FOSL2 promotes leptin gene expression in human and mouse adipocytes

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The adipocyte-derived hormone leptin is a critical regulator of many physiological functions, ranging from satiety to immunity. Surprisingly, very little is known about the transcriptional pathways that regulate adipocyte-specific expression of leptin. Here, we report studies in which we pursued a strategy integrating BAC transgenic reporter mice, reporter assays, and chromatin state mapping to locate an adipocyte-specific *cis*-element upstream of the leptin (*LEP*) gene in human fat cells. Quantitative proteomics with affinity enrichment of protein-DNA complexes identified the transcription factor FOS-like antigen 2 (FOSL2) as binding specifically to the identified region, a result that was confirmed by ChIP. Knockdown of *FOSL2* in human adipocytes decreased *LEP* expression, and overexpression of *Fosl2* increased *Lep* expression in mouse adipocytes. Moreover, the elevated *LEP* expression observed in obesity correlated well with increased *FOSL2* levels in mice and humans, and adipocyte-specific genetic deletion of *Fosl2* in mice reduced *Lep* expression. Taken together, these data identify FOSL2 as a critical regulator of leptin expression in adipocytes.

Introduction

Obesity and its associated complications have become a global epidemic. In the United States, two-thirds of the adult population is obese or overweight, resulting in approximately 240,000 excess deaths annually (1). Major research efforts have identified the adipose tissue itself as an important regulator of metabolism, in large part exerted through a variety of hormones and cytokine-like molecules collectively called adipokines (2). Leptin, among the first adipocyte-derived hormones to be identified, is a dominant regulator of food intake and energy expenditure, acting primarily through various sites in the central nervous system (3–5). Besides its role in metabolism, leptin participates in various other physiological activities, including reproduction, thyroid function, bone density, and immunity (6, 7). Recombinant leptin has been successfully used to treat insulin resistance in patients with lipodystrophy (8), but enthusiasm for the use of leptin as a weight loss agent waned after the disappointing results of early trials (9). More recently, however, leptin has demonstrated improved weight loss activity when combined with the pancreatic polypeptide amylin (10), and hopes have once again been raised that leptin may one day fulfill its promise as a clinically effective antiobesity therapy.

The expression of leptin (encoded by the *LEP* gene in humans, *Lep* in mice) is considered to be adipocyte specific, although low levels of leptin expression have been reported in brain, pituitary, trophoblast, stomach, mammary epithelial cells, liver, chondrocytes, and muscle (6, 11–13). The physiological role of leptin derived from these sites, if any, is currently unclear. *LEP* gene expression in adipocytes is regulated by a variety of physiological and experimental stimuli, including fasting and feeding, insulin, glucocorticoids, thiazolidinediones, and leptin itself, to name a few (6, 14, 15).

Although its physiological functions have been extensively studied, very little is known about the transcriptional regulation of leptin, particularly its adipocyte-specific expression. The proximal promoter of the *LEP* gene has been characterized (16, 17); besides a classic TATA box, binding sites have been identified for C/EBP, Sp-1, GR, and CREB, as well as an E-box element that may bind SREBP1c (18–21). However, a transgenic reporter driven by a 762-bp leptin proximal promoter cassette showed lower levels of expression in adipocytes than in a variety of non-adipose tissues (22), suggesting that this region is insufficient to confer tissue specificity. Recent work has highlighted the importance of distal enhancers for transcriptional gene regulation and tissue-specific gene expression (23, 24). We therefore pursued a strategy integrating BAC transgenic reporter mice, luciferase reporter assays, and genome-wide chromatin state mapping to identify an adipocyte-specific *cis*-regulatory element with enhancer activity upstream of the leptin gene. We then used a quantitative proteomics strategy to identify FOS-like antigen 2 (FOSL2) as a key transcription factor that binds to this region. FOSL2 (also called Fra-2) belongs to the AP-1 transcription factor family that includes the various isoforms of Fos and Jun. Knockdown of FOSL2 in mature human adipocytes decreased *LEP* gene expression, and ChIP-PCR confirmed direct binding of FOSL2 to our identified *cis*-element. Furthermore, manipulations of FosL2 levels in various murine systems in vivo and in vitro are consistent with the hypothesis that this factor plays a major role in leptin expression.

Results

Gross mapping of cis-regulatory elements using BAC transgenic mouse lines. The *Lep* gene is located in a region devoid of other annotated transcripts. To locate candidate *cis*-elements regulating tissue-specific *Lep* gene expression, we generated multiple BAC transgenic lines in which EGFP was inserted into the starting ATG of murine *Lep* (Figure 1). These BACs together cover approximately

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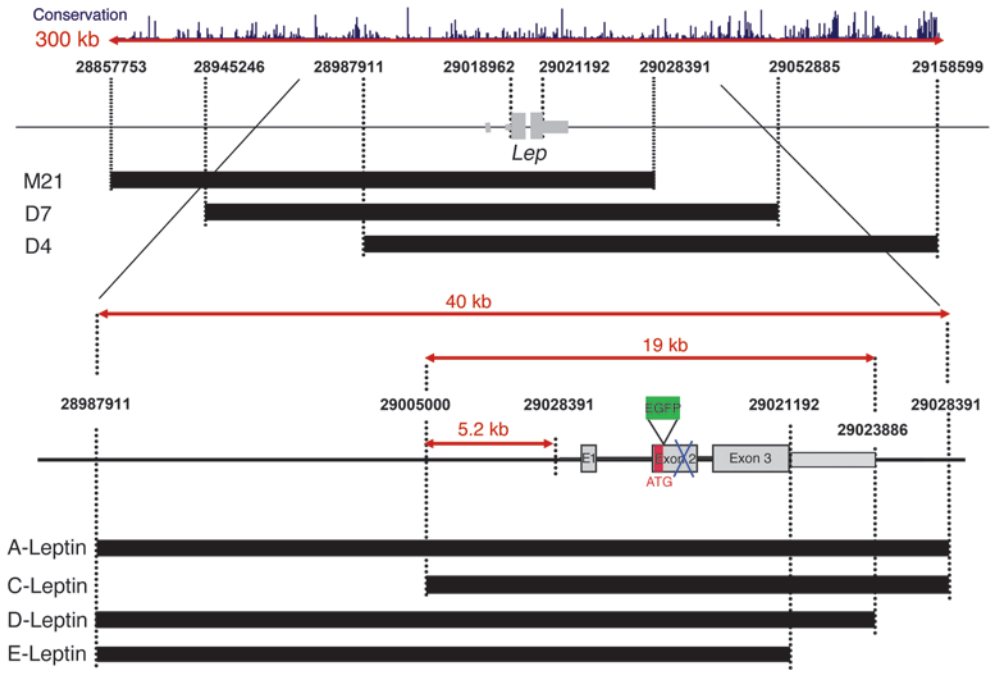

Figure 1
Gross mapping of *cis*-regulatory elements for adipocyte-specific *Lep* gene expression using BAC transgenic mouse lines. Top: Map of the *Lep* locus on mouse chromosome 6, showing areas of mouse-human conservation and regions covered by the 3 engineered BACs (M21, D7, and D4). Numbers refer to genomic coordinates for the beginning and end of each BAC, as well as the starting ATG of *Lep* (in exon 2) and the terminal GAA (in exon 3). There are no other known transcripts within this 300,000-bp region. Bottom: Maps of "trimmed" BACs. Numbers represent genomic coordinates of the ends of 4 new modified BACs, as well as exon 1, the terminal GAA in exon 3, and the transcriptional stop site.

300 kb of sequence flanking the *Lep* locus. With few exceptions, we observed adipose tissue–specific EGFP expression in all founders (Figure 2), although there were some differences likely reflecting the consequences of random insertion of the BAC into the genome. We narrowed down the region required for adipose-specific expression by sequential "BAC trimming" (25). Using this approach, we demonstrated that a region containing the three *Lep* exons, both introns, and 5.2 kb of 5′ flanking sequence was sufficient to drive adipocyte-specific EGFP expression. Of note, truncating the 3′ flanking sequence led to a diminution of the magnitude of EGFP expression, and although we did not pursue this in the present study, it seems likely that there are additional elements of interest in this region.

An adipocyte-specific enhancer is located 4.5 kb upstream of the human leptin gene. We decided to pursue further analysis on the human *LEP* gene, in part because we are ultimately interested in the application of transcriptional regulation in humans to facilitate future therapy. To fine-map possible regulatory elements in humans, we performed a "tiling" PCR across the proximal 10 kb upstream of the transcriptional start site (TSS), as well as through both introns of *LEP*. We extended our analysis further 5′ than indicated by the murine BAC analysis to cover orthologous regions in the human. Altogether, 24 overlapping 1-kb amplicons (except for the second intron, which was covered in a single amplicon of 2,293 bp) were cloned downstream of a luciferase reporter cassette that also contained the first 527 bp of the human *LEP* promoter (Figure 3A). These constructs were transfected into mature human adipocytes differentiated from human adipose stromal cells (hASCs). These cells express high levels of *LEP* in the mature state, in sharp contrast to 3T3-L1 adipocytes, which express *Lep* mRNA at quite low levels (ref. 26 and Figure 3B). Of the 24 constructs tested in the human adipocytes, only one acted as an enhancer in the luciferase assay, displaying a very robust induction relative to basal promoter activity. Based on our alphabetical naming system for constructs, we designated this area upstream region E, or UpE (chr7:127664032–127664989), located around 4.5 kb upstream of the *LEP* TSS (Figure 3C). To test whether the UpE region exhibits differentiation-dependent enhancer activity, we performed an analogous set of experiments in undifferentiated pre-adipocytes. UpE showed no significant activity in pre-adipocytes, but there was a 10-fold induction of enhancer activity after differentiation (Figure 3D).

We next sought the minimal region required for the activity of the UpE region using deletion mutants. Cutting the 3′ 493 bases from the 958 bp UpE region (bp 1–465) led to a minor, nonsignificant diminution of enhancer activity, but the removal of an additional 30-bp segment (bp 1–435) had a very large effect (Figure 3E). Removal of a further 48 base pairs had a small additional effect on reporter activity that was not significant. By introducing single base pair mutations at various positions within the 30-bp segment (bp 435–465), we identified two specific mutations that also abrogated enhancer activity ($1–465^{2M}$). We conclude that the 1–465 fragment of the larger 1-kb UpE region contains several sites required for full enhancer activity, and that the identified 30-bp fragment is a particularly important area in this regard.

Chromatin state mapping identifies a differentiation-dependent enhancer mark coincident with the UpE region. Genomic regions that act as enhancers are typically "marked" by various histone modifications, such as H3K4me1 and H3K27Ac (27). The latter in particular has been shown to be associated with active enhancers (28). We sought to determine whether the UpE region showed histone modifications consistent with a role as a differentiation-dependent enhancer in human adipocytes. To that end, we utilized chromatin state maps of human adipocytes in which multiple histone modifications were mapped using ChIP-Seq at several time points during the time course of differentiation (29). For the purposes of the current study, we focused on H3K4me3 (a promoter mark), H3K4me1 and H3K27ac (enhancer marks), and H3K36me3 (a mark of transcriptional elongation) (27). As shown in Figure 4, there was particular enrichment of H3K4me3 at the TSS and H4K36me3 in the gene body in mature cells, but not in pre-adipocytes, consistent

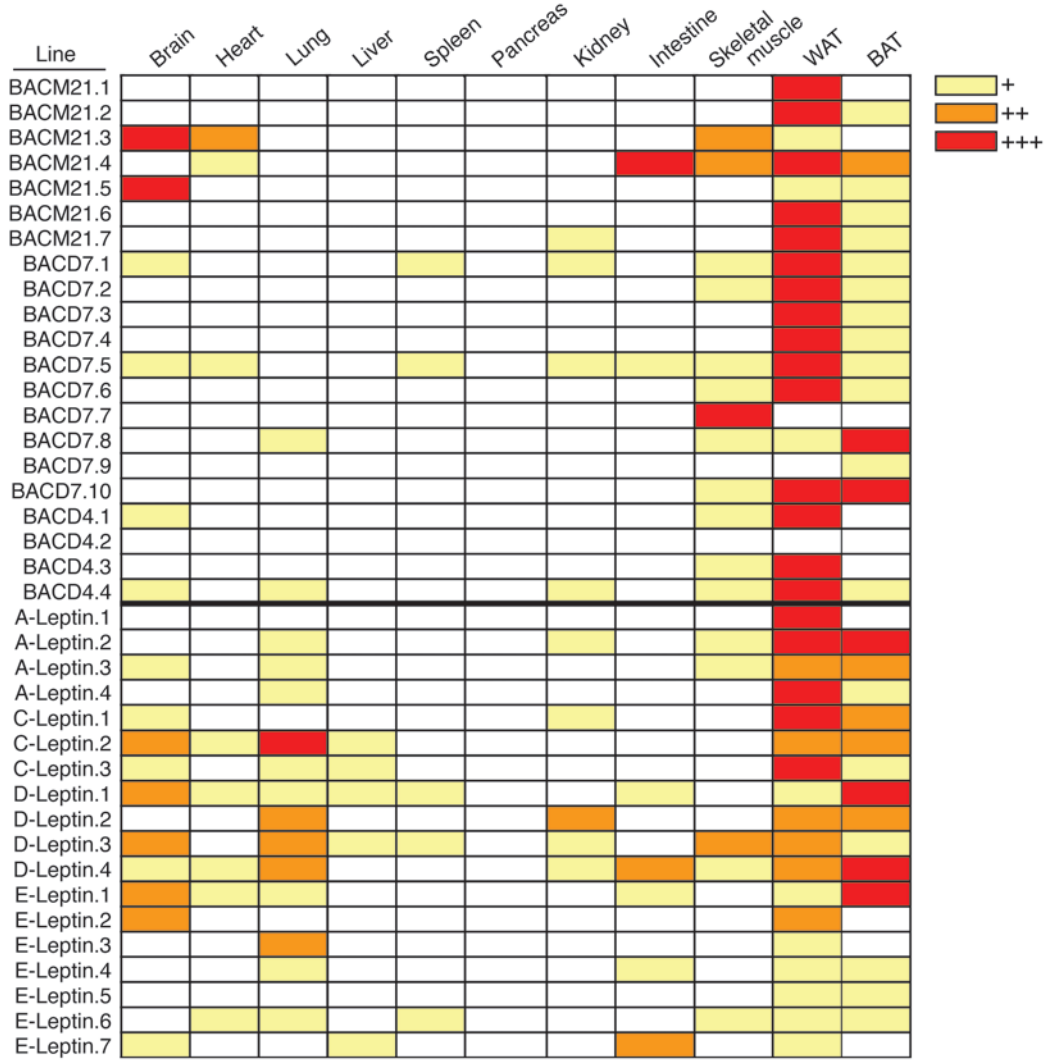

Figure 2
Summary of EGFP expression in multiple BAC transgenic lines. EGFP was quantified in tissues by RT-qPCR and normalized to cyclophilin. Tissues were harvested from transgenic founders at 8–10 weeks of age. Single, double, and triple plus signs indicate relative intensity of EGFP expression.

with the increase in *LEP* gene expression seen after differentiation (Figure 3B). There was a single H3K4me1 peak upstream of the *LEP* start site in pre-adipocytes, coinciding precisely with the UpE region. This peak persisted in the mature adipocytes, and a new H3K27ac peak appeared at the same locus. These data are consistent with the identification of UpE as an enhancer region. Furthermore, the presence of an H3K4me1 mark at this site in pre-adipocytes suggests that the region is "poised" to act as an enhancer prior to activation, which occurs during adipogenesis (28).

Proteome-wide screening for adipocyte nucleoproteins that bind UpE identifies members of the Fos/Jun transcription factor family. We wished to identify specific transcription factor(s) that bind relevant portions of the UpE region in human adipocytes. Such a task has traditionally been challenging. Motif analysis is of limited use, due to false positives (many motifs are found frequently in the genome, only a small fraction of which are actually bound by the predicted factor), as well as false negatives (of the approximately 1,500 human transcription factors [ref. 30], very few have a characterized binding motif). Motif finding in the 958-bp UpE region using the MULAN algorithm (31) and the TRANSFAC database (http://www.gene-regulation.com/pub/databases.html), for example, identified hundreds of putative motifs. In the 30-bp region of interest, there were fewer motifs, of course, some of which were interesting (AR, PPARA, NFE2L1) (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI58431DS1). However, knockdown and other validation studies involving these factors showed no effect on *LEP* expression (data not shown).

We therefore employed an unbiased approach utilizing mass spectrometry–based quantitative proteomics combining stable isotope labeling by amino acids in cell culture (SILAC) with pull-downs using biotinylated DNA as affinity baits (32). Mature human adipocytes were labeled with either "heavy" arginine and lysine bearing ^{13}C and ^{15}N or with "light" natural isotope versions of these amino acids. We mixed heavy nuclear extracts with WT bait and light extracts with mutant bait (we designate this the "forward" experiment) and determined the identity and relative amounts of bound proteins using mass spectrometry (Supplemental Figure 3). The WT bait contained the sequence of the critical 30-bp region of UpE identified in the luciferase assay, with an additional 15 bp of flanking sequence on both sides. The mutant DNA bait had the identical sequence, except that the same single base pair mutations that abolished enhancer activity in the luciferase reporter assay were introduced. We then repeated the experiment by interchanging nuclear extracts and baits, so that heavy extracts

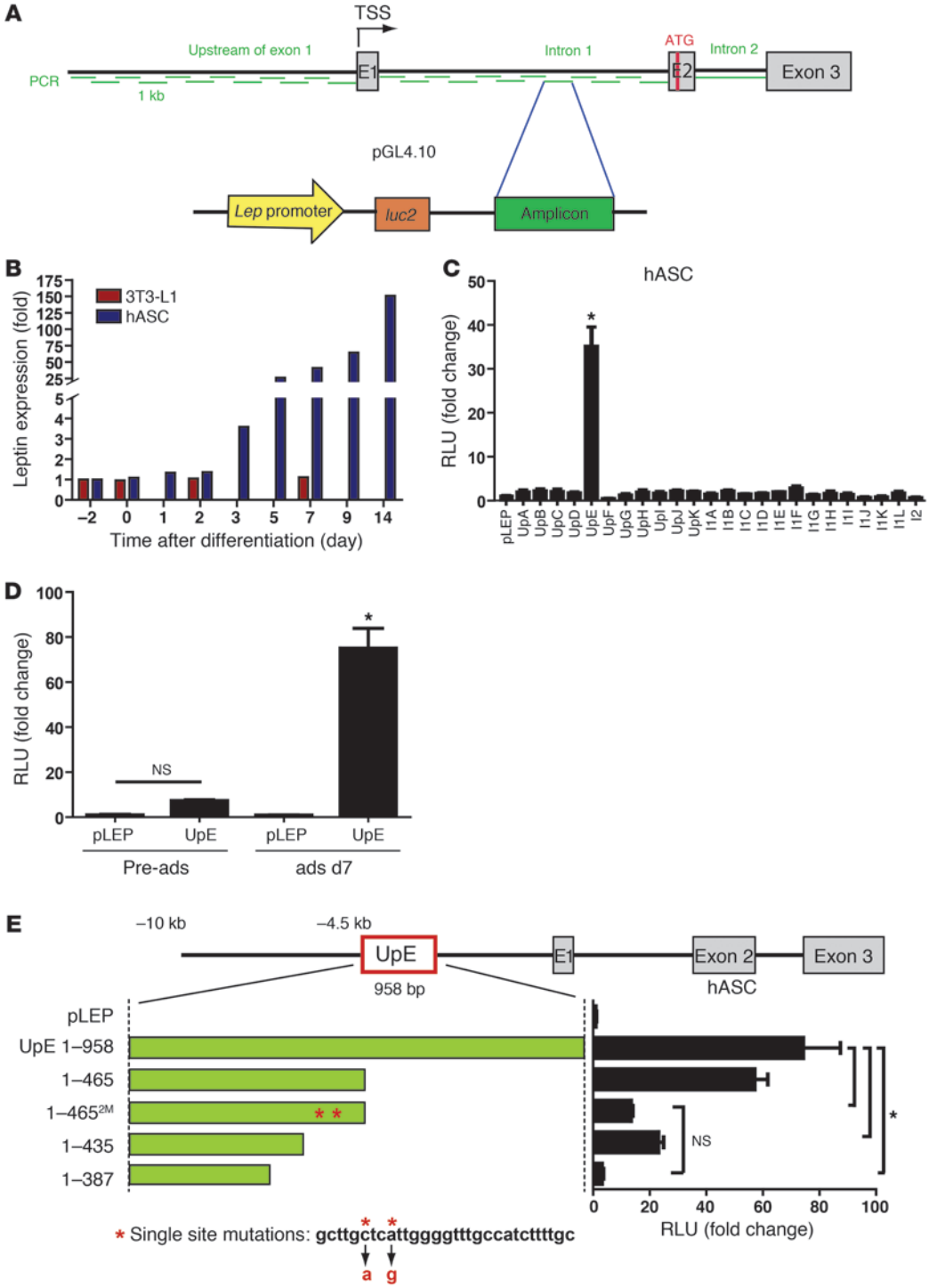

Figure 3

A 1-kb region 4.5 kb upstream of the leptin gene acts as an adipocyte-specific enhancer. (**A**) Tiling PCR across 10 kb upstream (UpA–J), the first intron (I1A–K), and the second intron (intron 2) was performed, and the resulting amplicons were cloned into pGL4 with the *Lep* promoter in the 5′ position. E1, exon 1. (**B**) Comparison of *Lep* mRNA expression during adipogenesis in 3T3-L1 and hASCs using Affymetrix arrays. (**C**) Luciferase activity of tiled reporter constructs in human adipocytes. Data are shown as relative luciferase units (RLU) and expressed as mean ± SEM. *$P < 0.05$. (**D**) Comparison of luciferase activity of element UpE in human pre-adipocytes (Pre-ads) and adipocytes (ads). Data are shown as RLU and expressed as mean ± SEM. *$P < 0.05$. (**E**) Luciferase activity of different deletion mutants of the enhancer element UpE in human adipocytes. Numbers indicate the length in base pairs. Data are shown as RLU and expressed as mean ± SEM. *$P < 0.05$.

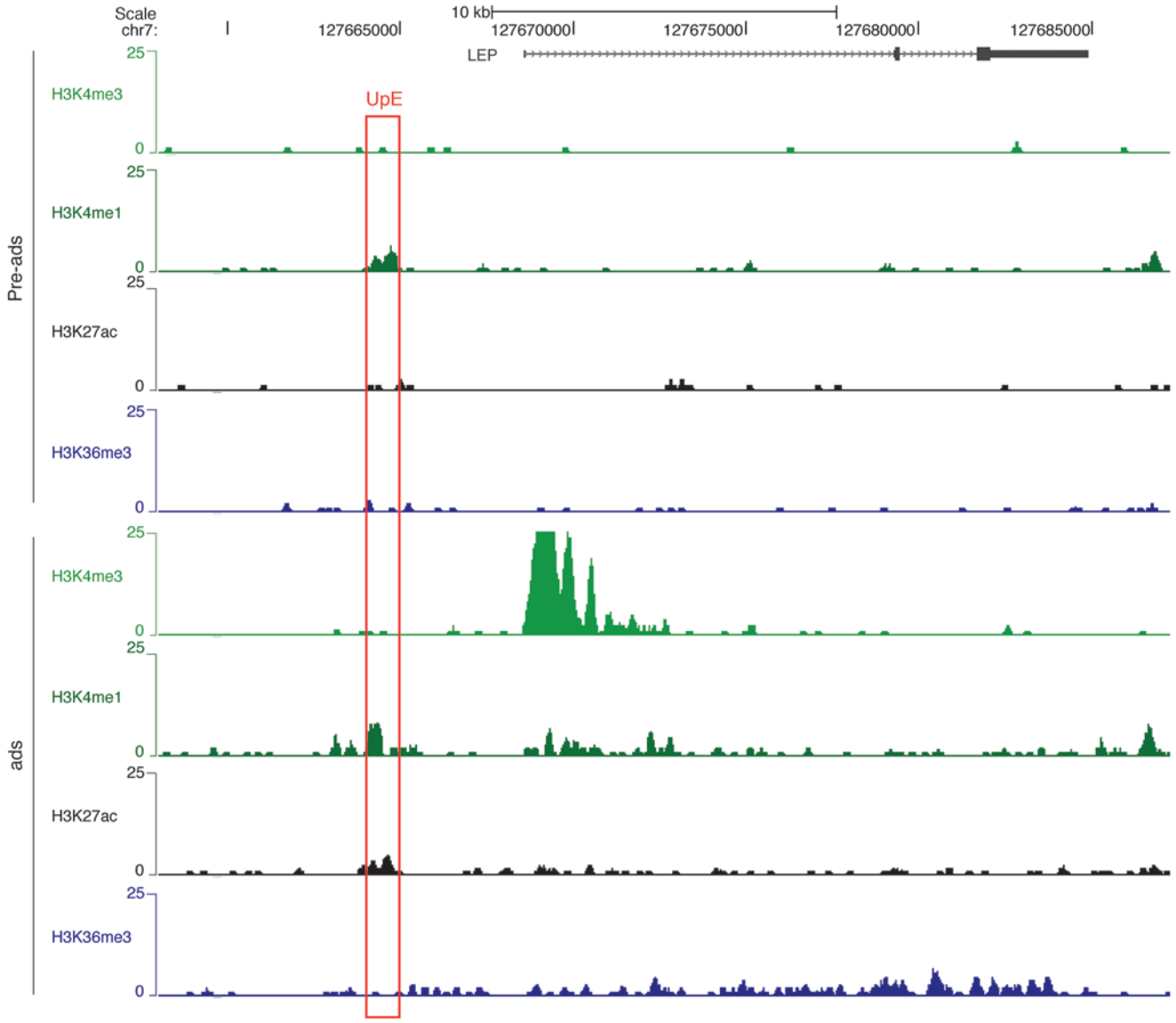

Figure 4
Chromatin state mapping near the *Lep* gene during hASC adipogenesis. Histograms of ChIP fragments across the *Lep* locus, normalized to fragments per 10 million aligned reads, for each of the profiled histone modifications in hASC pre-adipocytes and mature adipocytes (day 9 after induction). H3K4me3 marks promoter activity; H3K4me3 marks poised enhancers; H3K27ac marks active enhancer activity; and H3K36me3 marks active transcription. All histograms are shown on the same scale, and high values were truncated as necessary. The red box corresponds to the region delineated by UpE.

were mixed with mutant bait and light extracts with WT bait (designated the "reverse" experiment). The results are shown as a scatter plot (Figure 5A), in which proteins that bound the mutant with the same affinity as the WT bait cluster around the origin, while proteins that bound the WT bait with a higher affinity are found in the upper-right quadrant. SILAC ratios of specific binders should invert between forward and reverse experiments, providing a stringent filter for contaminating proteins and artifacts from nuclear lysate preparation. Overall, 11 proteins were found to preferentially associate with the WT bait (combined significance $< 1.0 \times 10^{-2}$) (ref. 32 and Supplemental Methods), of which 10 are nucleic acid binding by GO term, 7 are DNA binding by GO term, and only 4 represented known transcription factors (Supplemental Table 1 and Methods).

In order to provide an additional level of filtering, we repeated the entire SILAC experiment using immortalized mouse embryonic fibroblasts (MEFs) overexpressing PPARγ that were differentiated into mature adipocytes. Unlike 3T3-L1 cells, these MEF-derived adipocytes express leptin (Supplemental Figure 2). In this experiment, we identified 5 proteins that bound preferentially to the WT probe, of which 4 are nucleic acid binding by GO term, 4 are DNA binding by GO term, and only two were known transcription factors (Supplemental Figure 4). When the mouse and human data were compared, the only proteins identified in both experiments

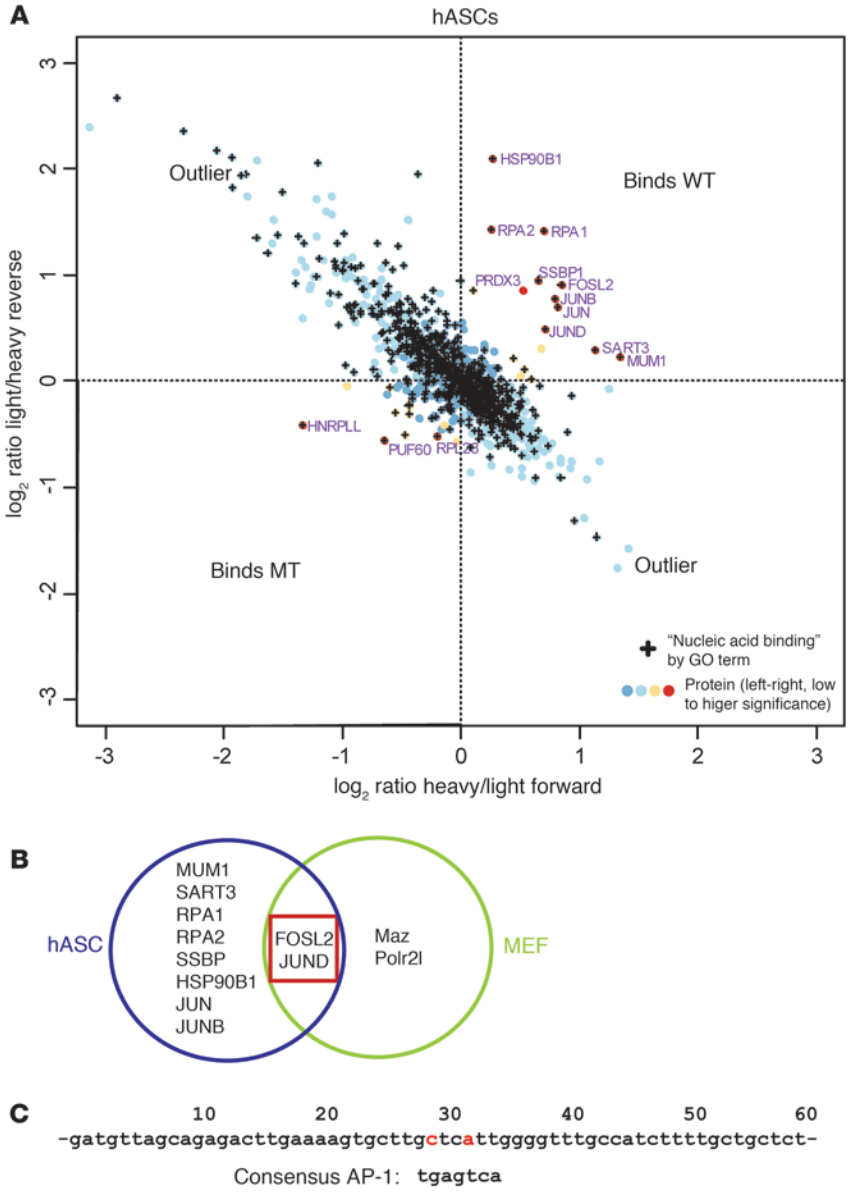

Figure 5
Proteome-wide screening for adipocyte nucleoproteins that specifically bind enhancer element UpE using SILAC. (**A**) Scatter plot of the results from the SILAC experiments in human adipocytes. (**B**) Venn diagram of identified nuclear proteins. The "nucleic acid binding" term includes all proteins bearing the following GO terms: nucleic acid binding, nucleosome binding, nucleotide binding, nucleoside binding, and DNA binding. WT, WT bait, MT, mutant bait. (**C**) DNA sequence of the WT bait and the AP-1 consensus motif, which is known to bind members of the Fos/Jun transcription factor family. The base pairs altered in the mutant bait are depicted in red.

were FOSL2 and JUND, both of which belong to the AP-1 transcription factor family (Figure 5B). Manual reexamination of the probe DNA sequence revealed a degenerate AP-1 consensus motif, and the point mutations we introduced are predicted to disrupt that motif (Figure 5C).

Increased levels of LEP gene expression correlate with increased levels of FOSL2 gene expression. Our quantitative proteomic analysis pointed strongly to a role for AP-1 family members as regulators of *LEP* gene expression. To gain support for this idea, we assessed whether *FOSL2* and *LEP* expression is associated in a variety of physiological and pathological contexts. We found such an association during hASC adipogenesis, for example, in which *FOSL2* mRNA levels rose concordantly with *LEP* mRNA (Figure 6A). Furthermore, obese mice express elevated *Lep*; we therefore looked at white adipose tissue harvested from high-fat diet–fed animals as well as from *db/db* mice, which lack leptin receptor and develop severe obesity in the presence of high leptin levels. In both models, the expression of *Fosl2* was increased compared with that in chow-fed WT mice (Figure 6B). Different adipose depots express different levels of *Lep* mRNA, and these differences reflected differences in *Fosl2* expression (Figure 6C). To investigate whether this correlation also applies to human biology, we measured *LEP* and *FOSL2* gene expression in subcutaneous adipose samples of subjects with different BMIs. We found a significant correlation between *FOSL2* and *LEP* expression (Figure 6D), between *LEP* and BMI (Figure 6E), and between *FOSL2* and BMI (Figure 6F). Similarly, stimulation of LEP expression by dexamethasone in mature human adipocytes in culture was accompanied by a rise in FOSL2 expression (Supplemental Figure 5). Taken together, these data establish a consistent correlation between FOSL2 and LEP expression in multiple biological contexts, ranging from development to pathophysiology.

FOSL2 is a key transcriptional regulator of LEP gene expression in vitro and in vivo. We next sought more direct proof that FOSL2 is a key transcriptional regulator of *LEP* gene expression in adipocytes.

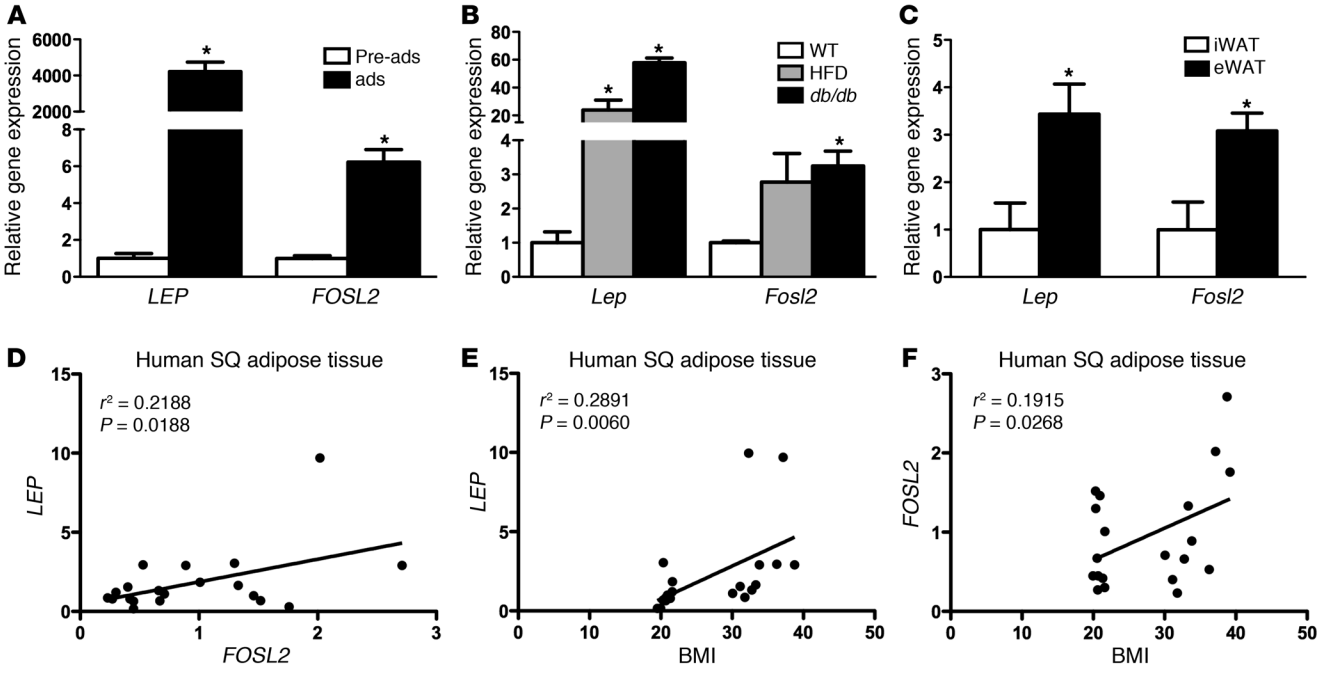

Figure 6

Increased levels of *LEP* gene expression correlate with increased levels of *FOSL2* gene expression. (**A**) *FOSL2* and *LEP* mRNA expression in human pre-adipocytes (pre-ads) and adipocytes (ads) 13 days after differentiation. Gene expression was assessed by RT-qPCR. Data are shown as mRNA levels relative to cyclophilin expression, expressed as mean ± SEM. **P* < 0.05. (**B**) Subcutaneous white adipose tissue was harvested from mice, and gene expression was assessed by RT-qPCR. Data are shown as mRNA levels relative to cyclophilin expression, expressed as mean ± SEM. **P* < 0.05. HFD, high-fat diet. (**C**) Adipose tissue was harvested from the inguinal (iWAT) and epididymal (eWAT) fat depots of mice, and gene expression was assessed by RT-qPCR. Data are shown as mRNA levels relative to cyclophilin expression, expressed as mean ± SEM. **P* < 0.05. (**D**–**F**) Subcutaneous (SQ) white adipose tissue was collected from human subjects with different BMIs, and gene expression was assessed by RT-qPCR. Data are shown as mRNA levels relative to cyclophilin expression.

First, we confirmed that FOSL2 is required for the enhancer activity associated with UpE, using a luciferase assay with the UpE construct in HeLa cells treated with negative control siRNA or siRNA against *FOSL2*. As predicted, knockdown of FOSL2 significantly decreased the enhancer activity of UpE (Supplemental Figure 6A). We also performed RNAi-mediated knockdown of *FOSL2* in mature human adipocytes using a pool of 4 different siRNAs against *FOSL2*. This approximately 50% decrease in *FOSL2* mRNA resulted in a significant decrease in *LEP* mRNA by approximately 50% relative to negative control siRNA (Figure 7A). We confirmed that this was not an off-target effect by testing two distinct shRNA sequences against Fosl2, both of which were able to reduce leptin synthesis (Supplemental Figure 6B).

To establish a role for Fosl2 in leptin expression in vivo, we employed different mouse models. Global *Fosl2*-knockout mice die shortly after birth and could not be used in this study (33). We therefore compared serum leptin levels in heterozygous ($Fosl2^{+/-}$) and WT ($Fosl2^{+/+}$) mice and found significantly lower leptin levels in the $Fosl2^{+/-}$ sera (Figure 7B), despite no differences in body weight or fat pad mass (data not shown). We also studied adipocyte-specific male *Fosl2*-knockout mice, generated by crossing $Fosl2^{fl/fl}$ mice with tamoxifen-inducible aP2-Cre^{ERT} mice. Nineteen days after the first injection of tamoxifen, the adipocyte-specific *Fosl2*-knockout mice demonstrated a significant reduction in serum leptin levels (Figure 7C) but did not show a difference in body weight. As a final test, we isolated calvarial cells from mice in which *Fosl2* was ablated in osteoblast precursor cells (using osterix-Cre) and differentiated them into adipocytes in culture. These cells can be differentiated into osteoblasts or adipocytes depending on the culture conditions. Under conditions favoring adipogenesis, loss of Fosl2 resulted in significantly lower *Lep* mRNA levels compared with cells derived from WT mice (Figure 7D).

To assess whether FOSL2 binds directly to UpE, we performed ChIP followed by quantitative PCR (qPCR) in mature human adipocytes. Significant enrichment relative to the IgG control was found at UpE but not at an irrelevant upstream region (UpK) that did not act as an enhancer in our other assays (Figure 7E). There was no comparable enrichment of FOSL2 noted in human pre-adipocytes.

FOSL2 overexpression in pre-adipocytes increased LEP gene expression in mature adipocytes. The loss-of-function studies described above establish a requirement for FOSL2 in normal adipose leptin expression. We next sought to determine whether FOSL2 was sufficient to force the expression of leptin. Lentiviral overexpression of *FOSL2*, *JUN*, *JUNB*, or *JUND* or co-overexpression of *FOSL2* with *JUN*, *JUNB*, or *JUND* in mature human adipocytes was not able to significantly increase *LEP* gene expression (Supplemental Figure 7). However, overexpression of Fosl2 in MEFs prior to differentiation led to significantly increased *Lep* mRNA expression (Figure 8A) and secreted Lep levels (Supplemental Figure 8A) after these cells were converted into adipocytes. Importantly, this was not due to an overall effect on adipogenic efficiency, as we used a MEF cell line overexpressing PPARγ and determined equivalent degrees of differentiation, as

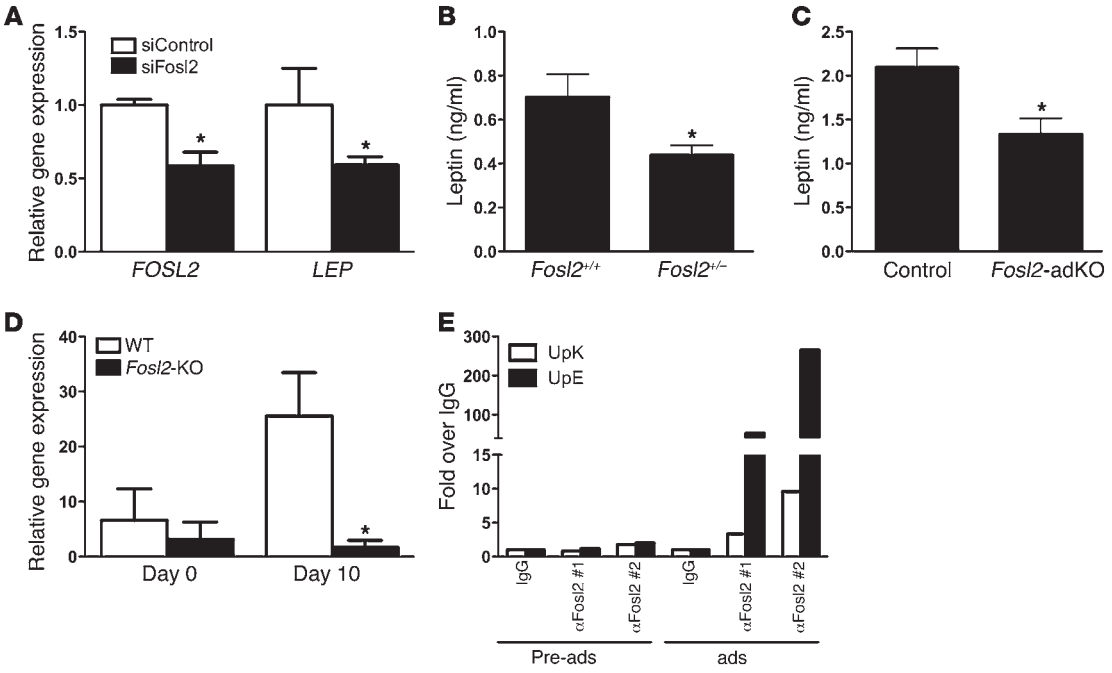

Figure 7

FOSL2 is an important regulator of *LEP* gene expression. (**A**) Seven days after differentiation, human adipocytes were transfected with pooled siFOSL2 or siControl. Forty-eight hours later, mRNA was prepared, and gene expression was assessed by RT-qPCR. Data are shown as mRNA levels relative to cyclophilin expression, expressed as mean ± SEM. **P* < 0.05. (**B**) Serum leptin levels were measured in WT (*Fosl2*$^{+/+}$) and Fosl2 heterozygous (*Fosl2*$^{+/-}$) mice using ELISA. Data are shown as mean ± SEM. **P* < 0.05. (**C**) Serum leptin levels were measured in WT and adipocyte-specific *Fosl2*-knockout mice (*Fosl2*-adKO) 19 days after tamoxifen treatment using ELISA. Data are shown as mean ± SEM. **P* < 0.05. (**D**) Calvarial osteoblast precursors were harvested from WT or osteoblast precursor–specific knockout mice and differentiated into adipocytes. At day 0 and day 10 after differentiation, mRNA was prepared, and gene expression was assessed by RT-qPCR. Data are shown as mRNA levels relative to 36B4 expression, expressed as mean ± SEM. **P* < 0.05. (**E**) ChIP-qPCR assay of FOSL2 binding to the enhancer UpE or negative control region UpK with hASC pre-adipocytes or adipocytes 12 days after differentiation. Data were normalized to input and are shown as fold induction relative to IgG control.

shown by gene expression (Supplemental Figure 8, B–D) and lipid accumulation (Supplemental Figure 8, E and F).

We wished to extend these gain-of-function experiments to an in vivo context, but global Fosl2-transgenic mice (*Fosl2-tg*) experience systemic fibrosis and inflammation, rendering them unsuitable for metabolic study (34). However, calvarial cells isolated from these mice showed elevated *Lep* expression compared with WT cells; this effect was apparent even before differentiation and reached significance at day 10 (Figure 8B). The level of differentiation at this time point assayed by gene expression of *Pparg* and *Fabp4* (aP2) was not significantly different.

These data indicate that Fosl2 is sufficient to drive leptin synthesis, if it is provided early in the differentiation process, and suggest that Fosl2 may induce additional factors that cooperate in *Lep* expression without affecting the overall level of differentiation.

Discussion

Because of its enormous physiological significance as well as its potential biomedical importance, leptin has become one of the most intensively studied molecules of the past few decades. As part of this effort, several groups have attempted to understand the transcriptional mechanisms underlying *LEP* gene expression in adipocytes. Characterization of the proximal promoter identified several elements, including a C/EBP site, Sp-1 site, GRE, CREB, and an E-box element that may bind SREBP1c (18–21). In addition, the transcription factor activator protein–2β (AP-2β) was recently shown to inhibit *LEP* expression by binding directly to the promoter (35). Others have noted demethylation of CpG islands in the leptin promoter during adipogenesis coinciding with the onset of *LEP* expression (36), and SNPs have been identified in the porcine leptin promoter that correlate with expression (37, 38). These studies have all been biased by an exclusive focus on the proximal promoter and/or a concentration on prespecified adipocyte transcription factors.

We sought a more unbiased strategy based on the integration of data from BAC transgenic reporter mice covering 300 kb of the *Lep* flanking region, luciferase reporter assays, and genome-wide chromatin state mapping. These approaches allowed us to focus on an adipocyte-specific *cis*-regulatory element located 4.5 kb upstream of the *LEP* TSS. SILAC DNA pull-downs and mass spectrometry identified FOSL2 as a likely cognate transcription factor, and RNAi-based knockdown confirmed a requirement for this factor in *LEP* expression.

FOSL2, also called Fra-2, belongs to the AP-1 transcription factor family that includes FOS, FOSB, FOSL1, JUN, JUNB, and JUND proteins. These transcription factors share a basic leucine zipper (bZIP) domain and require dimerization before binding to DNA. Fos-Jun family members play diverse and important roles in cel-

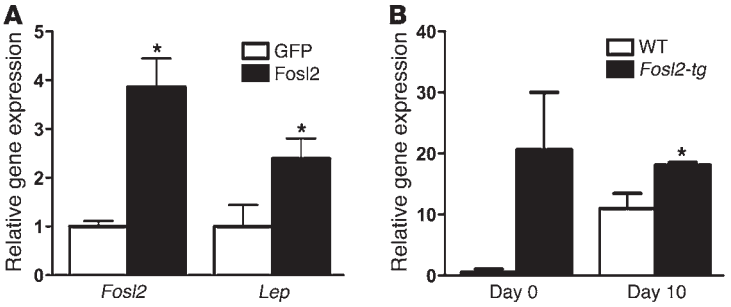

Figure 8

Fosl2 overexpression in pre-adipocytes increased *Lep* gene expression in mature adipocytes. (**A**) MEF pre-adipocytes lentivirally transduced to overexpress either Fosl2 or GFP were differentiated into adipocytes following overexpression of PPARγ. Seven days after differentiation, mRNA was prepared, and gene expression was assessed by RT-qPCR. Data are shown as mRNA levels relative to cyclophilin expression, expressed as mean ± SEM. **P* < 0.05. (**B**) Calvarial osteoblast precursors were harvested from WT or Fosl2 transgenic mice (*Fosl2-tg*) and differentiated into adipocytes. At day 0 and day 10 after differentiation, mRNA was prepared, and gene expression was assessed by RT-qPCR. Data are shown as mRNA levels relative to 36B4 expression, expressed as mean ± SEM. **P* < 0.05.

lular proliferation, differentiation, and oncogenesis (39). Fosl2 is expressed in a wide range of tissues during development (40, 41), and *Fosl2*$^{-/-}$ mice die within a week of birth (33). Loss-of-function studies have implicated Fosl2 in the development of mesenchymal cell types such as chondrocytes, osteoblasts, and osteoclasts (42–44). The role of FOSL2 in adipocytes has been less well explored, although osteoblasts from *Fosl2*$^{-/-}$ mice display increased levels of the adipogenic markers Cebpα, Cebpβ, and Pparγ and increased adipogenesis in vitro (42). Leptin expression was not addressed in those studies. Other AP-1 proteins (Fos, FosB, Fra-1, Jun, JunB) have been implicated in the early phase of differentiation of 3T3-L1 adipocytes (45), and Fos was identified as part of a nuclear complex that regulates the expression of certain adipocyte-specific genes, such as aP2, adipsin, and GPD, during adipocyte differentiation (46). Interestingly, transgenic mice overexpressing ΔFosB from the enolase 2 promoter display increased bone formation and reduced adiposity, but this effect was not likely contained within adipose tissue, as the phenotype was not seen when ΔFosB was expressed from the aP2 promoter (47, 48). A recent study has reported a lipodystrophic phenotype in Fra1 (Fosl1) transgenic mice (49).

Although FOSL2 itself is not an adipocyte-specific transcription factor, AP-1 factors have been shown to exert distinct tissue-specific effects due to a range of mechanisms that include differential expression, composition, and orientation of the DNA-binding dimers, posttranslational modifications, and interaction with ancillary proteins (50). Our data indicate that one possible mechanism of *LEP* gene expression by FOSL2 is the differentiation-dependent regulation of *FOSL2* mRNA levels. *FOSL2* was upregulated in hASCs during adipogenesis and in the subcutaneous adipose tissue of high-fat diet–fed and *db/db* mice, both of which have increased *Lep* levels compared with WT mice on chow diet. Differential phosphorylation of FOSL2 in adipocytes could also play a role in promoting a tissue-specific effect (51). Similarly, interactions with ancillary proteins provide another level of regulation that could mediate the adipocyte-specific actions of FOSL2. The MULAN search algorithm identified a PPARγ binding motif in the 5′ end of UpE (265–288 bp), coinciding with a PPARγ peak at that locus determined by ChIP-Seq (ref. 29 and data not shown). PPARγ could act as an adipocyte-specific "pioneer" factor that opens up the FOSL2 binding site during differentiation, thereby making it accessible. This is consistent with the H3K4me1 mark present at UpE in pre-adipocytes.

To further determine the biological significance of FOSL2 in regulating *LEP* gene expression, we utilized genetic mouse models. Unfortunately, *Fosl2*-deficient mice die shortly after birth (33). MORE-cre Fosl2 mice have been used to study osteoclast formation in newborn mice, but the lifespan and metabolic status of these mice were not reported (43). Therefore, we compared serum leptin levels in *Fosl2* heterozygous and WT mice and found them to be significantly reduced. In addition, we generated adipocyte-specific *Fosl2*-knockout mice, which showed a similar reduction in serum leptin levels. Calvarial cells from osteoblast-specific knockout mice differentiated into adipocytes in culture also have significantly lower *Lep* expression than cells from WT mice.

Interestingly, our results point toward a model in which multiple *cis*-regulatory elements participate in driving adipocyte-specific *LEP* gene expression. For example, in our BAC transgenic reporter mouse model, truncation of the 3′ flanking sequence resulted in a significant reduction in EGFP expression in adipose tissue, indicating additional *cis*-regulatory elements in this region. Although knockdown of FOSL2 in mature hASCs significantly decreased *LEP* expression, gain-of-function studies with overexpressed FOSL2 alone or in combination with JUN proteins in mature adipocytes did not result in a significant increase in *LEP* expression. This suggests that FOSL2 is required but not sufficient to regulate adipocyte-specific *LEP* expression. Such complex models of transcriptional regulation have been well described for other genes, such as the α-globin and β-globin loci in erythroid cells (52). Notably, overexpression of Fosl2 in immature adipocytes was associated with higher leptin levels after differentiation. This was seen despite no alteration in the general level of adipogenesis. This suggests that Fosl2 may induce additional factors during differentiation that are also required for full leptin expression, an effect that cannot be mimicked by adding in Fosl2 after differentiation has been completed.

There is some indication from this study and from the literature that FOSL2 could be involved in physiological regulation of *LEP* expression, distinct from the developmental regulation we focus on here. We found that *Fosl2* was upregulated in obese mice with increased *Lep* levels compared with WT mice on chow diet. In human subjects, *FOSL2* expression was correlated with BMI and *LEP* expression. In addition, adipose depots with higher *Lep* expression displayed elevated *Fosl2* expression. Stimulation of human adipocytes with dexamethasone increased both *LEP* and *FOSL2* expression. A study on gene expression profiles in Atlantic salmon adipose–derived stromal-vascular fraction during differentiation into adipocytes reports the upregulation of AP-1 factors (c-Jun, c-Fos, JunB1, JunB2, and JunD), with a peak at the end of adipogenesis when the lipid load was the highest and genes involved in ER stress and ROS stress were also upregulated (53). Similarly, stress through serum deprivation was reported to upregulate leptin in multipotent mesenchymal stromal cells (54). Hypoxia is another stressor in the adipose depots in obesity, when fat mass

rapidly expands beyond the capabilities of its blood supply. In this situation, HIF-1α is induced (55). In 3T3-L1 and 3T3-F442A adipocytes as well as in human PAZ6 adipose cells, hypoxia induced leptin expression (56–58). In human skin-derived fibroblasts, breast cancer cell lines, and a trophoblast cell line, this phenomenon was found to be mediated by HIF-1α binding and transactivation of the leptin promoter (59–61). Interestingly, Fosl2 affects HIF-1α through transcriptional modulation of the HIF-1α prolyl hydroxylase PHD2 (43), suggesting that an additional layer of regulation between Fosl2 and leptin could exist. Interestingly, overexpression of *FOSL2* before differentiation results in upregulation of Lep expression in mature adipocytes, as shown using MEF pre-adipocytes or isolated osteoblastic precursors from Fosl2 transgenic mice. This difference could be explained by a role of Fosl2 in determination or a model in which Fosl2 upregulates an intermediate in pre-adipocytes, which feeds into a feed-forward loop that is not existent in mature adipocytes.

As a final note, we believe that our approach integrating multiple levels of analysis can be used as a template for defining transcriptional pathways that are difficult to deduce using more traditional means.

Methods

Oligonucleotides and antibodies. All primers, probes, and antibodies used are listed in Supplemental Table 2.

Cell culture. Human subcutaneous adipose tissue was obtained from consenting healthy donors undergoing liposuction. Cells from 6–8 donors were used; all were female, with a mean age of 44 years and an average BMI of 28.9 kg/m^2. hASCs were isolated, cultured, and differentiated as described in Supplemental Methods and elsewhere (29). For stimulation, mature human adipocytes were incubated with either dexamethasone (10 nM) or vehicle for 16 hours, and gene expression was assessed with RT-qPCR.

Calvariae were sequentially digested for 30 minutes in modified α-MEM containing 0.1% collagenase and 0.2% dispase. Cells isolated by fractions 2–3 were combined as an osteoblastic cell population, expanded for 2 days in α-MEM with 10% FCS, and replated at a density 5×10^5 cells/well, and medium was supplemented with 1 mM dexamethasone (Sigma-Aldrich) and 10 mg/ml insulin (Sigma-Aldrich) for adipocyte culture. After 10 days, RNA was extracted.

Animal studies. Mice were kept under 14-hour light/10-hour dark cycles at constant temperature (22°C) with free access to food and water. Mice were fed either a standard diet (Rodent Diet 8664, Harlan Teklad) or high-fat diet (D12331i, Research Diets Inc.). For RNA expression studies, animals were sacrificed and tissues harvested and stored at –80°C until analysis. Samples from mice fed a high-fat diet or from *db/db* mice were provided by Xun Wang and Mariana Sarto and by Christian Bjorbaek (Beth Israel Medical Center, Boston, Massachusetts, USA), respectively.

Human subjects. Human subcutaneous adipose tissue was obtained from healthy donors with different BMIs. A total of 22 samples from donors were collected; all were female, with a mean age of 40.1 ± 10.5 years and an average BMI of 27.2 ± 7.2 kg/m^2 ranging from 19.52 to 39.17 kg/m^2.

Generation of transgenic reporter mice. An EGFP cassette was inserted into the *Lep* start codon of three murine BACs (RP24-369M21, RP24-69D4, RP24-D7) using recombineering techniques (62, 63). The BACs were obtained from the BACPAC Resource Center at the Children's Hospital Oakland Research Institute. Deletions in BACs containing the EGFP cassette were created by "BAC trimming" (25). The recombineered BACs were injected into FVB blastocysts to generate transgenic offspring. Founders were sacrificed at 8–10 weeks of age, and tissues were harvested for RNA expression analysis.

Generation of Fosl2-tg, heterozygous, adipocyte-specific knockout, and osteoblast precursor–specific knockout mouse models. The generation of the heterozygous *Fosl2*$^{+/-}$, *Fosl2*$^{fl/fl}$, and *Fosl2-tg* mice has been reported previously (33, 34). For the latter, *Fosl2* was expressed under the control of the ubiquitous major histocompatibility complex class I antigen H-2K^b promoter. Generation of the adipocyte-specific and osteoblast precursor–specific *Fosl2*-knockout mice is described in detail in Supplemental Methods.

ChIP-qPCR, ChIP-Seq, and sequence analysis. Details of hASC ChIP-Seq analysis have been described in detail elsewhere (29). For ChIP-PCR, the EZ-ChIP kit (Millipore) with anti-FOSL2 rabbit polyclonal antibody (Santa Cruz Biotechnology Inc.), anti-FOSL2 MaxPab rabbit polyclonal antibody (Abnova), and normal rabbit IgG (Santa Cruz Biotechnology Inc.) were used, and 1 μl of eluted DNA was amplified using SYBR Green Master Mix in a 7900HT Real-Time PCR system (Applied Biosystems). Values were normalized to input and expressed as fold increase relative to IgG control.

Reporter assays. Reporter assays are described in detail in Supplemental Methods.

RNA preparation and expression analysis. Total RNA was harvested from cells or tissues using TRIzol (Invitrogen) according to the manufacturer's instructions. First-strand cDNA was generated using a RETROscript kit (Ambion), and qPCR was performed using SYBR Green Master Mix in a 7900HT Real-Time PCR system (Applied Biosystems). mRNA quantities were normalized to cyclophilin B after determination by the comparative Ct method (64). Published Affymetrix arrays data were used in this study (GEO GSE20752) (29).

Determination of leptin protein levels in mouse serum cell culture supernatant. Leptin levels in mouse serum and cell culture supernatant were measured with the mouse leptin ELISA kit from Crystal Chem Inc. according to the manufacturer's instructions.

SILAC and DNA-protein interaction screen with SILAC-labeled nuclei extracts. Reporter assays are described in detail in Supplemental Methods.

Knockdown and overexpression. For knockdown studies, mature human adipocytes (day 7 after induction) were transfected with FlexiTube pooled siRNA containing 4 different siRNAs against *FOSL2* (25 nM) or AllStars Negative Control siRNA (25 nM) (QIAGEN) using HiPerFect transfection reagent (QIAGEN). RNA was harvested 48 hours later. MEFs were transduced with viral supernatant from HEK293 cells transfected with pLKO.1 vector (Sigma-Aldrich) containing different shRNAs. For overexpression studies, mature human adipocytes or MEF pre-adipocytes were transduced with viral supernatant from HEK293 cells transfected with pCDH vector (gift from William A. Chutkow, Brigham and Women's Hospital, Boston, Massachusetts, USA) containing the relevant transcription factor cDNA cloned into EcoRI and BamHI sites.

Statistics. Data analysis was performed using GraphPad Prism 5 software. As appropriate, 1-tailed Student's *t* test or 1-way ANOVA followed by Tukey's multiple comparison as a post-hoc test were applied. Significance was assigned to differences with a P value less than 0.05.

Study approval. Animal studies were approved by the Institutional Animal Care and Use Committee of Beth Israel Deaconess Medical Center and the Spanish National Cancer Center. Human studies were approved by the Pennington Biomedical Research Center Institutional Review Board (protocol PBRC24030), and informed consent was obtained from all human subjects.

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1. Flegal KM, Graubard BI, Williamson DF, Gail MH. Sources of differences in estimates of obesity-associated deaths from first National Health and Nutrition Examination Survey (NHANES I) hazard ratios. *Am J Clin Nutr*. 2010;91(3):519–527.
2. Rosen ED, Spiegelman BM. Adipocytes as regulators of energy balance and glucose homeostasis. *Nature*. 2006;444(7121):847–853.
3. Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM. Positional cloning of the mouse obese gene and its human homologue. *Nature*. 1994; 372(6505):425–432.
4. Halaas JL, et al. Weight-reducing effects of the plasma protein encoded by the obese gene. *Science*. 1995;269(5223):543–546.
5. Belgardt BF, Brüning JC. CNS leptin and insulin action in the control of energy homeostasis. *Ann N Y Acad Sci*. 2010;1212:97–113.
6. Margetic S, Gazzola C, Pegg GG, Hill RA. Leptin: a review of its peripheral actions and interactions. *Int J Obes Relat Metab Disord*. 2002;26(11):1407–1433.
7. Friedman JM. Leptin at 14 y of age: an ongoing story. *Am J Clin Nutr*. 2009;89(3):973S–979.
8. Ebihara K, et al. Efficacy and safety of leptin-replacement therapy and possible mechanisms of leptin actions in patients with generalized lipodystrophy. *J Clin Endocrinol Metab*. 2007;92(2):532–541.
9. Heymsfield SB, et al. Recombinant leptin for weight loss in obese and lean adults. *JAMA*. 1999; 282(16):1568–1575.
10. Ravussin E, et al. Enhanced weight loss with pramlintide/metreleptin: an integrated neurohormonal approach to obesity pharmacotherapy. *Obesity (Silver Spring)*. 2009;17(9):1736–1743.
11. Goiot H, Laigneau JP, Devaud H, Sobhani I, Bado A. Similarities and differences in the transcriptional regulation of the leptin gene promoter in gastric and adipose cells. *FEBS Lett*. 2005;579(9):1911–1916.
12. Moreno-Aliaga MJ, Swarbrick MM, Lorente-Cebrian S, Stanhope KL, Havel PJ, Martinez JA. Sp1-mediated transcription is involved in the induction of leptin by insulin-stimulated glucose metabolism. *J Mol Endocrinol*. 2007;38(5):537–546.
13. Simopoulou T, et al. Differential expression of leptin and leptin's receptor isoform (Ob-Rb) mRNA between advanced and minimally affected osteoarthritic cartilage; effect on cartilage metabolism. *Osteoarthritis Cartilage*. 2007;15(8):872–883.
14. Kallen CB, Lazar MA. Antidiabetic thiazolidinediones inhibit leptin (ob) gene expression in 3T3-L1 adipocytes. *Proc Natl Acad Sci U S A*. 1996; 93(12):5793–5796.
15. Harris RB, Ramsay TG, Smith SR, Bruch RC. Early and late stimulation of ob mRNA expression in meal-fed and overfed rats. *J Clin Invest*. 1996; 97(9):2020–2026.
16. de la Brousse FC, Shan B, Chen JL. Identification of the promoter of the mouse obese gene. *Proc Natl Acad Sci U S A*. 1996;93(9):4096–4101.
17. Gong DW, Bi S, Pratley RE, Weintraub BD. Genomic structure and promoter analysis of the human obese gene. *J Biol Chem*. 1996;271(8):3971–3974.
18. He Y, Chen H, Quon MJ, Reitman M. The mouse obese gene. Genomic organization, promoter activity, and activation by CCAAT/enhancer-binding protein alpha. *J Biol Chem*. 1995;270(48):28887–28891.
19. Hwang CS, Mandrup S, MacDougald OA, Geiman DE, Lane MD. Transcriptional activation of the mouse obese (ob) gene by CCAAT/enhancer binding protein alpha. *Proc Natl Acad Sci U S A*. 1996;93(2):873–877.
20. Miller SG, et al. The adipocyte specific transcription factor C/EBPalpha modulates human ob gene expression. *Proc Natl Acad Sci U S A*. 1996;93(11):5507–5511.
21. Kim JB, et al. Nutritional and insulin regulation of fatty acid synthetase and leptin gene expression through ADD1/SREBP1. *J Clin Invest*. 1998; 101(1):1–9.
22. Chen XL, Hartzell DL, McGraw RA, Hausman GJ, Dean RG. Analysis of a 762-bp proximal leptin promoter to drive and control regulation of transgene expression of growth hormone receptor in mice. *Biochem Biophys Res Commun*. 1999;262(1):187–192.
23. Heintzman ND, et al. Histone modifications at human enhancers reflect global cell-type-specific gene expression. *Nature*. 2009;459(7243):108–112.
24. Visel A, et al. ChIP-seq accurately predicts tissue-specific activity of enhancers. *Nature*. 2009; 457(7231):854–858.
25. Hill F, Benes V, Thomasova D, Stewart AF, Kafatos FC, Ansorge W. BAC trimming: minimizing clone overlaps. *Genomics*. 2000;64(1):111–113.
26. MacDougald OA, Hwang CS, Fan H, Lane MD. Regulated expression of the obese gene product (leptin) in white adipose tissue and 3T3-L1 adipocytes. *Proc Natl Acad Sci U S A*. 1995;92(20):9034–9037.
27. Bernstein BE, et al. The NIH Roadmap Epigenomics Mapping Consortium. *Nat Biotechnol*. 2010; 28(10):1045–1048.
28. Creyghton MP, et al. Histone H3K27ac separates active from poised enhancers and predicts developmental state. *Proc Natl Acad Sci U S A*. 2010; 107(50):21931–21936.
29. Owyang SY, Luther J, Kao JY. Helicobacter pylori: beneficial for most? *Expert Rev Gastroenterol Hepatol*. 2011;5(6):649–651.
30. Vaquerizas JM, Kummerfeld SK, Teichmann SA, Luscombe NM. A census of human transcription factors: function, expression and evolution. *Nat Rev Genet*. 2009;10(4):252–263.
31. Ovcharenko I, et al. Mulan: multiple-sequence local alignment and visualization for studying function and evolution. *Genome Res*. 2005;15(1):184–194.
32. Ong SE, et al. Identifying the proteins to which small-molecule probes and drugs bind in cells. *Proc Natl Acad Sci U S A*. 2009;106(12):4617–4622.
33. Eferl R, Zenz R, Theussl HC, Wagner EF. Simultaneous generation of fra-2 conditional and fra-2 knock-out mice. *Genesis*. 2007;45(7):447–451.
34. Eferl R, et al. Development of pulmonary fibrosis through a pathway involving the transcription factor Fra-2/AP-1. *Proc Natl Acad Sci U S A*. 2008; 105(30):10525–10530.
35. Fuke T, et al. Transcription factor AP-2beta inhibits expression and secretion of leptin, an insulin-sensitizing hormone, in 3T3-L1 adipocytes. *Int J Obes (Lond)*. 2010;34(4):670–678.
36. Melzner I, et al. Leptin gene expression in human preadipocytes is switched on by maturation-induced demethylation of distinct CpGs in its proximal promoter. *J Biol Chem*. 2002;277(47):45420–45427.
37. Stachowiak M, et al. Polymorphism of the porcine leptin gene promoter and analysis of its association with gene expression and fatness traits. *Biochem Genet*. 2007;45(3–4):245–253.
38. Liu D, Hu Y, Yang X, Liu Y, Wei S, Jiang Y. Identification and genetic effects of a novel polymorphism in the distal promoter region of porcine leptin gene. *Mol Biol Rep*. 2011;38(3):2051–2057.
39. Eferl R, Wagner EF. AP-1: a double-edged sword in tumorigenesis. *Nat Rev Cancer*. 2003;3(11):859–868.
40. Foletta VC, Sonobe MH, Suzuki T, Endo T, Iba H, Cohen DR. Cloning and characterisation of the mouse fra-2 gene. *Oncogene*. 1994;9(11):3305–3311.
41. Carrasco D, Bravo R. Tissue-specific expression of the fos-related transcription factor fra-2 during mouse development. *Oncogene*. 1995;10(6):1069–1079.
42. Bozec A, Bakiri L, Jimenez M, Schinke T, Amling M, Wagner EF. Fra-2/AP-1 controls bone formation by regulating osteoblast differentiation and collagen production. *J Cell Biol*. 2010;190(6):1093–1106.
43. Bozec A, et al. Osteoclast size is controlled by Fra-2 through LIF/LIF-receptor signalling and hypoxia. *Nature*. 2008;454(7201):221–225.
44. Karreth F, Hoebertz A, Scheuch H, Eferl R, Wagner EF. The AP1 transcription factor Fra2 is required for efficient cartilage development. *Development*. 2004; 131(22):5717–5725.
45. Stephens JM, Butts MD, Pekala PH. Regulation of transcription factor mRNA accumulation during 3T3-L1 preadipocyte differentiation by tumour necrosis factor-alpha. *J Mol Endocrinol*. 1992; 9(1):61–72.
46. Distel RJ, Ro HS, Rosen BS, Groves DL, Spiegelman BM. Nucleoprotein complexes that regulate gene expression in adipocyte differentiation: direct participation of c-fos. *Cell*. 1987;49(6):835–844.
47. Sabatakos G, et al. Overexpression of DeltaFosB transcription factor(s) increases bone formation and inhibits adipogenesis. *Nat Med*. 2000;6(9):985–990.
48. Rowe GC, Choi CS, Neff L, Horne WC, Shulman GI, Baron R. Increased energy expenditure and insulin sensitivity in the high bone mass DeltaFosB transgenic mice. *Endocrinology*. 2009;150(1):135–143.
49. Luther J, et al. Elevated Fra-1 expression causes severe lipodystrophy. *J Cell Sci*. 2011;124(pt 9):1465–1476.
50. Chinenov Y, Kerppola TK. Close encounters of many kinds: Fos-Jun interactions that mediate transcription regulatory specificity. *Oncogene*. 2001; 20(19):2438–2452.
51. Murakami M, Ui M, Iba H. Fra-2-positive autoregulatory loop triggered by mitogen-activated protein kinase (MAPK) and Fra-2 phosphorylation sites by MAPK. *Cell Growth Differ*. 1999;10(5):333–342.
52. Sexton T, Bantignies F, Cavalli G. Genomic interactions: chromatin loops and gene meeting points in transcriptional regulation. *Semin Cell Dev Biol*. 2009;20(7):849–855.
53. Todorcevic M, Skugor S, Krasnov A, Ruyter B. Gene

expression profiles in Atlantic salmon adipose-derived stromo-vascular fraction during differentiation into adipocytes. *BMC Genomics*. 2010;11:39.
54. Sanchez C, Oskowitz A, Pochampally RR. Epigenetic reprogramming of IGF1 and leptin genes by serum deprivation in multipotential mesenchymal stromal cells. *Stem Cells*. 2009;27(2):375–382.
55. Halberg N, et al. Hypoxia-inducible factor 1alpha induces fibrosis and insulin resistance in white adipose tissue. *Mol Cell Biol*. 2009;29(16):4467–4483.
56. Grosfeld A, Zilberfarb V, Turban S, Andre J, Guerre-Millo M, Issad T. Hypoxia increases leptin expression in human PAZ6 adipose cells. *Diabetologia*. 2002;45(4):527–530.
57. Lolmede K, Durand de Saint Front V, Galitzky J, Lafontan M, Bouloumie A. Effects of hypoxia on the expression of proangiogenic factors in differentiated 3T3-F442A adipocytes. *Int J Obes Relat Metab Disord*. 2003;27(10):1187–1195.
58. Wang B, Wood IS, Trayhurn P. Hypoxia induces leptin gene expression and secretion in human preadipocytes: differential effects of hypoxia on adipokine expression by preadipocytes. *J Endocrinol*. 2008;198(1):127–134.
59. Ambrosini G, Nath AK, Sierra-Honigmann MR, Flores-Riveros J. Transcriptional activation of the human leptin gene in response to hypoxia. Involvement of hypoxia-inducible factor 1. *J Biol Chem*. 2002;277(37):34601–34609.
60. Grosfeld A, Andre J, Hauguel-De Mouzon S, Berra E, Pouyssegur J, Guerre-Millo M. Hypoxia-inducible factor 1 transactivates the human leptin gene promoter. *J Biol Chem*. 2002;277(45):42953–42957.
61. Cascio S, et al. Mechanism of leptin expression in breast cancer cells: role of hypoxia-inducible factor-1alpha. *Oncogene*. 2008;27(4):540–547.
62. Testa G, et al. Engineering the mouse genome with bacterial artificial chromosomes to create multipurpose alleles. *Nat Biotechnol*. 2003;21(4):443–447.
63. Copeland NG, Jenkins NA, Court DL. Recombineering: a powerful new tool for mouse functional genomics. *Nat Rev Genet*. 2001;2(10):769–779.
64. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. *Nat Protoc*. 2008;3(6):1101–1108.