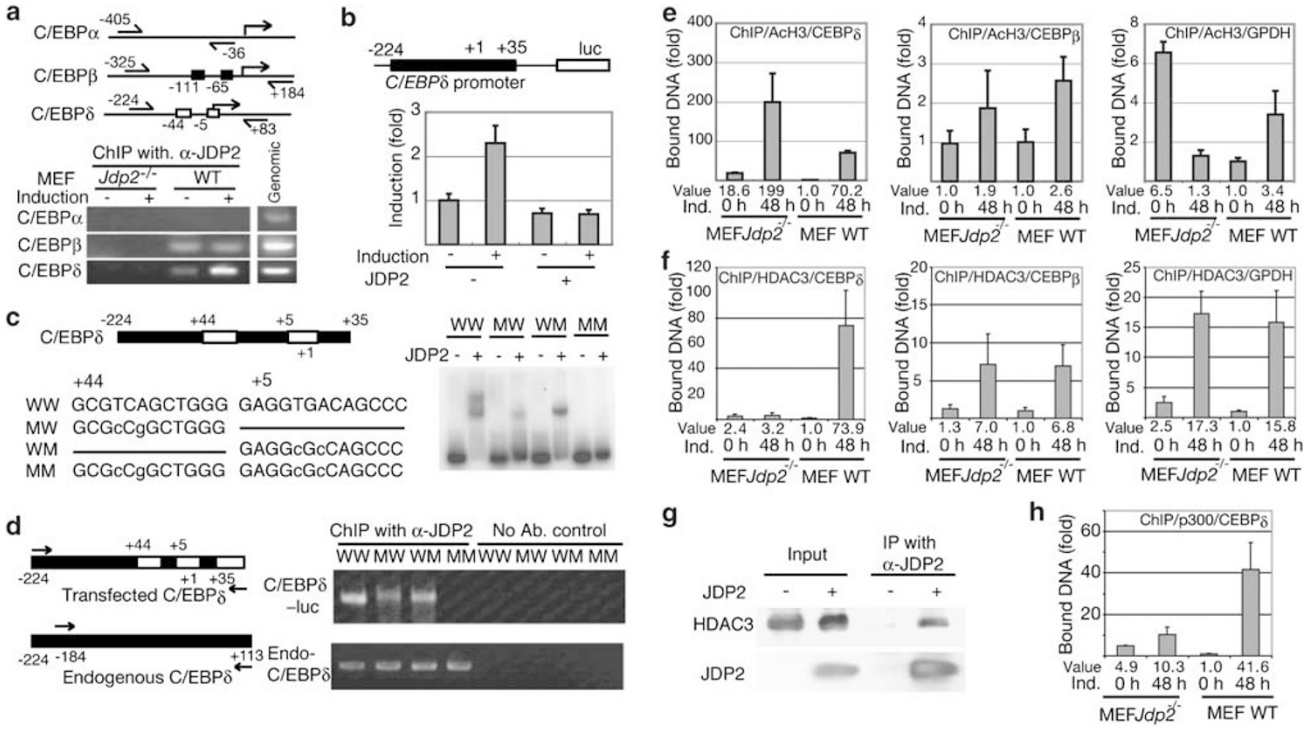


Figure 6 JDP2 negatively regulates expression of the *C/EBPδ* gene. (**a**) ChIP assays with JDP2-specific antibodies of lysates of *Jdp2*$^{-/-}$ and wild-type (WT) MEFs with or without exposure to adipogenic stimuli. DNA fragments of the *C/EBPα*, *C/EBPβ* and *C/EBPδ* genes were detected by PCR. The positions of primers are indicated by arrows in the schematic structure of each promoter. The black and white boxes represent CRE[35] and JDP2-binding sites,[36] respectively. (**b**) Transactivation assay. The *C/EBPδ* promoter–luciferase construct was introduced into the *Jdp2*$^{-/-}$ MEFs with or without the JDP2 expression vector. Cells were exposed to adipogenic stimuli for 12 h. Then promoter activities were examined by the luciferase assay. (**c**) Gel shift assays using purified recombinant JDP2 and the *C/EBPδ* promoter probe (nucleotides (nts) −224 to +35) with or without mutations in JDP2-binding sites (white boxes). (**d**) ChIP assays with JDP2-specific antibodies (α-JDP2). *Jdp2*$^{-/-}$ MEFs were transfected with a pGL3-tagged WT or mutant *C/EBPδ* promoter fragment and the JDP2 expression vector. The mutants are depicted schematically in (**c**). The arrows indicate the positions of primers used for the PCR. Precipitated DNA fragments were detected by PCR with primers specific for the exogenous and endogenous (ChIP control) *C/EBPδ* promoter. (**e**, **f** and **h**) ChIP assays with antibodies against (**e**) acetyl H3, (**f**) HDAC3 and (**h**) p300 and the same cell extracts as those in the experiment for which results are shown in (**a**). The fragments of *C/EBPδ* (**e**, **f** and **h**), *C/EBPβ* and *GPDH* (**e** and **f**) genes were detected by real-time PCR. The relative levels were normalized by reference to the input DNA. (**g**) Co-immunoprecipitation of the complex of HDAC3 and JDP2. Cos-1 cells were transfected with the expression vector(s) for HDAC3 or for HDAC3 and JDP2. Cell lysates were subjected to immunoprecipitation (IP) with JDP2-specific antibodies and analyzed by Western blotting with HDAC3-specific or JDP2-specific antibodies, respectively

We also examined the acetylation of histone H3 on the *C/EBPβ* and *C/EBPδ* genes after 48 h of adipocyte induction by ChIP assays with antibodies against acetylated histone H3 (Figure 6e). The level of acetylated histone H3 at the promoter region of the *C/EBPδ* gene increased in response to adipogenic stimulation of *Jdp2*$^{-/-}$ MEFs (18-fold increase without stimulation and 200-fold increase with stimulation). Moreover, in WT MEFs, less extensive acetylation of histone H3 on the promoter of the *C/EBPδ* gene was evident during such differentiation (70-fold increase with stimulation). By contrast, the amount of acetylated H3 on the promoter of the *C/EBPβ* gene was much less significantly affected by the stimulation that induced the differentiation of adipocytes (1.9-fold increase in *Jdp2*$^{-/-}$ MEFs with induction and 2.6-fold increase in WT MEFs with induction). In addition, no repression of histone acetylation at the *C/EBPβ* gene was observed in WT MEFs (Figure 6e).

We reported previously that JDP2 functions as a repressor by recruiting histone deacetylase 3 (HDAC3) to the promoter region of the *c-jun* gene in F9 cells.[18] Moreover, we showed also that JDP2 can act directly to inhibit the histone acetyltransferase (HAT) activity induced by p300 in undifferentiated F9 cells and that it induces chromatin assembly.[24] In the presence of RA, JDP2 and HDAC3 were excluded from the differentiation response element (DRE) site and p300 HAT was recruited to this site during the RA-induced differentiation of F9 cells. Therefore, we next examined the involvement of HDAC3 and p300 in adipocyte differentiation.

Our ChIP assays with antibodies against HDAC3 showed that the recruitment of HDAC3 to the *C/EBPδ* promoter was markedly enhanced in the presence of differentiation-inducing stimuli in WT MEFs (74-fold) but not in *Jdp2*$^{-/-}$ MEFs (3.2-fold). By contrast, the recruitment of HDAC3 to the *C/EBPβ* gene was unaffected by expression of JDP2 during differentiation (seven-fold in each case; Figure 6f). Co-immunoprecipitation (co-IP)assay and Western blotting revealed that JDP2 associated with HDAC3 in Cos-1 cells *in vivo* (Figure 6g), a result that was consistent with results reported previously in a different system.[18]

Additional ChIP assays with antibodies against p300 demonstrated that p300, recruited to the promoter of the *C/EBPδ* gene (Figure 6h), probably contributed to histone acetylation via its histone acetyltransferase (HAT) activity. The p300 protein recruited to the *C/EBPδ* promoter was more

abundant in WT MEFs (42-fold incerase) than in *Jdp2*$^{-/-}$ MEFs (10-fold) during the differentiating, suggesting that the extent of histone acetylation might depend mainly on the presence of JDP2 and HDAC3 but not of p300.

Discussion

We have shown here that JDP2 acts as a repressor of adipocyte differentiation via the repression of histone acetylation, under physiological conditions. We and others reported that JDP2 is an AP-1 transcriptional repressor[15,17–20] with histone–chaperone activity that inhibits histone acetylation by recruiting HDAC3.[24] JDP2 also inhibited the RA-dependent differentiation of embryonic carcinoma F9 cells.[18] These findings were obtained from the studies *in vitro* and the overexpression studies *in vivo*. In the present study, we generated *Jdp2*-deficient mice to examine the effects of endogenous JDP2 protein. We found that *Jdp2*$^{-/-}$, *Jdp2*$^{+/-}$ and *Jdp2*$^{+/+}$ mice were born at appropriate frequencies without any apparent physical defects, an indication that JDP2 is not essential for the regulation of differentiation and cell proliferation during embryogenesis. Then, we prepared MEFs to study the effects of JDP2 on cell differentiation and proliferation. We used, as a model, an adipocyte differentiation system, whose signalling cascade had been well characterized, and found that MEFs lacking JDP2 were more susceptible than WT MEFs to the stimulation that induced the differentiation to adipocytes. This observation was supported by the results of the rescue experiments in which introduction of the recombinant Adenovirus JDP2 vector in 3T3-L1 preadipocytes and MEFs blocked adipocyte differentiation (Figure 3c and e).

The C/EBPδ and C/EBPα mRNAs were overexpressed in *Jdp2*$^{-/-}$ MEFs during the initial stages of adipocyte differentiation, whereas C/EBPβ and PPARγ mRNAs were less affected by the absence of JDP2 (Figure 5b). C/EBPα is known to be the downstream target of C/EBPδ and C/EBPβ,[25] therefore, it can be speculated that the augmentation of expression of the *C/EBP*α gene might have been due to indirect effects, which were probably caused by the enhanced expression of the *C/EBP*δ gene. In fact, the binding of JDP2 to the *C/EBP*δ and *C/EBP*β genes was detected but there was no obvious binding to the *C/EBP*α gene (Figure 6a). In addition, two AP-1-like sequences that were targets of JDP2 by gel shift and ChIP assays were found (Figure 6c and d). Our results indicated that JDP2 was able to bind to the *C/EBP*δ promoter to repress the transcription of the *C/EBP*δ gene.

Our JDP2-deficient mice did not have abundant adipose cells. In other words, they did not have a thick layer of fat tissue, perhaps because the mass of adipose tissue might be determined by more complex factors such as cytokines and hormones, via as yet unknown mechanisms, and by energy status. We found that the adipose tissue of scapulae from young *Jdp2*$^{-/-}$ mice consisted mostly of white adipocytes, whereas the majority of cells were brown adipocytes in WT mice. This observation suggested that JDP2 might play a role in adipogenesis *in vivo*.

Recently, it has been reported that the activation of Wnt signalling inhibited the development of brown adipocytes by mediating the PGC1-α-UCP1 cascade.[26] In addition to C/EBPs, as shown in the present studies, JDP2 might be a target of such signal networks. Other report suggested that the absence of retinoblastoma protein (Rb) switched the differentiation of MEF cells from white to brown adipocytes,[27] suggesting that the differentiation of WT MEFs to adipocytes is a good model for white adipocyte differentiation but not brown adipocyte differentiation. We also observed that PGC-1α, a marker of brown adipocyte, was not induced after induction of adipocyte differentiation in *Jdp2*$^{-/-}$ MEFs and WT MEFs (data not shown). Then, we analyzed the levels of mRNA of PGC-1α in Adeno-JDP2-infected 3T3-L1 cells. 3T3-L1 cells are also known to be a model for white adipocyte differentiation; however, we could detect small amount of PGC-1α mRNA by real-time RT-PCR, as the others reported elsewhere,[28] probably because quite minor population of 3T3-L1 cells can differentiate to brown adipocyte. The expression of PGC-1α was increased after adipocyte differentiation and the levels of expression were not remarkably different between Adeno-JDP2- and Adeno-lacZ-infected 3T3-L1 cells (Figure 3d). So, we speculate that JDP2 might exclusively inhibit the differentiation of white adipocytes but not brown adipocytes. In consequence, brown adipocytes are preferentially developed in WT mice scapulae, whereas white adipocyte appeared in *Jdp2*$^{-/-}$. However, further studies are required to clarify the role of JDP2 in the molecular mechanism that directs fibroblast and mesenchymal cells to differentiate into white or brown adipocytes.

How JDP2 might inhibit transcription of the *C/EBP*δ gene? It seems possible that, in response to signals that lead to differentiation, the *C/EBP*δ promoter might recruit transcription factors, including the coactivator p300, that mediate the acetylation of histones associated with the *C/EBP*δ gene to stimulate transcription of this gene. In the presence of JDP2, the acetylation of histone is inhibited and the expression of the *C/EBP*δ gene is suppressed during adipocyte differentiation, even in the presence of differentiation-inducing signals. Decreased expression of C/EBPδ results in less effective differentiation to adipocytes, a conclusion that is consistent with a previous report that deletion of the *C/EBP*δ gene results in the impairment of adipocyte differentiation.[29] By contrast, HDAC3 was not recruited by JDP2 to the promoter region of the *C/EBP*β gene, although the recruitment of HDAC3 to the *C/EBP*β gene was slightly enhanced during differentiation. In addition, the acetylation of histone H3 on the *C/EBP*β gene was less affected by differentiation-inducing stimuli. These observations suggest that histone acetylation might not play an important role in the transcriptional regulation of the *C/EBP*β gene and the HDAC3 might not be involved in such regulation. This hypothesis explains why the presence of JDP2 on the promoter had no effect on transcription.

In conclusion, we propose that JDP2 acts as a negative molecular switch in some, but not all, types of differentiation via the regulation of the expression of specific genes in concert with histone deacetylase HDAC3, as shown in the present study of adipocyte differentiation. Further investigations are necessary to clarify the molecular networks involved in the rearrangement of chromatin that is associated with such differentiation.

Materials and Methods

Plasmids, antibodies and reagents. A fragment of the *C/EBPδ* gene (nts −224 to + 35; DDBJ no. X61800) was amplified by genomic PCR and inserted in the pGL3(R2.2)-basic vector (Promega, Madison, WI, USA) to generate pGL3C/EBPδ. The coding region of the *Jdp2* gene was inserted at the *Xho*I/*Eco*RI site of the pPyCAG-*Bst*XI-IRES-Zeocin-pA vector (kindly provided by Dr Hitoshi Niwa, Center for Developmental Biology, Kobe Institute, RIKEN, Kobe, Japan) to generate pPyZmJDP2. pcDNA3flagHDAC3 was provided by Dr Ed Seto (Moffitt Cancer Center, Tampa, FL, USA). The monoclonal antibody directed against JDP2 was prepared as described elsewhere.[18] Acetylated histone H3-specific antibody (#06-599) and HDAC3-specific antibody (#06-890) were obtained from Upstate (Charlottesville, VA, USA). p300-specific antibody (sc-584) was obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA).

Construction of the targeting vector and generation of *Jdp2* mutant mice genomic DNA (11 kb) containing the first exon of the *Jdp2* gene was obtained by screening a mouse lambda FIX II library generated from 129/sv mice. The targeting vector (pTV-2) was constructed by subcloning a 5.5-kbp *Eco*RV/*Xba*I fragment (located in the 5′-upstream region of the gene) and a 2.3-kbp *Pst*I/*Sal*I fragment (located in intron 1) in the pPNT vector[30] as the 5′- and 3′-homologous regions, respectively. The *Not*I-linearized targeting vector was introduced by electroporation into E14tg2a ES cells[31] and transformed cells were selected in the presence of 200 μg/ml G418. Resistant colonies were subjected to Southern blotting analysis. ES clones were injected into 3.5-day-old C57BL/6J blastocytes, which were then transferred to pseudopregnant female recipients. The resulting chimeras were mated with C57BL/6J mice and germline transmission was monitored in terms of coat color and by Southern blotting analysis. F1 mice that were heterozygous for the disrupted *Jdp2* gene were mated to generate homozygous *Jdp2*-null mice (JDP2$^{-/-}$ mice).

Genotyping of *Jdp2*$^{-/-}$ mutant mice. Genotypes were determined by PCR and confirmed by Southern blotting analysis. The WT *Jdp2* allele was detected by use of a forward primer (5′-TATGGGTGATGACCTGCTGT-3′) and a reverse primer (5′-CAGGATCTCGCAAGCTTGTT-3′) derived from the 5′-upstream region and exon 1 of the *Jdp2* gene, respectively, as shown in Figure 1a. A reverse primer (5′-TCCTCGTGCTTTACGGTATC-3′) specific for a gene for aminoglycoside-3′-phosphotransferase (neomycin resistance gene) was designed to detect the *Jdp2* gene-targeted allele in combination with the forward primer of the WT, as described above.

Histology. Interscapular adipose tissue was dissected out from 3-month-old *Jdp2*$^{-/-}$ and WT mice and fixed by immersion in ice-cold 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4) for 6 h. The tissues were rinsed with 0.1 M sodium phosphate buffer, dehydrated with an ethanol series, embedded in polyester wax (BDH, Poole, England) and sectioned serially at 4 mm thickness. The sections were dewaxed with three changes of ethanol, rehydrated with distilled water, counterstained with Mayer's hematoxylin solution (Merck, Darmstadt, Germany) and photographed under a light microscope (Axiophoto2; Carl Zeiss, Jena, Germany).

Immunostaining. The sections were treated in 0.05 M sodium citrate buffer (pH 6.0) for 5 min at 115°C in an autoclave, immersed in methanol containing 0.2% sodium azide and 0.6% H_2O_2 to inactivate endogenous peroxidase for 30 min, incubated with avidin–biotin blocking solution (Vector Laboratory, Burlingame, CA, USA) for 30 min, with PBS that containing 5% normal horse serum and 1% BSA for 30 min, and with rabbit polyclonal antibodies against PGC-1α (Calbiochem, San Diego, CA, USA) at 1:300 dilution overnight. After reaction with biotinylated antibodies against rabbit IgG for 30 min (Vector Laboratory), the sections were incubated with avidin–biotin peroxidase complex for 30 min. After washing in PBS and distilled water, the sections were incubated in a solution of diaminobenzidine (Dako Japan, Kyoto, Japan) until staining had reached the desired intensity.

Induction of adipocyte differentiation. Confluent MEFs were induced to differentiate to adipocytes by incubation in differentiation medium (DMEM containing 10% FCS supplemented with 10 μM troglitazone, 0.5 mM isobutylmethylxanthine (IBMX), 0.25 μM dexamethasone and 10 μg/ml insulin) for 2, 3 or 4 days.[32] Then cells were maintained for 7 days in standard growth medium supplemented with 5 μg/ml insulin (Insulin-GM). MEFs that differentiated to adipocytes were detected by light microscopy and eventually stained with Oil-Red.

Adenoviral expression of JDP2 and adipocyte induction. The Adeno-JDP2 vector was constructed by inserting JDP2 cDNA into pAxCAwt[33] and infectious viral particles were produced and purified as described elsewhere.[34] 3T3-L1 cells or *Jdp2*$^{-/-}$ MEFs were distributed in 24 wells at 1×10^5 cells/well to generate nearly confluent culture. After 12 h, Adeno-JDP2 or Adeno-lacZ was added at a multiplicity of infection (MOI) of 10. Two days after infection, the cells were washed, incubated with differentiation medium for 2 days and then cultured for 1 week with Insulin-GM. Insulin-GM was replaced by fresh medium every 2 days.

ChIP assays. ChIP assays were performed according to the manufacturer's instructions (ChIP assay kit; Upstate). Confluent MEFs were cultured in differentiation medium for 48 h, crosslinked by treatment with formaldehyde, lysed in lysis buffer and sonicated to generate DNA fragments of approximately 2000 bp (for IP with JDP2-specific and p300-specific antibodies) or 300 bp (for IP with acetyl histone H3-specific and HDAC3-specific antibodies). Alternatively, MEF cells were transfected with JDP2 expression vector and mutant or WT C/EBPδ promoter fragment containing partial pGL3 vector derived sequence (from 53 to 248 bp, Promega vector data), exposed to the differentiation medium for 12 h, and crossliked, lysed and sonicated as described above. Immunoprecipitated fragments of DNA were analyzed by semi-quantitative PCR or real-time PCR with specific primers as follows: C/EBPα, 5′-TCCAAACGCTCCCCAACCTC-3′ and 5′-TTATAGAGGGTCGGGCATCG-3′; C/EBPβ, 5′-TAGCTGGAGGAACGATCTGT-3′ and 5′-TTGGCCACTTCCATGGGTCT-3′; C/EBPδ, 5′-GTCGGGGCCAAATCCAGATT-3′ and 5′-TAGAAGGCCGCGGGTTCTGT-3′ and transfected C/EBPδ, 5′-CTCCGGTCTCCGACCCACTGGGGCCGGGGC-3′ and 5′-GCATAGGTGATGTCCACCTC-3′.

Real-time RT-PCR. Confluent MEFs were cultured in the differentiation medium for 48 or 72 h and total RNA were extracted and purified with an RNeasy Mini Kit™ (Qiagen, Hilden, Germany). Alternatively, differentiated or undifferentiated MEFs or 3T3-L1 cells were lysed by TES (10 mM Tris–HCl (pH 8.0), 1 mM EDTA, 1% SDS), sonicated for 15 s and extracted twice with phenol/chloroform and once with chloroform. Then RNA was precipitated in ethanol (for experiments for which the results are shown in Figure 2b and d). The relative levels of specific transcripts were analyzed with PRISM™ 7700 (ABI, Foster City, CA, USA). For Figure 5, we used the SuperScript™ III Platinum one-step qRT-PCR system (Invitrogen, Groningen, The Netherlands) supplemented with SyberGreen. For Figures 2b and 3d, the samples were reverse transcribed by SuperScript™ III and amplified with SYBR PCR mix (Applied Biosystems). Without reverse transcription, samples were used to determine levels of genomic DNA as input controls. The sets of primers were as follows: PPARγ2, 5′-ATGCTGTTATGGGTGAAACT-3′ and 5′-CTTGGAGCTTCAGGTCATATTTGTA-3′; C/EBPα, 5′-GTCGGTGGACAAGAACAGCA-3′ and 5′-CCTTCTGTTGCGTCTCCACG-3′; C/EBPβ, 5′-GACGGTGGACAAGCTGAGCG-3′ and 5′-CCTTGTGCTGCGTCTCCAGG-3′; C/EBPδ, 5′-AAAGTGCAGGCTTGTGGACT-3′ and 5′-TTACTCCACTGCCCACCTGT-3′; PGC1-α, 5′-CGGTCTTAGCACTCAGAACC-3′ and 5′-AAGCTCTGAGCAGGGACGTC-3′; aP2, 5′-GTACTCTAAGTCCAGTGATC-3′ and 5′-TCTGACCGGATGGTGACCAA-3′; and JDP2, 5′-CGCTGACATCCGCAACATTG-3′ and 5′-CATCTGGCTGCAGCGACTTT-3′. The primers for amplification of the cDNA for GPDH were provided as TaqMan™ EZ ET-PCR Core Reagents (ABI).

Levels of TGs. Cells were lysed in TES0.1 buffer (10 mM Tris–HCl, pH 7.4, 1 mM EDTA and 0.1% SDS). Levels of TGs were determined by the triglyceride *E*-test (Wako, Tokyo, Japan) and normalized by reference to the concentration of protein in the respective cell extracts.

Luciferase assay. The pGL3 C/EBPδ vector was introduced into *Jdp2*$^{-/-}$ MEFs with or without the JDP2 expression vector (pPyZmJDP2) in *N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES)-buffered saline (BBS) by the calcium phosphate method. Transfected cells were washed, cultured for 24 h and then incubated in differentiation medium or in normal growth medium for a further 12 h. Cells were lysed and subjected to the luciferase assay according to the instructions from Promega.

Gel shift assays. The ^{32}P-labelled double-stranded DNA probe was incubated for 15 min with 1.5 μg of His-tagged JDP2 that has been expressed in *E. coli* and 1 μg of poly dI : dC in TGE buffer (50 mM Tris, 2 mM EDTA and 380 mM glycine) that contained 2.5% glycerol. The DNA–protein mixture was fractionated on a 4% polyacrylamide gel prepared in TGE buffer and bands were visualized by

autoradiography. The sequences of the probes used in this assay corresponded to nts −224 to + 35 of the *C/EBPδ* promoter sequence, with or without the mutations indicated in Figure 6c.

Co-IP. Semiconfluent Cos-1 cells in 10-cm-dishes were transfected with 10 μg of pcDNA3HDAC3 and 10 μg of pPyZmJDP2 or the empty vector. Transfected cells were lysed in NETx-200 buffer (200 mM NaCl, 10 mM Tris–HCl, pH 7.4, 1 mM EDTA and 0.1% Triton-X 100) and lysates were clarified by centrifugation. Each supernatant was incubated with JDP2-specific antibodies and then incubated with beads of protein A–Sepharose (Pharmacia, Uppsala, Sweden). The beads were pelleted, washed and boiled with loading buffer for SDS-PAGE. The eluted proteins were analyzed by Western blotting with antibodies specific for HDAC3 and JDP2.

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