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X-box binding protein 1 is a novel key regulator of peroxisome proliferator-activated receptor γ2

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X-box binding protein 1 (XBP1), a transcription factor of the unfolded protein response, plays various roles in many biological processes. We examined its pro-adipogenic activity and target genes during adipogenic differentiation in wild-type and genetically modified 3T3-L1 cells. Signalling pathways that contribute to *Xbp1* mRNA splicing, and the correlation of the transcriptionally active XBP1 isoform (XBP1s) level with body mass index and the level of peroxisome proliferator-activated receptor γ2 (PPARγ2) in human adipose tissues were also examined. The mRNA and nuclear protein expression levels of XBP1s increased immediately following hormonal induction of adipogenesis, reaching a peak at 6 h. Results from cDNA microarray and gene expression analyses using genetically modified cells indicated that PPARγ2 was a principal target of XBP1s. The XBP1s-specific binding motif, which is distinct from the CCAAT/enhancer-binding protein α binding site, was identified in the PPARγ2 promoter by site-directed mutagenesis. Fetal bovine serum, insulin, 3-isobutyl-1-methylxanthine and dexamethasone contributed independently to *Xbp1* mRNA splicing. In human subcutaneous adipose tissues, the levels of both *Xbp1s* and *Pparγ2* mRNA increased proportionally with body mass index, and there was a significant positive correlation between the two genes. These data suggest for the first time that positive regulation of PPARγ2 is a principal mechanism of XBP1s-mediated adipogenesis in 3T3-L1 cells.

Introduction

Obesity is an established risk factor for many pathological conditions such as insulin resistance, type 2 diabetes, cardiovascular disease and cancer [1]. Therefore, it is imperative to understand the molecular events underlying extensive fat expansion and the resulting alteration of the normal physiological function of adi-

Abbreviations
Ap2, adipocyte protein 2; ATF, activating transcription factor; BIP/Grp78, binding immunoglobulin protein/78 KDa glucose-regulated protein; BMI, body mass index; C/EBP, CCAAT/enhancer binding protein; ChIP, chromatin immunoprecipitation; CHOP, C/EBP homologous protein; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IRE1, inositol requiring enzyme 1; LPL, lipoprotein lipase; NCS, new-born calf serum; PERK, pancreatic ER kinase; PPAR, peroxisome proliferator-activated receptor; UPR, unfolded protein response; XBP1, X-box binding protein 1; XBP1-KD, XBP1 knockdown; XBP1s, spliced isoform of XBP1; XBP1s-OE, XBP1s over-expression; XBP1t, total XBP1; XBP1u, unspliced isoform of XBP1.

pose tissue. Adipogenesis is a differentiation process by which fibroblast-like preadipocytes become lipid-laden mature adipocytes, and more than 100 transcription factors, coactivators and repressors have been reported to be involved in this process [2]. Among these, peroxisome proliferator-activated receptor γ (PPARγ) is becoming recognized as the master regulator of adipogenesis [3,4].

PPARγ is a ligand-activated transcription factor that belongs to the nuclear hormone receptor superfamily. In the presence of ligands, PPARγ heterodimerizes with members of the retinoid X receptor family, and activates target gene expression by binding to the PPAR response element. In adipocytes, PPARγ is the only known factor that is necessary and sufficient for induction of adipogenesis [5]. In addition, adipose-specific activation of PPARγ exerts systemic insulin-sensitizing and anti-inflammatory effects, suggesting a role in maintaining the normal function of mature adipocytes [6]. These findings underline the need to elucidate the mechanisms involved in the regulation of PPARγ in adipose tissue. Although, the regulatory roles of some adipogenic transcription factors have been reported [7], detailed mechanisms remain largely unknown.

Activation of the unfolded protein response (UPR) in adipose tissue contributes to adipocyte differentiation and weight gain [8], and all three arms of the UPR [pancreatic ER kinase, inositol requiring enzyme 1 (IRE1) and activating transcription factor 6 (ATF6)] are known to participate in adipogenesis [9–11]. Among these, the most extensively studied pathway is IRE1α–X-box binding protein 1 (XBP1) signalling. XBP1 is a member of the bZIP family of transcription factors, and the transcriptionally active isoform of XBP1 (XBP1s) is synthesized after IRE1α-mediated splicing of *Xbp1* mRNA. During the UPR, XBP1s restores homeostasis in the endoplasmic reticulum, and also plays a role in many processes such as cell differentiation [12–16], regulation of immune responses [17], hepatic lipogenesis [18] and lactation [19]. Recently, a possible role of XBP1s in adipogenesis has been suggested based on the increase in XBP1s during *in vitro* adipogenesis [8,10,19,20] and the suppression of adipogenesis by depletion of XBP1s [8,10,19,21]. In the mechanistic studies, CCAAT/enhancer-binding protein α (C/EBPα) was identified as a target of XBP1s [10], and we also suggested that suppression of Wnt10b/β-catenin signalling is a possible pro-adipogenic mechanism of XBP1s [21]. However, despite numerous attempts, the mechanism of how XBP1 affects adipogenic differentiation is still unknown.

Here we have shown for the first time that regulation of PPARγ is the principal mechanism of XBP1s-mediated adipogenic differentiation in 3T3-L1 cells. We also provide evidence suggesting the possible involvement of XBP1s in fat mass expansion *in vivo*.

Results

XBP1s is prerequisite for adipogenesis

Chemical induction of adipocyte differentiation in 3T3-L1 cells significantly increased expression of *Xbp1s* mRNA in two phases (Fig. 1A, left). The level of *Xbp1s* mRNA immediately increased 3 h after induction, and reached a peak (almost a 12-fold increase) at 6 h; thereafter, it sharply decreased to the control level until day 4. A second increase, but much less potent and gradual, was observed from day 6. The temporal pattern for the total *Xbp1* mRNA level (*Xbp1t*) was similar to that of *Xbp1s*, but the overall range was smaller (Fig. 1A, right). In the nuclear fraction, where XBP1s is predominantly localized and transcriptionally active [22], XBP1s protein expression was also prominent at 3 and 6 h, and then rapidly decreased but was still observed until day 2 (Fig. 1B). When we differentiated human adipose tissue-derived stromal cells and rat bone marrow mesenchymal stem cells into adipocytes, XBP1s levels also significantly increased (data not shown).

To investigate the function of XBP1s during adipogenic differentiation, we prepared 3T3-L1 cells with genetic modification of *Xbp1*. In the 3T3-L1 cells in which *Xbp1* is silenced (XBP1-KD), the level of *Xbp1* mRNA decreased by almost 80%, and protein was not detected (Fig. 1C). When these cells were differentiated for 8 days, intracellular fat accumulation was suppressed (Fig. 1D,E) and the mRNA levels of *Xbp1t* and *Xbp1s* were significantly decreased (Fig. 1F). Moreover, transcription of *ERdj4*, a specific target gene for XBP1s [23], was also significantly suppressed, indicating remarkable loss of transcriptional activity of XBP1s in XBP1-KD cells (data not shown). In agreement with these findings, the expression of all adipogenic genes that we tested was also significantly down-regulated by XBP1 knockdown (Fig. 1G). In cells over-expressing XBP1s (XBP1s-OE), a significant acceleration of triacylglycerol accumulation rate was observed on day 6, but the overall change was less dramatic than in XBP1-KD cells (Fig. 1H–J). Finally, replenishment of XBP1s in XBP1-KD cells successfully reversed the lipid depletion (Fig. 1K) and the expression of adipogenic genes (Fig. 1L). Although the importance of XBP1s in *in vitro* adipogenesis has been suggested previously [10,21], our findings from XBP1s over-expression experiments in wild-type and XBP1-KD cells and the quantitative

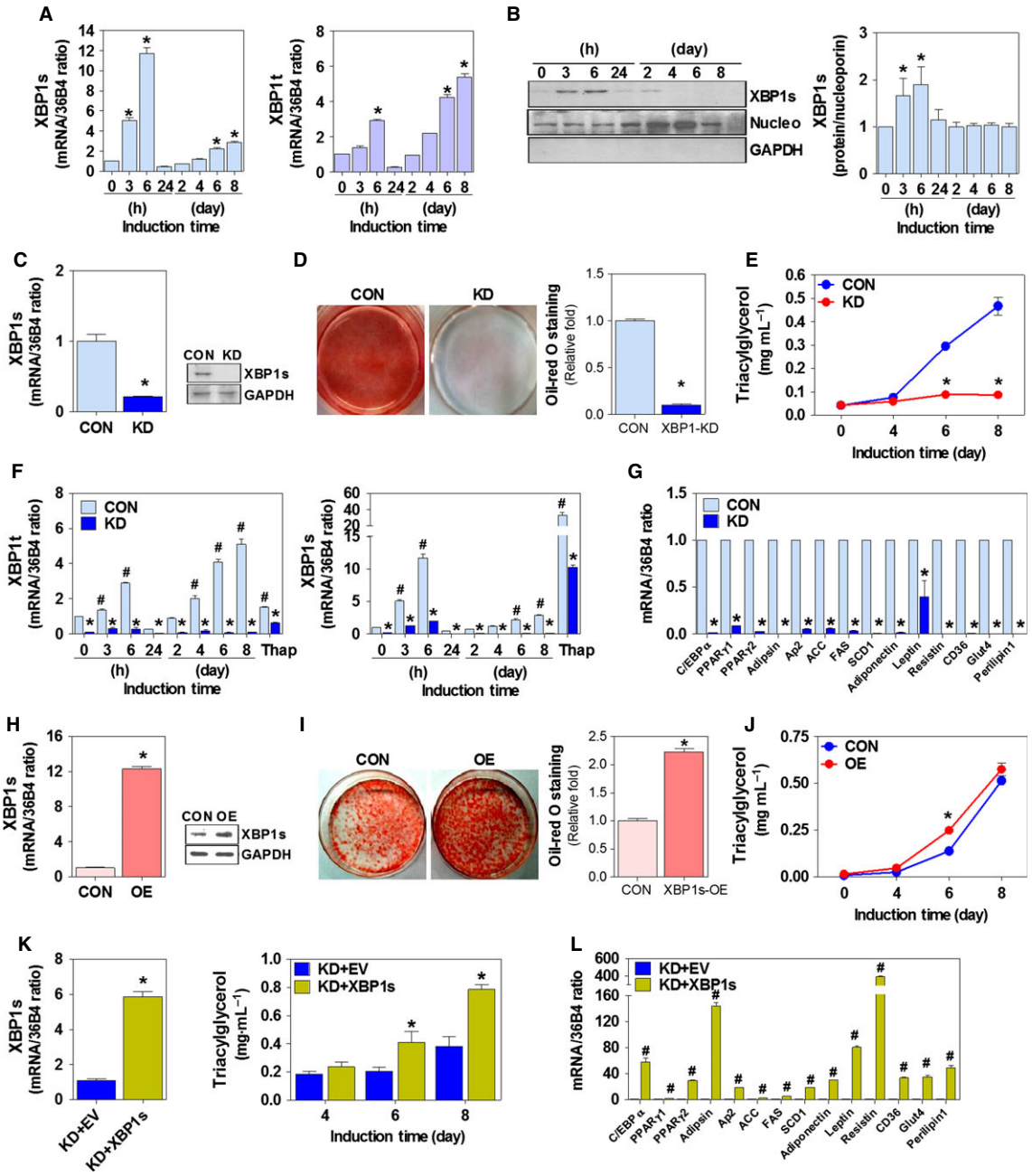

Fig. 1. XBP1 is necessary for adipocyte differentiation. Wild-type and genetically modified 3T3-L1 preadipocytes were differentiated into adipocytes, and lipid content and gene expression levels were examined. (A, B) Cytoplasmic *Xbp1s* and *Xbp1t* mRNAs and nuclear XBP1s protein levels in wild-type cells. For immunoblot analysis (B), nucleoporin (Nucleo) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as nuclear and cytoplasmic controls, respectively. (C) *Xbp1s* mRNA and protein levels in control (CON) and XBP1-KD (KD) cells. The effect of *Xbp1* depletion on intracellular lipid accumulation was assessed by Oil Red O staining (at day 8) (D) and triacylglycerol content measurement (E). (F) Levels of *Xbp1t* and *Xbp1s* in control and XBP1-KD cells. Thapsigargin (Thap, 100 nM, 6 h) was used as a positive control for Xbp1 induction and transcriptional activation. (G) Expression of adipogenic genes in XBP1-KD cells at day 8 after induction. (H–J) *Xbp1* mRNA and protein levels in control (CON) or XBP1s-OE (OE) cells (H). The effect of XBP1s over-expression on adipogenesis was assessed by Oil Red O staining (at day 6) (I) and triacylglycerol content measurement (J). (K, L) Effect of XBP1s replenishment on adipogenesis and gene expression in XBP1-KD cells. Intracellular triacylglycerol content during adipogenic differentiation (K) and mRNA levels of adipogenic genes at day 8 (L) were measured in XBP1-KD cells transfected with vectors carrying XBP1s (KD + XBP1s) or empty vector (KD + EV). Immunoreactivities and Oil Red O staining results were quantified by densitometry as described in Experimental procedures. Values are means ± SEM of three independent experiments (*$P < 0.05$, compared to CON or KD + EV; $^{\#}P < 0.05$, compared to 0 h CON).

measurement of intracellular triacylglycerol contents more reliably suggest that XBP1s is an essential pro-adipogenic transcription factor, especially in the very early time period of differentiation.

PPARγ2 is a specific target of XBP1s

Because XBP1s is a typical transcription factor, we attempted to identify its target gene(s) through which it exerts its pro-adipogenic activity. In comparative cDNA microarray analysis, expression of a total of 862 genes was found to be altered more than twofold in XBP1-KD cells, and the genes involved in lipid metabolism and transcription regulation are listed in Table 1. Among these genes, PPARγ was considerably down-regulated (5.0-fold) in XBP1-KD cells, and, moreover, as shown in XBP1-KD cells (Fig. 1G), expression of its target genes such as those encoding adiponectin, stearoyl CoA desaturase 1, lipoprotein lipase, acetyl CoA acetyltransferase, perilipin 1, acetyl CoA carboxylase and CD36 was also remarkably reduced (Table 1).

During the differentiation period, PPARγ2 expression was XBP1s-dependent. In control cells, *Pparγ2* transcription was time-dependently induced from day 2, and increased more than 400-fold by day 8 (Fig. 2A). Interestingly, when we examined *Pparγ2* mRNA levels within the first 24 h, they began to increase immediately after the expression of *Xbp1s* mRNA reached a peak, suggesting a possible correlation between them (Fig. 2C). Expression of PPARγ2 protein also increased in a time-dependent manner during adipogenic differentiation (Fig. 2B). However, in XBP1-KD cells, PPARγ2 expression was significantly suppressed (Fig. 2A,B). Moreover, PPARγ-specific target genes such as those encoding adipsin, Ap2 and lipoprotein lipase were also significantly down-regulated (Fig. 2D). In addition to PPARγ2, mRNA expression levels of *Pparγ1* (Fig. 2B,E) and PPARα (Fig. 2E) also decreased in XBP1-KD cells.

PPARγ is induced through a complex network of transcription factors such as C/EBPβ, C/EBPδ and Kruppel-like factor 5 during adipogenesis [7]. To rule out the possibility that XBP1s indirectly up-regulates PPARγ2 expression, we examined the effect of XBP1 silencing on expression of these genes. However, as shown in Fig. 2F, no significant changes in their expression were observed. These findings strongly indicate that PPARγ is a candidate target of XBP1s during adipogenesis of 3T3-L1 cells.

Table 1. List of genes found to be differentially expressed by cDNA microarray analysis. The numbers in the fold column were calculated by dividing the XBP1-KD cell value by the wild-type 3T3-L1 cell value. Only genes that belong to the lipid metabolism and transcription regulation pathways are listed.

Fold change	Gene ID	Protein name	Gene abbreviation
Lipid metabolism			
> −10	67800	Diacylglycerol *O*-acyltransferase 2	*Dgat2*
> −10	11450	Adiponectin, C1Q and collagen domain-containing	*Adipoq*
> −10	20249	Stearoyl CoA desaturase 1	*Scd1*
−10	14245	Lipin 1	*Lpin1*
−10	14555	Glycerol-3-phosphate dehydrogenase 1 (soluble)	*Gpd1*
−10	16956	Lipoprotein lipase	*Lpl*
−10	11812	Apolipoprotein C-I	*Apoc1*
−5	16890	Lipase, hormone-sensitive	*Lipe*
−3.3	212862	Choline phosphotransferase 1	*Chpt1*
−3.3	13350	Diacylglycerol *O*-acyltransferase 1	*Dgat1*
−2.5	11364	Acyl CoA dehydrogenase, medium chain	*Acadm*
−2.5	11370	Acyl CoA dehydrogenase, very long chain	*Acadvl*
−2.5	11752	Annexin A8	*Anxa8*
−2.5	78894	Acetoacetyl CoA synthetase	*Aacs*
−2.5	110460	Acetyl CoA acetyltransferase 2	*Acat2*
−2.5	12896	Carnitine palmitoyltransferase 2	*Cpt2*
−2	12908	Carnitine acetyltransferase	*Crat*
2.1	15211	Hexosaminidase A	*Hexa*
3	329910	Acyl CoA thioesterase 11	*Acot11*
9.7	16803	Lipopolysaccharide-binding protein	*Lbp*
Transcription regulation			
−5	19016	Peroxisome proliferator activated receptor γ	*Pparγ*
−2.5	19015	Peroxisome proliferator activator receptor δ	*Pparδ*
−2.5	22337	Vitamin D receptor	*Vdr*
−2.5	170826	Peroxisome proliferative activated receptor γ, coactivator 1β	*Pparγc1β*
2.8	16477	Jun-B oncogene	*Junb*
2.8	15902	Inhibitor of DNA binding 2	*Idb2*
2.9	13653	Early growth response 1	*Egr1*

Table 1. (Continued).

Fold change	Gene ID	Protein name	Gene abbreviation
4.2	19017	Peroxisome proliferative activated receptor γ, coactivator 1α	*Pparγ1α*
4.5	20847	Signal transducer and activator of transcription 2	*Stat2*
4.9	13654	Early growth response 2	*Egr2*

XBP1s binds to a specific DNA motif in the PPARγ2 promoter

To examine whether PPARγ2 is a direct target of XBP1s, we performed a promoter analysis and chromatin immunoprecipitation (ChIP) assay. XBP1s significantly stimulated PPARγ2 promoter activity with a similar potency to C/EBPα, which is a positive control for PPARγ2 promoter activity (Fig. 3A) [24]. In the ChIP assay, XBP1s binding to the PPARγ2 promoter increased more than eightfold at 6 h after the induction, at which time XBP1s expression was highest (Fig. 1A); thereafter, it decreased slightly but levels that were approximately fourfold higher remained until day 6 (Fig. 3B). Next, we identified the CCACG motif in the PPARγ2 promoter (−369 bp/−373 bp) as the XBP1s binding site (Fig. 3C) using deletion mutation analysis of consensus *cis*-regulatory elements for XBP1s binding [25], and the specificity of this motif to XBP1s was confirmed using a PPARγ2 promoter construct with a mutated XBP1s binding sequence (Fig. 3D).

Expression of C/EBPα was significantly down-regulated in XBP1-KD cells (Fig. 3E), as previously reported [10]. ChIP analysis also showed direct binding of XBP1s to the C/EBPα promoter on day 6 (Fig. 3F). Therefore, it is plausible that the effect of XBP1s on PPARγ2 may occur through binding of C/EBPα, instead of XBP1s, to the motif that we identified. However, when the C/EBP-specific consensus sequence in *Pparγ2* was mutated [26], PPARγ2 promoter activity was significantly suppressed by C/EBPα, but not by XBP1s (Fig. 3G). Moreover, C/EBPα-mediated PPARγ2 promoter activity was not altered by mutation of the XBP1s binding sequence (Fig. 3D). These findings clearly indicate that XBP1s stimulates PPARγ2 transcription by directly binding to the XBP1s-specific sequence motif, which is distinct from the C/EBPα binding site, in the PPARγ2 promoter. In addition, binding of XBP1s to the PPARγ2 promoter appeared to be considerably faster than that to the C/EBPα promoter (Fig. 3B,F), suggesting that PPARγ2 may be an earlier and more significant target than C/EBPα for the pro-adipogenic activity of XBP1s.

Xbp1 mRNA splicing is induced by insulin-, cAMP- and glucocorticoid-mediated signalling pathways

Because *Xbp1s* mRNA levels increased in response to a hormone mixture (insulin, dexamethasone and 3-isobutyl-1-methylxanthine, a phosphodiesterase inhibitor), we examined whether the individual components may induce *Xbp1* mRNA splicing. Unexpectedly, culture medium containing 10% fetal bovine serum (FBS) alone, but not newborn calf serum (NCS), significantly induced *Xbp1* mRNA splicing in the absence of hormones (Fig. 4A); therefore, we used 10% NCS in the experiments. When post-confluent preadipocytes were treated for 6 h at the same concentrations as used for adipogenic induction, a significant and dose-dependent increase were observed (Fig. 4B). These findings indicate that signalling pathways mediated by insulin, cAMP and glucocorticoid and by unknown factor(s) in FBS may be involved in the splicing of *Xbp1* mRNA.

Xbp1s is positively correlated with body mass index (BMI) in human adipose tissues

The *Pparγ2* mRNA level of adipose tissue increased in obesity [27,28]. Given our results showing the XBP1s-mediated positive regulation of PPARγ2, we investigated the possible correlation of *Xbp1s* with BMI. Using subcutaneous adipose tissues obtained from 26 healthy females, we measured *Xbp1s* mRNA levels and their relationship to BMI. Both *Xbp1s* and *Pparγ2* mRNA levels increased proportionally to BMI ($r = 0.40$, $P < 0.05$; $r = 0.34$, $P < 0.05$, respectively) (Fig. 5A,B). We then examined the correlation between the two genes. When we plotted the mRNA levels of *Xbp1s* versus those of *Pparγ2* in each subject, a significant positive correlation was observed ($r = 0.32$, $P < 0.05$) (Fig. 5C). Taken together, these results suggest that the XBP1s–PPARγ2 signalling pathway may be involved in the expansion of adipose tissue.

Discussion

PPARγ is the master regulator of adipogenesis, and here we provide strong evidence that XBP1s specifically regulates the expression of PPARγ2 in adipocytes. Given that C/EBPα and Wnt10b, two important

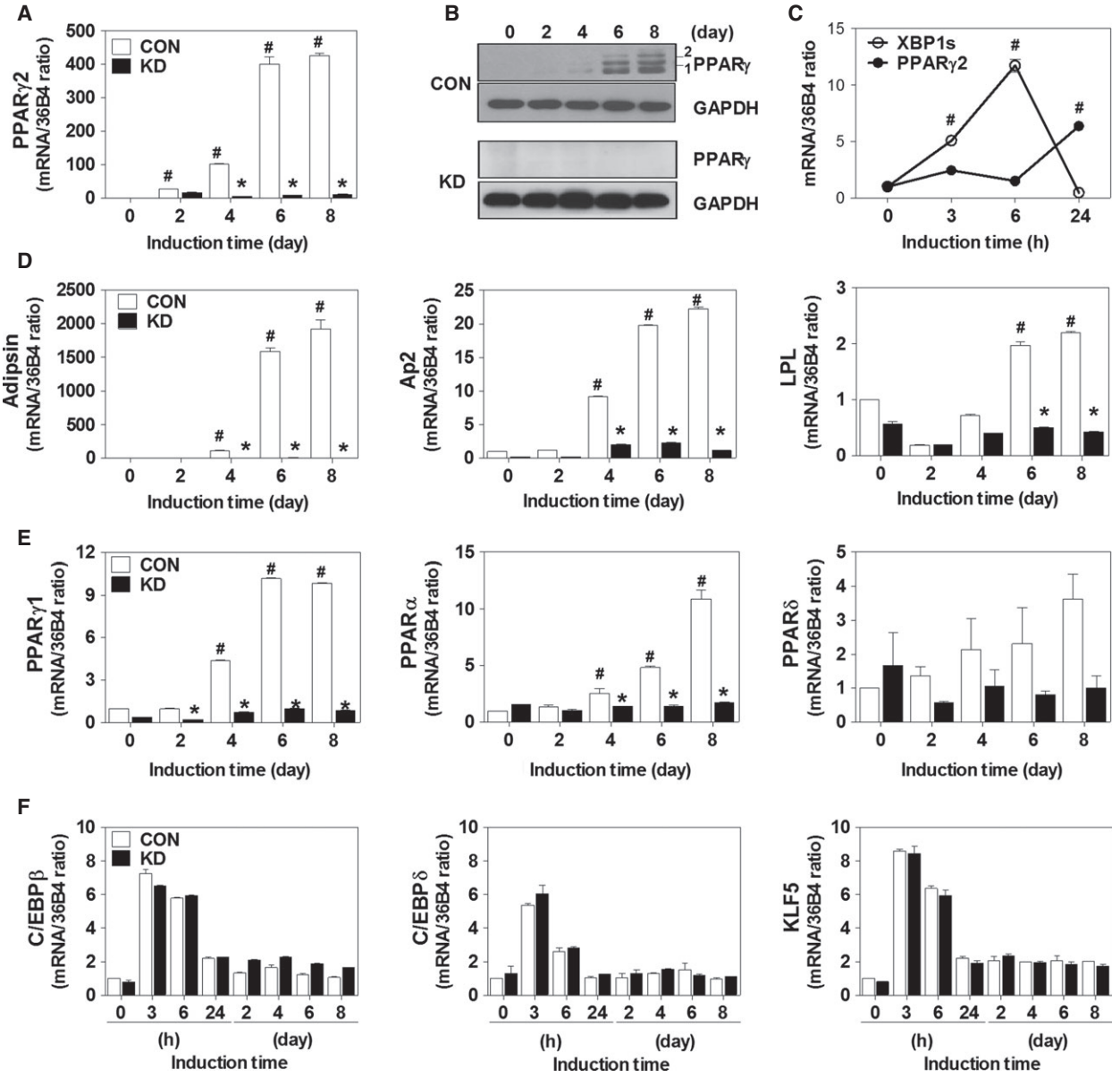

Fig. 2. PPARγ2 expression is regulated by XBP1s. Control (CON) or XBP1-KD (KD) cells were differentiated, and gene expression levels were compared. (A, B) PPARγ2 mRNA and protein levels during adipogenesis. (C) Temporal patterns of *Xbp1s* and *Pparγ2* mRNA expression in wild-type cells for the first 24 h. (D) Transcription levels of typical target genes of PPARγ. (E) Expression of *Pparγ1*, *Pparα* and *Pparδ* mRNA. (F) Transcription levels of well-known upstream regulators for PPARγ during adipogenesis. Values are means ± SEM of three independent experiments (*$P < 0.05$, compared to CON; $^{\#}P < 0.05$, compared to 0 h CON). Ap2, adipocyte protein 2; KLF5, Krueppel-like factor 5; LPL, lipoprotein lipase.

regulators for adipogenesis, are target genes of XBP1s [10,21], it is plausible that XBP1s may direct adipogenesis by regulation of pro- and anti-adipogenic signalling pathways. Moreover, to our knowledge, *in vitro* adipogenesis is always suppressed by depletion of XBP1s [10,19,21]. Taken together, these findings imply that XBP1s may also play roles in adipose tissue formation *in vivo*. However, Hotamisligil *et al.* recently reported conflicting results on XBP1s; adipocyte-specific deletion of XBP1s suppressed adipogenesis in 3T3-L1 cells, but did not affect adipocyte formation in the mouse model [19]. The differential effects of XBP1s in adipogenesis were not elucidated in the present work. However, as adipogenesis is a complex

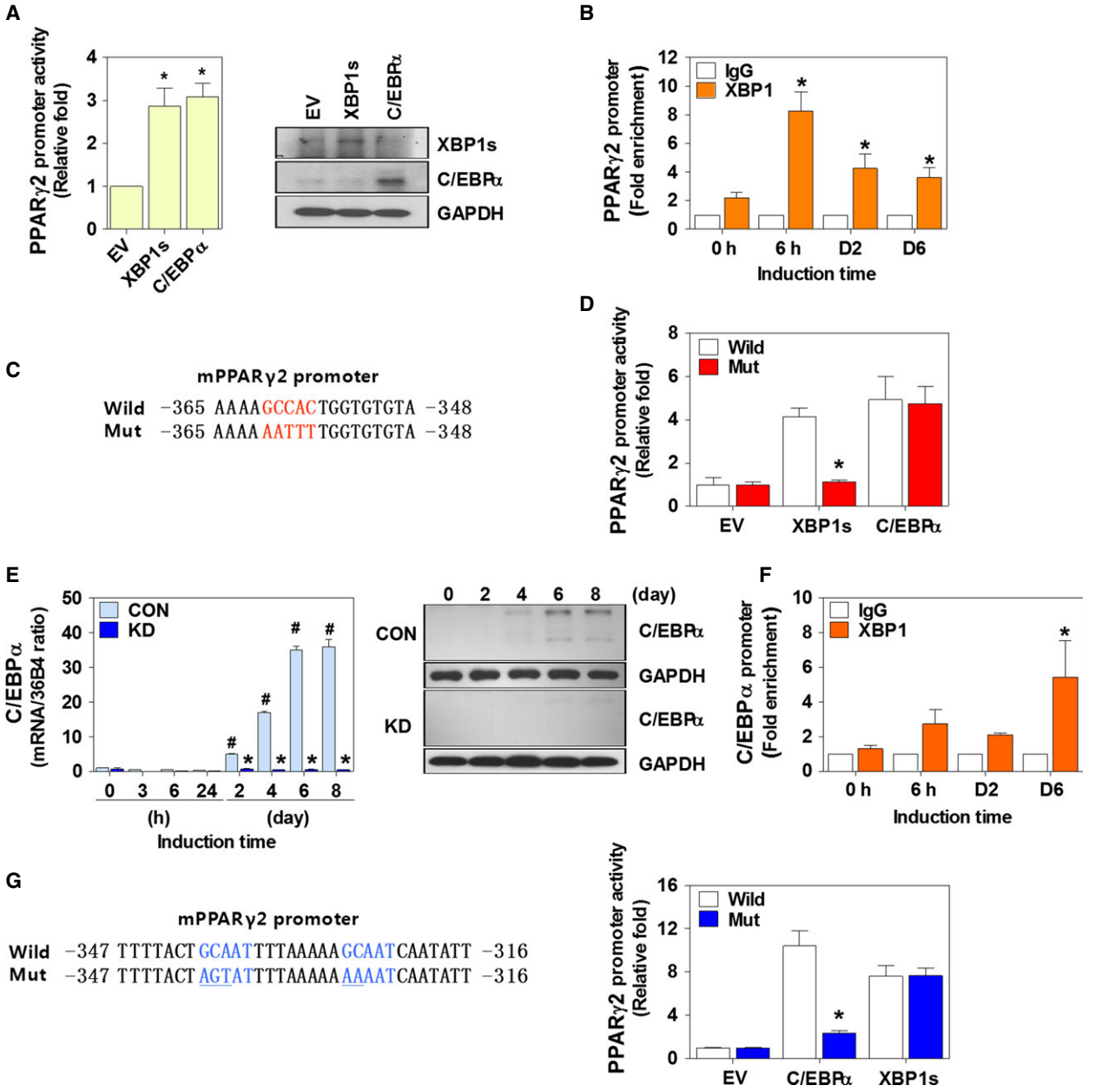

Fig. 3. XBP1s binds to a specific DNA motif in the PPARγ2 promoter region. The XBP1s-specific DNA motif involved in the regulation of PPARγ2 expression was identified using promoter analysis and ChIP assays. (A) PPARγ2 promoter and immunoblot analyses in 3T3-L1 cells transfected with empty vector (EV) or plasmids expressing XBP1s or C/EBPα for 3 days. (B) ChIP assay of the PPARγ2 promoter. (C) DNA sequence of the normal (Wild) or mutated (Mut) XBP1s binding sites in the PPARγ2 promoter. (D) PPARγ2 promoter analysis using reporter constructs with a normal (Wild) or mutated (Mut) XBP1s binding motif. (E) Expression of C/EBPα mRNA (left) and protein (right) in control (CON) or XBP1-KD (KD) cells. (F) ChIP assay of the C/EBPα promoter. (G) PPARγ2 promoter analysis using reporter constructs with normal (Wild) or mutated (Mut) C/EBP binding sites. Values are means ± SEM of three independent experiments (*$P < 0.05$, compared to CON; $^{\#}P < 0.05$, compared to 0 h CON).

process performed by a complicated transcriptional network, and XBP1s is not a final executor of adipogenic differentiation but one of the upstream regulators, it is reasonable to assume that loss of XBP1 in adipocytes may be successfully compensated by other (upstream) players *in vivo*. In addition, XBP1 is involved in multiple signalling pathways in adipogenesis, e.g. transcription of *Pparα*, which contributed to

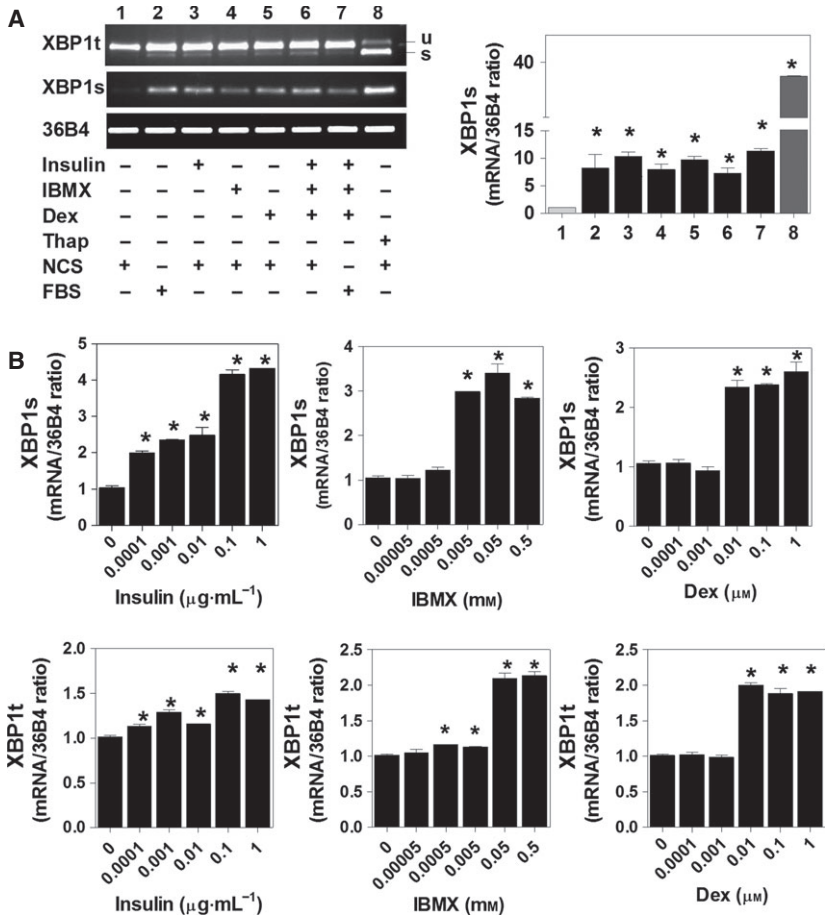

Fig. 4. *Xbp1* mRNA splicing is induced by insulin-, 3-isobutyl-1-methylxanthine- and glucocorticoid-mediated signalling pathways. 3T3-L1 cells were cultured to confluency and treated with the indicated reagents for 6 h, and the expression of total (Xbp1t), unspliced (Xbp1u) and spliced (Xbp1s) forms of *Xbp1* was analysed. (A) RT-PCR analysis (left) and real-time PCR analysis (right). The concentration of each component is the same as that used in the adipogenic induction medium. Thapsigargin (Thap; 100 nM) was used as a positive control. (B) Real-time PCR results of dose-dependent experiments. Values are means ± SEM of three independent experiments (*$P < 0.05$, compared to untreated control). Dex, dexamethasone; IBMX, 3-isobutyl-1-methylxanthine; NCS, newborn calf serum; FBS, fetal bovine serum.

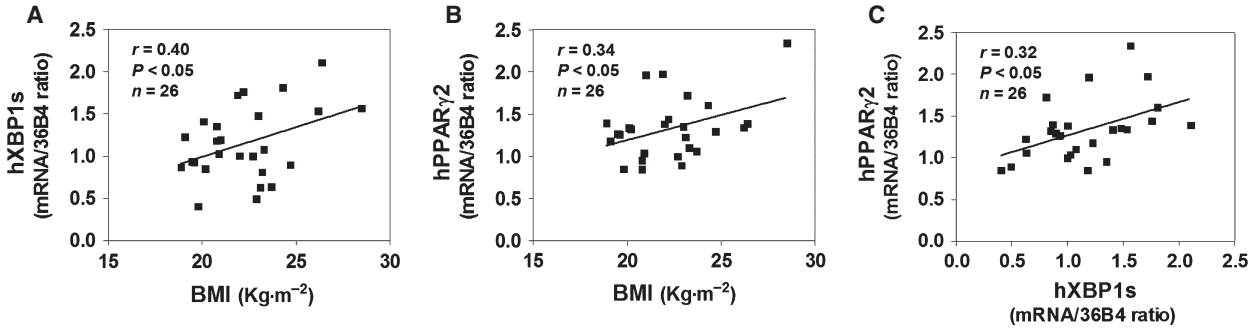

Fig. 5. *Xbp1s* levels are positively correlated with BMI and *Pparγ2* levels in human adipose tissues. Expression levels of *Xbp1s* (A) and *Pparγ2* (B) mRNAs were measured in subcutaneous adipose tissues and plotted against the BMI. (C) Correlation between the expression levels of *Xbp1s* and *Pparγ2* mRNAs in each subject. Values are means ± SEM of three independent experiments.

suppression of adipocyte hypertrophy by increasing fatty acid oxidation [29], was also XBP1-dependent in our results (Fig. 2E). Moreover, PPARγ is a potential target gene for many pro-adipogenic transcription factors such as C/EBPβ, C/EBPδ and Kruppel-like factor 5 that are not involved in XBP1s-induced adipogenesis (Fig. 2F). Recently, PPARγ was also recognized as a target for activating transcription factor 4 (ATF4) [30]. Although these findings suggest the possible *in vivo* role of XBP1s, further evidence is necessary to confirm whether the *in vitro* activity of XBP1s is applicable to adipose tissue development.

Both *Xbp1t* and *Xbp1s* were immediately but transiently induced, with a peak level at 6 h after the induction, and this was followed by a significant increase in *Pparγ2* mRNA within the first 24 h. Once expressed, the PPARγ2 level may be maintained through autoactivation [31] or mutual stimulation by C/EBPα [7]. Therefore, although the *Xbp1* mRNA increase was transient, the PPARγ2 level continued to increase until preadipocytes matured into adipocytes. The temporal pattern of *Xbp1s* expression also raised the question of how *Xbp1* was rapidly induced or activated during this early time of adipogenesis, and we therefore examined the effect of individual components of the hormone cocktail used for adipogenic induction. Sha *et al.* [10] have previously reported that 3-isobutyl-1-methylxanthine increases the total level of *Xbp1* mRNA, but they did not examined the effect on transcriptionally active XBP1s. In this study, consistent with the expression pattern of *Xbp1s* mRNA, we provide evidence for the first time that 6 h exposure of 3T3-L1 cells to individual hormonal components markedly increased *Xbp1s* mRNA, suggesting that signalling pathways amplified by FBS, insulin, glucocorticoids and cAMP are key regulators. In particular, the effect of insulin on *Xbp1* mRNA splicing is noteworthy. Insulin is a potent adipogenic hormone [32], and maintains the proper function of mature adipocytes through regulation of PPARγ [6,33]. Moreover, it also increases nuclear translocation of XBP1s [34]. We found that insulin substantially increased *Xbp1* mRNA splicing even at 0.1 ng·mL^{-1}. These findings strongly indicate that insulin regulates the transcriptional activity of XBP1s by increasing its production and nuclear translocation, and thereby contributes to induce adipocyte differentiation and maintenance of insulin sensitivity in insulin-sensitive cells such as adipocytes. In agreement with this finding, XBP1s has previously been suggested to play roles in insulin signalling in the pancreas [35,36] and the liver [37]. It will be interesting to explore the possible relationship between insulin and XBP1 signalling pathways.

Our data also suggest a possible role of XBP1 in non-adipose tissues. Although PPARγ protein is most abundant in adipose tissue, it is also expressed at high constitutive levels in the intestinal tract, liver, lung and immunological system [38–41]. Moreover, PPARγ1, which appeared to be XBP1s-dependent in our findings, is ubiquitously expressed [42]. In these non-adipose tissues, PPARγ plays a role in various functions such as tumorigenesis [43], cholesterol homeostasis and inflammation in macrophages [40], and fibrosis in the liver and pancreas [41,44]. It has been also linked to circadian control during adipogenesis, osteogenesis and hepatic steatosis [45,46]. Given the increasing evidence showing the importance of PPARγ in many pathophysiological functions, it will be also interesting to examine the roles of XBP1s in PPARγ-mediated signalling pathways other than adipogenesis in non-adipose tissues.

The UPR contributes to adipocyte differentiation [8]. Currently, the involvement of UPR regulators such ATF6α, IRE1α and CEBP homologous protein (CHOP) in adipocyte differentiation has been reported [9–11]. While this manuscript was in preparation, Yu *et al.* reported that ATF4 is a positive regulator of adipogenesis via up-regulation of C/EBPβ and PPARγ in 3T3-L1 cells [30]. Here we have shown that XBP1s induces the expression of PPARγ but not C/EBPβ. These findings indicate that many UPR proteins are involved in adipogenesis through their differential regulation of adipogenic transcription network. We also examined UPR gene expression during adipogenesis, and found that the mRNA levels of the genes encoding ATF4, binding immunoglobulin protein/78 KDa glucose-regulated protein (BiP/Grp78) and CHOP were not altered by XBP1s knockdown, suggesting that the pro-adipogenic role of XBP1s may be independent of UPR in general (data not shown). The UPR is also linked to obesity and obesity-related metabolic disorders [47]. For example, it is activated in adipose tissue of obese animals and humans [48], and causes weight gain in mice [8]. Our findings show for the first time that *Xbp1s* mRNA expression is proportional to BMI, and is also positively correlated with *Pparγ2* expression in human adipose tissues. In addition, we observed that *Xbp1s* mRNA levels in fatty tissue increased with body weight in a high-fat diet-fed mouse model (data not shown). PPARγ activation in adipocytes is recognized as sufficient for maintaining systemic insulin sensitivity [6]. Accumulating evidence also indicates that adipose PPARγ protects non-adipose tissues against excessive lipid overload by the expansion of adipose tissue mass, and consequently improves the metabolic profile especially in metabolically healthy obese subjects [49].

Together with our results showing a possible link between insulin and XBP1s, these findings suggest that XBP1s may participate in maintaining systemic insulin sensitivity by the regulation of PPARγ during adipogenesis as well as in mature adipocytes *in vivo*. More research is necessary for further understanding of the function of the XBP1s in mature adipocytes or adipose tissue.

In summary, we found strong evidence that UPR-, insulin-, glucocorticoid- or cAMP-mediated signals induce XBP1s, which stimulates adipogenesis by up-regulating PPARγ2 expression (Fig. 6). Our results from *in vivo* experiments also suggest possible involvement of XBP1s–PPARγ signalling pathways in adipose tissue expansion.

Experimental procedures

Culture and adipocyte differentiation of 3T3-L1 preadipocytes

Mouse 3T3-L1 preadipocytes (American Type Culture Collection, Manassas, VA, USA) were cultured and differentiated into adipocytes as described previously [50]. Briefly, cells were maintained for 2 days in high-glucose (4.5 $g \cdot L^{-1}$) Dulbecco's modified Eagle's medium containing 10% NCS (Gibco, Green Island, NY, USA). Two days after reaching confluence (day 0), adipogenic differentiation was induced for 2 days in medium containing insulin (1 $\mu g \cdot mL^{-1}$; Sigma, St Louis, MO, USA), 3-isobutyl-1-methylxanthine (0.5 mM; Sigma) and dexamethasone (1 μM; Sigma). Then the cells were cultured for 2 days in Dulbecco's modified Eagle's medium containing 10% FBS (Gibco) and insulin (1 $\mu g \cdot mL^{-1}$), and thereafter the medium was changed to Dulbecco's modified Eagle's medium/10% FBS until day 8.

Oil Red O staining and quantification of intracellular triacylglycerol content

After washing cells at room temperature for 10 min twice with PBS, cells were fixed with 10% formalin in distilled water for 10 min, rinsed with distilled water, and stained for 10 min with 0.3% Oil Red O (Sigma) as previously described [51]. Intracellular triacylglycerol levels were determined using a serum triglyceride determination kit (Sigma) according to the manufacturer's instructions. Briefly, cells were washed with PBS and lysed using RIPA buffer [20 mM Tris/HCl, pH 7.5, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1% nonidet P-40 (Sigma) and proteinase inhibitors], and then incubated on ice for 10 min. The resulting lysates were cleared by centrifugation at 16 000 ***g*** at 4 °C for 10 min, and 10 μL of glycerol standard or samples were mixed with 500 μL of the reagents. The triacylglycerol concentration was calculated using the initial and final absorbances at 540 nm, and normalized to total cellular protein. For statistical analysis, images of each dish were captured using an inverted microscope and analysed using OPTIMAS 6.5 software (Meyer Instruments Inc., Houston, TX, USA).

Genetic manipulation of *Xbp1*

XBP1-KD cells were prepared using retroviral vectors expressing an shRNA targeting mouse *Xbp1* (SFGneo-iXBP1) as described previously [52]. Briefly, 293T cells were transfected for 48 h with the viral vectors using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), and virus-containing medium was collected. For infection, Polybrene (hexadimethrine bromide; 8 $\mu g \cdot mL^{-1}$; Sigma) was added to the filter-sterilized virus-loaded medium and applied to proliferating cells for 2 days. For preparation of XBP1s-OE cells, recombinant plasmids expressing mouse XBP1s (pcDNA3.1-mXBP1s) were transfected using Lipofectamine 2000. Genetically engineered cells were selected using antibiotics G418 (1 $mg \cdot mL^{-1}$; Gibco).

RNA isolation and real-time PCR

Total RNA was extracted using Trizol reagent (Invitrogen), and cDNA was synthesized using a PrimeScript™ RT kit (Takara, Shiga, Japan). The real-time PCR analysis was performed using an ABI 7300 thermal cycler (Applied Biosystems, Foster City, CA, USA) with SYBR Green Q-PCR Master Mix (Takara). The $\Delta\Delta C_T$ method was used to calculate the relative amount of mRNAs with 36B4 as internal controls [21]. The primer sequences are listed in Table 2.

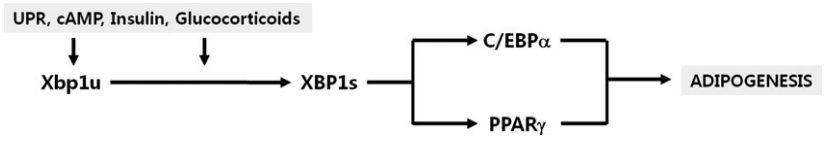

Fig. 6. Diagram of XBP1s-mediated adipogenesis. Transcription of *Xbp1u* and its splicing into *Xbp1s* mRNA is induced by the unfolded protein response (UPR), cAMP, insulin or glucocorticoids. XBP1s then directly induces C/EBPα and PPARγ, which cooperate to activate adipogenic differentiation.

Table 2. Primer sequences for RT-PCR and real-time PCR analysis.

	Primer sequences (5′ → 3′)			
Name	Sense	Antisense	Size (bp)	Gene ID
mAcc	GGGCACAGACCGTGGTAGTT	CAGGATCAGCTGGGATACTGAGT	106	NM_133360
mAdiponectin	TGTGTATCGCTCAGCGTTCAGTGT	AGAGAACGGCCTTGTCCTTCTTGA	224	NM_009605
mAdipsin	CATGCTCGGCCCTACATGG	CACAGAGTCGTCATCCGTCAC	129	NM_013459
mAp2	CATGGCCAAGCCCAACAT	CGCCCAGTTTGAAGGAAATC	101	NM_024406
mC/ebpα	CCCACTTGCAGTTCCAGAT	CTGTTCTTGTCCACCGACTT	250	NM_007678
mC/ebpβ	ACAAGCTGAGCGACGAGTACAAGA	GCAGCTGCTTGAACAAGTTCCG	201	NM_009883
mC/ebpδ	CTCTTCAACAGCAACCACAAAG	CCAAGCTCACCACTGTCTG	196	NM_007679
mCd36	CAGGTCTATCTACGCTGTGTTC	CAGGCTTTCCTTCTTTGCATTT	200	NM_001159558
mFas	TATCCTGCTGTCCAACCTCAGCAA	TCACGAGGTCATGCTTTAGCACCT	95	NM_007988
mGlut4	TCGTGGCCATATTTGGCTTTGTGG	TAAGGACCCATAGCATCCGCAACA	190	NM_009204
mKlf5	AGTTCGACAAACCAGACGGCAGTA	GGCATGCCCTGGAACTGTTTCATT	113	NM_009769
mLeptin	GATGGACCAGACTCTGGCAG	AGAGTGAGGCTTCCAGGACG	197	NM_008493
mLpl	ACGAGCGCTCCATTCATCTCTTCA	TCTTGCTGCTTCTCTTGGCTCTGA	180	NM_008509
mPerillipin1	ATGTCAATGAACAAGGGCCCAACC	TGGTGCTGTTGTAGGTCTTCTGGA	133	NM_175640
mPparα	AAGAACCTGAGGAAGCCGTTCTGT	AGCTTTGGGAAGAGGAAGGTGTCA	245	NM_001113418
mPparγ1	TGAAAGAAGCGGTGAACCACTG	AGAGGTCCACAGAGCTGATTCC	91	NM_001127330
mPparγ2	CGCTGATGCACTGCCTATGA	AGAGGTCCACAGAGCTGATTCC	101	NM_011146
mPparδ	GCCTCCATCGTCAACAAAGA	CGGTCTCCACACAGAATGATG	200	NM_011145
mResistin	ACCCACGGGATGAAGAACCTTTCA	ATGGCTTCATCGATGGGACACAGT	104	NM_022984
mScd1	GAAGAAGACATCCGTCCTGAAA	GCGCTGGTCATGTAGTAGAAA	209	NM_009127
mXbp1s	CTGAGGTCCGCAGCAGGT	TGTCAGAGTCCATGGGAAGA	66	NM_013842
mXbp1t	AGCAGCAAGTGGTGGATTTGGAAG	AAGAGGCAACAGTGTCAGAGTCCA	299	NM_013842
rXbp1s	TCTGCTGAGTCCGCAGCAGG	CTCTAAGACTAGAGGCTTGG	321	NM_001271731
hPparγ2	ATTGACCCAGAAAGCGATTC	CAAAGGAGTGGGAGTGGTCT	154	NM_005037
hXbp1s	GGTCTGCTGAGTCCGCAGCAGG	AGTTCATTAATGGCTTCCAGCT	280	NM_001079539
36b4	GCTCCAAGCAGATGCAGCA	CCGGATGTGAGGCAGCAG	143	NM_007475
mCebpα promoter	TGTTGGCTGGAAGTGGGTGACTTA	TGTGACTTTCCAAGGCGGTGAGT	204	12606
mPparγ2 promoter	CAGATGTGTGATTAGGAGTTTCAACC	CAGTAGTTGGAATTACCAGAGCAGAG	190	19016

Xbp1 mRNA splicing

Xbp1 mRNA splicing was examined using RT-PCR and real-time PCR analyses as described previously [53]. For RT-PCR, the unspliced isoform of *Xbp1* (*Xbp1u*) and *Xbp1s* were amplified together using primers covering a 26 nucleotide intron (483 bp/508 bp) and flanking exon fragments (expected sizes: 273 bp for *Xbp1s* and 299 bp for *Xbp1u*). Amplified fragments were separated by electrophoresis on a 3.5% agarose gel, visualized by ethidium bromide staining, and detected using a Gel Doc EZ system (Bio-Rad, Hercules, CA, USA). Thapsigargin (100 nM; Sigma) was used as a positive control. The primer sequences are listed in Table 2.

Immunoblot analysis

Cells were lysed in RIPA buffer, and 30 μg of protein were separated by 10% SDS/PAGE, and transferred onto a nitrocellulose membrane (Schleicher & Schell, Dassel, Germany). The membranes were incubated with mouse antisera against GAPDH (1 : 500; Sigma) and nucleoporin (1 : 500; BD Biosciences, Franklin Lakes, NJ, USA), or with rabbit antisera against XBP1 (1 : 200; Biovision, Mountain View, CA, USA), C/EBPα (1 : 500; Santa Cruz, Dallas, TX, USA) and PPARγ (1 : 500; Santa Cruz). Antibody binding was detected using horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG (1 : 2000; Sigma), and the immunoreactive bands were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech, Little Chalfont, UK). For statistical analysis, immunoreactivities were quantified by densitometry using a Gel Doc EZ system (Bio-Rad).

cDNA microarray analysis

Wild-type or XBP1-KD 3T3-L1 cells were differentiated for 8 days, and total RNAs were isolated using Trizol (Invitrogen). The purity and quality of RNA was assessed using an Agilent Bioanalyzer 2100 (Agilent, Santa Clara, CA, USA), and the RNA was reverse-transcribed to cDNAs. Biotinylated cRNA was prepared using an Illumina TotalPrep RNA amplification kit (Ambion, Austin, TX, USA), loaded onto mouse WG-6 version 2.0 Sentrix

Expression BeadChip kits (Illumina, San Diego, CA, USA), and hybridized at 58 °C for 18 h. The arrays were scanned on an Illumina BeadArray reader at 532 nm (Cy3), and data from each sample were extracted using GENOME STUDIO software (Illumina) and then analysed using GENPLEX 3.0 (Istech, Seoul, Korea). Differentially expressed genes (fold change ≥ 2) in XBP1-KD cells were categorized according to biological pathways using the PANTHER protein classification system (http://www.pantherdb.org).

PPARγ2 promoter analysis

Cells were seeded into 24-well plates at a density of 2×10^4/well, and transfected with reporter plasmids (50 ng) and effector or empty vectors (0.5 μg). After 48 h, cells were lysed, and the luciferase activities were measured using a dual luciferase assay kit (Promega, Madison, WI, USA). Plasmid expressing mouse PPARγ2 (−609 bp/+52 bp) (pGL3-mPPARγ2 promoter-Luc) was used as a reporter, and mouse C/EBPα-expressing plasmids (pcDNA3.1-mC/EBPα) were used as a positive control. Plasmids were transfected with Lipofectamine, and transfection efficiency was normalized using 5 ng of *Renilla*-expressing pRL-CMV-luc (Promega).

Site-directed mutagenesis

Binding motifs on pGL3-mPPARγ2 promoter-Luc were mutated using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). Mutagenesis was performed by PCR amplification using *Pfu* polymerase (Stratagene) followed by digestion with *Dpn*I (Fermentas, Glen Burnie, MD, USA) to remove the templates. The mutagenic primer sequences were 5′-GGTTTTCTATTTTAAAAAATTTTGGTGTGTATTTTACTGC-3′ (XBP1s-Mut) and 5′-GGTGTGTATTTTACTAGTATTTTAAAAAAAAATCAATATTGAAC-3′ (C/EBP-Mut). Mutation in each site was confirmed by sequencing.

Chromatin immunoprecipitation assay

Cells were differentiated for 6 h, 2 days or 6 days, cross-linked with formaldehyde, and sonicated to generate 200 bp/1000 bp DNA fragments. The fragmented chromatin samples were immunoprecipitated with rabbit antibody against mouse XBP1 (5 μg; M-186X; Santa Cruz), and reverse-cross-linked, purified, and analysed by real-time PCR according to the manufacturer's instructions (Millipore, Billerica, MA. USA). Primers for the amplicons of mouse *Pparg2* (–261 bp/–81 bp) or *Cebpa* (–454 bp/–251 bp) are listed in Table 2. DNA samples immunoprecipitated with rabbit IgG were used as negative controls.

Collection of human adipose tissue samples

The experiments were undertaken with the understanding and written consent of each subject according to the 'Act on Legal Codes for Biomedical Ethics and Safety' and the Declaration of Helsinki. All procedures and collections were performed under a research protocol that was reviewed and approved by the Institutional Review Board of the Catholic University of Korea, College of Medicine (CUMC09U010). Subcutaneous adipose tissues were obtained from 26 healthy Korean female subjects (BMI 18–28 $kg \cdot m^{-2}$, age 24–50 years) who were undergoing elective liposuction at a local cosmetic clinic (Suwon, Gyonggi-do, Korea), and immediately frozen at −80 °C until analysis.

Statistical analysis

All values are means ± SEM from at least three independent experiments. Data were analysed using one- or two-way ANOVA followed by Tukey's multiple comparison test and simple linear regression analysis. Statistical analyses were performed using GRAPHPAD PRISM 5 for Windows (Graphpad Prism Software, La Jolla, CA, USA). In all cases, statistical significance was set at $P < 0.05$.

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Author contributions

The work presented here was carried out in collaboration between all authors. OJK and YMC defined the research theme, interpreted the results and prepared the manuscript. YMC, SNK and DHK carried out the laboratory experiments and analyzed the data. NSJ, KSK and JBS prepared human adipose tissue samples. AHL and SWJ discussed analyses and interpretation.

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