Defective adipocyte differentiation in mice lacking the C/EBP β and/or C/EBP δ gene

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**To investigate the role of C/EBP family members during adipocyte differentiation *in vivo*, we have generated mice lacking the C/EBP β and/or C/EBP δ by gene targeting. Approximately 85% of C/EBP β ($-/-$)- δ ($-/-$) mice died at the early neonatal stage. By 20 h after birth, brown adipose tissue of the interscapular region in wild-type mice contained many lipid droplets, whereas C/EBP β ($-/-$)- δ ($-/-$) mice did not accumulate droplets. In addition, the epididymal fat pad weight of surviving adult C/EBP β ($-/-$)- δ ($-/-$) mice was significantly reduced compared with wild-type mice. However, these adipose tissues in C/EBP β ($-/-$)- δ ($-/-$) mice exhibit normal expression of C/EBP α and PPAR γ , despite impaired adipogenesis. These results demonstrated that C/EBP β and C/EBP δ have a synergistic role in terminal adipocyte differentiation *in vivo*. The induction of C/EBP α and PPAR γ does not always require C/EBP β and C/EBP δ , but co-expression of C/EBP α and PPAR γ is not sufficient for complete adipocyte differentiation in the absence of C/EBP β and C/EBP δ .
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Introduction

There are two types of adipose tissue in the body, termed white adipose tissue (WAT) and brown adipose tissue (BAT). WAT stores excess energy as triglycerides and releases free fatty acids in response to energy requirements at other sites. BAT is involved in non-shivering and diet-induced thermogenesis through the uncoupling of oxidative phosphorylation, which is carried out by the action of a BAT-specific uncoupling protein (UCP). WAT is distributed in a number of locations throughout the body. In contrast, BAT is restricted to specific areas. WAT consists of adipocytes having a single fat droplet within the cells, whereas BAT has a multilocular disposition of fat droplets, i.e. a number of individual droplets within each adipocyte.

Adipocyte differentiation is a complex process involving a cascade of expression of many transcription factors and adipocyte-specific genes. Accumulating evidence suggests that two families of transcription factors, CCAAT/

enhancer-binding proteins (C/EBPs) and peroxisome proliferator-activated receptors (PPARs), are important regulators of adipocyte gene expression and differentiation. The C/EBP family belongs to the large family of basic leucine zipper (bZip) transcription factors. All members of the C/EBP family have a C-terminal leucine zipper domain for dimerization and a basic domain for DNA binding, respectively. To date, six members have been cloned and characterized: C/EBP α , C/EBP β (also known as NF-IL6, LAP, AGP/EBP, IL-6DBP or NF-M) (Akira *et al.*, 1990; Chang *et al.*, 1990; Descombes *et al.*, 1990; Poli *et al.*, 1990; Cao *et al.*, 1991; Katz *et al.*, 1993; Ness *et al.*, 1993), C/EBP γ (Ig/EBP or GPE1BP), C/EBP δ (NF-IL6 β , CRP3 or CELF) (Cao *et al.*, 1991; Kageyama *et al.*, 1991; Williams *et al.*, 1991; Kinoshita *et al.*, 1992), C/EBP ϵ (CRP1) and CHOP (gadd153). These proteins can both homodimerize and heterodimerize with each other and bind to the same C/EBP consensus sequences. Among them, three members of the C/EBP family, α , β and δ , are expressed in both WAT and BAT (Cao *et al.*, 1991; Manchado *et al.*, 1994; our unpublished data) and have been analyzed extensively with regard to their role in adipogenesis. Pre-adipocyte cell line 3T3-L1 cells differentiate into mature adipocytes when cultured in the presence of methylisobutylxanthine (MIX), dexamethasone (DEX), insulin (INS) and fetal bovine serum (FBS). These three C/EBP isoforms are expressed at specific periods during the differentiation of 3T3-L1 cells (Cao *et al.*, 1991; Yeh *et al.*, 1995). Ectopic expression of C/EBP α in 3T3-L1 pre-adipocytes blocked mitosis and induced adipocyte differentiation in the absence of hormonal stimulants. The blocking of C/EBP α induction in 3T3-L1 cells by antisense RNA inhibits adipocyte differentiation (Lin and Lane, 1992). Although NIH-3T3 fibroblasts cannot differentiate into adipocytes even in the presence of hormonal stimulants, forced expression of C/EBP α in NIH-3T3 cells results in adipose conversion in the absence of hormonal stimulants (Freytag *et al.*, 1994; Lin and Lane, 1994). Recently, mice with a targeted disruption of the C/EBP α gene have been generated. These mice have a severely reduced mass of both BAT and WAT, demonstrating that C/EBP α is essential for *in vivo* adipocyte differentiation (Wang *et al.*, 1995). Similarly, ectopic expression of C/EBP β , and to a lesser extent of C/EBP δ , can convert NIH-3T3 cells into adipogenic cells even in the absence of hormonal stimulants (Yeh *et al.*, 1995). Overexpression of a truncated and dominant-negative form of C/EBP β inhibits adipocyte conversion of 3T3-L1 cells (Yeh *et al.*, 1995). These results indicated that C/EBP β and C/EBP δ are also involved in adipocyte differentiation.

PPARs consist of PPAR α , γ and δ , and belong to the nuclear hormone receptor superfamily of ligand-activated transcription factors (Kliwer *et al.*, 1994; Mangelsdorf

et al., 1995). Of these members, only PPAR γ is expressed almost exclusively in adipose tissue (Chawla *et al.*, 1994). Ectopic expression of PPAR γ in NIH-3T3 fibroblasts leads to adipogenic conversion in the presence of its ligands thiazolidinedione or prostaglandin derivatives (Forman *et al.*, 1995; Kliewer *et al.*, 1995). When PPAR γ and C/EBP α are expressed together ectopically in NIH-3T3 fibroblasts, they synergistically accelerate the conversion into adipocytes (Tontonoz *et al.*, 1994b). Although the other two members are expressed to some extent in adipose tissue, PPAR α is less adipogenic than PPAR γ , and PPAR δ cannot stimulate adipogenesis (Burn *et al.*, 1996).

In the course of differentiation of adipocytes from 3T3-L1 pre-adipocytes, three C/EBP isoforms and PPAR γ are expressed sequentially: C/EBP β and δ are induced early and temporally in the process of differentiation, followed by PPAR γ . C/EBP α is expressed at the late stage of differentiation (Cao *et al.*, 1991; Yeh *et al.*, 1995). A large body of evidence points to a model of a cascade of transcription factors involved in adipogenesis as follows. At the onset of differentiation, C/EBP β and C/EBP δ are elevated in response to hormonal stimulation. Then, C/EBP β and δ synergistically stimulate the expression of C/EBP α and PPAR γ , both of which contain C/EBP-binding sites in their promoters (Christy *et al.*, 1989; Cao *et al.*, 1991; Wu *et al.*, 1995, 1996; Zhu *et al.*, 1995). C/EBP α activates several adipocyte-specific genes such as the stearoyl CoA desaturase (SCD1), insulin-responsive glucose transporter-4 (GLUT4), 422/aP2, phosphoenolpyruvate carboxykinase (PEPCK) and UCP genes which have functional C/EBP-binding sites in the promoter region (Christy *et al.*, 1989; Park *et al.*, 1990; Yubero *et al.*, 1994).

PPAR γ also transactivates the 422/aP2, PEPCK and lipoprotein lipase (LPL) genes whose promoters contain regulatory elements of PPAR γ (Tontonoz *et al.*, 1994a, 1995; Schoonjans *et al.*, 1996). Furthermore, reciprocal gene activation between C/EBP α and PPAR γ is demonstrated by the fact that ectopic expression of either transcription factor alone induces expression of the other. Therefore, at the final stage of adipocyte differentiation, PPAR γ and C/EBP α may cooperatively induce adipogenic genes to establish the mature adipocyte phenotype.

However, this scenario is established mainly from *in vitro* experiments using pre-adipocyte and fibroblast cell lines, and it remains to be determined whether it is applicable to *in vivo* adipocyte differentiation. To investigate the *in vivo* role of C/EBP β and C/EBP δ genes in adipocyte differentiation, we have generated C/EBP β - and/or C/EBP δ -deficient mice by gene targeting. Our results demonstrated that C/EBP β and C/EBP δ are essential for terminal differentiation of brown and white adipocytes *in vivo*.

Results

Generation of C/EBP β and C/EBP δ knockout mice and C/EBP β -C/EBP δ double knockout mice

Details as to the generation of C/EBP β knockout mice have been described previously (Tanaka *et al.*, 1995). Briefly, to construct the targeting vector for inactivation of the C/EBP β gene, the DNA-binding domain and leucine zipper domain were deleted and replaced by a neomycin

phosphotransferase (NEO) gene derived from pMC1Neopoly(A) (Thomas and Cappechi, 1987) (Figure 1A). Five homologous recombinant clones were obtained by PCR screening and subsequent Southern blot analysis using probe A (Figure 1B). Genotypic analysis of progeny of intercrosses between heterozygotes is shown in Figure 1C.

To construct the targeting vector for disruption of the C/EBP δ gene, a Balb/c mouse genomic DNA library was screened using the human C/EBP δ cDNA as a probe (Kinoshita *et al.*, 1992). One clone containing the C/EBP δ gene, composed of one exon, was obtained. A 9.5 kb fragment spanning from 1 kb 5' of the C/EBP δ transcriptional initiation site to 7.5 kb 3' of the end of the exon was subcloned into pBluescriptII KS(+) plasmid vector. To inactivate the C/EBP δ gene, the promoter region, transcription start site and transactivation domain were deleted and replaced by NEO derived from pMC1Neopoly(A) (Thomas and Cappechi, 1987). The MC1-herpes simplex virus thymidine kinase (HSV-TK) (Mansour *et al.*, 1988) was ligated at the 3' end of the homologous region.

The targeting vector was introduced into E14-1 embryonic stem cells by electroporation, and transfectants were selected with G418 and gancyclovir. Resistant clones were screened by PCR using primer pairs, one of which is located in the homologous 5' region outside the targeting vector and the other is in the NEO gene (Figure 1A). Homologous recombination events were confirmed further by Southern blot analysis using probe B (Figure 1B). Two homologous recombinant clones were obtained from 342 G418- and gancyclovir-resistant colonies. These two clones were injected into C57BL/6 blastocysts, and chimeric mice were obtained. Both of these clones gave a germline transmission of mutant alleles. C/EBP δ homozygous (-/-) mice were obtained by intercrosses between C/EBP δ heterozygous (+/-) females and males (Figure 1C). C/EBP δ (-/-) mice of both sexes were fertile. However, C/EBP β (-/-) females were infertile. To generate C/EBP β -C/EBP δ double knockout mice, we first produced C/EBP β (+/-)-C/EBP δ (+/-) mutants by mating C/EBP β (+/-) with C/EBP δ (-/-) mice. Then, these mice were crossed to C/EBP δ (-/-) to generate C/EBP β (+/-)-C/EBP δ (-/-) mutants. C/EBP β (-/-)-C/EBP δ (-/-) mice were finally obtained by intercrosses between C/EBP β (+/-)-C/EBP δ (-/-) females and males. Northern blot analysis confirmed the absence of any detectable C/EBP β mRNA in C/EBP β (-/-) mice and C/EBP δ mRNA in C/EBP δ (-/-) mice (Figure 1D).

C/EBP β -C/EBP δ double knockout mice died at the early neonatal stage

To determine the frequency of each of the knockout mice in heterozygote intercrosses, we analyzed the genotype of mice at 4 weeks of age. C/EBP δ (-/-) mice were obtained at the expected Mendelian ratio from C/EBP δ (+/-) parents (Table I), but both C/EBP β (-/-) offspring, from the cross of C/EBP β (+/-) mutants, and C/EBP β (-/-)-C/EBP δ (-/-) offspring, from C/EBP β (+/-)-C/EBP δ (-/-) mutants, were obtained at a frequency lower than the expected Mendelian ratio (Table I). The percentage of C/EBP β (-/-) mice was 16.2%, while that of C/EBP β (-/-)-C/EBP δ (-/-) mice was only 3.8%. Since genotype analysis at embryonic stages 15.5 and 18.5 d.p.c. revealed no embryonic death in either C/EBP β (-/-) or C/EBP β (-/-)-C/EBP δ (-/-) mice, we next

Fig. 1. The generation of mice lacking C/EBP β and C/EBP δ . (A) Diagram of targeting vector, wild-type allele and mutated allele. C/EBP β and C/EBP δ are intron-less genes. The solid box represents the exon of the C/EBP β or C/EBP δ gene. The broken lines represent the site of C/EBP β or C/EBP δ gene deletion and neomycin phosphotransferase (NEO) cassette insertion. The arrowheads represent the primers used for PCR analysis of screening. Probes used for Southern blot analysis are shown as a closed bar; probe A for C/EBP β and probe B for C/EBP δ . Abbreviations for the restriction enzyme sites are: (B) *Bam*HI; (E) *Eco*RI; (H) *Hind*III; (X) *Xho*I. (B) Southern blot analysis of targeted C/EBP β and C/EBP δ clones. C/EBP β : genomic DNA, isolated from five homologous recombinant clones, was digested with *Bam*HI and hybridized with probe A. This probe detects the 7.0 and 5.5 kb fragments corresponding to the wild-type and mutant allele, respectively. The introduction of an additional *Bam*HI site in the NEO insertion results in a smaller fragment in correctly targeted clones. C/EBP δ : genomic DNA, isolated from two homologous recombinant clones, was digested with *Eco*RI and hybridized with probe B. This probe detects the 7.0 and 2.5 kb fragments corresponding to the wild-type and mutant allele, respectively. The introduction of an additional *Eco*RI site in the NEO insertion results in a smaller fragment in correctly targeted clones. Lane W is wild-type ES cells, and the numbers represent different clones. (C) Southern blot analysis of progeny of C/EBP β or C/EBP δ heterozygote intercrosses. Genomic tail DNA was extracted from progeny of C/EBP β or C/EBP δ heterozygote intercrosses. They were digested with *Bam*HI and hybridized with probe A for C/EBP β knockout mice, or digested with *Eco*RI and hybridized with probe B for C/EBP δ knockout mice. (D) Northern blot analysis of lung of C/EBP β and C/EBP δ knockout mice. RNA was extracted from lung of wild-type, C/EBP β (-/-) and C/EBP δ (-/-) mice. Ten μ g of total RNA were electrophoresed and hybridized with C/EBP β cDNA as a probe for wild-type and C/EBP β (-/-) mice (left panel) or C/EBP δ cDNA as a probe for wild-type and C/EBP δ (-/-) mice (right panel). C/EBP β expression is not detected in the lung of C/EBP β (-/-) mice, and C/EBP δ expression is not detected in the lung of C/EBP δ (-/-) mice.

observed the fate of each newborn mouse. The initial survival of C/EBP β (-/-) neonates was good; the percentage of C/EBP β (-/-) mice at 10 h after birth was 22.4%, which is almost equal to the expected Mendelian ratio. Further observation of these neonates showed that ~35% of C/EBP β (-/-) neonates died within 24 h after birth, with no further deaths thereafter (Table I). In the case of C/EBP β (-/-)·C/EBP δ (-/-) mice, some had died at birth, as the percentage of these mice at 10 h after birth was only 13.0%, while others died within 24 h, a somewhat earlier time point than for C/EBP β (-/-) mice. Consequently, 85% of double knockout mice died at the perinatal or early postnatal stage (Table I). However, C/EBP β (-/-) and C/EBP β (-/-)·C/EBP δ (-/-) neonates showed no gross abnormalities in appearance and could suckle.

Lipid accumulation is impaired in interscapular BAT from C/EBP β (-/-)·C/EBP δ (-/-) mice

BAT is found only at certain sites in the body, such as in interscapular, perirenal and retroperitoneal regions, of

which the interscapular BAT deposit is the largest. BAT develops during fetal life and becomes identifiable at the late fetal stage as a mass with small lobules. At the early stage of development, BAT is composed of only pre-adipocytes, where the lipid droplet distribution is dispersed. Subsequently, these cells store triglycerides and become multilocular, typical of brown adipocytes. Both C/EBP β and C/EBP δ are expressed in BAT. We examined interscapular BAT microscopically in newborn wild-type, C/EBP β (-/-), C/EBP δ (-/-) and C/EBP β (-/-)·C/EBP δ (-/-) mice. As shown in Figure 2, interscapular BAT from wild-type mice had accumulated many lipid droplets by 20 h after birth. In contrast, lipid accumulation in the adipocytes of interscapular BAT from C/EBP β (-/-) mice was significantly reduced compared with that from wild-type mice. Lipid accumulation in C/EBP δ (-/-) BAT was only slightly impaired. Interestingly, C/EBP β (-/-)·C/EBP δ (-/-) BAT could not accumulate fat droplets (Figure 2A).

Adipose tissue is composed of several types of cells that are at different stages of differentiation. Pre-adipocytes are defined as cells with no lipid accumulation in the

Table 1. Genotype of progeny of heterozygote intercrosses

C/EBP β (+/-)×C/EBP β (+/-)				
Stage	Genotype			Total
	β (+/+)	β (+/-)	β (-/-)	
4-Week	61 (26.8%)	130 (57.0%)	37 (16.2%)	228
Newborn	19 (25.0%)	40 (52.6%)	17 (22.4%)	76
18.5 d.p.c.	15 (19.0%)	48 (60.8%)	16 (20.2%)	79
15.5 d.p.c.	11 (19.6%)	34 (60.7%)	11 (19.6%)	56

C/EBP δ (+/-)×C/EBP δ (+/-)				
Stage	Genotype			Total
	δ (+/+)	δ (+/-)	δ (-/-)	
4-Week	39 (25.3%)	73 (47.4%)	42 (27.3%)	154

C/EBP β (+/-)· δ (-/-)×C/EBP δ (+/-)· δ (-/-)				
Stage	Genotype			Total
	β (+/+)· δ (-/-)	β (+/-)· δ (-/-)	β (-/-)· δ (-/-)	
4-Week	71 (39.2%)	103 (53.6%)	7 (3.8%)	228
Newborn	17 (31.5%)	30 (55.6%)	7 (13%)	54
18.5 d.p.c.	12 (25.5%)	25 (53.2%)	10 (21.3%)	47
15.5 d.p.c.	9 (29.0%)	13 (41.9%)	9 (29.0%)	31

cytoplasm. Pre-adipocytes differentiate into immature adipocytes which contain only small lipid droplets in the cytoplasm, and mature adipocytes whose cytoplasm is filled with well-developed lipid droplets (Ailhaud *et al.*, 1992). To evaluate the extent of differentiation of each adipocyte in this tissue in more detail, we stained lipid components in the adipocytes with Oil-Red-O, which revealed lipid droplets as red-colored deposits. In wild-type mice, all cells in this tissue have lipid droplets, and the cytoplasm of each adipocyte is filled with numerous large droplets. This represents a multilocular lipid distribution typical of mature brown adipocytes (Figure 2B). In contrast, lipid droplets in the adipocytes of C/EBP β (-/-) mice are distributed more sparsely and each droplet is smaller than that of wild-type mice, although almost all cells have these small droplets (Figure 2B). This suggested that differentiation of the cells in C/EBP β (-/-) adipose tissue is arrested while the adipocyte is immature. The intracytoplasmic lipid content of C/EBP δ (-/-) adipocytes is almost the same or somewhat reduced compared with that of wild-type mice (Figure 2B). However, the majority of the cells in C/EBP β (-/-)· δ (-/-) BAT contain no lipid droplets, although some cells have one or two droplets in the cytoplasm (Figure 2B). This finding suggests that the differentiation is blocked at the pre-adipocyte stage in the majority of the cells of interscapular BAT from C/EBP β (-/-)· δ (-/-) mice.

Decreased expression of UCP1 but not C/EBP α and PPAR γ in interscapular BAT from C/EBP β (-/-)· δ (-/-) mice

A number of genes are expressed specifically in adipose tissue. It is suggested that the timing of the expression of these genes is essential for the acquisition and maintenance of adipocyte phenotype. LPL is expressed at the early stage of differentiation, whereas expression of aP2 and PEPCCK is detected during the late or terminal stage of

differentiation. UCP1 is the functional marker of terminally differentiated brown adipocytes. To examine the expression of these adipocyte-specific genes, we performed Northern blot analysis of RNA isolated from BAT with several adipogenic cDNAs as probe. RNA was extracted from interscapular fat of 18.5 d.p.c. mice embryos to exclude the effect of differences of feeding. As shown in Figure 3A, UCP1 mRNA expression in C/EBP β (-/-)· δ (-/-) BAT was markedly reduced, and was almost undetectable in two of three C/EBP β (-/-)· δ (-/-) embryos. Figure 3B shows a densitometric analysis of Figure 3A. Densitometric values are normalized to β -actin, and each bar represents the mean $\pm$ SD of three independent pairs of embryos. UCP1 expression in C/EBP β (-/-), C/EBP δ (-/-) and C/EBP β (-/-)· δ (-/-) BAT was 31.2, 46.2 and 8.7%, respectively. aP2 mRNA expression is slightly reduced in C/EBP β (-/-)· δ (-/-) BAT (52.3% of wild-type embryo). PEPCCK and LPL expression was indistinguishable between wild-type and the three mutant embryos.

We further analyzed the expression of C/EBP α and PPAR γ , the transcription factors whose expression is shown to be induced by C/EBP β and C/EBP δ . In contrast to the hypothesis of previous studies using *in vitro* culture systems, the expression of C/EBP α and PPAR γ in interscapular BAT of C/EBP β (-/-), C/EBP δ (-/-) and C/EBP β (-/-)· δ (-/-) mice is comparable with that in wild-type mice (Figure 3B). This finding shows that C/EBP α and PPAR γ can be induced *in vivo* in the absence of C/EBP β and C/EBP δ expression.

Embryonic fibroblasts from C/EBP β (-/-)·C/EBP δ (-/-) mice could not differentiate into adipocytes

To demonstrate that these phenotypes are not a secondary effect of systemic growth defects but rather the primary effect of a lack of C/EBP β and C/EBP δ in adipocytes and to investigate the difference in the results obtained from the present analyses of knockout mice and previous *in vitro* culture systems, we prepared primary embryonic fibroblasts from each knockout mouse and examined their differentiation properties, since cell lines frequently used for *in vitro* adipocyte differentiation such as 3T3-L1 and 3T3-F442A pre-adipose cells are derived from embryonic fibroblasts. These cell lines can differentiate into adipocytes in the presence of MIX, DEX, INS and FBS (standard differentiation induction medium). The embryonic fibroblasts from each knockout mouse were cultured to confluence and then treated with the standard differentiation induction medium. As shown in Figure 4A, embryonic fibroblasts from wild-type mice differentiated into adipocytes and accumulated many lipid droplets in response to hormonal stimulants. The differentiation frequency of embryonic fibroblasts from C/EBP β (-/-) mice is significantly reduced as compared with that of wild-type embryonic fibroblasts. C/EBP δ (-/-) embryonic fibroblasts exhibited slightly reduced differentiation. In contrast, C/EBP β (-/-)· δ (-/-) embryonic fibroblasts can hardly differentiate into adipogenic cells and accumulate very few lipid droplets. Low magnitude observations with Oil-Red-O staining demonstrated clear differences in adipocyte phenotype (Figure 5B).

There are specific marker genes for each stage of adipocyte differentiation. For example, LPL and fatty acid

Fig. 2. Histological examination of interscapular BAT of newborn mice. Transverse section of interscapular region of brown adipose tissue in 20-h-old wild-type (upper left column), C/EBPβ(-/-) (upper right column), C/EBPδ(-/-) (lower left column) and C/EBPβ(-/-)•C/EBPδ(-/-) mice (lower right column) are shown. (A) Hematoxylin and eosin staining (original magnification, $\times 40$), (B) Oil-Red-O staining (original magnification, $\times 400$)

transporter are expressed at the pre-adipocyte stage and are the earliest markers of differentiation, while aP2 and GLUT4 are expressed at the immature adipocyte stage, and PEPCK and adiponin at the mature stage (Ailhaud

et al., 1992). These findings were based mainly on *in vitro* experiments using a 3T3-L1 pre-adipocyte differentiation system. We next examined the expression of adipocyte-specific genes during the course of differentiation. Adipo-

Fig. 3. Expression of adipogenic genes in interscapular BAT of 18.5 d.p.c. embryos. **(A)** Northern blot analysis of adipocyte-specific genes and adipogenic transcription factors in interscapular BAT. RNA was extracted from the interscapular fat pad of 18.5 d.p.c. embryos. Total RNA (10 μ g per lane) was separated by electrophoresis, and hybridized with the indicated cDNAs as probe. LPL, lipoprotein lipase; aP2, adipocyte P2; PEPCK, phosphoenolpyruvate carboxykinase; UCP1, uncoupling protein 1; PPAR γ , peroxisome proliferator-activated receptor γ ; C/EBP α , CCAAT/enhancer-binding protein α . **(B)** Quantitative analysis of expression of adipogenic genes in interscapular BAT. Densitometric values of the data shown in (A) were measured by the BAS2000 image analyzing system. Data are normalized to β -actin and calculated as fold intensity. Each bar represents the mean $\pm$ SD of three embryos.

cyte differentiation of wild-type embryonic fibroblasts accompanied the expression of all the adipocyte markers examined, which is indicative of a completely mature state. In contrast, cells from C/EBP β (-/-) mice expressed LPL and aP2 but not PEPCK, and represent immature adipocytes. Furthermore, C/EBP β (-/-)· δ (-/-) cells only expressed LPL, the earliest marker of differentiation, which corresponds to pre-adipocytes. C/EBP δ (-/-) cells

appeared to be slightly impaired in differentiation, but were considered to mature as normal because they expressed PEPCK, a very late marker of differentiation. 5,8,11,14-Eicosatetraenoic acid (ETYA) is an activator of PPAR γ and can accelerate PPAR γ -mediated adipogenesis in certain culture conditions. However, ETYA did not affect the differentiation in our system (data not shown). These findings indicate that C/EBP β and C/EBP δ synergistically accelerate adipocyte differentiation, and ablation of both of these genes leads to almost complete inhibition of adipocyte differentiation.

We examined the expression of C/EBP α and PPAR γ . C/EBP α expression was reduced markedly in C/EBP β (-/-)· δ (-/-) embryonic fibroblasts, although C/EBP α expression in C/EBP β (-/-) or C/EBP δ (-/-) fibroblasts was comparable with that of wild-type fibroblasts. PPAR γ expression was reduced slightly in C/EBP δ (-/-) fibroblasts and decreased markedly in C/EBP β (-/-) and C/EBP β (-/-)· δ (-/-) fibroblasts.

Taken together, adipocyte differentiation both in interscapular BAT and in cultured embryonic fibroblasts treated with adipogenic hormones was apparently blocked, but the expression of adipocyte-specific genes and of adipogenic transcription factors was quite different; in embryonic fibroblasts derived from C/EBP β (-/-)· δ (-/-) mice, the expression of genes induced following the expression of C/EBP β and C/EBP δ genes was severely impaired. In contrast, in the case of interscapular BAT, transcription factors such as C/EBP α and PPAR γ and the late differentiation markers, aP2, PEPCK and adipsin, but not UCP1, were induced normally.

Development of epididymal WAT in C/EBP β (-/-)· δ (-/-) mice

Approximately 85% of C/EBP β (-/-)· δ (-/-) mice died within 24 h after birth. The few mice that survived beyond this period were of normal appearance and exhibited a normal rate of mortality in adulthood. WAT cannot be detected macroscopically during embryogenesis and at birth, and is considered to function mainly as an energy store and in the regulation of metabolism in later life. We examined the abnormalities of WAT in surviving adult mice. WAT can be detected in epididymal, parametrial, inguinal and subcutaneous regions within the body. Since it is plentiful and can be dissected easily from the surrounding tissues, we prepared epididymal WAT from each mouse, and measured its weight. As shown in Figure 6A, the epididymal WAT weight of C/EBP β (-/-)· δ (-/-) mice is significantly lower than that of wild-type mice (~30% of wild-type). The WAT weight of C/EBP β (-/-) or C/EBP δ (-/-) mice tended to be lower, but not significantly so. Reduced WAT volume is not due to the secondary effect of systemic growth retardation, since the body weight values of these mice were not significantly different (data not shown). Figure 6B shows the appearance of representative epididymal WAT from wild-type and C/EBP β (-/-)· δ (-/-) mice. Histologically, adipocytes in epididymal WAT from C/EBP β (-/-)· δ (-/-) mice were normal, with a typical unilocular or signet-ring appearance (data not shown).

We examined the expression of adipocyte-specific genes in epididymal WAT. As shown in Figure 7A, there were no differences in the expression of LPL and aP2 between wild-type, C/EBP β (-/-), C/EBP δ (-/-) and C/EBP β (-/-)·

Fig. 4. Adipogenic differentiation from primary embryonic fibroblasts. Primary embryonic fibroblasts from each knockout mouse were cultured in the presence of standard differentiation induction medium (containing 0.5 mM MIX, 1 μ M DEX, 5 μ g/ml INS and 10% FBS). After 8 days of differentiation, cells were observed by light microscopy with (B) or without (A) Oil-Red-O staining. Cells from wild-type (upper left column), C/EBP β (-/-) (upper right column), C/EBP δ (-/-) (lower left column) and C/EBP β (-/-)·C/EBP δ (-/-) mice (lower right column) are shown. The original magnification is $\times 20$ for (A) and $\times 10$ for (B). This experiment was repeated five times using cells prepared from five different embryos, and similar results were obtained.

δ (-/-) mice. C/EBP α and PPAR γ were also expressed in C/EBP β (-/-), C/EBP δ (-/-) and C/EBP β (-/-)· δ (-/-) mice to an extent similar to that in wild-type mice. These

findings suggest that maturation of epididymal adipocytes of C/EBP β (-/-)· δ (-/-) mice is almost normal, despite a reduced volume of epididymal WAT.

Fig. 5. Northern blot analysis of primary embryonic fibroblasts during adipogenic differentiation. Primary embryonic fibroblasts from each knockout mouse were differentiated in the presence of the hormonal stimulants described in Materials and methods. RNA was extracted from these cells at day 0, 2, 5 and 8 of differentiation. Ten μ g of total RNA were subjected to Northern blot analysis using the probes as indicated.

Discussion

Remarkable progress has been made recently in elucidating the molecular basis of adipose differentiation. The process involves the sequential expression of transcription factors, C/EBP β , C/EBP δ , PPAR γ and C/EBP α . However, this scenario is based mainly on cultured cell systems in which adipogenic hormones can induce mature differentiation of pre-adipocyte cell lines. *In vivo*, adipocyte development is not fully understood. In this study, we have generated mice lacking C/EBP β and/or C/EBP δ , and analyzed the *in vivo* role of these transcription factors in adipocyte differentiation.

C/EBP β and C/EBP δ genes are essential for neonatal development

Unexpectedly, 35% of C/EBP β ($-/-$) mice and 85% of C/EBP β ($-/-$)-C/EBP δ ($-/-$) mice died at the early neonatal stage. In the case of C/EBP α gene targeting, C/EBP α knockout mice died within 8 h of birth. These mice had little lipid accumulation in interscapular BAT and no detectable lipid depot in inguinal WAT. This phenotype resembles that of C/EBP β ($-/-$)-C/EBP δ ($-/-$) mice. In addition, C/EBP α ($-/-$) mice showed severe hypoglycemia due to the defective induction of liver enzymes essential for glucose metabolism. This defect may be the major cause of neonatal death of C/EBP α ($-/-$) mice. Furthermore, histological abnormalities of liver and lung were detected in C/EBP α ($-/-$) mice (Flodby *et al.*, 1996). C/EBP β ($-/-$), C/EBP δ ($-/-$) and C/EBP β ($-/-$)-C/EBP δ ($-/-$) mice showed no histological abnormalities in these tissue and did not suffer from hypoglycemia (data not shown). These findings suggest that the cause of neonatal death of C/EBP β ($-/-$)-C/EBP δ ($-/-$) mice is quite different from that of C/EBP α ($-/-$) mice. Interestingly, transgenic ablation of BAT in mice leads to early neonatal death (Ross *et al.*, 1993). When diphtheria toxin A chain is expressed specifically in adipose cells using the aP2

gene promoter, transgenic mice with high levels of toxin expression died within 24 h of birth, although mice with low expression survived. The expression of the UCP1 gene in interscapular BAT of these transgenic mice was decreased in accordance with the expression of transgene. These results suggest that thermogenic activity in BAT is essential for the survival of newborn mammals. The high mortality of C/EBP β ($-/-$)-C/EBP δ ($-/-$) mice may be due to the defective differentiation of BAT and its resulting dysfunction as a thermogenic organ. The marked decrease of UCP1 gene expression in C/EBP β ($-/-$)-C/EBP δ ($-/-$) BAT may support this speculation. However, UCP1 itself is not an essential factor for adipocyte development and thermogenic activities in neonates because UCP1 knockout mice suffered neither neonatal death nor impaired adipocyte differentiation of interscapular BAT (Enerbäck *et al.*, 1997). Other adipocyte-specific factors that are regulated by C/EBP β or/and C/EBP δ may be essential for neonatal survival. Further study such as the generation of mice lacking C/EBP β and C/EBP δ in an adipose tissue-specific manner will be required to clarify this point.

Defects in adipocyte maturation in C/EBP β ($-/-$)-C/EBP δ ($-/-$) mice despite normal expression of C/EBP α and PPAR γ

Previous experiments proposed that adipocyte development is regulated by many transcription factors which are expressed sequentially. C/EBP β and C/EBP δ are expressed early on in differentiation and synergistically activate the transactivation of both the PPAR γ and C/EBP α genes. Then both PPAR γ and C/EBP α directly induce adipogenic genes, which results in the adipocyte phenotypes (Cao *et al.*, 1991; Yeh *et al.*, 1995; Wu *et al.*, 1996). Indeed, many adipogenic genes harbor the DNA-binding sites for both PPAR γ and C/EBP α . If this hypothesis is true, the expression of PPAR γ and C/EBP α and the subsequent induction of adipogenic marker genes would be markedly reduced in C/EBP β ($-/-$)-C/EBP δ ($-/-$) mice. In the *in vitro* study of adipocyte differentiation using primary embryonic fibroblasts derived from C/EBP β ($-/-$)-C/EBP δ ($-/-$) mice, the expression of both PPAR γ and C/EBP α was severely reduced to almost undetectable levels, and the expression of several adipogenic marker genes was also impaired in line with defective lipid accumulation, which agrees with the above hypothesis. However, *in vivo*, these transcription factors were expressed normally in neonatal BAT of C/EBP β ($-/-$)-C/EBP δ ($-/-$) mice despite the defective lipid accumulation. This finding demonstrated that PPAR γ and C/EBP α can be induced in the absence of C/EBP β and δ . In *in vitro* differentiation experiments, the only differentiation stimuli are MIX, DEX, INS and FBS; however, *in vivo*, there are many other adipogenic factors such as growth hormone, IGF-1, prostaglandins and β 3-adrenergic ligand. With the restricted number of stimuli *in vitro*, PPAR γ and C/EBP α are induced according to a proposed cascade of transcription factors, but *in vivo*, C/EBP α and PPAR γ may be induced by other transcription factors. It is important to identify such transcription factors for alternative pathways that lead to C/EBP α or PPAR γ expression.

Despite normal expression of PPAR γ and C/EBP α , C/EBP β ($-/-$)-C/EBP δ ($-/-$) mice have almost the same phenotype as C/EBP α ($-/-$) mice in terms of interscapular BAT, i.e.

Fig. 6. Epididymal WAT development of adult mice. **(A)** The epididymal fat pad weight of 8-week-old wild-type, $C/EBP\beta(-/-)$, $C/EBP\delta(-/-)$ and $C/EBP\beta(-/-)\cdot\delta(-/-)$ mice was measured. Each point represents the mean weight of both sides of the fat pads in a single mouse. The bar represents mean $\pm$ SD of five to seven different mice. **(B)** Appearance of epididymal fat pad of wild-type and $C/EBP\beta(-/-)\cdot\delta(-/-)$ mice. Peritoneal cavity (upper) and resected epididymal fat pad (lower) of 8-week-old wild-type and $C/EBP\beta(-/-)\cdot\delta(-/-)$ mice are shown. Ep, epididymal fat pad; T, testis.

impairment of lipid accumulation and a reduced level of UCP1 expression. Interestingly, $C/EBP\beta$ and $C/EBP\delta$ were expressed normally in $C/EBP\alpha(-/-)$ mice. These findings indicate that loss of $C/EBP\alpha$ function cannot be compensated by $C/EBP\beta$ and $C/EBP\delta$, and vice versa.

The expression of adipogenic marker genes, with the exception of UCP1, was almost normal, despite defective adipocyte maturation in $C/EBP\beta(-/-)\cdot\delta(-/-)$ BAT. Furthermore, mice lacking the *aP2* gene exhibit normal adipocyte development (Hotamisligil *et al.*, 1996) and the mass of interscapular BAT in mice lacking UCP1 is significantly greater with increased deposition of triglyceride in each adipocyte (Enerbäck *et al.*, 1997). These findings indicate that the expression of the adipogenic marker genes is not associated with the maturity of adipocyte differentiation.

Decreased WAT volume in $C/EBP\beta(-/-)\cdot\delta(-/-)$ mice

WAT is the organ that stores energy as triglycerides. Excess energy storage in fat tissue leads to obesity, and

increased fat volume is a hallmark of this. Mild obesity reflects an increase in the size of each adipocyte (hypertrophic obesity), while more severe obesity or obesity arising in childhood typically involves increased fat cell number (hyperplastic obesity) (Spiegelman and Flier, 1996). Since hyperplastic obesity is closely related to many clinical diseases such as coronary heart disease, non-insulin-dependent diabetes mellitus, hypertension and hyperlipidemia, controlling the adipose cell number in the body may lead to the prevention of such diseases. An intriguing finding is the decreased epididymal WAT weight in $C/EBP\beta(-/-)\cdot\delta(-/-)$ mice, despite normal histology and normal expression of the adipogenic genes. Since individual $C/EBP\beta(-/-)\cdot\delta(-/-)$ mice have variations in the expression of adipogenic genes in BAT, as shown in Figure 3, the fact that only a few $C/EBP\beta(-/-)\cdot\delta(-/-)$ mice survived to adulthood implies that surviving knockout mice exhibit a somewhat mild phenotype. Nevertheless,

adult C/EBP β (-/-)· δ (-/-) mice exhibit a decreased weight of epididymal WAT. This is considered to be due to the reduced number of adipocytes in C/EBP β (-/-)· δ (-/-) WAT, since epididymal WAT of C/EBP β (-/-)· δ (-/-) mice is histologically normal and the size of each adipocyte differs little from that of wild-type mice (data not shown). This suggested that C/EBP β and δ are involved in the increase in the number of adipocytes after birth, and that inhibition of C/EBP β and C/EBP δ at the appropriate times may serve as a treatment for obesity.

Conclusion

Our present study demonstrated that C/EBP β and C/EBP δ can synergistically promote terminal adipocyte differentiation both *in vitro* and *in vivo*. The defects of adipocyte

differentiation observed in C/EBP β (-/-)· δ (-/-) mice are summarized in Table II. In *in vitro* differentiation systems using primary embryonic fibroblasts, cells from C/EBP β (-/-)· δ (-/-) mice neither differentiate into mature adipocytes nor express C/EBP α and PPAR γ . This suggests that adipocyte differentiation *in vitro* proceeds according to the proposed transcriptional regulatory cascade in which adipogenic transcription factors such as C/EBP family members and PPARs are activated sequentially. However, *in vivo*, C/EBP α and PPAR γ can be induced without expression of C/EBP β and C/EBP δ . In addition, despite normal expression of C/EBP α or PPAR γ , adipogenesis is severely impaired in C/EBP β (-/-)· δ (-/-) mice. These findings demonstrated that the induction of C/EBP α and PPAR γ does not always require C/EBP β and C/EBP δ , but co-expression of C/EBP α and PPAR γ is not sufficient for complete adipocyte differentiation in their absence. Further studies to identify the target genes that are regulated by C/EBP β and C/EBP δ using our knockout mice and embryonic fibroblasts derived from them will produce a clearer picture of adipocyte differentiation.

Materials and methods

Construction of targeting vector

Construction of the targeting vector for disruption of the C/EBP β gene was as described (Tanaka *et al.*, 1995).

To construct the targeting vector for disruption of the C/EBP δ gene, a Balb/c mouse genomic DNA library, packaged in EMBL3 (Clontech), was screened using the human C/EBP δ cDNA as a probe (Kinoshita *et al.*, 1992). One clone containing the C/EBP δ gene, composed of one exon, was isolated. A 9.5 kb genome fragment spanning from 1 kb 5' of the C/EBP δ transcription initiation site to 7.5 kb 3' of the exon was subcloned into pBluescriptII KS(+) plasmid vector. To inactivate the C/EBP δ gene, a 0.8 kb fragment containing the promoter region, transcription start site and transactivation domain was deleted and replaced by a 1.1 kb *XhoI*-*SalI* fragment of NEO derived from pMC1Neopoly(A) (Thomas and Cappechi, 1987). Homologous regions 5' and 3' of NEO were 1.1 and 7.7 kb, respectively. For negative selection against random integration, the MC1-HSV-TK (Mansour *et al.*, 1988) was ligated at the 3' end of the homologous region.

ES cell culture and generation of mutant mice

The strategy for culturing and electroporation of E14-1 embryonic stem cells and screening of homologous recombinant clones was as described

Fig. 7. Quantitative analysis of adipogenic gene expression in epididymal WAT of adult mice. RNA was isolated from the epididymal fat pad of 8-week-old mice. Ten μ g of total RNA were subjected to Northern blot analysis using the probes indicated. Densitometric values of each band were measured with a BAS2000 image analyzing system. Data were normalized to β -actin and calculated as fold intensity relative to β -actin. Each bar represents the mean $\pm$ SD of three mice.

Table II. Summary of defects in adipocyte differentiation observed in C/EBP β (-/-)· δ (-/-) mice

	Embryo	Newborn	Adult
Cell or tissue	primary embryonic fibroblasts <i>in vitro</i> differentiation system	interscapular BAT <i>in vivo</i>	epididymal WAT <i>in vivo</i>
Defects	blockade of differentiation from pre-adipocytes to adipocytes	severely reduced lipid accumulation	reduced volume of fat pad
Expression of transcription factors	C/EBP α ↓ PPAR γ ↓	C/EBP α → PPAR γ →	C/EBP α → PPAR γ →
Expression of adipogenic marker genes	LPL→ aP2↓ PEPCK↓	LPL→ aP2→ or ↓ PEPCK→ UCP1↓	LPL→ aP2→ adipsin→
Comments	The expression pattern of transcription factors and adipogenic marker genes agrees with the cascade model of adipocyte differentiation previously proposed.	C/EBP β (-/-)· δ (-/-) mice have defects in BAT development and UCP1 expression, as observed in C/EBP α (-/-) mice (Wang <i>et al.</i> , 1995).	This phenotype might be somewhat underestimated because only adult mice which survived beyond the neonatal stage due to incomplete penetrance were analyzed.

(Tanaka *et al.*, 1995). The generation of chimeras and mutant mice was essentially as described (Kawabe *et al.*, 1994). To generate C/EBP β -C/EBP δ double knockout mice, we first generated C/EBP β (+/-)-C/EBP δ (+/-) mice by mating C/EBP β (+/-) with C/EBP δ (+/-) mice. Then, these mice were crossed to C/EBP δ (-/-), and C/EBP β (+/-)-C/EBP δ (-/-) mice were obtained. C/EBP β (-/-)-C/EBP δ (-/-) mice were generated by intercrosses between C/EBP β (+/-)-C/EBP δ (-/-) males and females. In the experiments described here, we use wild-type and C/EBP β (-/-) mice which were obtained by intercrosses between C/EBP β (+/-)-C/EBP δ (-/-) males and females, and C/EBP δ (-/-) and C/EBP β (-/-)-C/EBP δ (-/-) mice which were obtained by intercrosses between C/EBP β (+/-)-C/EBP δ (-/-) males and females.

Preparation of primary embryonic fibroblasts and induction of adipogenic differentiation

Primary embryonic fibroblasts were harvested from 14.5 d.p.c. embryos. Cells were cultured at 37°C in α -modified Eagle's medium (α MEM; Gibco/BRL) supplemented with 10% heat-inactivated FBS (Hyclone). Cells were plated to 24-well or 60 mm plastic dishes and propagated to confluence. Two days later, medium was replaced with standard differentiation induction medium containing 0.5 mM MIX (Sigma), 1 μ M DEX (Sigma), 5 μ g/ml INS (Sigma) and 10% FBS (Hyclone). This medium was renewed every other day. After 8 days, the appearance of cytoplasmic lipid accumulation was observed by bright-field microscopy with or without Oil-Red-O staining. The Oil-Red-O staining was performed as follows; cells were washed with phosphate-buffered saline (PBS), and then stained with 60% filtered Oil-Red-O stock solution [0.15 g of Oil-Red-O (Sigma) in 50 ml of isopropanol] for 30 min at 37°C. Cells were washed first with 60% isopropanol and then briefly with water and visualized. To prepare RNA for Northern blotting, cells were harvested at days 0, 2, 5 and 8 of differentiation.

Northern blot analysis

Total RNA was isolated from the interscapular fat pad of 18.5 d.p.c. embryos or adult mice, epididymal fat pad of adult mice and differentiating embryonic fibroblasts by using TRIzol reagent (Gibco/BRL). Ten μ g of each RNA sample were electrophoresed through denaturing formaldehyde-agarose gel, and transferred to Hybond-N⁺ nylon membrane (Amersham). The probes were labeled by random priming using the Megaprime DNA labeling system (Pharmacia) and [α -³²P]dCTP (Dupont NEN Research Product). cDNAs used as a probe were as follows; murine C/EBP α , C/EBP β , C/EBP δ (which were prepared from genomic DNA), adipsin, aP2, LPL, UCP1, PPAR γ , β -actin and rat PEPCK (which were amplified by PCR from adipose tissue cDNA using specific primers). For quantitative analysis for comparing each knockout mouse, the intensity of the hybridized band of each lane was measured with a BAS2000 image analyzing system, and densitometric values were normalized to β -actin. Mean values and standard deviations are shown.

Histological analysis

At 20 h after birth, neonates were fixed separately in Bouin's reagent or 20% formaldehyde in PBS. Genomic DNA was prepared from the tail of each neonate, and PCR analysis was performed to determine the genotype. After PCR analysis, mice with the desired genotype were subjected to the following steps. For HE staining, neonates, which were fixed in Bouin's reagent, were embedded in paraffin, sectioned transversely at 2 μ m intervals, and stained with hematoxylin and eosin. For fat staining, neonates, which were fixed in 20% formaldehyde in PBS, were frozen, sectioned transversely at 10 μ m intervals, and stained with Oil-Red-O. All sections were examined by light microscopy.

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References

Aihaud, G., Grimaldi, P. and Nègre, R. (1992) Cellular and molecular aspects of adipose tissue development. *Annu. Rev. Nutr.*, **12**, 207–233.
Akira, S., Isshiki, H., Sugita, T., Tanabe, O., Kinoshita, S., Nishio, Y., Nakajima, T., Hirano, T. and Kishimoto, T. (1990) A nuclear factor for

IL-6 expression (NF-IL6) is a member of a C/EBP family. *EMBO J.*, **9**, 1897–1906.
Burn, R.P., Tontonoz, P., Forman, B.M., Ellis, R., Chen, J., Evans, R.M. and Spiegelman, B.M. (1996) Differential activation of adipogenesis by multiple PPAR isoforms. *Genes Dev.*, **10**, 974–984.
Cao, Z., Umek, R.M. and McKnight, S.L. (1991) Regulated expression of three C/EBP isoforms during adipose conversion of 3T3-L1 cells. *Genes Dev.*, **5**, 1538–1552.
Chang, C.J., Chen, T.T., Lei, H.Y., Chen, D.S. and Lee, S.C. (1990) Molecular cloning of a transcription factor, AGP/EBP, that belongs to members of the C/EBP family. *Mol. Cell. Biol.*, **10**, 6642–6653.
Chawla, A., Schwarz, E.J., Dimaculangan, D.D. and Lazer, M.A. (1994) Peroxisome proliferator-activated receptor (PPAR) γ : adipose-predominant expression and induction early in adipocyte differentiation. *Endocrinology*, **135**, 798–800.
Christy, R.J., Yang, V.W., Ntambi, J.M., Geiman, D.E., Landschultz, W.H., Friedman, A.D., Nakabeppu, Y., Kelly, T.J. and Lane, M.D. (1989) Differentiation-induced gene expression in 3T3-L1 preadipocytes: CCAAT/enhancer binding protein interacts with and activates the promoters of two adipocyte-specific genes. *Genes Dev.*, **3**, 1323–1335.
Christy, R.J., Kaestner, K.H., Geiman, D.E. and Lane, M.D. (1991) CCAAT/enhancer binding protein gene promoter: binding of nuclear factors during differentiation of 3T3-L1 preadipocytes. *Proc. Natl Acad. Sci. USA*, **88**, 2593–2597.
Descombes, P., Chojkier, M., Lichtsteiner, S., Falvey, E. and Schibler, U. (1990) LAP, a novel member of the C/EBP gene family, encodes a liver-enriched transcriptional activator protein. *Genes Dev.*, **4**, 1541–1551.
Enerbäck, S., Jacobson, A., Simpson, E.M., Guerra, C., Yamashita, H., Harper, M.-E. and Kozak, L.P. (1997) Mice lacking mitochondrial uncoupling protein are cold-sensitive but not obese. *Nature*, **387**, 90–94.
Flodby, P., Barlow, C., Kylefjord, H., Åhrlund-Richter, L. and Xanthopoulos, K.G. (1996) Increased hepatic cell proliferation and lung abnormalities in mice deficient in CCAAT/enhancer binding protein α . *J. Biol. Chem.*, **271**, 24753–24760.
Forman, B.M., Tontonoz, P., Chen, J., Brun, R.P., Spiegelman, B.M. and Evans, R.M. (1995) 15-Deoxy- $\Delta^{12,14}$ -prostaglandin J2 is a ligand for the adipocyte determination factor PPAR γ . *Cell*, **83**, 803–812.
Freytag, S., Paielli, D.L. and Gilbert, J.D. (1994) Ectopic expression of the CCAAT/enhancer binding protein α promotes the adipogenic program in a variety of mouse fibroblastic cells. *Genes Dev.*, **8**, 1654–1663.
Hotamisligil, G.S., Johnson, R.S., Distel, R.J., Ellis, R., Papaioannou, V.E. and Spiegelman, B.M. (1996) Uncoupling of obesity from insulin resistance through a targeted mutation in aP2, the adipocyte binding protein. *Science*, **274**, 1377–1379.
Kageyama, R., Sasai, Y. and Nakanishi, S. (1991) Molecular characterization of transcription factors that bind to the cAMP responsive region of the Substance P precursor gene. *J. Biol. Chem.*, **266**, 15525–15531.
Katz, S., Kowenz-Leutz, E., Müller, C., Meese, K., Ness, S.A. and Leutz, A. (1993) The NF-M transcription factor is related to C/EBP β and plays a role in signal transduction, differentiation and leukemogenesis of avian myelomonocytic cells. *EMBO J.*, **12**, 1321–1332.
Kawabe, T., Naka, T., Yoshida, K., Tanaka, T., Fujiwara, H., Suematsu, S., Yoshida, N., Kishimoto, T. and Kikutani, H. (1994) The immune responses in CD40-deficient mice: impaired immunoglobulin class switching and germinal center formation. *Immunity*, **1**, 167–178.
Kinoshita, S., Akira, S. and Kishimoto, T. (1992) A member of the C/EBP family, NF-IL6 β , forms a heterodimer and transcriptionally synergizes with NF-IL6. *Proc. Natl Acad. Sci. USA*, **87**, 1473–1476.
Kliwer, S.A., Forman, B.M., Blumberg, B., Ong, E.S., Borgmeyer, U., Mangelsdorf, D.J., Umesono, K. and Evans, R.M. (1994) Differential expression and activation of a family of murine peroxisome proliferator-activated receptors. *Proc. Natl Acad. Sci. USA*, **91**, 7355–7359.
Kliwer, S.A., Lenhard, J.M., Willson, T.M., Patel, I., Morris, D.C. and Lehmann, J.M. (1995) A prostaglandin J2 metabolite binds peroxisome proliferator-activated receptor γ and promotes adipocyte differentiation. *Cell*, **83**, 813–819.
Lin, F.-T. and Lane, M.D. (1992) Antisense CCAAT/enhancer-binding protein RNA suppresses coordinate gene expression and triglyceride accumulation during differentiation of 3T3-L1 preadipocytes. *Genes Dev.*, **6**, 533–544.
Lin, F.-T. and Lane, M.D. (1994) CCAAT/enhancer binding protein α is sufficient to initiate the 3T3-L1 adipocyte differentiation program. *Proc. Natl Acad. Sci. USA*, **91**, 8757–8761.

- Manchado,C., Yubero,P., Viñas,O., Iglesias,R., Villarroya,F., Mampel,T. and Giralt,M. (1994) CCAAT/binding proteins α and β in brown adipose tissue: evidence for a tissue-specific pattern of expression during development. *Biochem. J.*, **302**, 695–700.
- Mangelsdorf,D.J. *et al.* (1995) The nuclear receptor superfamily: the second decade. *Cell*, **83**, 835–839.
- Mansour,S.L., Thomas,K.R. and Capecchi,M.R. (1988) Disruption of the proto-oncogene *int-2* in mouse embryo-derived stem cells: a general strategy for targeting mutations to non-selectable genes. *Nature*, **336**, 348–352.
- Ness,S.A., Kowenz-Leutz,E., Casini,T., Graf,T. and Leutz,A. (1993) Myb and NF-M: combinatorial activators of myeloid genes in heterologous cell types. *Genes Dev.*, **7**, 749–759.
- Park,E.A., Nizielski,S.E., Hakimi,P., Cao,Z., Moorman,A. and Hanson,R.W. (1992) Relative role of members of the C/EBP family of transcription factors in controlling transcription of the gene for phosphoenolpyruvate carboxykinase. *J. Biol. Chem.*, **267**, 613–619.
- Poli,V., Mancini,F.P. and Cortese,R. (1990) IL-6DBP, a nuclear protein involved in interleukin-6 signal transduction, defines a new family of leucine zipper proteins related to C/EBP. *Cell*, **63**, 643–653.
- Ross,S.R., Graves,R.A. and Spiegelman,B.M. (1993) Targeted expression of a toxin gene to adipose tissue: transgenic mice resistant to obesity. *Genes Dev.*, **7**, 1318–1324.
- Schoonjans,K., Peinado-Onsurbe,J., Lefebvre,A.M., Heyman,R.A., Briggs,M., Deeb,S., Staels,B. and Auwerx,J. (1996) PPAR α and PPAR γ activators direct a distinct tissue specific transcriptional response via a PPRE in the lipoprotein lipase gene. *EMBO J.*, **15**, 5336–5348.
- Spiegelman,B.M. and Flier,J.S. (1996) Adipogenesis and obesity: rounding out the big picture. *Cell*, **87**, 377–389.
- Tanaka,T. *et al.* (1995) Targeted disruption of the NF-IL6 gene discloses its essential role in bacteria killing and tumor cytotoxicity by macrophages. *Cell*, **80**, 353–361.
- Thomas,K.R. and Capecchi,M.R. (1987) Site-directed mutagenesis by gene targeting in mouse embryonic stem cells. *Cell*, **51**, 503–512.
- Tontonoz,P., Hu,E., Graves,R.A., Budavari,A.I. and Spiegelman,B.M. (1994a) mPPAR γ 2: tissue-specific regulator of an adipocyte enhancer. *Genes Dev.*, **8**, 1224–1234.
- Tontonoz,P., Hu,E. and Spiegelman,B.M. (1994b) Stimulation of adipogenesis in fibroblasts by PPAR γ 2, a lipid-activated transcription factor. *Cell*, **79**, 1147–1156.
- Tontonoz,P., Hu,E., Davine,J., Beale,E.G. and Spiegelman,B.M. (1995) PPAR γ 2 regulates adipose expression of the phosphoenolpyruvate carboxykinase gene. *Mol. Cell. Biol.*, **15**, 351–357.
- Wang,N.-D., Finegold,M.J., Bradley,A., Ou,C.N., Abdelsayed,S.A., Wilde,M.D., Tayler,L.R., Wilson,D.R. and Darlington,G.J. (1995) Impaired energy homeostasis in C/EBP α knockout mice. *Science*, **269**, 1108–1112.
- Williams,S.C., Cantwell,C.A. and Johnson,P.A. (1991) A family of C/EBP-related proteins capable of forming covalently linked leucine zipper dimers *in vitro*. *Genes Dev.*, **5**, 1553–1567.
- Wu,Z., Xie,Y., Bucher,N.L.R. and Farmer,S.R. (1995) Conditional ectopic expression of C/EBP β in NIH-3T3 cells induces PPAR γ and stimulates adipogenesis. *Genes Dev.*, **9**, 2350–2363.
- Wu,Z., Bucher,N.L.R. and Farmer,S.R. (1996) Induction of peroxisome-proliferator activated receptor γ during the conversion of 3T3 fibroblasts into adipocytes is mediated by C/EBP β , C/EBP δ , and glucocorticoids. *Mol. Cell. Biol.*, **16**, 4128–4136.
- Yeh,W.-C., Cao,Z., Classon,M. and Mcknight,S.L. (1995) Cascade regulation of terminal adipocyte differentiation by three members of the C/EBP family of leucine zipper protein. *Genes Dev.*, **9**, 168–181.
- Yubero,P., Manchado,C., Cassard-Doulcier,A.-M., Mampel,T., Viñas,O., Iglesias,R., Giralt,M. and Villarroya,F. (1994) CCAAT/enhancer binding protein α and β are transcriptional activators of the brown fat uncoupling protein gene promoter. *Biochem. Biophys. Res. Commun.*, **198**, 653–659.
- Zhu,Y., Qi,C., Korenberg,J.R., Chen,X.-N., Noya,D., Rao,M.S. and Reddy,J.K. (1995) Structural organization of mouse peroxisome-proliferator activated receptor γ (mPPAR γ) gene: alternative promoter use and different splicing yield two mPPAR γ isoforms. *Proc. Natl Acad. Sci. USA*, **92**, 7921–7925.

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