

Fra-2/AP-1 controls adipocyte differentiation and survival by regulating PPAR γ and hypoxia

J Luther^{1,3}, K Ubieta^{1,3}, N Hannemann¹, M Jimenez², M Garcia¹, C Zech¹, G Schett¹, EF Wagner² and A Bozec^{*,1}

Adipocyte cell number is a crucial factor for controlling of body weight and metabolic function. The regulation of adipocyte numbers in the adult organism is not fully understood but is considered to depend on the homeostasis of cell differentiation and apoptosis. Herein, we show that targeted deletion of the activator protein (AP-1)-related transcription factor Fra-2 in adipocytes *in vivo* (Fra-2 ^{Δ adip} mice) induces a high-turnover phenotype with increased differentiation and apoptosis of adipocytes, leading to a decrease in body weight and fat pad mass. Importantly, adipocyte cell numbers were significantly reduced in Fra-2 ^{Δ adip} mice. At the molecular level, Fra-2 directly binds to the PPAR γ 2 promoter and represses PPAR γ 2 expression. Deletion of Fra-2 leads to increased PPAR γ 2 expression and adipocyte differentiation as well as increased adipocyte apoptosis through upregulation of hypoxia-inducible factors (HIFs). These findings suggest that Fra-2 is an important checkpoint to control adipocyte turnover. Therefore, inhibition of Fra-2 may emerge as a useful strategy to increase adipocyte turnover and to reduce adipocyte numbers and fat mass in the body.

Cell Death and Differentiation (2014) 21, 655–664; doi:10.1038/cdd.2013.198; published online 24 January 2014

Alterations in adipose tissue lead to severe metabolic disturbances. During obesity, white adipose tissue expansion is based on both cellular hypertrophy (an increase in adipocyte volume) and hyperplasia (an increase in adipocyte cell number). White adipose tissue becomes hypoxic during obesity that leads to adipose tissue dysfunction.¹ The response of most cells to hypoxia is to induce a program of gene expression that is regulated by hypoxia-inducible factors (HIFs), basic helix-loop-helix/PAS transcription factors consisting of a α -subunit (HIF1 α) and a β -subunit (HIF1 β).² HIF target genes are involved in several physiological processes such as angiogenesis, glucose metabolism and cell survival.³

In accordance, targeted deletion of HIF1 α in adipocytes has an impact on dietary obesity and associated pathologies such as glucose intolerance.^{4,5} However, the role of HIFs during adipocyte differentiation and survival remains defined incompletely to date. Moreover, despite that adipocyte hypertrophy prevails in obesity, the adipocyte number variation in adult age remains under debate.¹ Apoptosis is a normal phenomenon of cell death for the purpose of maintaining homeostasis. The apoptotic molecular machinery such as the mitochondrial and the caspase pathways are present in adipocyte and not different from other cells. It is reported that several adipokines play roles in induction of adipocyte apoptosis.⁶ However, the question remains of how apoptosis is regulated in adipocytes at the cellular and molecular levels.

Mesenchymal stem cells (MSCs) can differentiate into adipocytes following the induction of lineage-specific transcription factors C/EBP β (CCAAT/enhancer binding protein- β) that induce the expression of PPAR γ 2 (peroxisome proliferator-activated receptor- γ 2) and C/EBP α controlling the late stages of adipogenesis.⁷ Another transcription factor family that can influence adipocyte commitment is the activator protein-1 (AP-1) transcription factor complex. This complex consists of a variety of dimers composed of members of the Fos, Jun and activating transcription factor (ATF) families.⁸ Analyses of mice genetically modified for Fos proteins have underlined the important functions of Fos in osteoblast and adipocyte differentiation.⁸ Mice overexpressing Fos-related antigen 1 (Fra-1) or the short isoform of FosB (Δ FosB) show impaired adipocyte differentiation^{9–11} but enhanced osteoblast differentiation.^{12–14}

Interestingly, the second Fra family member, Fos-related antigen 2 (Fra-2), regulates bone mass via affecting HIF expression. Thus, mice overexpressing Fra-2 develop an osteosclerotic phenotype, whereas the absence of Fra-2 leads to a defect of osteoblast differentiation¹⁵ and a giant osteoclast phenotype induced by the activation of HIF1 α .¹⁶ Furthermore, leptin is a transcriptional target of Fra-2,¹⁷ suggesting that Fra-2 may indeed represent an important transcription factor for adipocytes. Herein, we show that Fra-2 deletion in adipocytes affects body and fat pad mass by the regulation of adipocyte number. Mechanistically, Fra-2

¹Department of Internal Medicine 3, University of Erlangen-Nuremberg, Erlangen, Germany and ²Genes, Development and Disease Group, Cancer Cell Biology Program, Spanish National Cancer Centre (CNIO), Madrid, Spain

*Corresponding author: A Bozec, Department of Internal Medicine 3 and Institute of Clinical Immunology, University of Erlangen-Nuremberg, 91054 Erlangen, Germany. Tel: +49 9131 8529002; Fax: +49 9131 8529111; E-mail: aline.bozec@uk-erlangen.de

³These authors contributed equally to this work.

Keywords: AP-1/Fra-2; HIFs; adipocyte; apoptosis

Abbreviations: AP-1, activator protein-1; ATF, activating transcription factor; Bad, Bcl-2-associated death promoter; Bax, Bcl-2-associated X protein; Bcl2, B-cell lymphoma 2; Bcl-xl, B-cell lymphoma-extra large; BNIP3, BCL2/adonovirus E1B 19 kDa protein-interacting protein 3; BNIP3L, BCL2/adonovirus E1B 19 kDa protein-interacting protein 3-like; C/EBP, CCAAT/enhancer binding protein; Fabp4, fatty acid binding protein 4; Fra-1, Fos-related antigen 1; Fra-2, Fos-related antigen 2; Glut, glucose transporter; HIF, hypoxia-inducible factor; IBMX, 3-isobutyl-1-methylxanthine; Inos, nitric oxide synthase; MSC, mesenchymal stem cell; PPAR γ , peroxisome proliferator-activated receptor- γ ; Vegfx, vascular endothelial growth factor α

Received 11.6.13; revised 06.12.13; accepted 06.12.13; Edited by M Annicchiarico-Petruzzelli; published online 24.1.14

regulates transcriptionally PPAR γ 2 expression and adipocyte survival by the modulation of HIF expression.

Results

Inducible adipocyte-specific deletion of Fra-2. To obtain functional data for a role of Fra-2 in the adipose tissue, we generated adipocyte-specific Fra-2 knockout mice. Mice with the *Fabp4-CreERT* allele were crossed with mice carrying *Fra-2* floxed alleles to delete Fra-2 in adipocytes (Fra-2 Δ ^{adip}). As *Fabp4-CreERT* is a tamoxifen-inducible line, we were able to analyze the role of Fra-2 inactivation in adult mice. Injection of tamoxifen was performed at the age of 6 weeks and the analyses were performed 6 weeks later. Immunohistochemical (IHC) staining for Fra-2 of fat tissues showed decreased Fra-2 expression in adipocytes of white and brown adipose tissue, whereas no decrease in expression was found in the spleen (Supplementary Figure 1A). Furthermore, only very low levels of Fra-2 mRNA were expressed in the white and brown adipose tissue of Fra-2 Δ ^{adip} mice (Supplementary Figure 1B), whereas its expression in liver, muscle, spleen and lung was equal to wild-type littermate controls (Supplementary Figure 1B). These results indicate that Fra-2 deletion in Fra-2 Δ ^{adip} mice is specific to the adipose tissue.

Fra-2 deletion in adipocytes decreases body and fat pad weight. We first assessed body and fat pad weight in Fra-2 wild-type and mutant mice. Total body weight and percentage of body fat were significantly decreased in Fra-2 Δ ^{adip} mice compared with wild-type controls in conditions of normal diet (ND) (Figure 1a). Similarly, the ratio of fat pad weight to body weight was significantly lower in Fra-2 Δ ^{adip} mice than in controls (Figure 1b). When mice were fed a high-fat diet (HFD) for a period of 6 weeks for inducing obesity, similar changes were observed: Fra-2 Δ ^{adip} mice showed significantly lower body weight and fat pad weight than wild-type controls (Figures 1a and b).

Low adipocyte number but increased adipocyte size in Fra-2 Δ ^{adip} mice. Further histological analyses of the fat pads of Fra-2 Δ ^{adip} mice and respective controls showed that white adipocyte cell number was significantly decreased in Fra-2 Δ ^{adip} mice compared with controls when mice received ND (Figures 1c and d). Furthermore, analysis of adipocyte cell size showed a significant increase in cell size despite smaller cell number in Fra-2 Δ ^{adip} compared with controls (Figures 1c and d). HFD increased adipocyte size in both mutant and wild-type mice, reflecting hypertrophy of adipocytes that was significantly more pronounced in Fra-2 mutants than controls (Figures 1c and d).

Increased adipocyte apoptosis in Fra-2 Δ ^{adip} mice. To better understand the molecular mechanisms behind the fat phenotype observed in Fra-2 Δ ^{adip} mice and to determine why adipocyte numbers are low in Fra-2 mutants, we analyzed the proliferation and the apoptosis status of the fat pad. No differences could be detected in proliferation of adipocyte as assessed by Ki67 staining (data not shown). However, apoptosis assessed by TUNEL assay, annexin V flow

cytometry, cleaved caspase 3 staining and western blot revealed an increased rate of apoptosis in the fat pad of Fra-2 Δ ^{adip} mice (Figures 2a and b and Supplementary Figure 2A), suggesting that low fat pad mass and low adipocyte numbers in Fra-2 Δ ^{adip} mice depend on increased adipocyte apoptosis.

Increased hypoxia and HIF activation in adipose tissue of Fra-2 Δ ^{adip} mice. Our previous finding showing that Fra-2 is involved in the regulation of osteoclast apoptosis and that this effect depends on HIF1 α activation¹⁶ prompted us to investigate whether the increased apoptosis in Fra-2 Δ ^{adip} mice was based on HIF activation and tissue hypoxia. Indeed, *in vivo* analysis revealed increased hypoxia in the fat pad tissue of Fra-2 Δ ^{adip} mice compared with controls (Supplementary Figure 2B). IHC stainings of fat pad tissue showed an increased number of HIF1 α - and HIF2 α -positive cells in Fra-2 Δ ^{adip} mice compared with controls (Supplementary Figure 2B and Figure 2c). In addition, the molecular analyses of HIFs by quantitative PCR (qPCR) revealed increased expression of *HIF1 α* and *HIF2 α* in Fra-2 Δ ^{adip} fat pad, whereas no differences in *HIF1 β* levels were detected (Figure 2d). Moreover, expression of several HIF target genes such as *Glut1* (glucose transporter), *Inos* (nitric oxide synthase), *Vegfx* (vascular endothelial growth factor α) and *BNIP3* (*BCL2/adenovirus E1B 19 kDa protein-interacting protein 3*) was increased in Fra-2 Δ ^{adip} fat pads, whereas others such as *Bcl2* (*B-cell lymphoma 2*) and *BNIP3L* (*BCL2/adenovirus E1B 19 kDa protein-interacting protein 3-like*) were not altered (Figure 2e). These findings support the concept that Fra-2 regulates hypoxia and HIF expression in the adipose tissue that affects adipocyte numbers and fat pad size via regulation of apoptosis.

Hypoxia drives expression of HIFs and apoptosis-related genes in adipocytes. In order to determine whether hypoxia could induce apoptosis in a cell autonomous manner, adipocytes isolated from fat pads were placed in hypoxic chambers and analyzed for HIFs and HIF target gene expression. In addition, expression of apoptosis-related genes was analyzed. As shown in Figures 3a and b, *HIF1 α* , *HIF2 α* and their target genes *Inos* and *BNIP3* were increased in adipocytes placed in hypoxic chambers for 24 h. Moreover, *Bad* (*Bcl-2-associated death promoter*) and *Bax* (*Bcl-2-associated X protein*) mRNA levels in adipocytes increased within 12 h of hypoxia (Figure 3c), suggesting that hypoxic conditions lead to an imbalance of the Bcl-2 family in favor of the apoptotic pathway.

To determine whether apoptosis was dependent on the Bcl-2 family, overexpressing plasmids for B-cell lymphoma-extra large (Bcl-xl) were transfected in primary adipocyte exposed to normoxia or hypoxia 1% for 24 h. The annexin V flow cytometry analyses revealed increased apoptosis in adipocyte culture under hypoxia that can be rescued by the overexpression of Bcl-xl (Figure 3d), suggesting that the apoptosis induced by hypoxia in adipocytes is dependent on the Bcl-2 family.

Fra-2 regulates HIFs in the adipocyte in a cell autonomous manner. In order to determine whether

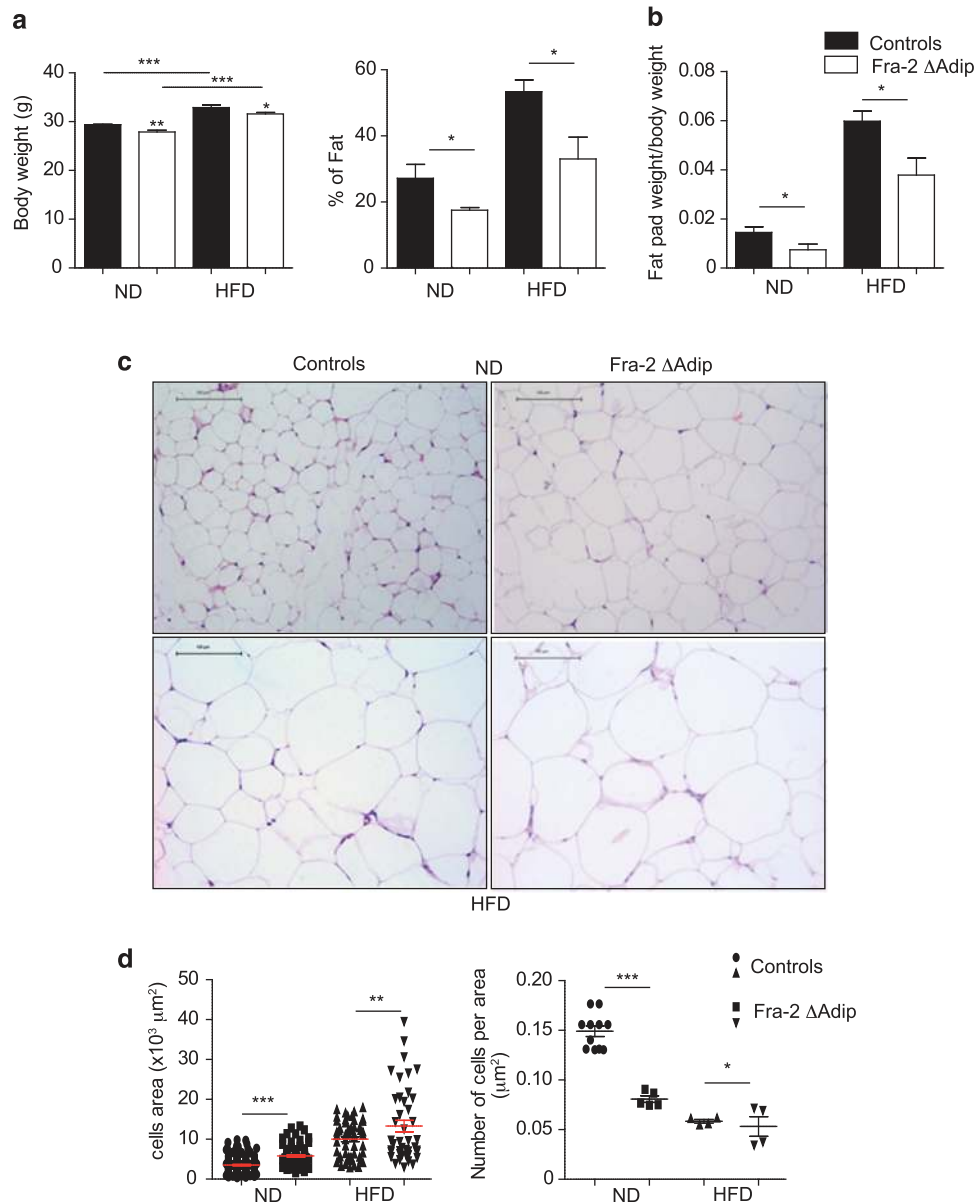


Figure 1 Fra-2 Δ adip mice have decreased body weight and adipocyte number. (a) Body weight of mice and fat content from controls and Fra-2 Δ adip mice at 6 weeks after tamoxifen injections with mice receiving normal diet (ND) or high-fat diet (HFD) ($n=4-5$). (b) Fat pad per body weight of Fra-2 Δ adip mice and littermate controls at 6 weeks after tamoxifen injections with mice receiving ND or HFD ($n=4-5$). (c) Hematoxylin and eosin staining of fat pad tissue from Fra-2 Δ adip mice and littermate controls at 6 weeks after tamoxifen injection receiving ND or HFD. Magnification $\times 20$, bars = 100 μm . (d) Adipocyte cells area and number of adipocyte per area of Fra-2 Δ adip mice and littermate controls at 6 weeks after tamoxifen injections with mice under ND or HFD ($n=4-10$). Bars represent mean values \pm S.D. Statistical analyses: * $P<0.05$, ** $P<0.01$, *** $P<0.001$

Fra-2 affects HIF expression in adipocytes, we analyzed HIF expression and HIF target gene expression on adipocyte-derived stem cells isolated from fat pads of Fra-2 Δ adip and wild-type controls. Interestingly, *HIF1 α* , *HIF2 α* and their targets *Vegf α* and *BNIP3* were significantly increased in the Fra-2 Δ adip cultures at day 7 of differentiation (Figure 3e). These data suggest that Fra-2 deletion leads to increased HIFs and HIF target gene expression in the adipose tissue *in vivo* and *in vitro*.

In order to determine whether apoptosis induced by hypoxia was dependent on HIF1 α or HIF2 α or both, knockdown of

HIF1 α and HIF2 α was performed *in vitro* by transfection of primary cells isolated from Fra-2 Δ adip and control fat pads. Interestingly, the knockdown of HIF1 α or HIF2 α was able to rescue the increased level of apoptosis observed in Fra-2 Δ adip adipocyte cultures under hypoxic conditions (Figure 3f). Moreover, to determine whether similar observations could be found *in vivo*, i.p. and hydrodynamic injections of shRNA against HIF1 α or HIF2 α were performed in Fra-2 Δ adip and littermate control mice. Interestingly, in wild-type condition, the knockdown of HIF1 α and HIF2 α was able to decrease the basal level of apoptosis in perigonadal fat tissue (Figure 3g).

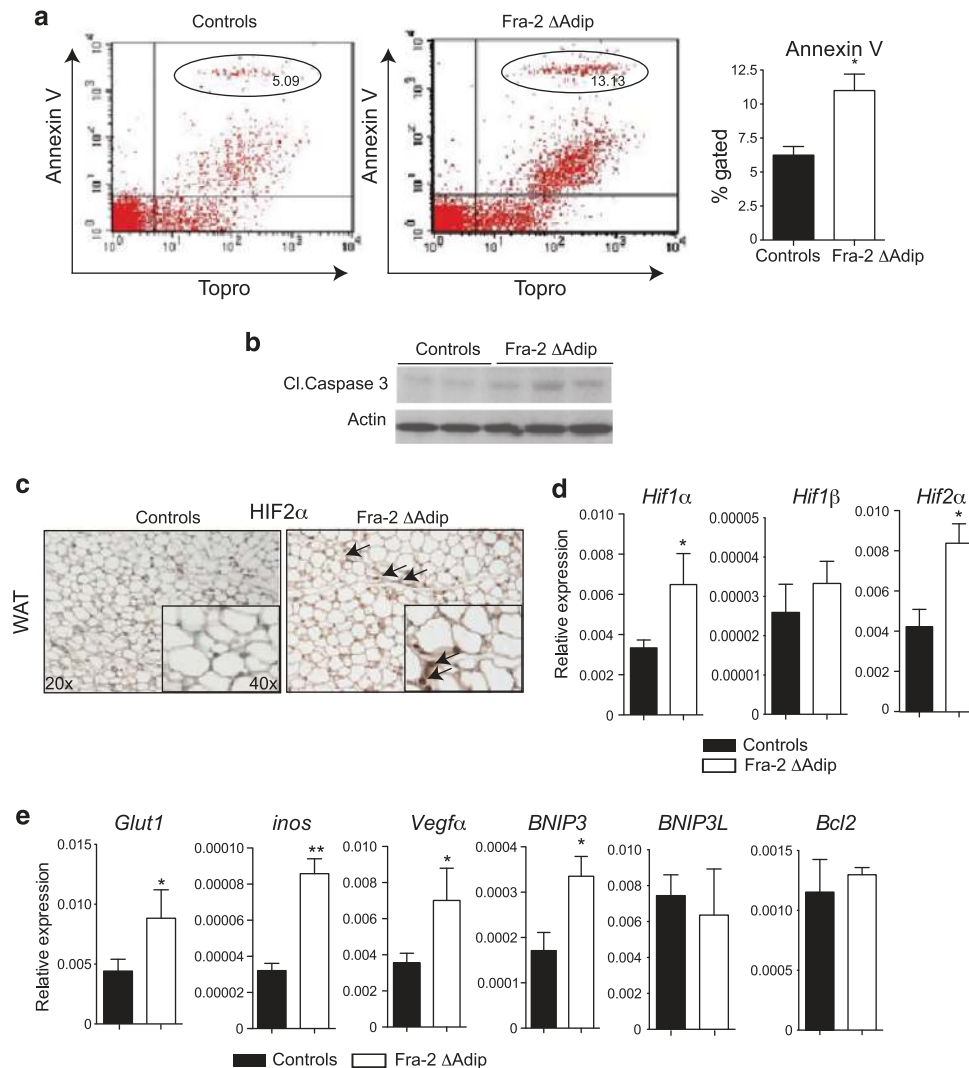


Figure 2 Fra-2 Δ adip fat pads are hypoxic and show higher apoptosis rates. (a) Quantification of apoptotic cells by FACS in perigonadal fat from Fra-2 Δ adip and littermate control mice at 6 weeks after tamoxifen injection ($n=4$). (b) Western blot for cleaved caspase-3 in perigonadal fat from Fra-2 Δ adip and littermate control mice at 6 weeks after tamoxifen injection. (c) HIF2 α staining in Fra-2 Δ adip and littermate controls fat pad at 6 weeks after tamoxifen injection. Magnification $\times 20$, insert $\times 40$. Black arrows indicate HIF-positive cells. (d) Real-time PCR analyses of *HIF1 α* , *HIF1 β* and *HIF2 α* in Fra-2 Δ adip and littermate controls fat pad at 6 weeks after tamoxifen injection ($n=4$). (e) Real-time PCR analyses of HIFs target genes *Glut1*, *Inos*, *Vegf α* , *BNIP3*, *BNIP3L* and *Bcl2* in Fra-2 Δ adip and littermate controls fat pad at 6 weeks after tamoxifen injection ($n=4$). Bars represent mean values \pm S.D. Statistical analyses: * $P<0.05$, ** $P<0.01$

In addition, no difference in apoptosis could be detected anymore in Fra-2 Δ adip and littermate control perigonadal fat after knockdown of HIF1 α or HIF2 α (Figure 3g), suggesting that HIF1 α and HIF2 α regulate apoptosis in white adipose tissue.

Increased PPAR γ 2 expression as the link between Fra-2 and HIF. To explain the molecular mechanisms of how Fra-2 regulates HIFs, we checked for differences in the regulation of genes involved in adipocyte differentiation among Fra-2 Δ adip mice and controls. *In vivo*, no significant changes could be detected in the expression of *C/ebp β* , *C/ebp α* , *C/ebp δ* , *Glut4*, *PPAR α* , *PPAR γ 1*, *adiponectin* and *resistin* (Supplementary Figure 2C and D). Interestingly, increase in mRNA levels of *PPAR γ total* and *PPAR γ 2* as well

as *Fabp4* (fatty acid binding protein 4) was observed in the fat pad from Fra-2 Δ adip mice fed ND and HFD as compared with respective wild-type littermate controls (Figure 4a and Supplementary Figure 2C). IHC analysis for PPAR γ expression confirmed its upregulation at the protein levels (Supplementary Figure 2E).

To determine whether the same phenotype could be observed in another white adipose tissue, subcutaneous fat from Fra-2 Δ adip and wild-type littermate control mice was analyzed. Indeed, similar phenotype, with increased adipocyte size, increased apoptosis, increased hypoxic markers and increased *Fabp4* and *PPAR γ 2* mRNA levels, was observed in the subcutaneous white fat from Fra-2 Δ adip mice when compared with littermate controls (Supplementary Figure 3), suggesting a common pathway in different white fat tissues.

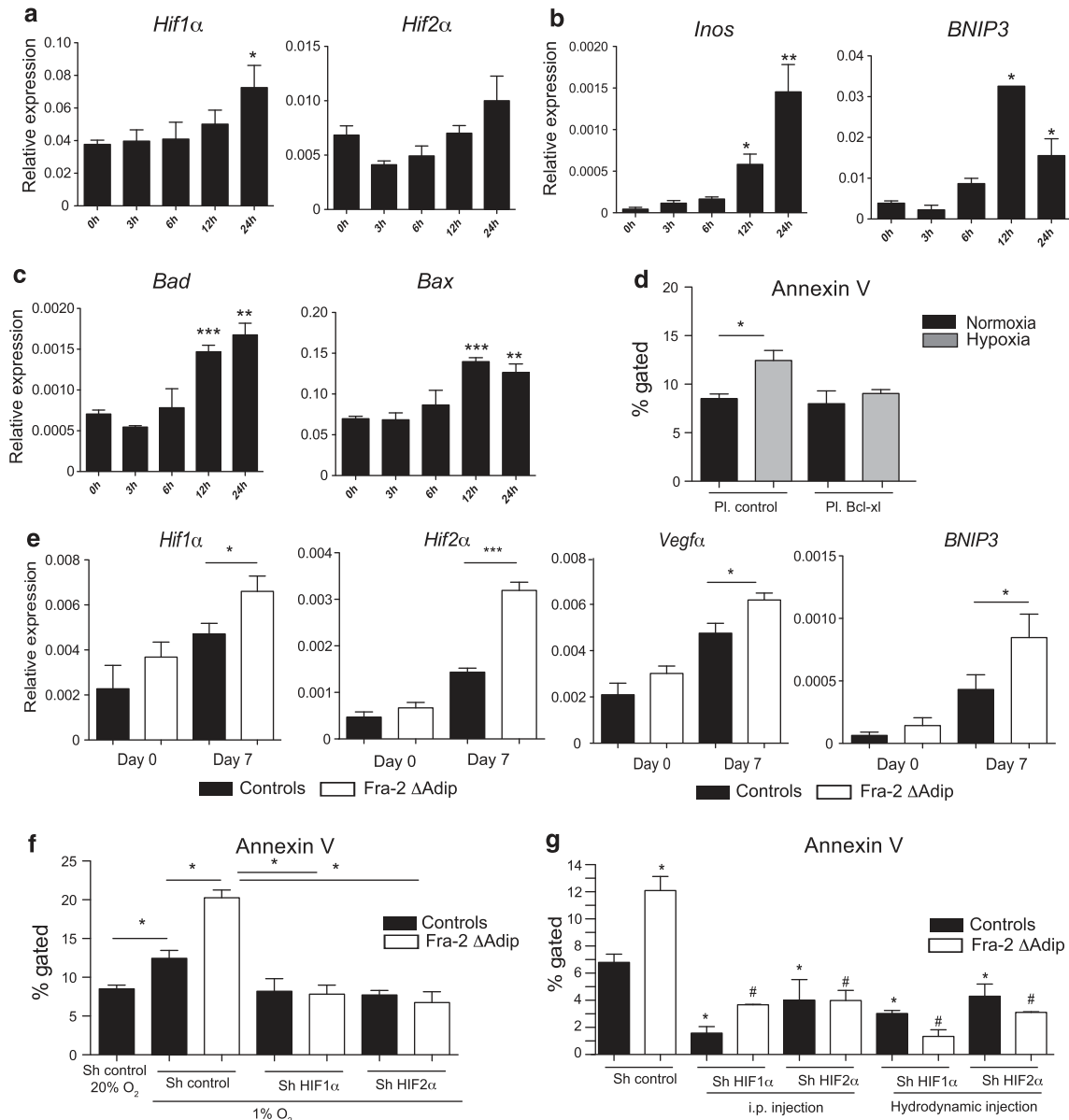


Figure 3 Hypoxia drives adipocyte apoptosis and Fra-2 regulates HIF expression in cell autonomous manner. (a and b) Real-time PCR analyses of *HIF1α* and *HIF2α* (a) and HIF targets *Inos* and *BNIP3* (b) in primary adipocytes placed in hypoxic chambers for 0, 3, 6, 12 and 24 h ($n=3$). (c) Real-time PCR analyses of *Bad* and *Bax* in primary adipocytes placed in hypoxic chambers for 0, 3, 6, 12 and 24 h ($n=3$). (d) Quantification of apoptotic cells by FACS in primary adipocytes transfected with control or overexpressing Bcl-xl plasmid under normoxia or hypoxia for 24 h ($n=3$). (e) Real-time PCR analyses of *HIF1α*, *HIF2α*, *Vegfa* and *BNIP3* in adipocytes of Fra-2^{ΔAdip} and controls cultures at days 0 and 7 of *in vitro* differentiation ($n=3$). Bars represent mean values \pm S.D. Statistical analyses: * $P<0.05$, ** $P<0.01$, *** $P<0.001$. (f) Quantification of apoptotic cells by FACS in primary adipocytes isolated from Fra-2^{ΔAdip} and controls fat pad and transfected with Sh control or Sh plasmid against HIF1α or HIF2α under hypoxia for 24 h ($n=3$). (g) Quantification of apoptotic cells by FACS in perigonadal tissue isolated from Fra-2^{ΔAdip} and control mice after i.p. or hydrodynamic injection with Sh control or Sh plasmid against HIF1α or HIF2α ($n=3$). * P -values compared with controls. # P -values compared with Fra-2^{ΔAdip} adipocytes. Statistical analyses: * or # $P<0.05$, ** $P<0.01$, *** $P<0.001$

Next, adipocyte differentiation and markers were analyzed when adipocyte stem cells from Fra-2^{ΔAdip} mice and respective controls were isolated from the fat pad and differentiated *in vitro*. Whereas *C/ebpα*, *C/ebpβ*, *C/ebpδ* and *PPARγ1* were not different among cells from mutant and control mice, the mRNA expression of Fra-2 was decreased and mRNA expression of *adiponectin*, *Fabp4*, *total PPARγ*, and *PPARγ2* was significantly increased in Fra-2^{ΔAdip} cells at day 7 of differentiation

(Supplementary Figure 4 and Figure 4b). In addition, cell differentiation was significantly increased in Fra-2^{ΔAdip} cultures compared with controls, potentially explaining the increased adipocyte size observed in Fra-2^{ΔAdip} mice (Figure 4c).

To determine whether PPARγ2 can regulate HIFs in adipocytes, inhibition of PPARγ2 activity by BADGE was performed (Figure 4d). Treatment with BADGE not only led to a decrease of the adipocyte marker *Fabp4* (Figure 4d),

but also to significant suppression of HIF2 α expression, whereas the expression of HIF1 α remained unchanged (Figure 4e).

Fra-2 leads to direct transcriptional repression of PPAR γ 2. As PPAR γ 2 essentially controls adipocyte differentiation as well as HIF activation in adipocytes,¹⁸

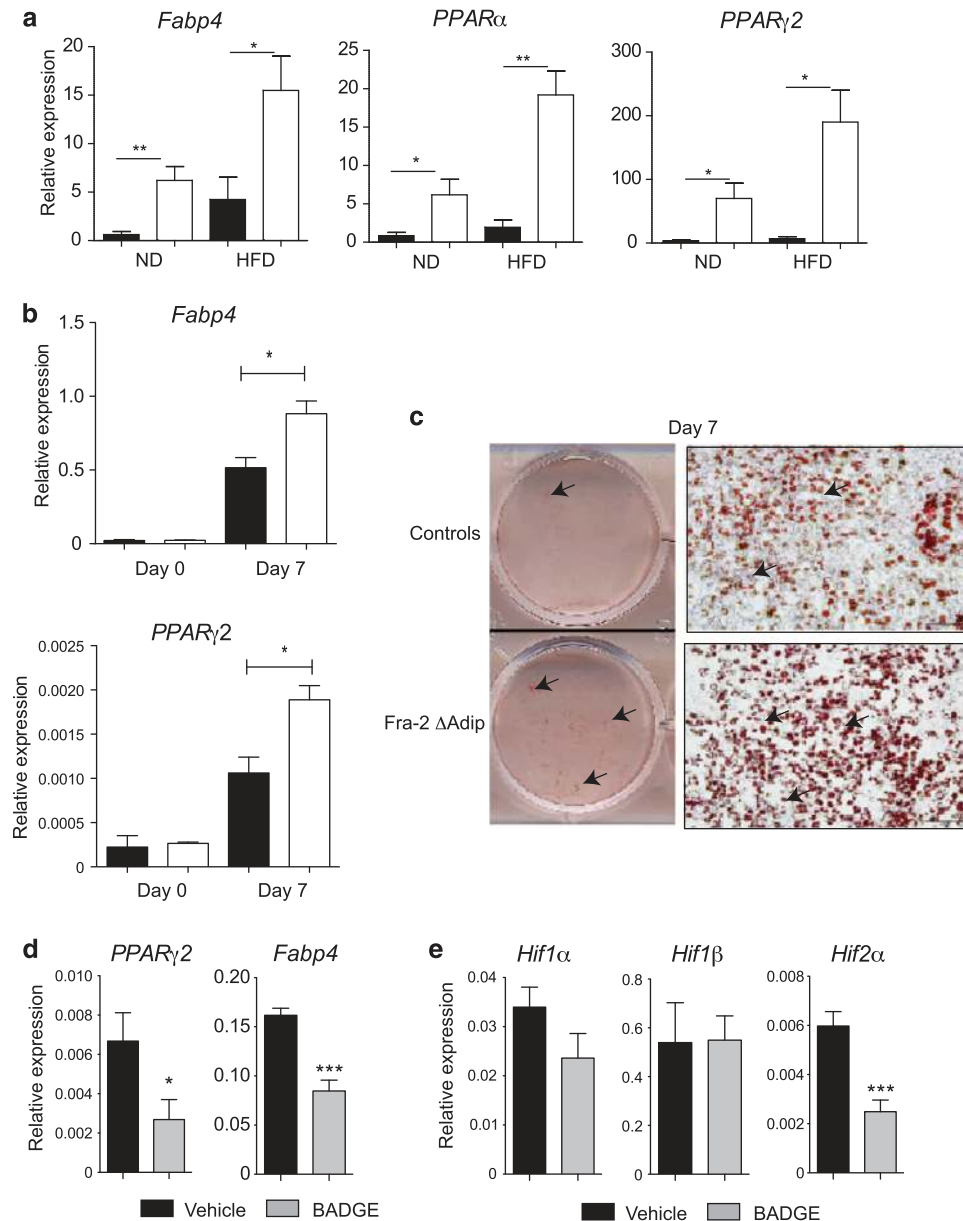
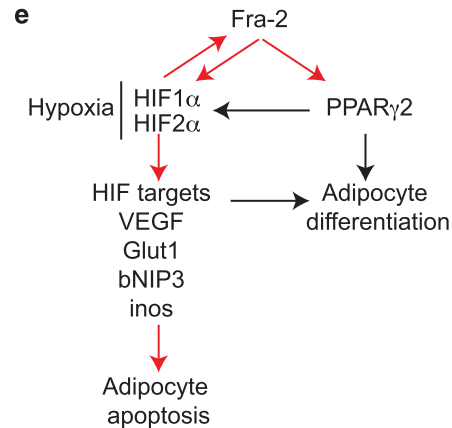
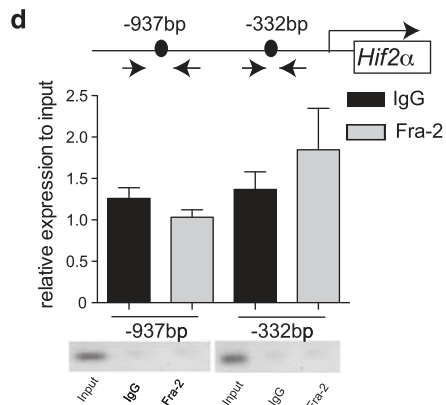
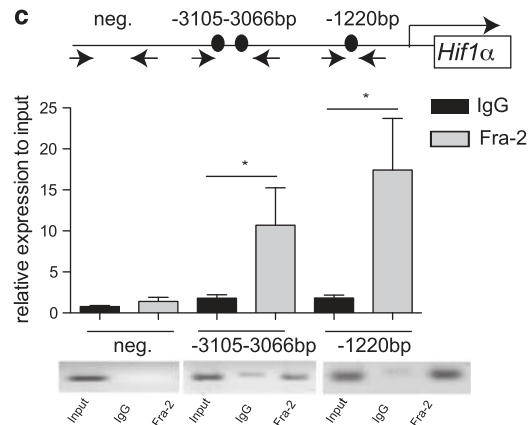
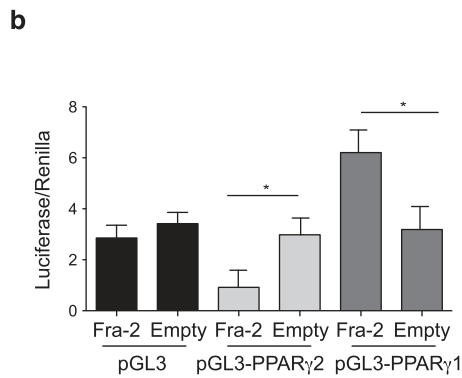
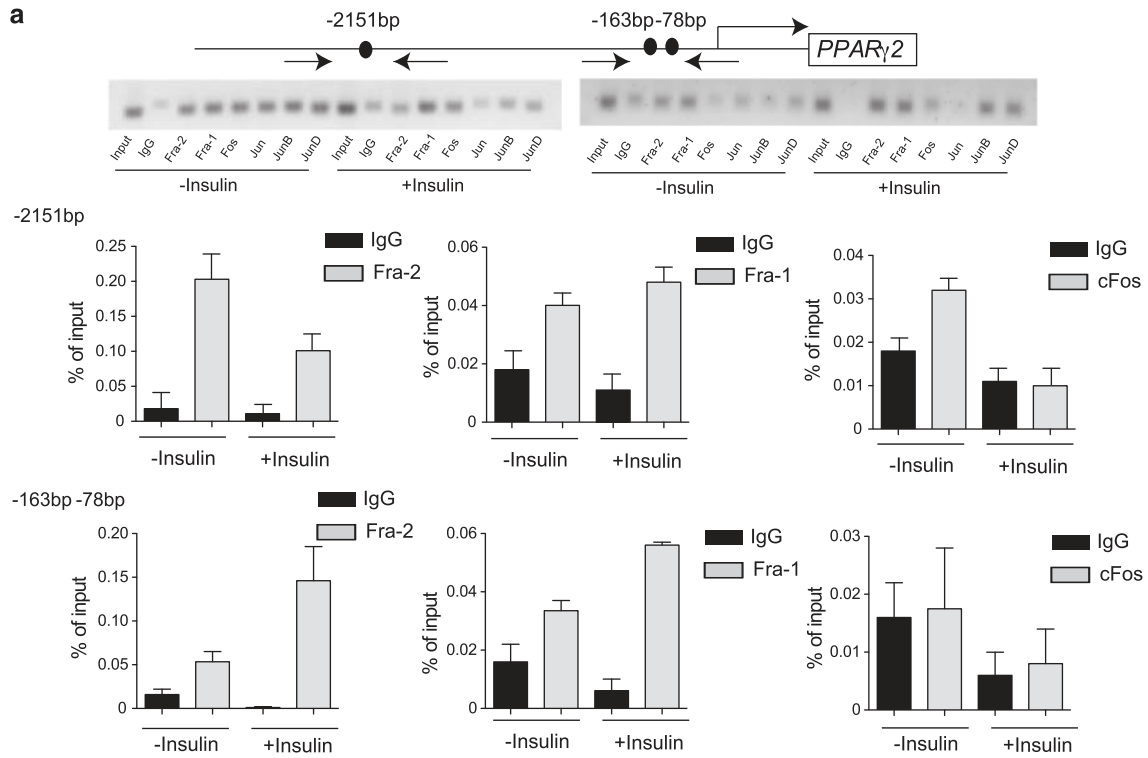


Figure 4 PPAR γ 2 is regulated by Fra-2 and can regulate HIF expression. (a) Real-time PCR analyses of *Fabp4*, *PPARα* and *PPARγ2* in fat pad tissue from Fra-2 Δ adip mice and littermate controls at 6 weeks after tamoxifen injections with mice receiving normal diet (ND) or high-fat diet (HFD) ($n = 4-5$). (b) Real-time PCR analyses of *Fabp4* and *PPARγ2* in adipocytes of Fra-2 Δ adip and controls cultures at days 0 and 7 of *in vitro* differentiation ($n = 3$). (c) Oil Red O staining of adipocytes derived from fat pad tissue of Fra-2 Δ adip mice and littermate controls at day 7 of *in vitro* differentiation. Bars represent 200 μ m. Arrows indicate adipocytes. (d and e) Real-time PCR analyses of *PPARγ2*, *Fabp4* (d) and *HIF1α*, *HIF1β* and *HIF2α* (e) in primary adipocytes treated with vehicle or BADGE ($n = 3$). Bars represent mean values \pm S.D. Statistical analyses: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Figure 5 PPAR γ 2 and HIF1 α are transcriptional targets of Fra-2. (a) Chromatin immunoprecipitation (ChIP) for PPAR γ 2 promoter. Arrows indicate primers amplifying fragments for the TRE elements. Chromatin isolated from 3T3L1 cells with insulin treatment for 24 h was immunoprecipitated with IgG and AP-1 antibodies. Real-time PCR analyses and loading of the gel are shown ($n = 2$). (b) Luciferase assay analyses of Fra-2 on PPAR γ 1 and PPAR γ 2 promoter; empty plasmids are shown for the control experiment. (c and d) ChIP for HIF1 α (c) and HIF2 α (d) promoters. Arrows indicate primers amplifying fragments for the TRE elements. Chromatin from 3T3L1 cells was immunoprecipitated with IgG and Fra-2 antibodies. Real-time PCR analyses and loading of the gel are shown ($n = 3$). (e) Scheme of Fra-2 actions in adipocytes. Red arrows indicate findings from the manuscript and black arrows indicate knowledge from the literature. Bars represent mean values \pm S.D. Statistical analyses: * $P < 0.05$



we reasoned that PPAR γ 2 was the key gene regulated by Fra-2 in adipocytes. We therefore assessed Fra-2 binding to the PPAR γ 2 promoter by chromatin immunoprecipitation (ChIP) analysis in 3T3L1 cell lines treated with insulin for 24 h. As shown in Figure 5a, specific primer pairs were used to amplify DNA fragments containing the AP-1 consensus TRE element. Fos and Jun binding to the PPAR γ 2 promoter was observed in two potential sites (Figure 5a and Supplementary Figure 5A). Interestingly, at the sites –163/–78 bp, the binding of Fra-2 and Fra-1 is increased after insulin treatment (Figure 5a), suggesting that Fra-2 can bind the PPAR γ 2 promoter and regulate its expression following insulin treatment.

Moreover, in DNA co-transfection experiments, Fra-2 expression vector decreased the activity of a luciferase reporter containing the human PPAR γ 2 promoter fragment including the two sites –163/–78 bp (Figure 5b). In contrast, an increase in the luciferase activity could be detected when DNA co-transfection experiments were performed with PPAR γ 1 promoter fragment (Figure 5b). These findings, together with the altered expression of PPAR γ 2 in Fra-2^{Adip} mutants, indicate that PPAR γ 2 is a direct transcriptional target of Fra-2.

Fra-2 and PPAR γ 2 can regulate HIFs and vice versa. In order to determine whether HIF1 α and HIF2 α could be transcriptionally regulated by Fra-2, we performed ChIP analysis in 3T3L1, on AP-1 consensus elements found in both promoters. Interestingly we could find that Fra-2 was able to bind the HIF1 α promoter at two putative TRE sequences, –3105/–3066 and –1220 bp, before the starting codon (Figure 5c), whereas no binding of Fra-2 could be detected on the HIF2 α promoter (Figure 5d), suggesting a direct transcriptional activation of HIF1 α by Fra-2.

To understand how HIF2 α can be regulated, we performed the ChIP for PPAR γ on the previously analyzed sequences. No PPAR γ binding could be detected on HIF1 α promoter (Supplementary Figure 5B). Surprisingly PPAR γ could bind the HIF2 α promoter at the site –937 bp (Supplementary Figure 5C). These results suggest that Fra-2 and PPAR γ can regulate HIF1 α and HIF2 α respectively.

To determine whether HIFs can regulate Fra-2 as a retro-control mechanism, its mRNA levels were analyzed in 3T3L1 cells exposed to hypoxia. Interestingly, Fra-2 mRNA level was upregulated in 3T3L1 cell culture in hypoxic conditions (Supplementary Figure 6), suggesting a compensatory mechanism on Fra-2 induced by hypoxia in adipocytes.

Discussion

The results presented in this study reveal a novel function of Fra-2/AP-1 in adipocytes, that is, to regulate fat pad mass and adipocyte number. Mechanistically, Fra-2 inhibits the expression of PPAR γ 2, a key regulator of adipocyte differentiation and homeostasis. By repressing PPAR γ 2, Fra-2 also represses HIFs that control hypoxia and adipocyte survival (see scheme in Figure 5e). The control of adipocyte numbers has been a matter of uncertainty since many years.¹ Here we

found a new pathway that is able to determine adipocyte number in adult age by the regulation of Fra-2 and HIFs.

It can be hypothesized that the regulation of the balance between adipocyte differentiation and death determines adipocyte numbers in the fat tissue. Differentiation of mesenchymal cells into adipocytes involves a cascade of transcription factors such as C/EBP α and C/EBP β that in turn activate PPAR γ , an essential gene for the activation of several adipocyte-specific genes.¹⁹ In addition, AP-1 proteins, such as Fra-1 and Δ FosB, have also been shown to regulate adipogenesis through C/EBP α or C/EBP β expression respectively, thereby decreasing adipocyte differentiation.^{10,11} Moreover, Fra-2 was shown to regulate leptin expression in adipocytes and osteocalcin in osteoblasts, both of which are involved in the control of body weight and fat distribution.^{15,17} By using *Fabp4-CreERT* tamoxifen-inducible line, we were able to analyze the role of Fra-2 inactivation in adult mice. The *Fabp4-Cre* lines were known to potentially have recombination in nonendothelial and nonmyocyte cells in the skeletal muscle.²⁰ Despite no difference in Fra-2 levels in other organs in the mutant mice, we could not exclude indirect effects induced by unwanted deletion. However, our biochemistry and *in vitro* culture studies directly link Fra-2 expression to adipogenesis by the transcriptional inhibition of PPAR γ 2 expression.

PPAR γ 2 is not only a master regulator of adipogenesis, but can also display a role in apoptotic signal transduction in adipocytes.⁶ Adipocytes express several molecules of the intrinsic and extrinsic apoptotic pathways. An excess of apoptosis can lead to lipodystrophic metabolic changes.²¹ However, induction of moderate amounts of adipocyte apoptosis in obese mice has shown metabolic improvement,²² suggesting that modulation of fat homeostasis by regulating differentiation and apoptosis might represent an instrument for controlling fat pad mass in the body. Interestingly, PPAR γ can have proapoptotic functions in adipocytes. Thus, PPAR γ is directly involved in the leptin-induced adipocyte apoptosis signal pathway.²³ Our observation that increased PPAR γ expression in the absence of Fra-2 not only enhanced adipocyte differentiation but also apoptosis support this concept.

During hypoxia and induction of HIF1 α , an intricate balance exists between factors that induce or counteract apoptosis. Previous studies of gain and loss of function of HIF1 α in adipocytes have revealed its role during adipocyte differentiation and diabetes.^{24,25} In addition, HIF2 α , which has independent functions of HIF1 α , increases during adipogenesis *in vitro*, indicating that its upregulation is necessary for execution of adipogenesis and maintenance of mature adipocyte functions.²⁶ Our results confirmed that increased HIF1 α and HIF2 α expression correlated with a cell-autonomous increase of adipocyte differentiation. In addition, we show that hypoxia and increased HIF expression induced adipocyte apoptosis that was dependent on Bcl-xl. Notably, HIF1 α can directly be regulated by Fra-2 and HIF2 α expression that, in turn, is directly regulated by PPAR γ , providing a link between adipocyte differentiation and apoptosis. Moreover, the alteration of Fra-2 expression by hypoxia as a feedback loop provides further insight into the regulation of adipocyte turnover.

In summary, our data add a new role of Fra-2 in adipose tissue homeostasis by regulating PPAR γ 2 and HIF expression. Modulation of Fra-2 expression in mesenchymal cells may emerge as potent tool to control adipocyte number in the body and may open new avenues to manage obesity and metabolic disease.

Materials and Methods

Mice. The generation of *Fra-2* floxed mice is described elsewhere.^{27,28} *Fabp4-CreERT* mice were described previously.²⁹ All mice were maintained on a mixed 129/C57Bl6 background. Littermate mice were used as controls in this study. Genotyping primers were as follows: Fra-2 lox forward: 5'-GAGGGAGTTGGGATAGAGTGGTA-3', Fra-2 lox reverse: 5'-GGACAGCAGGTGAGGAGTAGTA-3', Cre forward: 5'-CCAGAGACGGAAATCCATCGCTCG-3' and Cre reverse: 5'-CGGTCGATGCAACGAGTGATGAGG-3'. To delete Fra-2 *in vivo*, mutant and control mice were injected intraperitoneally with 1 mg tamoxifen (Sigma-Aldrich Chemie GmbH, München, Germany) for 5 consecutive days.

Intraperitoneal and hydrodynamic injection of shRNA HIFs. To downregulate the expression of HIF1 α and HIF2 α *in vivo*, intraperitoneal and hydrodynamic intravenous injections were performed with 200 μ l or 1 ml respectively with shRNA-HIF1 or shRNA-HIF2. After 24 h for the i.p. experiment and 72 h for the hydrodynamic experiment, mice were killed and fat pad adipocytes apoptosis was analyzed.

Histological analysis and immunohistochemistry. Mice were killed by CO $_2$ asphyxia and tissues were fixed in 3.7% PBS-buffered formaldehyde. For histological analysis, tissues were embedded in paraffin. Then, 5 μ m sections of white adipose tissue and 1 μ m sections of brown adipose tissue and spleen were used for histological analyses.

Immunohistochemical stainings for Fra-2, HIF1 α , HIF2 α (Abcam plc, Cambridge, UK), cleaved Caspase 3 (Cell Signaling Technology, Inc., Danvers, MA, USA) and PPAR γ (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) were performed on deparaffinized sections using Proteinase K as unmasking solution and according to the manufacturer's instructions for the Vectastain KIT (Vector Laboratories, Inc., Burlingame, CA, USA). TUNEL assay was performed according to the manufacturer's instructions using the *In Situ* cell death Detection kit (Roche Diagnostics Deutschland GmbH, Mannheim, Germany). Fluorescein is detected by anti-fluorescein antibody Fab fragments from sheep, conjugated with horseradish peroxidase (POD) (Roche Diagnostics Deutschland GmbH) and detected by DAB (Dako, Hamburg, Germany).

Apoptosis analyses by flow cytometry. Apoptosis and necrosis were quantified in adipocytes isolated from fat pad tissue, by Annexin V-FITC (Invitrogen, Life Technologies GmbH, Darmstadt, Germany) staining in the presence of TO-PRO-3 Iodide (Invitrogen). Adipocytes were incubated for 20 min at room temperature with 200 μ l Annexin V-FITC in Annexin-binding buffer, washed and resuspended in 200 μ l Annexin-binding buffer together with TO-PRO-3 Iodide and measured by flow cytometry.

Hypoxia analysis. Hypoxia stainings were performed using the hypoxiKit (Chemicon, Millipore, Billerica, MA, USA) according to the manufacturer's instructions.

RNA isolation and reverse transcription. Total RNA was isolated with the TRIzol or TriFast protocol (Invitrogen and Peqlab Biotechnologie GmbH, Erlangen, Germany, respectively). cDNA synthesis was performed using 0.50–2 μ g RNA with the cDNA Reagents from Applied Biosystems (Life Technologies GmbH).

Real-time PCR analyses. The qPCR reactions were performed using SYBR Green (Eurogentec Deutschland GmbH, Köln, Germany). Primers for genes analyses and primers for ChIP analyses used for real-time PCR are available upon request. The comparative CT method was used to quantify the amplified fragments. RNA expression levels were analyzed in duplicate and normalized to actin as housekeeping gene, whereas chromatin-bound fragments were normalized to the same fragments amplified from input chromatin.

Cell culture. The adipogenic cells were generated by culturing adipocyte stem cells isolated from fat pad tissue. Fra-2 deletion *in vitro* was induced by treating the cells with 3 μ g/ml tamoxifen for 2 days. Adipocyte differentiation was induced by addition of 5 μ g/ml insulin, 1 μ M dexamethasone and 500 μ M 3-isobutyl-1-methylxanthine (IBMX) to the confluent cultures.

Adipocyte stem cells isolated from fat pad tissue were cultured in 12-well plates until 70–80% of density. Cells were transfected with 1 μ g of shRNA HIF1 α , shRNA HIF2 α or overexpressing Bcl-xl plasmid diluted in the appropriate amount of Lipofectamine Reagent (Invitrogen). The transfected cells were incubated at 37 °C and 5% of CO $_2$. After 48 h, cells were placed in hypoxic chambers (1% O $_2$) for 24 h.

In 'hypoxic' experiments, cells were placed in hypoxic chambers (1% O $_2$) for 24 h.

In experiments using BADGE (Sigma), inhibitor of PPAR γ , the cells were treated with the adipogenic cocktail containing 100 μ M BADGE for 7 days. Medium was changed daily. Isolation of RNA and staining with Oil Red O was performed on day 0 (confluency) and day 7 after stimulation.

Oil Red O staining. Cells were fixed in 3.7% PBS-buffered formaldehyde for 1 h. Cells were stained with Oil Red O solution (0.3% Oil Red O in 60% isopropanol) for 2 h.

Chromatin immunoprecipitation. PPAR γ and HIF promoter analyses were performed by 'Tfsearch' web site (<http://www.cbrc.jp/research/db/TFSEARCH.html>).

For ChIP analyses, 3T3L1 cells were stimulated or not with 5 μ g/ml insulin for 24 h. ChIP was performed using the ChIP-IT Express kit (Active Motif, La Hulpe, Belgium) with antibodies against Fra-2, Fra-1, cFos, JunB, JunD (Santa Cruz Biotechnology, Inc.), cJun (Cell Signaling Technology, Inc.) and PPAR γ (Santa Cruz Biotechnology, Inc.), and control experiments were performed with IgG antibody. Bound fragments were quantified by real-time PCR.

Reporter assay. The promoter-reporter vector for human PPAR γ 1 and 2 were provided by J Auwerx (Ecole Polytechnique Fédérale in Lausanne, Switzerland). Control experiments were performed with a pGL3 empty plasmid. 6 \times 10 4 Hek cells/well were plated in 24-well dishes. Then, 1 μ g of the luciferase reporter construct, 0.2 μ g of the *Renilla* internal control (phRG-TK, Promega, Mannheim, Germany) and 1 to 2 μ g of empty or AP-1 expression vector were co-transfected in triplicate using lipofectamine (Invitrogen). Protein was isolated 24 h after transfection using Passive Lysis Buffer (Promega). Luciferase activity was quantified using self-made solutions.

Statistical analysis. All experiments were repeated at least three times and done in triplicate. Statistical analysis was performed using Student's *t*-test. **P*<0.05, ***P*<0.01 and ****P*<0.001 were accepted as significant. Data are shown as mean and the error bars represent S.D.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements. We are very grateful to Dr. Didier Soulat and Dr. Agi Grigoriadis for critically reading the manuscript and for helpful comments. We also thank Professor Backert and Dr. Jantsch for providing technical helps. This work was supported by Emmy Noether (DFG) grant to AB, and KU was funded by a Marie Curie international fellowship.

Author contributions

Julia Luther, Kenia Ubieta, Nicole Hannemann, Maria Jimenez, Mireia Garcia, Christine Zech and Aline Bozec collected the data; Georg Schett, Erwin F Wagner and Aline Bozec designed the study and wrote the manuscript.

1. Sun K, Kusminski CM, Scherer PE. Adipose tissue remodeling and obesity. *J Clin Invest* 2011; **121**: 2094–2101.
2. Huang LE, Gu J, Schau M, Bunn HF. Regulation of hypoxia-inducible factor 1 α is mediated by an O $_2$ -dependent degradation domain via the ubiquitin-proteasome pathway. *Proc Natl Acad Sci USA* 1998; **95**: 7987–7992.
3. Semenza GL. HIF-1 and mechanisms of hypoxia sensing. *Curr Opin Cell Biol* 2001; **13**: 167–171.
4. Jiang C, Qu A, Matsubara T, Chanturiya T, Jou W, Gavrillova O et al. Disruption of hypoxia-inducible factor 1 in adipocytes improves insulin sensitivity and decreases adiposity in high-fat diet-fed mice. *Diabetes* 2011; **60**: 2484–2495.

5. Krishnan J, Danzer C, Simka T, Ukrepec J, Walter KM, Kumpf S *et al*. Dietary obesity-associated Hif1alpha activation in adipocytes restricts fatty acid oxidation and energy expenditure via suppression of the Sirt2-NAD⁺ system. *Genes Dev* 2012; **26**: 259–270.
6. Herold C, Rennekampff HO, Engell S. Apoptotic pathways in adipose tissue. *Apoptosis* 2013; **18**: 911–916.
7. Lefterova MI, Lazar MA. New developments in adipogenesis. *Trends Endocrinol Metab* 2009; **20**: 107–114.
8. Wagner EF, Eferl R. Fos/AP-1 proteins in bone and the immune system. *Immunol Rev* 2005; **208**: 126–140.
9. Kveiborg M. The increased bone mass in DeltaFosB transgenic mice is independent of circulating leptin levels. *Endocrinology* 2002; **143**: 4304–4309.
10. Kveiborg M, Sabatakos G, Chiusaroli R, Wu M, Philbrick WM, Horne WC *et al*. FosB induces osteosclerosis and decreases adipogenesis by two independent cell-autonomous mechanisms. *Mol Cell Biol* 2004; **24**: 2820–2830.
11. Luther J, Driessler F, Megges M, Hess A, Herbolt B, Mandic V *et al*. Elevated Fra-1 expression causes severe lipodystrophy. *J Cell Sci* 2011; **124**: 1465–1476.
12. Jochum W, David JP, Elliott C, Wutz A, Plenk Jr H, Matsuo K *et al*. Increased bone formation and osteosclerosis in mice overexpressing the transcription factor Fra-1. *Nat Med* 2000; **6**: 980–984.
13. Kveiborg M, Chiusaroli R, Sims NA, Wu M, Sabatakos G, Horne WC *et al*. The increased bone mass in deltaFosB transgenic mice is independent of circulating leptin levels. *Endocrinology* 2002; **143**: 4304–4309.
14. Sabatakos G, Sims NA, Chen J, Aoki K, Kelz MB, Amling M *et al*. Overexpression of DeltaFosB transcription factor(s) increases bone formation and inhibits adipogenesis. *Nat Med* 2000; **6**: 985–990.
15. 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**: 1093–1106.
16. Bozec A, Bakiri L, Hoebertz A, Eferl R, Schilling AF, Komnenovic V *et al*. Osteoclast size is controlled by Fra-2 through LIF/LIF-receptor signalling and hypoxia. *Nature* 2008; **454**: 221–225.
17. Wrann CD, Eguchi J, Bozec A, Xu Z, Mikkelsen T, Gimble J *et al*. FOSL2 promotes leptin gene expression in human and mouse adipocytes. *J Clin Invest* 2012; **122**: 1010–1021.
18. Pino E, Wang H, McDonald ME, Qiang L, Farmer SR. Roles for peroxisome proliferator-activated receptor gamma (PPARgamma) and PPARgamma coactivators 1alpha and 1beta in regulating response of white and brown adipocytes to hypoxia. *J Biol Chem* 2012; **287**: 18351–18358.
19. Wu Z, Xie Y, Bucher NL, Farmer SR. Conditional ectopic expression of C/EBP beta in NIH-3T3 cells induces PPAR gamma and stimulates adipogenesis. *Genes Dev* 1995; **9**: 2350–2363.
20. Lee KY, Eguchi J, Bozec A, Xu Z, Mikkelsen T, Gimble J *et al*. Lessons on conditional gene targeting in mouse adipose tissue. *Diabetes* 2013; **62**: 864–874.
21. Trujillo ME, Pajvani UB, Scherer PE. Apoptosis through targeted activation of caspase 8 (“ATTAC-mice”): novel mouse models of inducible and reversible tissue ablation. *Cell Cycle* 2005; **4**: 1141–1145.
22. Fischer-Posovszky P, Wang QA, Asterholm IW, Rutkowski JM, Scherer PE. Targeted deletion of adipocytes by apoptosis leads to adipose tissue recruitment of alternatively activated M2 macrophages. *Endocrinology* 2011; **152**: 3074–3081.
23. Della-Fera MA, Qian H, Baile CA. Adipocyte apoptosis in the regulation of body fat mass by leptin. *Diabetes Obes Metab* 2001; **3**: 299–310.
24. Halberg N, Khan T, Trujillo ME, Wernstedt-Asterholm I, Attie AD, Sherwani S *et al*. Hypoxia-inducible factor 1alpha induces fibrosis and insulin resistance in white adipose tissue. *Mol Cell Biol* 2009; **29**: 4467–4483.
25. Majmundar AJ, Wong WJ, Simon MC. Hypoxia-inducible factors and the response to hypoxic stress. *Mol Cell* 2010; **40**: 294–309.
26. Wada T, Shimba S, Tezuka M. Transcriptional regulation of the hypoxia inducible factor-2alpha (HIF-2alpha) gene during adipose differentiation in 3T3-L1 cells. *Biol Pharm Bull* 2006; **29**: 49–54.
27. Eferl R, Zenz R, Theussl HC, Wagner EF. Simultaneous generation of fra-2 conditional and fra-2 knock-out mice. *Genesis* 2007; **45**: 447–451.
28. Eferl R, Hasselblatt P, Rath M, Popper H, Zenz R, Komnenovic V *et al*. Development of pulmonary fibrosis through a pathway involving the transcription factor Fra-2/AP-1. *Proc Natl Acad Sci USA* 2008; **105**: 10525–10530.
29. Imai T, Jiang M, Chambon P, Metzger D. Impaired adipogenesis and lipolysis in the mouse upon selective ablation of the retinoid X receptor alpha mediated by a tamoxifen-inducible chimeric Cre recombinase (Cre-ERT2) in adipocytes. *Proc Natl Acad Sci USA* 2001; **98**: 224–228.

Supplementary Information accompanies this paper on Cell Death and Differentiation website (<http://www.nature.com/cdd>)