Elevated Fra-1 expression causes severe lipodystrophy

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Summary

A shift from osteoblastogenesis to adipogenesis is one of the underlying mechanisms of decreased bone mass and increased fat during aging. We now uncover a new role for the transcription factor Fra-1 in suppressing adipogenesis. Indeed, *Fra1* (*Fosl1*) transgenic (*Fra1*tg) mice, which developed progressive osteosclerosis as a result of accelerated osteoblast differentiation, also developed a severe general lipodystrophy. The residual fat of these mice appeared immature and expressed lower levels of adipogenic markers, including the fatty acid transporter *Cd36* and the CCAAT/enhancer binding protein *Cebpa*. Consequently accumulation of triglycerides and free fatty acids were detected in the serum of fasting *Fra1*tg mice. Fra-1 acts cell autonomously because the adipogenic differentiation of *Fra1* transgenic primary osteoblasts was drastically reduced, and overexpression of Fra-1 in an adipogenic cell line blocked their differentiation into adipocytes. Strikingly, *Cebpa* was downregulated in the Fra-1-overexpressing cells and Fra-1 could bind to the *Cebpa* promoter and directly suppress its activity. Thus, our data add to the known common systemic control of fat and bone mass, a new cell-autonomous level of control of cell fate decision by which the osteogenic transcription factor Fra-1 opposes adipocyte differentiation by inhibiting C/EBPα.

Key words: Adipocytes, AP-1, Bone, C/EBPα, Fra-1

Introduction

A reciprocal interaction between bone and energy metabolism has been described, whereby a hormone secreted by adipocytes influences bone formation and a factor produced by osteoblasts regulates fat metabolism (Lieben et al., 2009; Rosen, 2008). The crucial factor in this systemic loop is leptin, because its deficiency causes obesity and increases bone formation in the mouse (Ducy et al., 2000). Leptin is produced by white adipocytes and acts via the hypothalamus to regulate appetite and to favor energy expenditure. Bone formation is also negatively regulated by leptin through a second hypothalamic pathway, the β-adrenergic sympathetic nervous system (Takeda et al., 2002). This pathway increases ATF-4-dependent expression of Esp (protein tyrosine phosphatase, receptor type, V; *Ptprv*) in osteoblasts, which leads to an inhibition of osteocalcin bioactivity. By contrast, insulin signaling in osteoblasts promotes the production of bioactive osteocalcin via acidification of the extracellular bone matrix as a consequence of increased bone resorption by osteoclasts (Ferron et al., 2010; Fulzele et al., 2010). In turn, osteocalcin, a hormone secreted by osteoblasts, modulates fat metabolism via the stimulation of pancreatic β-cell proliferation and insulin secretion and thus, can indirectly, via adiponectin, lower insulin resistance (Hinoi et al., 2008; Yoshizawa et al., 2009). Thus, a common neuroendocrine systemic co-regulation of bone and adipose mass is established.

In addition to this systemic regulation of bone and fat metabolism, a local control of cell fates balancing osteoblast and adipocyte differentiation, which is still poorly understood, must exist to integrate the systemic messages. Indeed, osteoblasts share with adipocytes a common mesenchymal progenitor, the mesenchymal stromal or stem cell (MSC) from which also arise other mesenchymal cell lineages such as chondrocytes, fibroblasts and myoblasts (Caplan, 2007). Mesenchymal cell fate decisions are driven by key transcription factors that confer identity to the cell. The major transcription factors regulating MSC differentiation to osteoblasts are β-catenin and Runx2, both of which are required for the differentiation to pre-osteoblasts and osterix that drives osteoblast maturation (Karsenty, 2008; Komori, 2006). Similarly, adipocyte differentiation occurs first by activation of C/EBPβ and C/EBPδ, resulting in expression of PPARγ2 and C/EBPα, which then regulate late stages of adipogenesis (Lefterova and Lazar, 2009). Furthermore, a number of additional factors such as bone morphogenic proteins (BMPs) and signaling, for instance through the WNT pathway, have been described to regulate cell fate decisions between osteoblasts and adipocytes by promoting commitment or differentiation into one lineage at the expense of the other (Takada et al., 2007). All these observations strongly argue in favor of a cell-autonomous locally controlled relationship between osteoblastogenesis and adipogenesis.

The heterodimeric transcription factor activator protein-1 (AP-1) formed by association of one of the four members of the Fos family of proteins (Fos, FosB, Fra-1 and Fra-2) to one of the three Jun members (Jun, JunB and JunD) (Zenz et al., 2008) is a key regulator of osteoblast differentiation (Eferl and Wagner, 2003; Karsenty, 2008; Wagner and Eferl, 2005). In particular, overexpression of Fos induces transformation of osteoblasts, resulting in osteosarcoma (Grigoriadis et al., 1993). In addition, mice lacking Fra-1 or JunB are osteopenic because of decreased osteoblast activity (Eferl et al., 2004; Kenner et al., 2004) and an increase in bone mass as a result of increased bone formation occurs in JunD-deficient mice (Kawamata et al., 2008) (V.M. and J.-P.D., unpublished data). Conversely overexpression of Fra-1 leads to the development of osteosclerosis (Jochum et al., 2000), a phenotype also observed in mice overexpressing ΔFosB, a splice variant of FosB (Kveiborg et al., 2004; Sabatakos et al., 2000). In both *Fra1* and *ΔfosB* transgenic mice, the phenotype appeared to be caused by a cell-autonomous increased osteoblast activity (Jochum et al., 2000; Kveiborg et al., 2004; Sabatakos et al., 2000). In addition to the bone phenotype, mice overexpressing ΔFosB also displayed a decreased mass of adipose tissue (Kveiborg et al., 2004). However, although overexpression of ΔFosB in adipogenic cells was originally described to inhibit their differentiation in vitro (Sabatakos et al., 2000), the same group later found that the decreased adipogenesis in vivo was not due to a cell-autonomous defect within the adipocytes but rather to increased energy expenditure and insulin sensitivity (Rowe et al., 2009). However, in contrast to Fra-1 deficiency (Eferl et al., 2004), no bone phenotype was reported in mice lacking FosB, nor was any adipose defect (Zenz et al., 2008). These observations suggest that an unidentified AP-1 member could be a key player in cell fate decisions driving the differentiation toward osteoblasts at the expense of adipocytes.

We hypothesized that this role could be carried by the pro-osteogenic transcription factor Fra-1. Indeed, we demonstrate here that overexpression of Fra-1 under control of the H2K promoter results, in addition to osteosclerosis, in a severe lipodystrophy. We further demonstrate that the reduced fat mass is caused by a cell-autonomous defect in adipocyte differentiation linked to a reduced expression of C/EBPα, a transcription factor that regulates adipocyte maturation. Thus, our results establish a role for Fra-1 in the suppression of adipogenesis.

Results

Severe lipodystrophy in mice overexpressing Fra-1

Mice overexpressing Fra-1 under the H2K promoter (H2-*fra-1*-LTR transgenic mice, here referred to as *Fra1*tg mice) were reported to be growth retarded (Jochum et al., 2000). We therefore compared the weight of the transgenic mice with that of their wild-type littermates and observed a progressive weight loss in the aging *Fra1* transgenic males and females (Fig. 1A,B). Magnetic resonance imaging (MRI) performed on 14-week-old males suggested a generalized loss of fat tissues in the *Fra1*tg mice (Fig. 1C). Although fat pads were still present in the young *Fra1*tg mice, they were strongly reduced in weight or even totally absent in aging transgenic mice (Fig. 1D), which suggested a progressive loss of the fat tissue in mice overexpressing Fra-1. To quantify the phenotype, we calculated the ratio of gonadal fat pad weight to body weight. A decreased ratio was observed in young (7–9 weeks old) *Fra1*tg males or females; in addition, although the ratio increased in aging wild-type animals (18–21 weeks old), it decreased in aging *Fra1*tg mice (Fig. 1E). The parametrial fat pads were totally absent in aging *Fra1*tg females, for which the ratio could not therefore be calculated, suggesting a stronger phenotype in the females (Fig. 1E). Both heart weight to body weight and spleen weight to body weight ratios were calculated to determine whether the decreased amount of fat tissue was due to a general tissue wasting. The ratio of spleen weight to body weight did not decrease in the aging transgenic mice (data not shown) and an increase in the ratio of heart weight to body weight was observed compared with the value in control mice (supplementary material Fig. S1A). To confirm a generalized lipodystrophic phenotype, we analyzed other adipose tissue depots. By determining the size of the subcutaneous fat layer in sections of the skin of *Fra1*tg compared with that of wild-type littermates (Fig. 1F), we observed a reduced amount of subcutaneous adipose tissue in *Fra1*tg mice. Furthermore, we measured the ratio of different adipose tissue depots to body weight in 6-week-old *Fra1*tg mice. In addition to the decreased ratio of the epididymal fat pad weight to body weight compared to wild-type littermates (supplementary material Fig. S1B), we also observed a decreased proportion of perirenal fat pad weight (supplementary material Fig. S1C). In addition to these white adipose tissues (WATs), brown adipose tissue (BAT) is present in mammals, which regulates energy expenditure and thermogenesis (Gesta et al., 2007). Interestingly, despite a clear increased expression of *Fra1* (data not shown), no histological change was observed in the BAT of newborn *Fra1*tg mice when compared with wild-type mice (supplementary material Fig. S1D) and no difference in the ratio of BAT weight to body weight could be observed (supplementary material Fig. S1E). This observation was confirmed by analyzing the levels of expression of markers for BAT differentiation namely *Pgc1a* (*Ppargc1a*), *Pgc1b* (*Ppargc1b*) and *Ucp1*, which were all unaffected by the increased Fra-1 expression (data not shown). Thus, a severe specific lipodystrophy develops in the osteosclerotic *Fra1*tg mice, which leads to an almost complete absence of white adipose tissue in aging animals.

Immature white adipose tissue in mice overexpressing Fra-1

We then performed histological analysis of the gonadal fat pads of 7–9 and 18-week-old *Fra1*tg and wild-type mice. We observed a clear reduction of the size of the adipocytes present in the adipose tissue of the male and female transgenic mice (Fig. 2A and data not shown). We quantified the cell density on sections of the fat pads of *Fra1*tg mice and related it to the cell density observed in the fat pads of sex- and age-matched wild-type mice. A significantly increased cell density was observed in both males and females overexpressing Fra-1, confirming the decreased size of the adipocytes (Fig. 2B). By binning the cell diameters of sections of white adipose tissue of *Fra1*tg mice and control littermates, we confirmed a shift in the distribution towards reduced cell diameters of *fra1*-tg compared with wild-type fat pads of 6- and 10-week-old mice (Fig. 2C,D). We next quantified the level of expression of *Ap2* (*Fabp4*) and *Glut4*, two typical markers for mature WAT in the fat pads of wild-type and *Fra1*tg mice. Although the level of *Ap2* mRNA was unaffected (Fig. 2E), Fra-1 overexpression resulted in a decreased expression of *Glut4* (Fig. 2F). A decrease in size of white adipocytes can be a sign of a transdifferentiation of WAT to BAT (Plum et al., 2007; Tsukiyama-Kohara et al., 2001). However, no increased expression of markers for BAT maturation was detected in the WAT of the *Fra1*tg mice, indicating that WAT transdifferentiation to BAT had not occurred (data not shown).

Thus *Fra1*tg mice appear to develop a general lipodystrophy linked to immature adipocytes in WAT deposits.

Normal food intake and insulin levels in *Fra1* transgenic mice

Decreased mass of adipose tissues can result from a decreased food intake. We thus compared the food consumption of *Fra1*tg mice with that of their wild-type littermates. When corrected for variation in the body weight, no significant difference in the food intake could be measured (Fig. 3A). Another common cause of lipodystrophy is a disturbance in insulin and glucose metabolism, which controls adipogenesis at the systemic level. We therefore analyzed the histology of the pancreas and measured the area occupied by the β-cells, the proliferation of β-cells and the levels of circulating insulin in *Fra1*tg and wild-type mice. We did not detect any significant changes in any of these parameters (Fig. 3B,C) despite a clear increased level of circulating osteocalcin (Fig. 3D) as a result of increased bone formation, and a decreased level of circulating leptin (Fig. 3E), which was due to decreased fat mass.

Normal glucose clearance but enhanced insulin response and increased serum triglyceride and non-esterified fatty acid levels in *Fra1* transgenic mice

In agreement with the unchanged level of circulating insulin, the level of circulating glucose was unaffected in normally fed mice

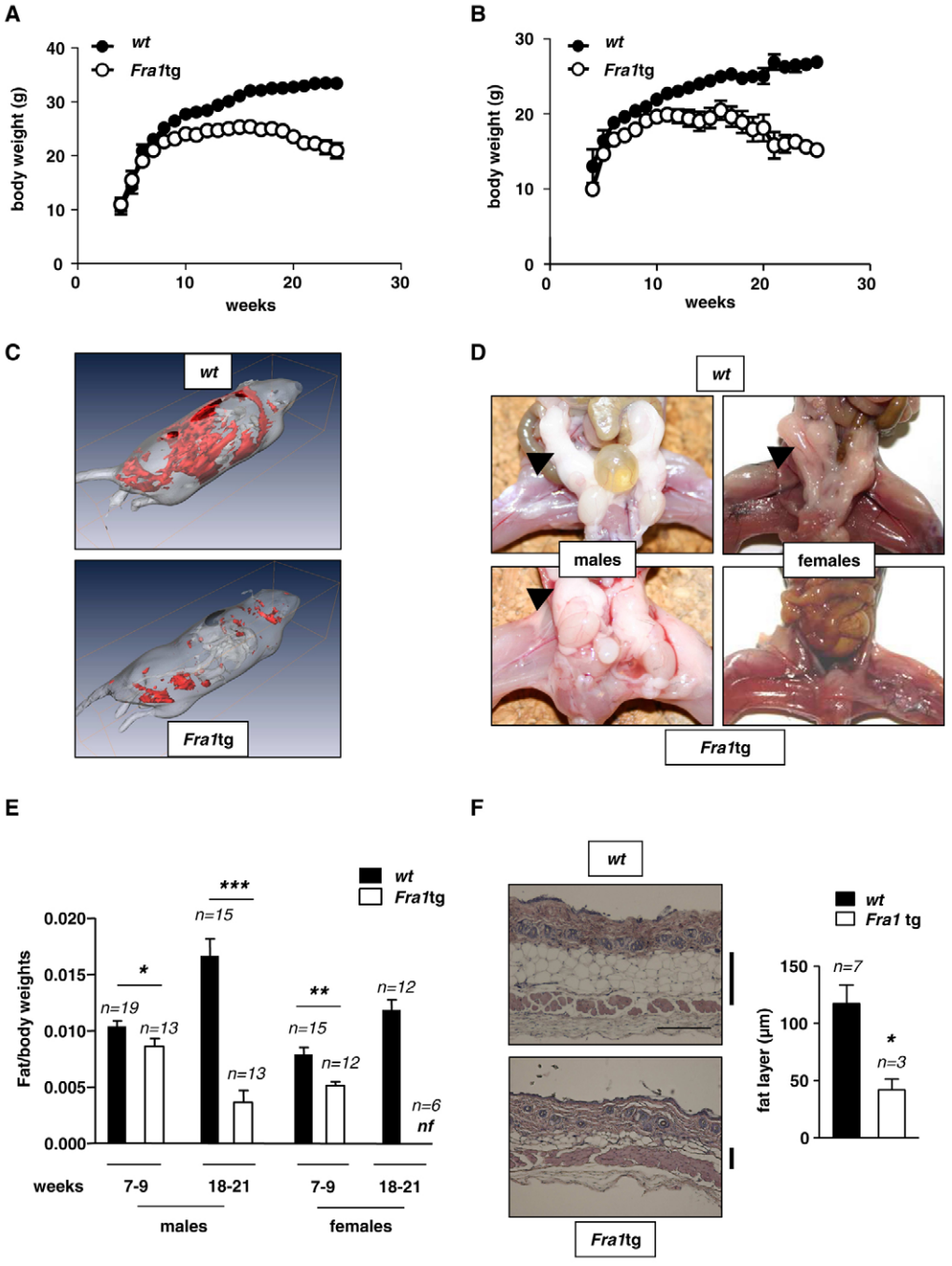

Fig. 1. Progressive lipodystrophy in *Fra1*tg mice. Weight curves *of Fra1*tg compared with wild-type mice, (**A**) males and (**B**) females (*n*=10). (**C**) MRI scans of a 14-week-old wild-type (*wt*) and *Fra1*tg male. Adipose tissues are shown in red. (**D**) The abdomen of 7-week-old *Fra1*tg and wild-type (*wt*) males and 18-week-old females; arrows indicate the fat pads. (**E**) Ratio of gonadal fat pad weight to body weight in *Fra1*tg mice and wild-type (*wt*); *nf*, fat pads not found. (**F**) Hematoxylin and eosin (H&E) staining of sections of the skin isolated from 18-week-old *Fra1*tg females compared with the wild type (*wt*). The vertical bars show the width of subcutaneous fat layer that is quantified on the graph. Data are the mean ± s.e.m. ***P<0.001; **P<0.01; *P<0.05. Scale bars: 200 µm (F).

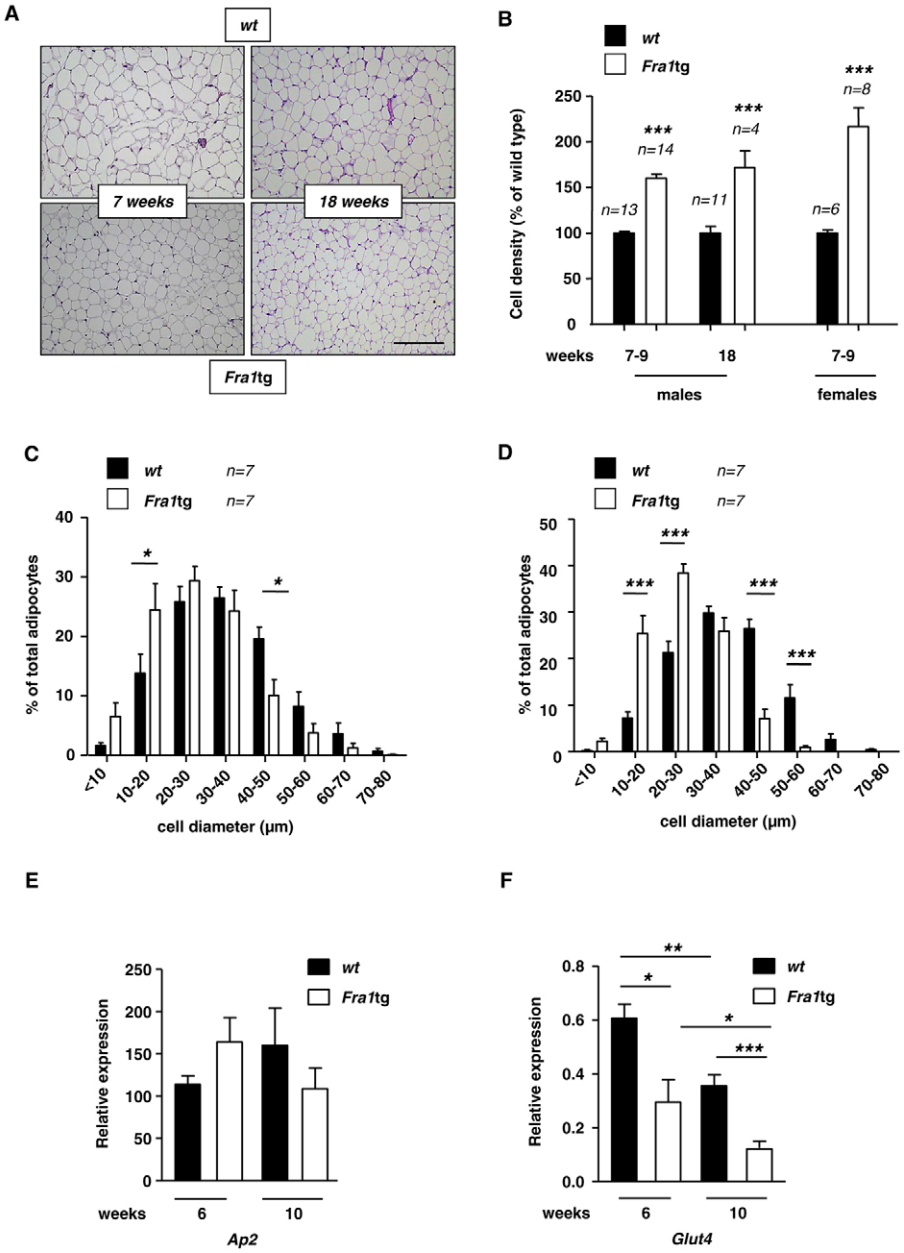

Fig. 2. Decreased adipocyte size in the fat of *Fra1*tg mice. (**A**) H&E staining of sections of gonadal fat pads isolated from *Fra1*tg males at 7 weeks and 18 weeks compared with the wild type (*wt*). Scale bar: 100 µm. (**B**) Cell density in the fat pad of *Fra1*tg and *wt* mice; age and sex are indicated. Distribution of cell diameters in the fat pad of 6-week-old (**C**) and 10-week-old (**D**) *Fra1*tg and *wt* mice. Quantitative PCR analysis of *Ap2* (**E**) and *Glut4* (**F**) expression in the fat pads of 6- and 10-week-old *Fra1*tg and *wt* males. Data are the mean ± s.e.m. ***P<0.001; **P<0.01; *P<0.05.

and a significant reduction in *Fra1*tg was only observed after starvation (supplementary material Fig. S2A). We then analyzed the capacity of *Fra1*tg mice to clear or to mobilize circulating glucose. Although no difference in glucose clearance was observed when glucose was intraperitoneally injected in *Fra1*tg and wild-type mice (supplementary material Fig. S2B), an increased sensitivity to insulin was measured (supplementary material Fig. S2C). Next, we analyzed the serum levels of triglycerides and free fatty acids that were both found to be unchanged in mice kept under normal diet. However, in agreement with a lipodystrophic phenotype, increased levels of circulating serum triglyceride and of non-esterified fatty acids were detected in the serum of *Fra1*tg compared with wild-type mice after starvation (supplementary material Fig. S2D,E). These changes in lipid metabolism prompted us to analyze the expression level of genes involved in lipogenesis, lipolysis and fatty acid uptake in white adipose tissue of 10-week-old *Fra1*tg compared with wild-type mice. Although we did not observe any difference in the expression of lipogenic genes acetyl CoA carboxylase 1 (*Acaca*) (data not shown) and fatty acid synthase (*Fasn*) (supplementary material Fig. S2F), the expression level of stearoyl CoA desaturase (*Scd1*) was decreased in *Fra1*tg mice as was the mRNA level of the lipolytic gene patatin-like phospholipase domain-containing 2 (*Pnpla2*) (supplementary material Fig. S2G,H). A decreased expression of lipoprotein lipase (*Lpl*) (supplementary material Fig. S2I) and of *Cd36* (supplementary material Fig. S2K), two genes encoding proteins essential for fatty acid uptake, was observed, as well as of the level of perilipin 1 (*Plin1*) (supplementary material Fig. S2J). A significantly decreased expression level of *Cd36* and *Plin*, and a slightly reduced level of *Lpl* was also observed in the epididymal fat pad of 6-week-old *Fra1*tg mice (data not shown). However, there was no change in the expression level of *Scd1* and *Pnpla2* in the white adipose tissue of 6-week-old *Fra1*tg mice.

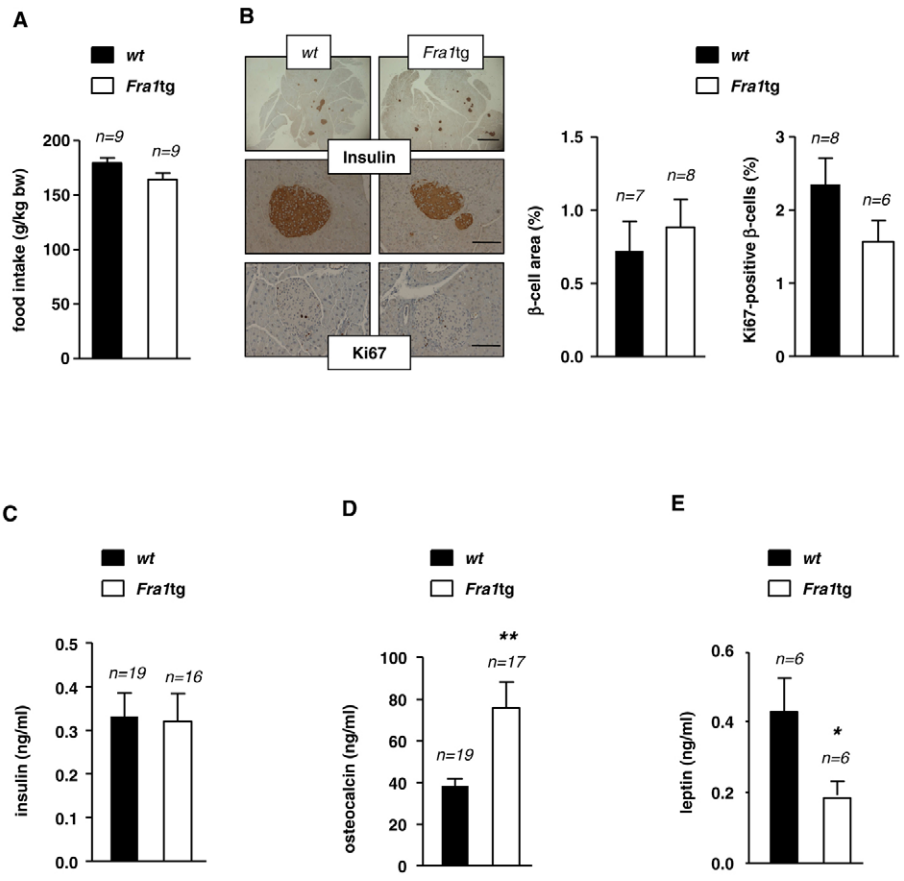

Fig. 3. Decreased leptin and normal insulin level in *Fra1*tg mice. (**A**) Food intake (g per kg of body weight per day) by 10-week-old females measured after acclimatization for 4 days in metabolic cages. (**B**) Immunohistochemical staining for insulin and Ki67 in sections of pancreas, quantification of the β-cell area reported to the tissue surface and β-cell proliferation. Scale bars: 50 µm (bottom two rows) and 1000 µm (top row). (**C**) Measurement of the level of circulating insulin in the serum of 10-week-old *Fra1*tg and wild-type (*wt*) females. Level of circulating osteocalcin (**D**) and leptin (**E**). Data are the mean ± s.e.m. $^{**}P<0.01$; $^{*}P<0.05$.

Adipose tissues are known to produce numerous cytokines, called adipokines, that can locally regulate insulin resistance or even exert lipolytic activity. We therefore measured the expression of Leptin (*Lep*), Adiponectin (*Adipoq*) and Resistin (*Retn*), as well as of the pro-inflammatory cytokines *Tnfa* and *Il6* in the fat tissue of *Fra1*tg and wild-type mice. Although a mild decrease in *Adipoq* expression and a marked decrease in *Lep* expression were observed as a consequence of the decreased maturity of the adipocytes (supplementary material Fig. S3A,B), the expression of *Retn*, which can confer insulin resistance, was not significantly affected by overexpression of Fra-1 (supplementary material Fig. S3C). Moreover, expression of *Il6* was unchanged (supplementary material Fig. S3D) and a decreased expression of the lipolytic cytokine *Tnfa* was observed in the fat tissue of *Fra1*tg mice (supplementary material Fig. S3E). In addition, no change in the number of apoptotic cells was detected by TUNEL staining in the WAT of *Fra1*tg mice (supplementary material Fig. S3F). Thus, the lipodystrophy should not be the consequence of a change in the levels of adipokines production.

A reduced amount of mesenchymal progenitor cells in the adipose tissue could explain the lipodystrophy of *Fra1*tg mice. We therefore analyzed the expression of markers for adipocyte progenitor cells, but no change in the levels of *Cd24a*, *Cd34*, *Ly6a* or *Itgb1* was detected (supplementary material Fig. S3G–J).

All these data suggest that, rather than a defect in the systemic control of fat mass, Fra-1 directly impairs adipocyte maturation, therefore leading to the lipodystrophic phenotype of the *Fra1*tg mice.

Decreased *Cebpa* expression in white adipose tissue overexpressing Fra-1

We thus investigated whether enhanced Fra-1 expression within the fat could cell autonomously affect adipocyte differentiation or maturation. Indeed, quantitative PCRs demonstrated a strong increased *Fra1* expression in the WAT of *Fra1*tg mice (Fig. 4A). Comparison of RT-PCRs using primers specifically amplifying the transcript encoded by the transgene or primers amplifying both the transcripts encoded by the transgene and the *Fra1* locus demonstrated that *Fra1* was expressed in wild-type adipose tissues and that the increased *Fra1* expression observed in the fat pads of *Fra1*tg mice was due to the expression of the transgene (Fig. 4B). Histoimmunological analysis of the tissue confirmed the expression of Fra-1 at the protein level in the fat of both wild-type and *Fra1*tg mice (Fig. 4C).

We next analyzed whether the decreased fat mass could be due to a change in the expression of transcription factors known to regulate or to be essential for adipogenesis. We first quantified the levels of mRNA encoding for transcription factors regulating early stages of WAT differentiation (White and Stephens, 2010). As expected and in agreement with the histology, the expression of *Cebpb* and *Cebpd*, genes encoding two regulators of the early stages of adipocyte differentiation were not significantly affected (Fig. 4D,E) nor were the expression of the glucocorticoid receptor (*Gr*) and of the sterol-responsive binding factor-1 (*Srebf1*) (Fig. 4F,G). However, when measuring the expression levels of transcription factors regulating adipocyte maturation, although no differences in the expression of *Pparg2* (Fig. 4H) was detected, the level of the *Cebpa* mRNA was significantly reduced in the fat pads

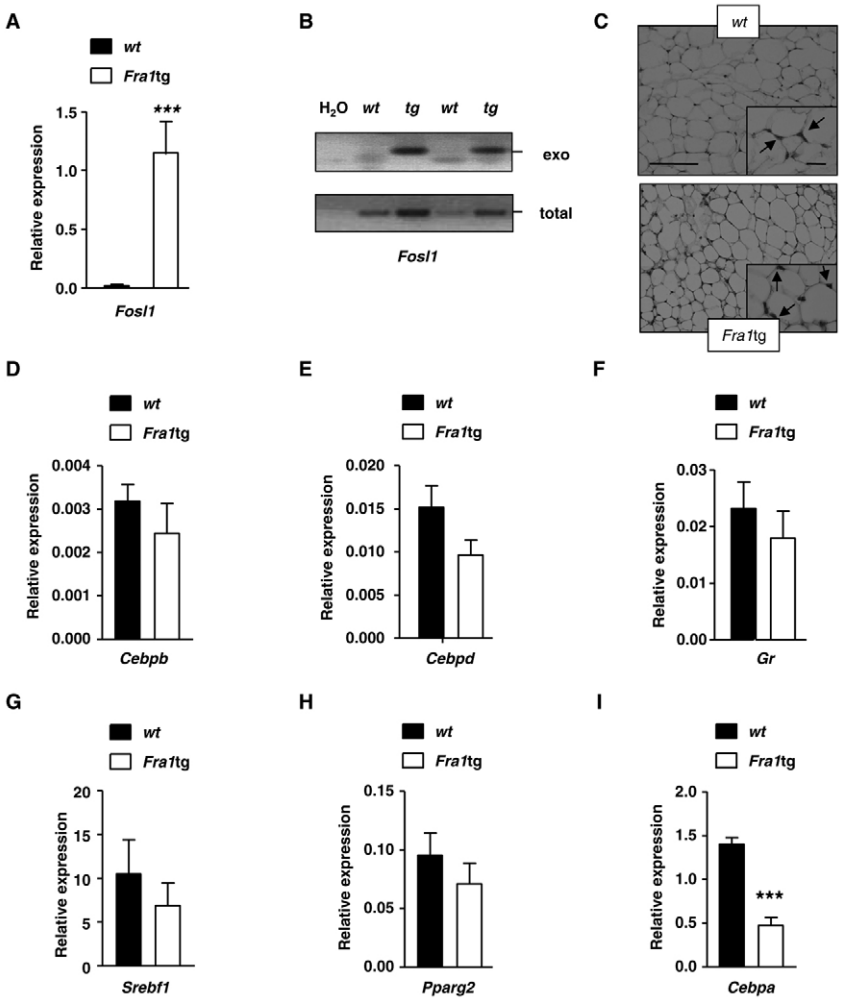

Fig. 4. Decreased *Cebpa* expression in the adipose tissue of *Fra1*tg mice. (**A**) Quantitative PCR analysis levels of *Fra1* (*Fosl1*) expression in the gonadal fat pad of 10-week-old *Fra1*tg males compared with the wild type (*wt*). (**B**) PCR analysis of *Fra1* expression in the fat pad of two *wt* and two *Fra1*tg (*tg*) mice. 'exo' indicates the specific amplification of the mRNA encoded by the transgene and ('total') of the mRNA encoded by the transgene and/or the endogene. (**C**) Immunohistochemical staining for Fra-1 in sections of white adipose tissue of 10-week-old *Fra1*tg males compared with *wt*. Arrows indicate Fra-1-positive nuclei. Scale bars: 100 µm and 20 µm (magnified insets). Quantitative PCR analysis of the expression of transcriptional regulators of adipocyte differentiation in the fat pads of 10-week-old *Fra1*tg males compared with the *wt*. (**D**) *Cebpb*, (**E**) *Cebpd*, (**F**) glucocorticoid receptor (*Gr*), (**G**) *Srebf1*, (**H**) *Pparg2* and (**I**) *Cebpa*. Data are the mean ± s.e.m. ***P<0.001.

of *Fra1*tg mice (Fig. 4I). These data were in agreement with the reduced size of the cells, as well as the decreased *Glut4* expression in vivo, which suggest a defect in the progression of adipocyte differentiation in *Fra1*tg mice. Interestingly, *Cebpa* was also found to be downregulated in bone, liver and muscle of *Fra1*tg mice (supplementary material Fig. S4B,D,F). In summary, elevated Fra-1 expression in adipose tissue is accompanied by reduced *Cebpa* expression, which might cause fat loss.

Cell-autonomous decreased adipogenesis of *Fra1* transgenic mesenchymal cells

The data discussed above suggested that a cell-autonomous defect in adipogenesis was the most likely cause of the lipodystrophy that developed in *Fra1*tg mice. To test this hypothesis, we performed adipocyte differentiation in vitro. Adipocytes and osteoblasts derive from common mesenchymal progenitor cells. These cells can be isolated from calvaria of newborn mice and cultured to differentiate into adipocytes when treated with an adipogenic cocktail composed of insulin, dexamethasone and IBMX (3-isobutyl-1-methylxanthine). It has already been published that primary osteoblasts (POBs) isolated from wild-type pups express Fra-1 and that Fra-1 overexpression cell-autonomously accelerated the differentiation into mature osteoblasts (Jochum et al., 2000). We isolated POBs from newborn wild-type and *Fra1*tg mice and induced them to differentiate into adipocytes. We first confirmed the increased expression of *Fra1* in POBs isolated from *Fra1*tg mice and the persistence of its expression following adipogenic stimulation (supplementary material Fig. S5A). After 15 days of treatment, Oil Red O staining was performed to reveal the accumulation of fat that characterized adipocytes. As expected, numerous Oil-Red-O-positive colonies were stained when wild-type POBs were cultured in presence of the adipogenic cocktail (Fig. 5A,B). By contrast, a decreased number of Oil-Red-O-positive colonies was seen in the culture of POBs overexpressing Fra-1 (Fig. 5A,B). The efficiency of differentiation was quantified by measuring the expression of *Glut4* and *Ap2*, two markers of adipocyte maturation that were both strongly upregulated in wild-type POBs stimulated by the adipogenic cocktail, but not in *Fra1*tg POBs (Fig. 5C,D). Factors regulating early adipogenesis (i.e. *Cebpb*, *Cebpd*, *Srebf1*, *Pref1* and *Gr*), osteoblastogenesis (*Runx2*, *Osx1* and *Pref1*), chondrogenesis (*Sox9*) or myogenesis (*Myod*) were not affected by overexpression of Fra-1 (Fig. 5E). By contrast, markers for later stages of adipogenesis, such *Cebpa* and *Pparg2* and consequently the PPARγ target gene *Ap2* as well as the C/EBPα target gene *Glut4* were strongly decreased (Fig. 5C,D,F). In addition, the induction of several genes characterizing functional mature adipocytes, namely *Scd1*, *Pnpla2*, *Plin1*, *Lpl* and *Cd36* was also inhibited in mesenchymal cells of *Fra1*tg mice after adipogenic differentiation (data not shown). Thus, overexpression

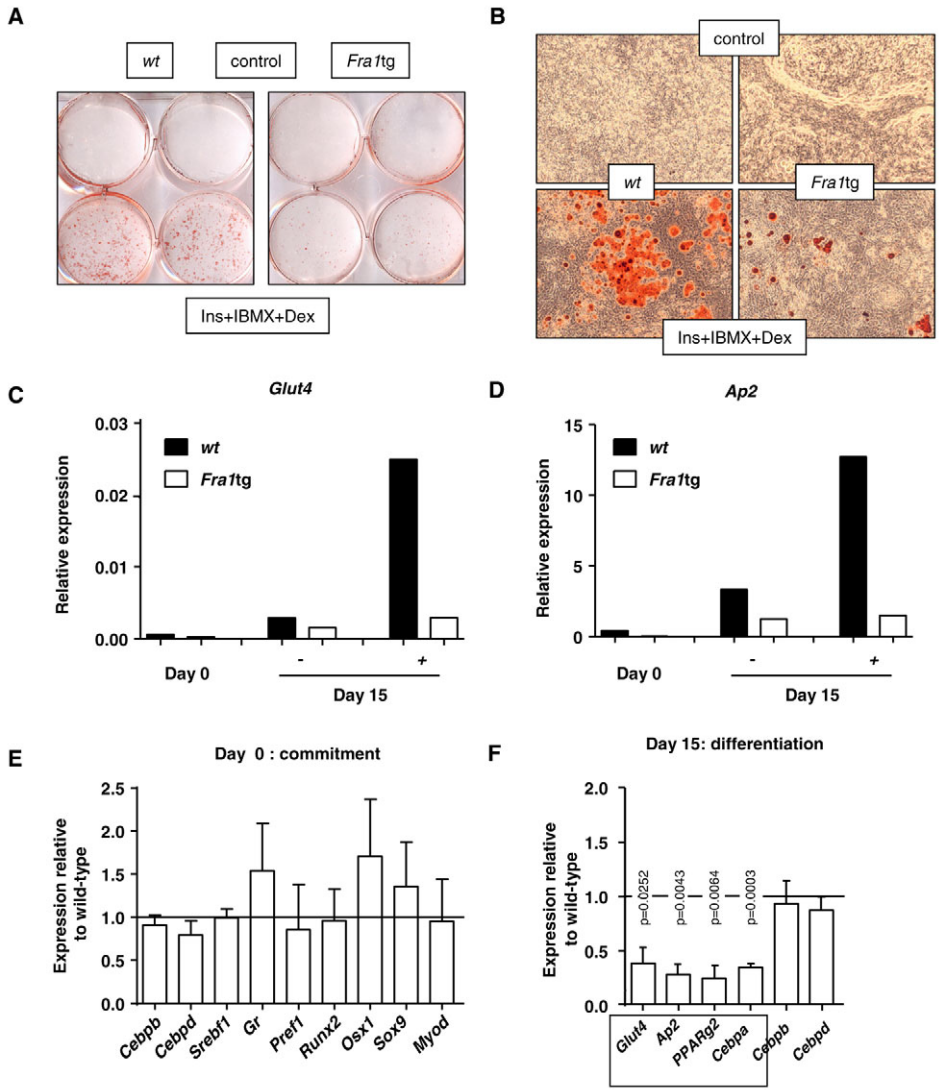

Fig. 5. Decreased adipogenic potential of mesenchymal cells isolated from *Fra1*tg mice. (**A**,**B**) Oil Red O staining of primary osteoblasts isolated from newborn wild-type or *Fra1*tg mice cultured for 15 days in adipogenic conditions (Ins+IBMX+Dex) compared with untreated cells (control). Quantitative PCR analysis of *Glut4* (**C**) and *Ap2* (**D**) expression during the course of differentiation; data from one representative experiment are shown; (–) control cell culture, (+) (Ins+IBMX+Dex) treated cell culture. (**E**) Quantitative PCR analysis of the expression of genes regulating the commitment of mesenchymal cells in five independent cultures, the data represent the ratio of the level measured in *Fra1*tg POBs reported to the level in wild-type cells at day 0 of the culture (i.e. confluent cells before adipogenic stimulation). (**F**) Relative expression of markers for adipogenesis in five independent cultures of *Fra1*tg POBs reported to the value measured for wild-type cells (± s.e.m.) cultured for 15 days in adipogenic conditions.

of Fra-1 did not modify mesenchymal cell commitment but rather blocked the progression toward adipocyte maturation that resulted in a marked cell-autonomous blockage of adipogenesis.

Normal responses of *Fra1* transgenic mesenchymal progenitors to adipogenic stimulation

Because adipogenic commitment was not altered, we hypothesized that undifferentiated mesenchymal progenitors overexpressing Fra-1 should respond to adipogenic stimulation, i.e. addition of the adipogenic cocktail containing insulin, IBMX and dexamethasone. We therefore compared the early response of mesenchymal progenitors isolated from *Fra1*tg mice or wild-type littermates to stimulation with insulin, IBMX or dexamethasone. No clear change in the kinetic or in the intensity of ERK activation by insulin stimulation was observed (supplementary material Fig. S5B). Similarly, phosphorylation of Creb and ATF1 in response to IBMX was unchanged (supplementary material Fig. S5C). Finally, the induction of *Gilz* and *Per1*, two genes known to be regulated by glucocorticoid (Shi et al., 2003; Yamamoto et al., 2005), in response to dexamethasone treatment was unaffected by Fra-1 overexpression (supplementary material Fig. S5D,E). Thus, a defect in early signaling events controlling adipogenesis cannot explain the decreased adipogenic properties of mesenchymal progenitor cells overexpressing Fra-1.

Fra-1 overexpression directly blocks adipogenesis

To verify a direct cell-autonomous effect of Fra-1 on adipocyte differentiation, we retrovirally stably overexpressed Fra-1 in an adipogenic cell line generated by sequentially passaging primary osteoblasts (Fig. 6A). An inhibition of the differentiation to mature adipocytes and accumulation of lipid droplets was seen in the cells overexpressing Fra-1 as indicated by a decreased amount of Oil-Red-O-positive cells (Fig. 6B). The block of adipogenic differentiation was confirmed by the strong reduction in the levels of induction of *Glut4* and *Ap2* in the Fra-1-overexpressing cells compared with control cells infected with the empty vector (Fig. 6C,D). In agreement with the results observed with the primary cells, no change in the expression of *Cebpb* and *Cebpd*, or in the expression of *Srebf1* and the glucocorticoid receptor was observed (Fig. 6E and data not shown). Again, the expression of transcription factors regulating adipocyte maturation, *Pparg2* and *Cebpa,* were strongly reduced in the Fra-1-overexpressing cell line induced to differentiate (Fig. 6E). Fra-1-mediated inhibition in adipogenesis and the decreased *Cebpa* expression were both confirmed in two

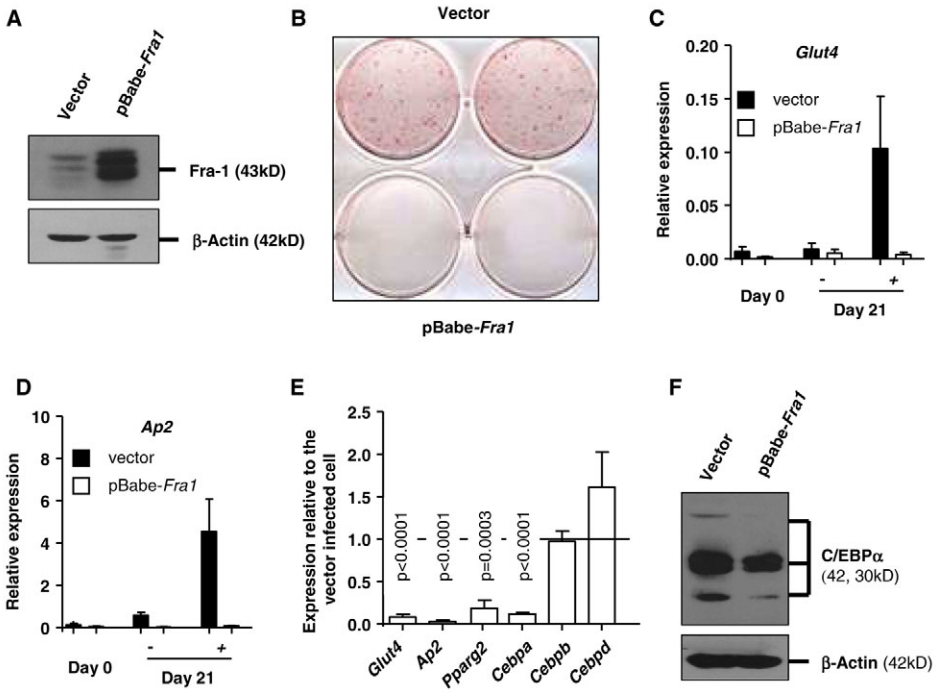

Fig. 6. Fra-1 overexpression in adipogenic cell line blocks adipogenesis by inhibiting C/EBPα expression. (**A**) Western blot analysis of Fra-1 expression in the adipogenic cell line infected with Fra-1 encoding virus (pBabe-*Fra1*) or empty vector (vector); β-actin is used as loading control. (**B**) Oil Red O staining of the infected cells cultured for 21 days in adipogenic condition. Quantitative PCR analysis of *Glut4* (**C**) and *Ap2* (**D**) expression during the course of differentiation of cells infected with the vector or pBabe-*Fra1*. (–) control cell culture; (+) Ins+IBMX+Dex-treated cell culture. Data are the mean ± s.e.m. of at least four different experiments. (**E**) Quantitative PCR analysis of the expression of markers for adipogenesis, the data represent the ratio of the level measured in cells overexpressing Fra-1 reported to the level in cells infected with the vector alone at day 21 of differentiation. (**F**) Western blot analysis of C/EBPα expression in Fra-1-overexpressing cells and cells infected with the vector alone; β-actin is used as loading control.

other independently infected cell lines (data not shown), as well as at the protein level by western analysis (Fig. 6F).

Similar experiments were performed by infecting the adipogenic cell line with a virus encoding a fusion of Fra-1 with the ligand binding domain of the human estrogen receptor. Upon induction with estradiol, the fusion protein accumulated in the nucleus (supplementary material Fig. S6A), where Fra-1 can then act as a transcription factor. Similarly to the cells infected with empty vector, Oil-Red-O-positive colonies were observed when the cells overexpressing the ER–Fra-1 fusion protein were cultured in the presence of the adipogenic cocktail (supplementary material Fig. S6B). As expected, although co-stimulation with the adipogenic cocktail and estradiol did not affect differentiation of the control infected cells, it inhibited adipocyte differentiation of ER–Fra-1-expressing cells (supplementary material Fig. S6B). The inhibition of adipogenesis was confirmed by analyzing *Glut4* expression (supplementary material Fig. S6C). These data demonstrate that Fra-1 directly inhibits adipogenesis.

Fra-1 directly inhibits the transcription of C/EBPα

The decreased level of C/EBPα observed in the fat tissues of *Fra1*tg mice as well as in the adipogenic cells overexpressing Fra-1, strongly suggest that Fra-1 inhibits adipogenesis by directly blocking C/EBPα expression. We took advantage of cells expressing the inducible ER–Fra-1 to determine the effect of short term activation of Fra-1 on the expression of *Cebpa* following adipogenic stimulation. A clear inhibition of *Cebpa* expression was observed in the cells expressing ER–Fra-1 stimulated with Estradiol (Fig. 7A), suggesting a direct repression of transcription of *Cebpa* by Fra-1. Interestingly, five potential AP-1 binding sites were identified in the promoter of the mouse *Cebpa* by sequence search analysis (Fig. 7B). We therefore analyzed the effect of overexpressing Fra-1 on the transcriptional activity of the mouse *Cebpa* promoter. To do so, a reporter construct linking 3359 base pairs of the *Cebpa* promoter to luciferase was co-transfected into the adipogenic cell line with an increased amount of vector expressing Fra-1. A dose-dependent significant decrease in *Cebpa* promoter activity was observed when the amount of vector expressing Fra-1 was increased (Fig. 7C). We performed deletion analysis to map the area of the promoter driving the repression by Fra-1. Deletion of the first 1011 N-terminal base pairs including three distal potential AP-1 binding sites did not abolish *Cebpa* repression by Fra-1 (Fig. 7D), nor did the further deletion that only left the proximal 269 C-terminal base pairs of the promoter (Fig. 7E). This 269 bp fragment of the *Cebpa* promoter contains a unique AP-1 binding site, suggesting that this proximal AP-1 binding would be sufficient to drive the repression of *Cebpa* transcription by Fra-1. We therefore determined whether Fra-1 could bind to the proximal AP-1 binding site of the *Cebpa* promoter. We performed chromatin immunoprecipitation using extract isolated from the adipogenic cell line and demonstrated that Fra-1 did indeed bind to this AP-1 binding site in both control cells and in cells overexpressing Fra-1 (Fig. 7F).

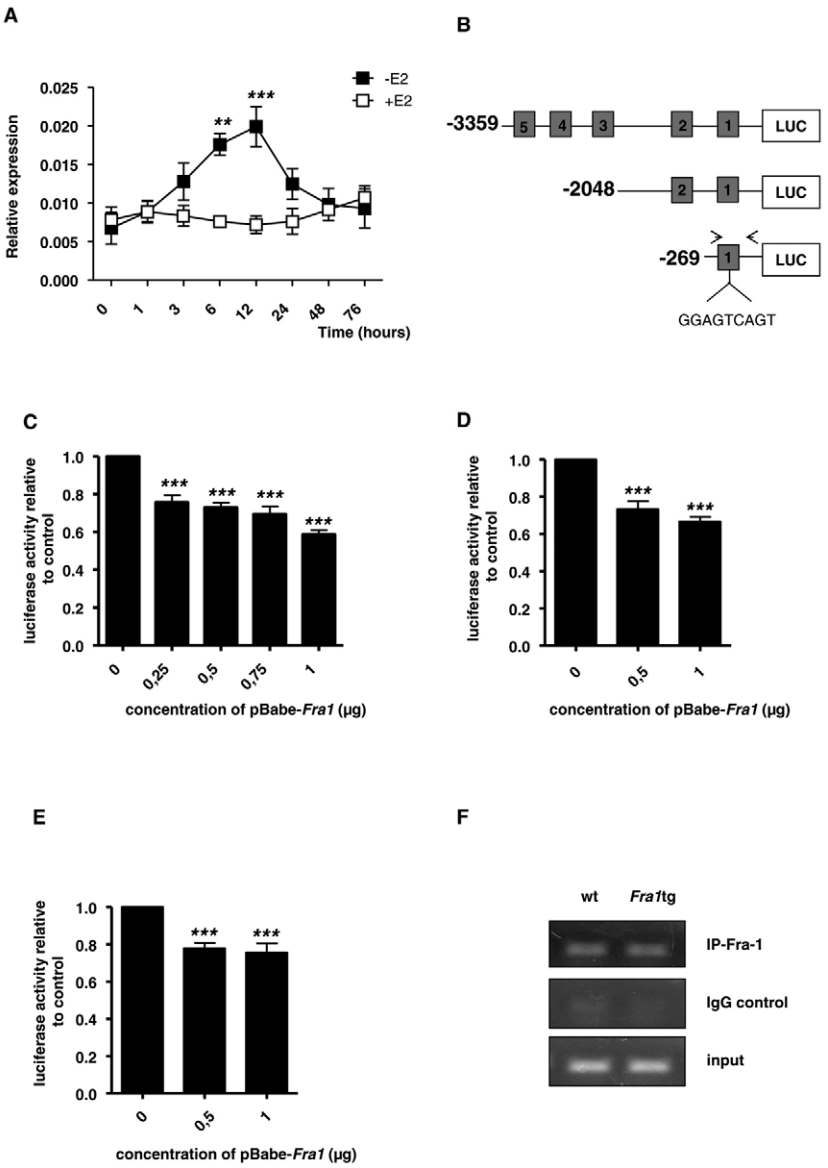

Fig. 7. Fra-1 directly inhibits the transcription of C/EBPα. (**A**) Quantitative PCR analysis of *Cebpa* expression in a cell line overexpressing a fusion protein of the estrogen ligand binding domain and Fra-1 (ER–Fra-1) cultured in the absence of estradiol (–E2) or stimulated for the indicated time with estradiol (+E2) 24 hours after adding the adipogenic cocktail. Data are the mean ± s.e.m. of three independent experiments. (**B**) Schematic representation of constructs of the *Cebpa* promoter cloned in front of the luciferase gene into pGL3-basic. Grey boxes represent potential AP-1 binding sites; arrows show localization of primers for ChIP experiments. (**C**) Luciferase activity in an adipogenic cell line transfected with vector containing the full-length construct and increasing concentrations of pBabe-*Fra1* relative to cells transfected with the empty vector. Luciferase activity in cells transfected with the promoter constructs containing the two proximal (**D**) or the first (**E**) proximal AP-1 binding site and 0, 0.5 and 1 μg of pBabe-*Fra1*. (**F**) Chromatin immoprecipitation (ChIP) for AP-1 binding site 1. PCR products are shown in the input, after immunoprecipitation with Fra-1 antibody (IP-Fra-1) or with isotype control (IgG control). ***P<0.001; **P<0.01.

Discussion

Although the systemic co-regulation of bone and fat mass is beginning to be understood (Lee and Karsenty, 2008; Rosen, 2008; Wong et al., 2008), the key regulators locally integrating this signaling are still largely unknown. We now show that mice overexpressing Fra-1, which develop a progressive osteosclerosis as a result of cell-autonomous increased osteoblast activity, also display a severely decreased amount of white adipose tissue. We characterized this lipodystrophic phenotype as being cell autonomous and probably caused by the direct transcriptional repression of the adipogenic transcription factor C/EBPα by Fra-1.

Thus, *Fra1* transgenic mice develop a severe decreased mass of white adipose tissue linked to a decreased size of adipocytes. We excluded the possibility that the phenotype was a consequence of a decreased food intake. We analyzed the potential metabolic alterations that are commonly associated with a lipodystrophic phenotype such as described for the A-ZIP/F transgenic mice (Moitra et al., 1998) or for the mice overexpressing a constitutively active form of SREBP-1 (Shimomura et al., 1998). *Fra-1*tg mice have normal levels of circulating insulin and normal β-cell proliferation; we also found that the glucose response was unaffected. Although a normal level of circulating glucose was measured in the fed animals, a decreased blood glucose level compared with wild-type littermates was only observed after fasting, which was most probably due to an increased sensitivity to insulin. As expected from reduced fat storage, *Fra1*tg mice show a reduced level of circulating leptin that might contribute to increased bone formation by osteoblasts (Ducy et al., 2000). However, this decreased level of leptin was not sufficient to affect the food intake of the *Fra1*tg mice, and although the role of leptin in regulating bone mass can be dissociated from its function in regulating appetite (Shi et al., 2008), leptin was not found to have a role in the very similar bone phenotype that developed in *ΔfosB* transgenic mice (Kveiborg et al., 2002). Osteocalcin was recently characterized as a hormone that regulates insulin production by increasing β-cell proliferation (Lee et al., 2007). Surprisingly, but similarly to the *ΔfosB*tg mice (Rowe et al., 2009), the increased circulating osteocalcin in *Fra1*tg mice did not result in an increased level of circulating insulin or in an increased number or volume of

insulin-positive β-cell islets in the pancreas. In agreement, proliferation of the β-cells of the pancreas was unaffected by overexpression of Fra-1. Other clinical features usually associated with severe forms of lipodystrophy, hypertriglyceridemia and a high level of serum non-esterified fatty acids were the only major metabolic changes observed in *Fra1*tg mice. The increased level of fatty acids and triglycerides in the serum of *Fra1*tg compared with wild-type mice can certainly be explained by the change in the expression of genes regulating lipolysis and fatty acid uptake. Indeed, we found a decreased level of expression of *Cd36*, a known C/EBPα target gene (Qiao et al., 2008) that regulates fatty acid uptake in peripheral tissues. This function is clearly demonstrated by the similar phenotype, that is hypertriglyceridemia and the increased serum free fatty acids that develop in CD36-deficient mice (Febbraio et al., 1999). Furthermore, a reduced, albeit insignificant, expression of *Lpl* was detected, which might contribute to the phenotype. Indeed, *Lpl*-knockout mice have a lean appearance and have an increased level of triglycerides as a result of the lack of removal of triglycerides from the plasma (Weinstock et al., 1995).

These results strongly suggest that a defect in the systemic regulation of adipogenesis would not be the major cause of the decreased fat mass of the *Fra1*tg mice. Interestingly, the very similar phenotype, including the cell-autonomous increased bone mass associated with the decreased fat mass, was also observed in two other mouse models, the ENO2-*ΔfosB* transgenic mice (Sabatakos et al., 2000) and the Sox8-deficient mice (Guth et al., 2009; Schmidt et al., 2005). In both cases, the fat phenotypes were shown to be associated with a cell-autonomous defect in adipocyte differentiation.

In vivo, early steps of adipogenesis still occur in young *Fra1*tg mice. This apparent discrepancy with the strong in vitro block of adipogenesis might be due to compensatory hormonal mechanisms, as suggested by the more pronounced phenotype observed in the females, as well as by the complete absence of fat observed in the aging mice. Although we did not find any change in FosB or Sox8 (data not shown) expression in the WAT of *Fra1*tg mice, a clear cell-autonomous defect in adipocyte differentiation was found in the *Fra1*tg mice. This conclusion is based on the results obtained in our three independent in vitro studies: (1) a reduced number of mature adipocytes with a decreased expression of late markers of adipogenesis such as *Cebpa*, *Pparg2*, *Glut4* and *Ap2*, as well as of markers of functional adipocytes such as *Scd1*, *Pnpla2*, *Lpl*, *Plin1* and *Cd36*, were generated in cultures of primary osteoblasts overexpressing Fra-1; (2) constitutive overexpression of Fra-1 also blocked the differentiation of an adipogenic cell line and similarly affected the expression of markers of adipocyte maturation; and (3) the estradiol-inducible activation of Fra-1 further supported a cell-autonomous adipocyte differentiation defect. In all cell culture systems, we found that Fra-1 did not affect any of the genes regulating the lineage commitment of mesenchymal cells, including adipocytes. This confirms the previous publication showing that Fra-1-dependent increased osteoblast differentiation was not due to increased Runx2 expression (Jochum et al., 2000). We are now extending this observation to the other mesenchymal lineages that share a common progenitor: chondrocytes, muscles and adipocytes. This result is in agreement with the idea that beyond expression of the commitment genes, their activities, often differentially regulated by the same cofactors, will determine the differentiation of the mesenchymal cells in the various lineages (Rosen and MacDougald, 2006). Based on the unchanged expression of all the regulators of the early steps of adipocytes differentiation in vivo or in vitro, our data suggest that Fra-1 regulates adipocyte maturation more than adipocyte determination. This is also illustrated in vivo by the decreased size of the adipocytes that histologically characterize immature adipocytes observed in the WAT of the younger *Fra1*tg mice, as well as by the decreased expression of the typical transcriptional regulator of adipocyte maturation, *Cebpa*, and of its known target genes *Glut4* and *Cd36*.

The inhibition of C/EBPα appears to be a key event mediating the adipocyte phenotype downstream of Fra-1. In agreement with this hypothesis, we consistently found a downregulation of *Cebpa* in our in vitro experiments that was concomitant with the repression of genes characterizing adipocyte maturation in Fra-1-overexpressing cells. C/EBPα is expressed during adipocyte maturation (Cao et al., 1991; Wu et al., 1998), when it cooperates with PPARγ to regulate the expression of mature adipocyte markers (Wu et al., 1999). In vivo, early post-natal lethality is observed in C/EBPα-deficient mice, which also display reduced white adipose tissue (Wang et al., 1995). The requirement of C/EBPα for development of WAT, but not BAT, was confirmed when the peri-natal lethality was improved by re-expressing C/EBPα in liver (Linhart et al., 2001). Interestingly, all studies published so far, suggest the key role of repressing C/EBPα expression in the potential modulation of adipocyte differentiation by AP-1. Indeed, a cell-autonomous blockage of adipogenesis by Fos or ΔFosB has also been associated with decreased C/EBPα expression (Abbott and Holt, 1997; Kveiborg et al., 2004). We also found that, although overexpressing Fos in the adipogenic cell line blocked adipogenesis (data not shown) and downregulated *Cebpa*, overexpression of Fra-2 did not block adipogenesis in vitro and did not interfere with *Cebpa* expression (data not shown). *Cebpa* as a potential downstream target of Fra-1 was also suggested by its downregulation in the liver, muscle and bone of *Fra1*tg mice. All these observations point to a key role for downregulation of C/EBPα by Fra-1 in the development of the lipodystrophy. A mechanism by which AP-1 could repress C/EBPα expression was proposed for mice overexpressing ΔFosB (Kveiborg et al., 2004). These authors suggested that the binding of ΔFosB to C/EBPβ would interfere with the transcriptional regulation of C/EBPα. We performed very similar experiments, but were unable to demonstrate a direct binding of Fra-1 to C/EBPβ (supplementary material Fig. S7) and therefore do not favor this model. By contrast, we demonstrated that Fra-1 can directly act as a transcriptional repressor of the promoter activity of C/EBPα. Indeed, we localized a minimal region in the proximal promoter of C/EBPα that confers the transcriptional repression and contains a potential AP-1 binding site, which is bound by Fra-1.

AP-1 acts as a sensor of changes affecting the cellular environment that it translates in proliferative, apoptotic or differentiating signals that probably depend on the composition of the dimer. These properties, combined with its known function in the development of mesenchymal tissues, make AP-1 a potential player in mesenchymal cell specification (Eferl and Wagner, 2003; Karsenty, 2008; Wagner and Eferl, 2005). Indeed, our work demonstrates that, in addition to the central nervous system and the hormonal systemic co-regulation of bone and adipose tissues, a local control of cell fate decisions is exerted by a Fos family component of AP-1, namely Fra-1. Based on the correlating bone phenotypes that develop in Fra-1-deficient and Fra-1-overexpressing mice, our data suggest that Fra-1 is a local integrator of the systemic signaling that promotes osteoblast formation, and

by blocking expression of C/EBPα, inhibits adipocyte differentiation. Thus, induction of Fra-1 activity will have therapeutic potential in metabolic diseases such as obesity and osteopenia.

Materials and Methods

Animals

The *Fra1* (H2-*fra-1*-LTR) transgenic mice (Jochum et al., 2000) were back-crossed into the C57BL/6 background by nine successive crossings. Animal experiments were approved by the local ethics committee.

Magnetic resonance imaging

MRI was performed on a 4.7 T BRUKER Biospec scanner with a free bore of 40 cm equipped with quadrature volume coil enabled homogenous excitation used as a receiver and transmitter coil. The rational of the MR scanning was to perform spin echo T2 weighted 3D datasets with and without fat suppression for anatomical characterization of the fat distribution in situ. A 3D RARE sequence (FOV 90×30×25 mm, matrix 256×128×128, resolution 0.352×0.234×0.195 mm coronal, TR=1000 ms, TE_{eff}=59.1, RARE partition=16, averages=14, total measurement time=3 hours 59 minutes, with fat suppression on and off) was used. For fat suppression, a frequency selective 90 degree Gaussian pulse is applied with 3.9 ms duration and 700.9 Hz bandwidth at a frequency offset of –3.5 p.p.m. with respect to water. Then, the datasets with fat suppression were subtracted from those without fat suppression. Consequently, the result contained the 3D fat compartment.

Histology

All tissues were fixed in 4% formaldehyde overnight at 4°C and embedded in paraffin. 5 µm sections (WAT), 2 µm sections (BAT) and 1 µm sections of the skin were stained with hematoxylin and eosin. 1 µm paraffin sections of the pancreas were unmasked using citrate buffer (20 minutes, 92°C) and stained with an anti-insulin or anti-Ki67 antibodies (Santa Cruz and BD, respectively) and 5 µm paraffin sections of WAT were unmasked using citrate buffer (1 hour, 95°C) and stained with an anti-Fosl1 antibody (Santa Cruz) and mounted in Roti-Histokitt II (Roth). β-cell area is the area of insulin stained cells positive in relation to total pancreas surface quantified using osteomeasure software (OsteoMetrics). TUNEL assay was performed according to the manufacturer's instructions (Roche). Images were acquired using a Nikon Eclipse 80i microscope with a Plan-Apo VC 100×/1.4 oil, 20×/0.75 or 2×/0.1 objective lens, equipped with Sony DXC-390P digital camera and NIS-Elements BR2.20 software.

Cell culture

Primary osteoblasts (POBs) were isolated as previously described (Jochum et al., 2000). The adipogenic cell line was generated by culturing POBs for at least 12 passages. Adipocyte differentiation was induced by addition of an adipogenic cocktail (5 µg/ml insulin, 10 µM dexamethasone and 500 µM IBMX) to the confluent cultures. In experiments using the *ER-Fra-1* inducible system, 1 µM of β-estradiol was added to the differentiation medium. Isolation of RNA and staining with Oil Red O was performed on day 0 (confluency) and day 15 (primary mesenchymal cells) or day 21 (cell lines) after stimulation.

Oil Red O staining

Dried cells were fixed in 4% formalin overnight. Cells were stained with Oil Red O solution (0.3% Oil Red O in 60% isopropanol) for 2 hours.

RNA extraction and quantitative PCR

Frozen tissue was homogenized with a Precellys 24 (Peqlab) and RNA isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. RNA was digested with DNaseI and reverse transcribed into cDNA using an oligo d(T) primer. qPCR was performed using SYBR Green I-dTTP (Eurogentec). Samples were analyzed in duplicate and normalized to the level of *Hprt* mRNA. Primer sequences are listed in supplementary material Table S1.

Stimulation

Cells were serum starved overnight (0.5% FBS) and treated with 5 µg/ml insulin or 500 µM IBMX for indicated periods. For dexamethasone response, non-serum starved cells were incubated in presence of 10 µM dexamethasone.

Protein extracts and western blotting

Protein extracts and nuclear extracts were prepared as previously described (David et al., 1998; David et al., 2002). Protein concentrations were measured using Bradford (Bio-Rad). Proteins were resolved on a 10% SDS polyacrylamide gel and transferred to a nitrocellulose membrane. The blots were probed with anti-Fra-1 and anti-C/EBPα (Santa Cruz), anti-phospho-p44/42 MAP Kinase (Thr202/Tyr204) and anti-phospho-CREB (Ser133) (Cell Signaling), anti-α-tubulin (Molecular Probes) and anti-β-actin (Sigma). As secondary antibody, anti-mouse/rabbit IgG HRP conjugate (Promega) was used. Bands were detected by ECL (Pierce).

Serum profiling

Blood was taken from anesthetized mice by cardiac puncture between 2 and 6 p.m. Leptin, insulin and osteocalcin concentrations were measured by ELISA (R&D, Crystal Chem and Tecomedical, respectively). A colorimetric assay (Sigma) was used to quantify serum triglyceride levels, non-esterified fatty acids were measured with a NEFA kit (Wako Diagnostics).

Glucose and insulin tolerance test

For the glucose tolerance test, D(+)-glucose (2 mg/g of body weight) in aqua ad injectabilia was injected intra-peritoneally in 9-week-old females after overnight fasting. The insulin tolerance test was performed with 10-week-old females after 6 hours of fasting; the mice were injected intra-peritoneally with 0.75 U/kg body weight of insulin (Lilly) in 0.9% NaCl. Blood samples were collected from the tail vein and glucose levels were measured at the indicated time points with a glucometer (Ascensia Elite, Bayer).

Immunoprecipitation

For immunoprecipitation, µMACS Protein A Micro Beads and µMACS columns (Miltenyi) were used. Protein was isolated with Triton X-100 lysis buffer (150 mM NaCl, 1% Triton X-100, 50 mM Tris-HCl, pH 8) supplemented with 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride and protease inhibitor cocktail, and precipitations were performed according to the manufacturer's instructions using 2 mg of protein extract. For western blotting, antibodies against C/EBPβ (Santa Cruz), FLAG tag (Cell Signaling) and Jun (Cell Signaling) were used.

Chromatin immunoprecipitation

ChIP experiments were performed with ChIP-IT™ Express kit (Active Motif) using the same antibody as for western blotting.

Luciferase activity measurement

Three fragments of the *Cebpa* promoter (–3359/+5), (–2048/+5) and (–269/+5) were cloned into pGL3-basic luciferase reporter. 0.5 µg of these promoter constructs, 0.15 µg phRL-TK and 0-1 µg pBabe-*Fra1* or empty vector were used for transfection with Lipofectamine 2000 (Invitrogen). Protein was extracted 36 hours after transfection using passive lysis buffer (Promega) and luciferase activity was measured with the Dual-Luciferase Reporter Assay System (Promega) in a Sirius Luminometer (Berthold).

Statistical analysis

Data are presented as means ± s.e.m. The statistic significance was determined by Mann Whitney test or one-way ANOVA (*P<0.05; **P<0.01; ***P<0.001) using GraphPad Prism software.

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