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Brief communication

MOLECULAR METABOLISM

Transcriptional regulation of adipocyte formation by the liver receptor homologue 1 (Lrh1)—Small hetero-dimerization partner (Shp) network

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ABSTRACT

Altered adipose tissue formation is a well-known effectors of obesity and T2D. Here, we describe the role of Lrh1 and its co-repressor Shp in the control of adipocyte formation. Expression of Lrh1 in the pre-adipocyte containing SVF is induced in obese mice models and humans while Shp expression is reduced. We demonstrate, that Lrh1 is an inhibitor of adipogenesis while Shp acts functions as an activator through repression of Lrh1 activity. This regulation is at least in part modulated by estradiol conversion through the regulation of Cyp19a1 gene expression. In vivo, loss of Lrh1 leads to induced adipogenesis, while loss of Shp causes uncontrolled activation of Lrh1 and reduced adipogenesis. As Shp expression has been linked to the development of obesity and metabolic disorders, it is possible that alterations of the Shp/Lrh1 network lead to changes in adipocyte formation, which might contribute to the development of obesity associated T2D.

Keywords Obesity; Diabetes; Adipogenesis; Adipocyte

1. INTRODUCTION

Obesity and the associated morbidity and mortality is an increasing problem of the westernized world [1]. The gain in adiposity results from an increase in both adipocyte number and volume of individual adipocytes [2]. White adipose tissue (WAT) accumulates rapidly after birth as a result of increased size of existing fat cells and proliferation of precursor pre-adipocyte cells. Thereafter, pre-adipocytes, which exist in close proximity to adipocytes respond to positive energy balance by proliferating and differentiating into adipocytes [3].

The balance between pro- and anti-adipogenic signaling molecules determines the fate of the pre-adipocytes. There is accumulating evidence that estrogen signaling plays an important role in the development and distribution of WAT. The testosterone converting enzyme aromatase is encoded by the *Cyp19a1* gene and catalyzes the final step of the conversion of C_{19} steroids into C_{18} estrogens. Estrogen receptor α (ERα) as well as ERβ are present in adipose tissue [4] and mediate the biological effects of estrogen [5,6]. Moreover, estrogen and genistein, both have inhibitory effects on adipogenesis in vitro [7]. Postmenopausal women as well as ovariectomized animals exhibit an increase of adipocytes, which can be prevented by estrogen replacement [4,8].

The liver receptor homologue-1 (Lrh1), an orphan member of the Ftz-F1 subfamily of nuclear receptors, plays an important role in bile acid homeostasis, reverse cholesterol transport and steroidogenesis [9]. Furthermore, it stimulates expression of aromatase through transactivation of promoter II activity of *Cyp19a1* gene [10]. Differentiation of cultured human breast adipose tissue is associated with rapid loss of Lrh1 and aromatase expression [11]. Previous studies showed that the small heterodimer partner (Shp, NR0B2), is a co-repressor of Lrh1 [12,13]. Shp is expressed in human adipose tissue [14] and mutations in Shp, which result in loss of its repressor activity, are associated with mild obesity [15].

We show here, that Shp and Lrh1 are part of a transcriptional network that control adipogenesis. Loss of Shp both in vitro and in vivo leads to decreased adipogenesis while loss of Lrh1 leads to an increased adipogenesis. Shp expression is reduced in adipose tissue of obese humans, suggesting that down-regulation of this factor might influence the progression of metabolic disorders.

2. EXPERIMENTAL PROCEDURES

2.1. Real-time PCR

mRNA was isolated and transcribed into cDNA using the Multi-MACS cDNA kit (Miltenyi). mRNA expression was normalized to 36b4. The primer sequences are available upon request.

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2.2. Adipocyte differentiation assays

3T3-L1 pre-adipocytes and primary cells from stromal-vascular fraction were cultured on collagen-coated plates in high-glucose DMEM supplemented with 10% fetal bovine serum. Adipogenesis was induced by induction medium (1 μg/ml insulin, 1 μM dexamethasone, 115 μg/ml isobutylmethylxanthine) for 2 days, followed by 2 days of insulin medium (1 μg/ml) and 2 days of normal medium. 3T3-L1 pre-adipocytes were transfected using lipofectamine (Invitrogen). Esterase inhibitor Fandrozol hydrochloride (FHC) was added at 20 and 100 ng/ml to the cells together with the differentiation cocktail.

2.3. Lentiviral infection

HEK293T cells were transfected using lipofectamine with lentiviral overexpression or control vector and the packaging vectors pMD2.G and psPAX2. Virus containing medium was collected 24 h after changing transfected cells to high BSA (1.1 g/100 ml) medium. 3T3-L1 adipocytes cells were infected for 24 h on the day after plating and 4 days before induction of differentiation with virus containing medium supplemented with polybrene (8 μg/ml).

2.4. Automated analysis of adipocyte differentiation

Differentiated cells were analyzed as described previously. Briefly, cells were fixed with 5% formaldehyde and stained with 4 μM Hoechst (nuclei), 5 μM Syto60 (cytosol) and 2 μM Bodipy (lipid droplets) [16]. Differentiation is presented as amount of differentiated cells divided by number of nuclei (relative differentiation).

2.5. PPARγ activity assay

PPARγ activity was measured by transfecting 3T3-L1 pre-adipocytes one day after plating with a reporter plasmid containing an ideal PPRE in addition to a renilla luciferase control vector. Luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega).

2.6. Animal studies

All mice were housed in specific pathogen-free conditions with a 12 h light–dark cycle and were allowed to free access to water and food. $Lrh1^{fl/fl}$ and $Shp^{fl/fl}$ mice were fed either a regular chow diet or a high-fat diet (Provimi Kliba AG) with 60% calories derived from fat. Only male animals were used. Subcutaneous adipose tissue was taken from the inguinal subcutaneous region while visceral adipose tissue was taken from the epididymal region.

2.7. Adenoviral injection into epididymal fat pads

Ad-Cre virus or Ad-Gfp control (40 μl, 5×10^9 PFU/ml per fat pad) was injected into both epididymal fat pad of five-week-old $Shp^{fl/fl}$ and $Lrh1^{fl/fl}$ mice. To place the injections exclusively into the epididymal fat pad, the intra-abdominal cavity was opened and the epididymal fat pads were exposed [17].

2.8. Serum measurements

Blood glucose was measured with a glycometer (Contour). Insulin was determined with the Insulin ELISA kit (Crystal Chem). Triglycerides were measured with the Trig/GB Kit and free fatty acids were measured with the NEAFA-HR kit (Wako). Serum measurements are part of the supplemental data.

2.9. Western blot

Tissue extracts were prepared as follows: tissue or cells were dounced in hypotonic buffer (10 mmol/l KCl, 1.5 mmol/l $MgCl_2$, 10 mmol/l HEPES pH 7.9), and hypertonic buffer (20 mmol/l HEPES/KOH pH 7.9, 25% glycerol, 0.2 mmol/l EDTA, 1.5 mmol/l $MgCl_2$, 1.2 M NaCl) was added, followed by incubation at 4 °C for 30 min. Protein concentration was determined using the DC protein assay (BioRad). Tissue lysates were subjected to 12% SDS–polyacrylamide gel electrophoresis and western blotting. Antibodies used: PPARγ was from Cell Signaling, Lrh1 was from Abcam, Cyp19a1 was from Abcam, Pref1 was from Santa Cruz and γ-Tub was from Sigma

2.10. Adipocyte and fat pad size

Adipose tissue was fixed in 5% paraformaldehyde over night before paraffin embedding and sectioning to 10 μm slices. H&E staining of sections was performed according to standard procedures. Microscopic pictures were taken and cell size was analyzed using Cell Profiler Software.

2.11. Ethical permissions

All mouse work was carried out in Zürich and was approved by the Kantonale Veterinäramt Zürich. The human work on obese vs. lean patients was performed in Bratislava. Informed consent was obtained from all patients, studied.

2.12. Statistical analysis

Results are given as mean±standard deviation. Statistical analyses were performed using a two-tailed Student's *t* test.

3. RESULTS

3.1. Lrh1 and Shp are associated with the development of obesity

As Lrh1 and Shp are both associated with cell differentiation [11,18] and mutations in Shp are associated with mild obesity [15], as well as with severe early-onset obesity [19], we aimed to analyze, whether Lrh1 and its co-repressor Shp directly affect adipogenesis. To assess if Shp and Lrh1 are expressed in either precursor cell and/or mature adipocytes we isolated both cell fractions from mouse visceral epididymal adipose tissue and quantified expression of Lrh1 and Shp. Purity of the control was assessed by Pparγ and A-Fabp as well as Pref1 expression. Interestingly, we could only detect Shp and Lrh1 expression in the stromal vascular fraction (SVF) of murine adipose tissue, but not in mature adipocytes (Figure. 1A and B). This finding suggests that Shp and Lrh1 may play a role in the differentiation of adipocytes, rather than in mature adipocyte function. Based on our assumption that Lrh1 and Shp could influence adipogenesis we compared the expression of both nuclear receptors in SVF derived from mice, challenged with a high fat diet. Interestingly, Shp mRNA expression was decreased in the SVF of these DIO mice, while we observed an increased expression of Lrh1 mRNA in DIO mice (Figure. 1C and D). To analyze whether the same effect could be observed in human patients we analyzed mRNA expression of Shp and Lrh1 in SVF from male Caucasian patients with a lean phenotype, obese phenotype as well as with an obese diabetic phenotype (Supplementary Table 1). Similar to mice, both Shp and Lrh1 were exclusively expressed in the SVF fraction, but not in mature adipocytes (data not shown). Shp expression was down-regulated approximately 3-fold in obese patients while a more than 10-fold decrease was observed in obese diabetic patients (Figure. 1E). Lrh1 expression, in contrast, was unchanged in obese patients and slightly increased in obese diabetic patients (Figure. 1E). Mmp9 and Hif1α, target genes known, to be upregulated in obese subjects served as a control (Supplementary Figure. 1A)

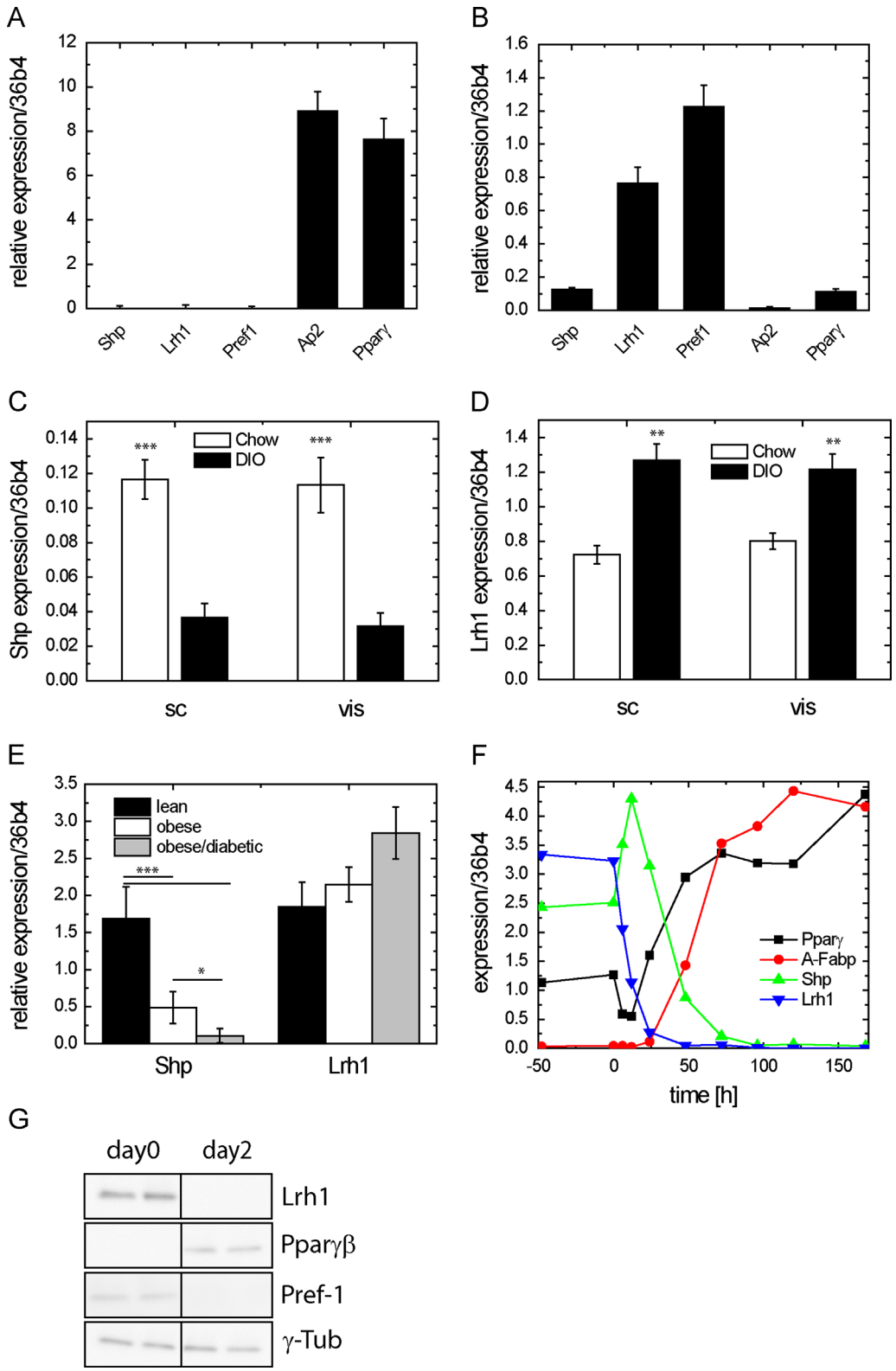

Figure 1: Shp and Lrh1 expression in adipose tissue. (A) mRNA expression levels of Shp, Lrh1 and marker genes in mature adipocytes ($n=6$). (B) mRNA expression levels of Shp, Lrh1 and marker genes in pre-adipocyte containing murine SVF ($n=6$). (C, D) mRNA expression levels of Shp and Lrh1 in subcutaneous (sc) and visceral (vis) SVF from adipose tissue of mice fed a 60% high fat diet for 12 weeks (DIO) ($n=6$–8). (E) mRNA expression levels of Shp and Lrh1 in subcutaneous SVF from adipose tissue of lean, obese and obese/diabetic human patients ($n=8$ per group). (F) mRNA expression levels of Shp and Lrh1 throughout adipocyte differentiation in 3T3-L1 cells ($n=6$). (G) Protein expression of Lrh1 and markers of adipocyte differentiation on day 0 and day 2 of induction of differentiation. -Tub was used as loading control. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

To assess, whether our findings are correlated with the expression of both factors during the course of adipocyte differentiation, we analyzed mRNA from differentiating 3T3-L1 pre-adipocytes at different time points (Figure. 1F and G). In accordance with our previous results, mRNA levels of Shp and Lrh1 was readily detectable before induction of adipogenesis, but declined during adipocyte maturation (Figure. 1F). We confirmed this finding on protein level for Lrh1; Shp protein expression in pre-adipocytes was below the detection limit (Figure. 1G). Lrh1 expression was reduced sharply after induction of differentiation, Shp expression was induced very shortly when adipocyte differentiation started, followed by a sharp decline in expression during adipocyte maturation (Figure. 1F).

3.2. Shp and Lrh1 control adipogenesis

Since we could show that the expression of Lrh1 and its co-repressor Shp, is regulated in pre-adipocytes from obese mice and obese patients, we assessed whether this regulation could modulate the process of adipocyte differentiation itself. To this end we transfected 3T3-L1 cells with various lentiviral constructs to either induce or reduce Shp expression. Transduction of the full length protein induced Shp more than 4-fold, while introducing the Shp shRNA virus, but not a scrambled shRNA virus, silenced endogenous Shp mRNA levels more than 4-fold (Supplementary Figure. 1B). Overexpression of Lrh1 led to an approximately 4-fold increase in mRNA expression levels (Supplementary Figure. 1B).

Induction of Shp expression led to a strong increase in the number of differentiated cells, while reduction of Shp expression led to a repression of adipocyte differentiation (Figure. 2A and B). These changes were independent of changes in cell number as measured by EDU staining (data not shown). Lrh1 in contrast was a repressor of adipocyte differentiation (Figure. 2A and B). Repression of Shp expression or induction of Lrh1 led to an increased Pref1 expression, demonstrating that cells are unable to enter the process of differentiation (Figure. 2C). Given the fact that Shp represses Lrh1 activity while Lrh1 induces the expression of Shp in a negative feedback loop, our results point to a Lrh1 mediated repression of adipogenesis which is relieved by increased Shp levels. To test the hypothesis, we co-expressed both proteins in 3T3-L1 cells and analyzed whether repression of Lrh1 by Shp might relieve the block of adipogenesis. As expected, Shp overexpression rescued the repression of adipogenesis exerted through Lrh1 (Figure. 2A and B).

To corroborate our findings in a different model system we used the primary pre-adipocyte containing SVF from $Shp^{fl/fl}$ and $Lrh1^{fl/fl}$ mice. Primary cells were isolated from these mice and differentiated, ex vivo, as described previously [16,20]. Ablation of Shp and Lrh1 expression was triggered by the addition of Ad-Cre to the culture 3 days prior to induction of differentiation. The expression of Cre recombinase in these cells led to a Cre mediated excision of the *Shp* or the *Lrh1* gene and to the complete loss of either Shp or Lrh1 expression (data not shown). Interestingly, genetic ablation of Shp inhibited differentiation of primary pre-adipocytes by approximately 35%. In contrast, genetic ablation of Lrh1 led to an induction of differentiation in primary stromal vascular cells by 50% (Figure. 2D). To substantiate these results we performed in vivo differentiation assays, in which SVF cells were implanted into the subscalpular region of mice in a matrigel pad [16]. In this experiment, we used SVF derived from $Shp^{fl/fl}$ and $Lrh1^{fl/fl}$ mice treated before implantation with either Ad-Cre or Ad-GFP. Similar to the in vitro results, loss of Shp expression by Cre mediated excision in the SVF led to a decrease in differentiation, while loss of Lrh1 expression resulted in a 2-fold induction of differentiation (Figure. 2E, Supplementary Figure. 2F).

To analyze whether the effect occurs upstream or downstream of the main transcription factor cascade we analyzed Pparγ mRNA and protein expression as well as activity. To this end we transfected cells with a peroxisome proliferator response element (PPRE) containing reporter plasmid together with Shp or with a shRNA targeting Shp. Silencing of Shp reduced the induction of PPARγ activity in these cells while overexpression of Shp led to the induction of PPARγ (Figure. 2F). Loss of Shp and overexpression of Lrh1 led to a decrease in PPARγ protein expression, while Shp overexpression increased the amount of PPARγ found in differentiated cells (Figure. 2C and G). Together these findings demonstrate that Shp is a modulator of adipogenesis and that it exerts its functions upstream or at the level of PPARγ.

3.3. Regulation of adipogenesis by Shp and Lrh1 is mediated in part through Cyp19a1 regulation

To quantify the expression of Shp and Lrh1 target genes that might regulate the process of adipocyte differentiation, we modulated either Shp or Lrh1 expression and analyzed mRNA expression during early time points of differentiation. One interesting target of Lrh1 is Cyp19a1, which encodes for the testosterone converting enzyme, aromatase, and which is important for the production of estradiol. Since it has been shown that estrogen signaling via ER1 inhibits adipocyte differentiation, changes in estrogen signaling could be a possible explanation for the regulation of adipogenesis. We could show that loss of Shp led to an enhanced expression of Cyp19a1 mRNA (Figure. 3A). Loss of either Shp expression or induction of Lrh1 expression led to an increase in Cyp19a1 protein levels, while overexpression of Shp reduced Cyp19a1 levels (Figure. 3B).

To analyze whether this target is also regulated in obesity we analyzed expression of Cyp19a1 in DIO mice. Similar to its regulatory factors, Cyp19a1 mRNA expression was strongly induced in DIO mice (Figure. 3C). Furthermore, we observed a specific expression of Cyp19a1 in SVF of adipose tissue, while no transcript could be detected in mature adipocytes (Figure. 3D). In line with this Cyp19a1 is highly expressed in undifferentiated 3T3-L1 cells and expression declines similar to Lrh1 early during differentiation (Figure. 3E). Consistent with our mouse findings we also observed a 2-fold up-regulation of Cyp19a1 in obese patients and a 5-fold increase in obese/diabetic patients (Figure. 3F).

To analyze whether the effects of Lrh1 and Shp on adipogenesis are mediated through Cyp19a1, we modulated Shp1 and Lrh1 expression and differentiated these cells in the presence or absence of the Cyp19a1 aromatase inhibitor, fadrozol hydrochloride (FC) [21]. Inhibition of aromatase led to increased differentiation of control cells (Figure. 3G). Furthermore, addition of FC could relieve the repression of adipogenesis by Lrh1 overexpression or Shp1 knockdown (Figure. 3G), albeit not completely. This effect was not observed when using a Shp mutant that cannot bind Lrh1 [22], indicating that Lrh1 is the main regulator of differentiation and that its activity is to a great extent regulated by the amount of Shp present in these cells (Figure. 3G). These findings were corroborated by protein expression studies demonstrating that induction of Pparγ expression and loss of Cyp19a1 and Lrh1 which normally is observed during differentiation is seen when cells overexpressing Lrh1 or a shRNA targeted against Shp are treated with FC (Figure. 3H). Taken together these data demonstrate that Lrh1 and Shp control adipogenesis and that the effect is at least partially mediated through regulation of Cyp19a1 and downstream estrogen signaling.

3.4. Shp and Lrh1 control adipogenesis in vivo

To assess the function of impaired differentiation in vivo, we infected visceral epididymal fat pads of both $Shp^{fl/fl}$ and $Lrh1^{fl/fl}$ mice with either Ad-Cre or Ad-Gfp by direct injection of the virus into the fat pads. This treatment led to an approximately 70–90% recombination in the adipose tissue stromal vascular fraction while mature adipocytes showed

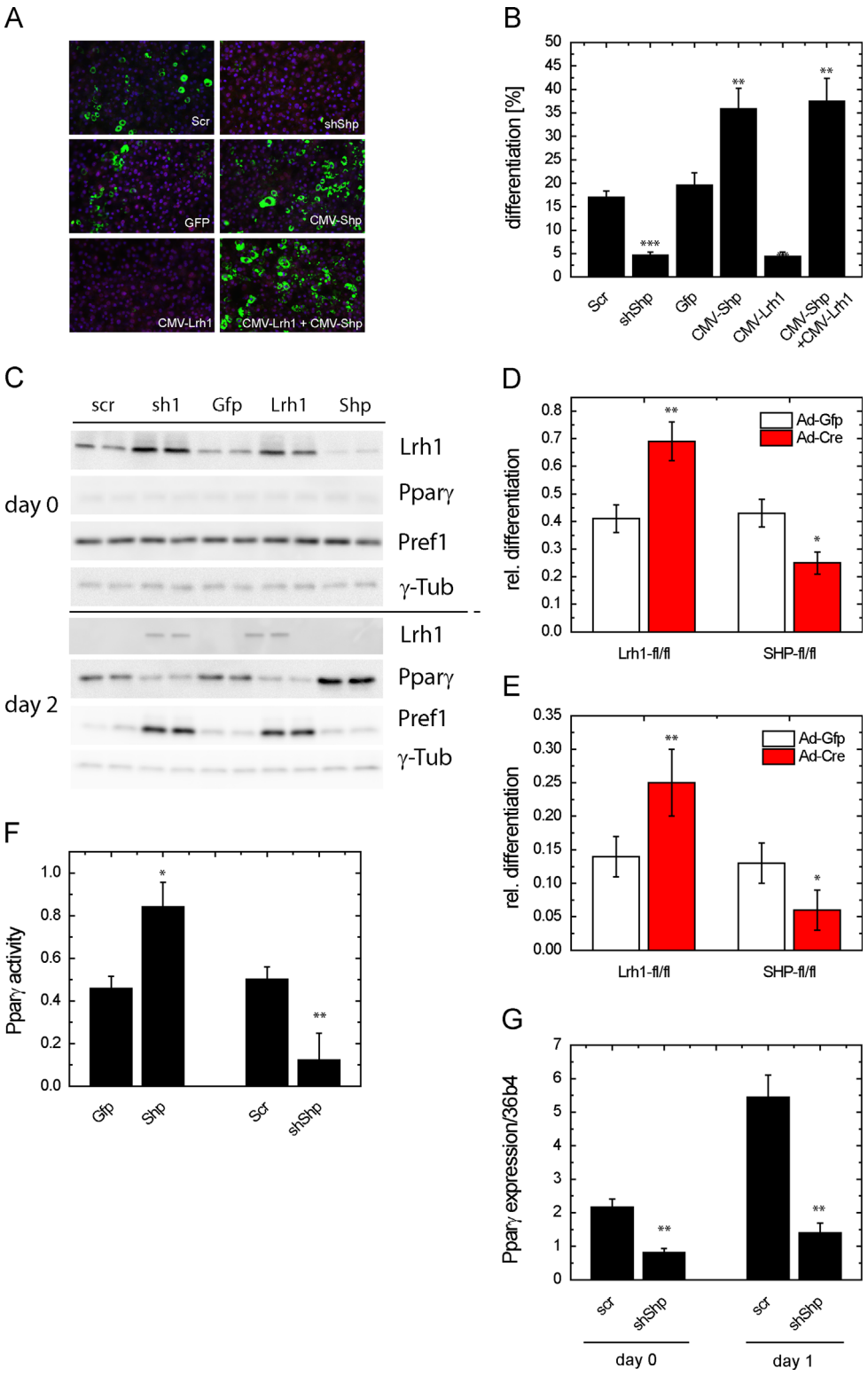

Figure 2: Shp and Lrh1 regulate adipogenesis. (A) Shp and L1rh1 expression was modulated in differentiating 3T3-L1 cell. Cells were stained for nuclei, cytosolic fraction and lipid droplets. Each image is a representation of 54 pictures (6 pictures per replicate) taken for each condition ($n=9$). (B) Quantification of differentiation using the cell profiler platform ($n=9$). (C) Protein expression of Lrh1 and markers of adipocyte differentiation on day 0 and day 2 of induction of differentiation in cells with modulated Shp and Lrh1 expression (Shp and Lrh1 denotes overexpression, Gfp served as a control; sh1 denotes knockdown of Shp, scr served as control). -Tub was used as loading control. (D) Differentiation of primary pre-adipocytes derived from Lrh1 or Shp floxed mice treated with either Cre-expressing adenovirus or Gfp-adenovirus as control ($n=6$). (E) In vivo differentiation of primary pre-adipocytes derived from Lrh1 or Shp floxed mice treated with either Cre-expressing adenovirus or Gfp-adenovirus as control implanted in C57Bl6 acceptor mice ($n=6$). (F) Ppar activity measured by luciferase activity assay in 3T3-L1 cells with modulated Shp expression ($n=9$). (G) mRNA expression levels of Ppar in 3T3-L1 cells with lentiviral mediated knockdown of Shp ($n=9$). $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

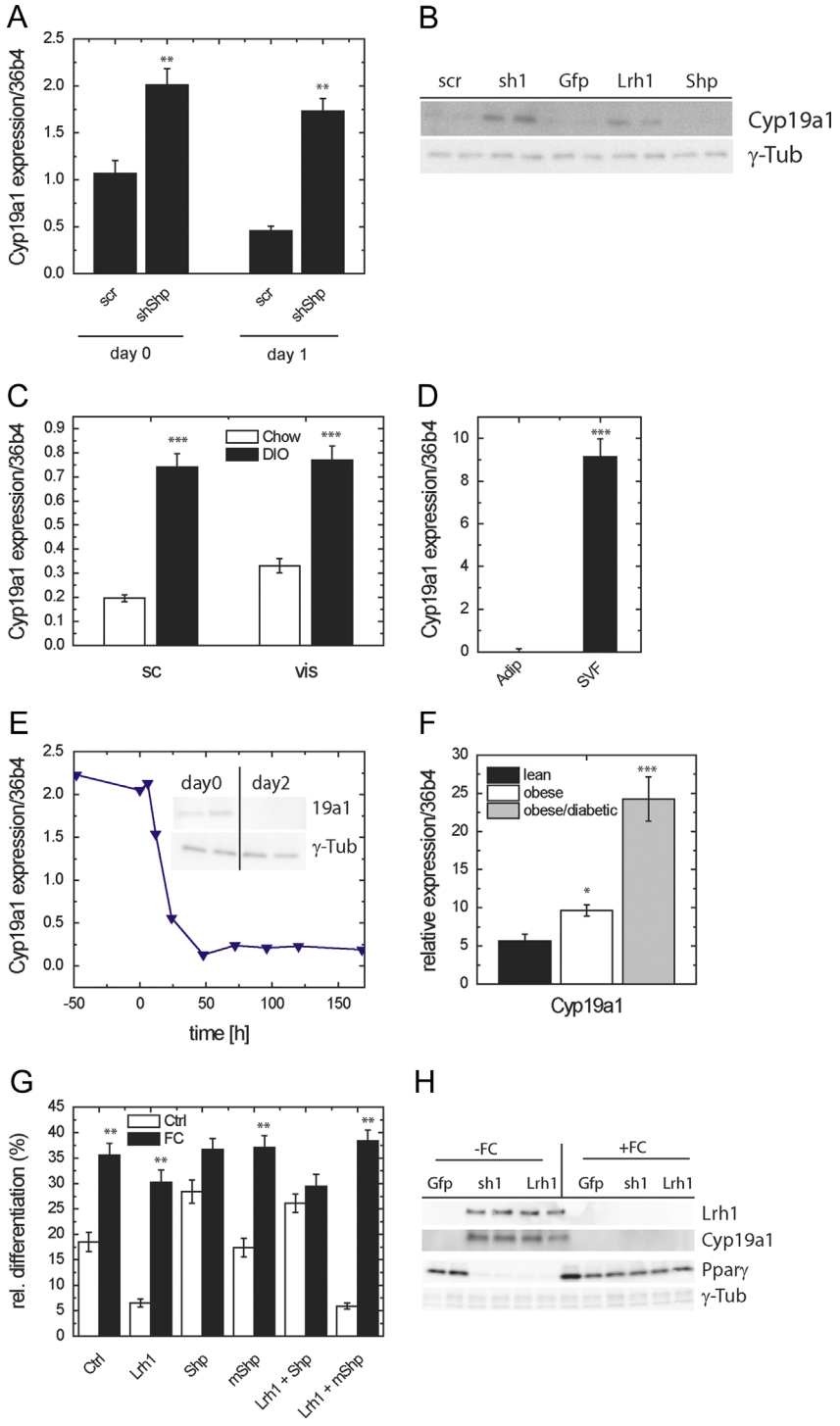

Figure 3: Regulation of adipogenesis by Shp and Lrh1 is mediated by Cyp19a1. (A) mRNA expression levels of Cyp19a1 in 3T3-L1 cells with lentiviral mediated knockdown of Shp ($n=9$). (B) Protein expression of Cyp19a1 in cells with modulated Shp and Lrh1 expression. -Tub was used as loading control. (C) mRNA expression levels of Cyp19a1 in subcutaneous (sc) and visceral (vis) SVF from adipose tissue of mice fed a 60% high fat diet for 12 weeks (DIO) ($n=6$–8). (D) mRNA expression levels of Cyp19a1 in pre-adipocyte containing murine SVF as well as in mature adipocytes ($n=6$). (E) mRNA expression levels of Cyp19a1 throughout adipocyte differentiation and protein expression of Cyp19a1 at day 0 and day 2 of differentiation (inset) in 3T3-L1 cells ($n=6$). (F) mRNA expression level Cyp19a1 in subcutaneous SVF from adipose tissue of lean, obese and obese/diabetic human patients ($n=8$). (G) Shp and Lrh1 expression was modulated in differentiating 3T3-L1 cell in the presence or absence of differing concentrations of fadrozol hydrochloride (FC). A Shp mutant (mShp) that cannot bind Lrh1 was used as a control. Cells were stained for nuclei, cytosolic fraction and lipid droplets. Quantification of differentiation using the cell profiler platform is shown ($n=9$). (H) Protein expression of Lrh1, Cyp19a1 and markers of adipocyte differentiation on day 2 of induction of differentiation in cells with modulated Shp and Lrh1 expression in the presence or absence of FC. -Tub was used as loading control. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

recombination of around 60–80% (Figure. 4A, Supplementary Figure. 2A). The efficacy of adenoviral transduction was confirmed by a 4-fold reduction in Lrh1 expression when the *Lrh1* gene is removed. Similar results were obtained for the Cre-mediated excision of the *Shp* gene as we observed an approximately 2-fold increase in Lrh1 expression when the *Shp* gene was excised (Figure. 4A). We also tested neighboring tissues for recombination;

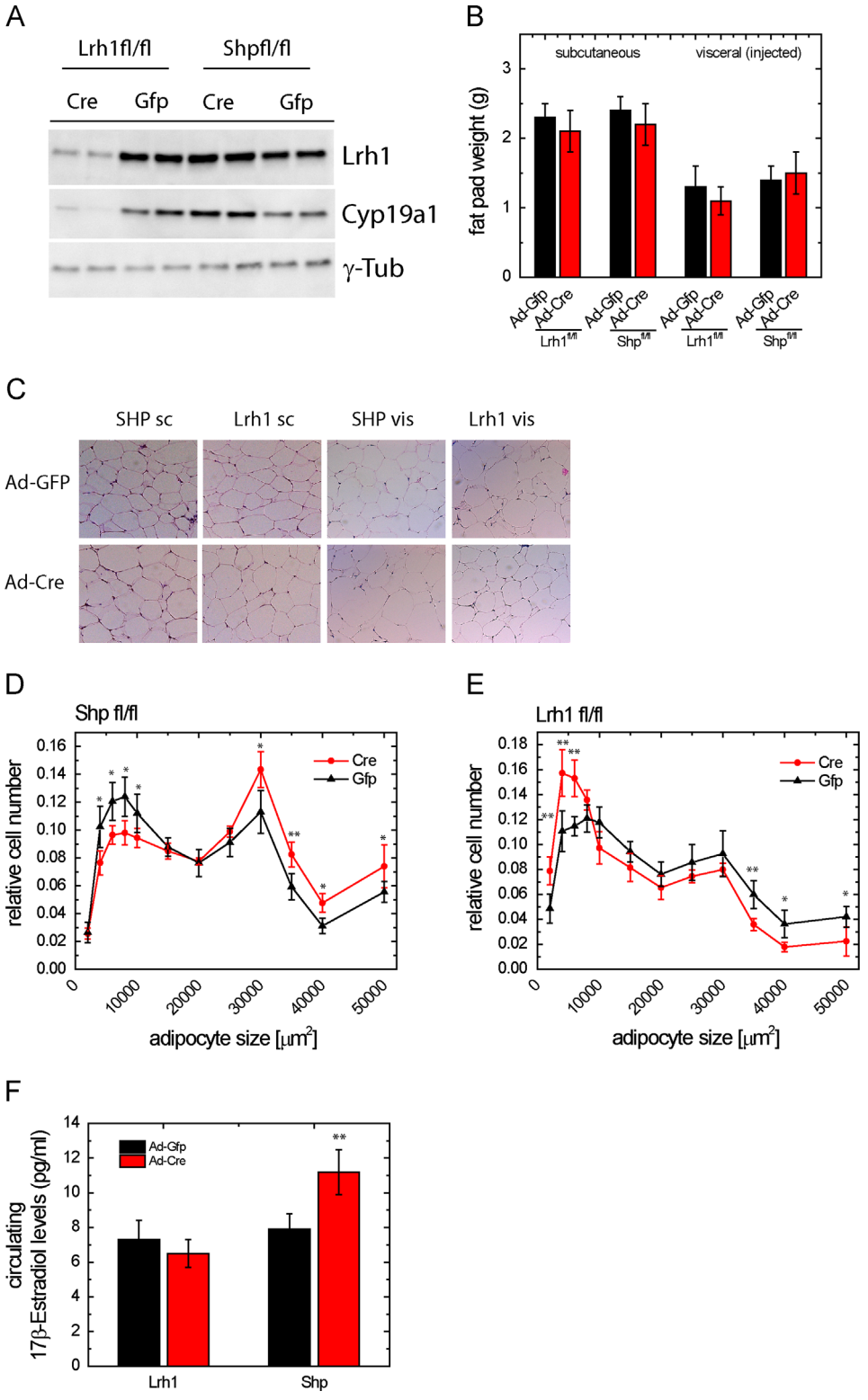

Figure 4: Shp and Lrh1 regulate adipocyte formation in vivo. (A) Protein expression of Lrh1, Cyp19a1 in *Shp* and *Lrh1* floxed mice injected with Ad-Gfp or Ad-Cre into both visceral fat pads, -Tub was used as loading control. (B) Fat pad weight of *Shp* and *Lrh1* floxed mice injected with Ad-Gfp or Ad-Cre into both visceral fat pads ($n=8$–11). (C–E) Paraffin sections of adipocyte tissue and adipocyte size quantification of *Shp* and *Lrh1* floxed mice injected with Ad-Gfp or Ad-Cre into both visceral fat pads ($n=8$–11). (F) Estrogen serum levels in *Shp* and *Lrh1* floxed mice injected with Ad-Gfp or Ad-Cre into both visceral fat pads. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

intestinal recombination as well as liver recombination was less than 5% (data not shown). After infection with adenovirus, mice were kept on a high fat diet for 6 weeks to induce adipose tissue growth. Analysis of adipose tissue depot size by CT scanning did not reveal any changes either in injected (visceral) fat depots or in non-injected (subcutaneous) fat pads (Figure. 4B). In contrast, analysis of the adipose tissue morphology in these

mice revealed that adipocyte size was markedly increased in the Ad-Cre infected fat pads compared to the Ad-Gfp infected mice in *Shp*$^{fl/fl}$ mice (Figure. 4C–E). The opposite was observed in *Lrh1*$^{fl/fl}$ mice where a marked decrease in adipocyte size in Ad-Cre vs. Ad-Gfp injected animals (Figure. 4C–E) was observed. No change in adipocyte size was seen in the subcutaneous adipose tissue depots of all animals (Supplementary Figure. 2B and C). Based on these findings, we calculated the approximate number of adipocytes in each fat pad, based on average adipocyte size and fat pad weight. From our calculation adipocyte numbers were decreased approximately 14% in *Shp*$^{fl/fl}$ mice infected with Ad-Cre compared to Ad-GFP, while numbers were increased by 22% in *Shp*$^{fl/fl}$ mice infected with Ad-Cre compared to Ad-GFP. In order to assess the expression of Lrh1/Shp target genes we also measured Cyp19a1 expression in both injected fat pad and non-treated fat pads of these animals. Similar to our data from cell culture experiments we observed decreased Cyp19a1 protein and mRNA expression in *Lrh1*$^{fl/fl}$ mice injected with Ad-Cre while in *Shp*$^{fl/fl}$ mice infected with Ad-Cre protein and mRNA expression of Cyp19a1 was increased (Figure. 4B, Supplementary Figure. 2E), while Shp levels were decreased in *Lrh1*$^{fl/fl}$ mice injected with Ad-Cre (Supplementary Figure. 2A). In accordance we also observed a tendency to decrease in circulating estrogen levels when Lrh1 expression was silenced, while estrogen was increased significantly when Shp was silenced (Figure. 4F). These small changes correlate with the reduced expression of Cyp19a1 in adipose tissue of the treated animals, while Pparγ mRNA expression remained unaffected (Supplementary Figure. 2D, data not shown).

Taken together we identify here Shp and Lrh1 as regulators of adipogenesis both in vitro and in vivo. Loss of Shp expression leads to a reduction in adipogenesis while loss of Lrh1 promotes the process. Given the fact that Shp itself as well as the putative downstream target Cyp19a1 are deregulated in obese mouse models and human patients, this signaling events might be the underlying cause for altered adipogenesis in obesity and altered metabolic control as a consequence of impaired adipose tissue adaptation.

4. DISCUSSION

The detailed understanding of the processes, regulating adipose formation and the identification of novel target genes are instrumental in combating obesity. It was previously shown that Shp is expressed in human adipose tissue [14] and that mutations in Shp are associated with mild obesity [15], as well as with severe early-onset obesity [19]. Lrh1 and Cyp19a1 are co-expressed at in human pre-adipocytes, but expression of both factors is rapidly down-regulated during differentiation [11]. This tight confinement of Lrh1 and aromatase expression to the pre-adipocyte fraction indicates that these factors may participate in a more general control of adipocyte differentiation, even though such a mechanism has not been described, so far. In addition it was shown previously that Shp controls Cyp19a1 expression in human pre-adipocytes via Lrh1 [23]. Taken together, these findings implicate Shp and Lrh1 as master regulators of Cyp19a1. While Lrh1 and Cyp19a1 expression was reduced sharply after induction of differentiation, Shp mRNA expression was induced early in differentiation and sharply declined during later stages of adipocyte maturation. Such a pattern was also observed in testis and Volle et al. suggested that this Shp peak could be implicated in germ cell differentiation [18]. Thus, it might be possible that short term Shp activation during the process of differentiation might be a more general concept applicable also to other cell types.

Most pro-adipogenic factors seem to function at least in part by activating expression or activity of PPARγ, the master regulator of adipogenesis [2]. We could show that a loss of Shp reduced the induction of PPARγ activity. This finding is consistent with a study, which showed that Shp increases the transcriptional activity of PPARγ by directly binding PPARγ and competing with nuclear receptor co-repressors for PPARγ [14,24]. We also observed that repression of Shp, led to the reduction of Pparγ mRNA and protein levels, while the expression of c-Myc was not affected. In accordance with that, it has been reported that in constitutively expressing Shp transgenic mice, the expression of endogenous Pparγ was up-regulated (3.5-fold). This suggests that Shp functions at an early time point of differentiation and that the reduction of Pparγ, is due to a reduction in expression rather than to a reduction of activity alone. Interestingly, in a recent study the presence of a functional PPAR response element (PPRE) in the human Shp promoter was identified, indicating a positive feedback mechanism [25].

A recent report showed that overexpression of Shp in 3T3L1 pre-adipocytes might lead to the inhibition of adipogenesis [26]. This is in contrast to our experiments, however it has to be noted that we mostly studied the reduction of Shp and even in the overexpression system we never achieved more than an 4–5-fold induction. In contrast the previous report demonstrated a nearly 100-fold induction of Shp in cells transduced with an adenovirus expressing Shp. Thus it might be well possible that at high levels Shp blocks adipogenesis maybe through binding to Pparγ as discussed above.

It has been shown that Lrh1 might be important for controlling the process of adipogenesis through regulation of Lipocalin-type prostaglandin D synthase (Lpgds) in 3T3-L1 cells [27]. Induction of Lpgds through Srebp1c activation led to the recruitment of Lrh1 to the promoter of Lpgds, however the contribution of Lrh1 to the process of adipogenesis was not addressed. Our data suggests, that Lrh1 functions as an inhibitor of differentiation, which could be due to impaired Lpgds activation. In addition to Lpgds, it has been shown, that Lrh1 stimulates expression of aromatase, through transactivation of the *Cyp19a1* gene promoter II activity [10]. Our data, demonstrating that inhibition of aromatase by FC led to an increase of differentiation and that the addition of FC could relieve the repression of adipogenesis by either Lrh1 overexpression or Shp knockdown, suggests that both factors regulate adipogenesis via estrogen signaling. It has been reported that E_2 signaling inhibits adipogenic differentiation in mouse pre-osteoblast-like cell lines [28], as well as in mouse [7,29] and human bone marrow cell lines [30]. These studies could be verified in ERα- and in aromatase-deficient mice [5,6], as well as in subjects with estrogen-deficiency [4,8], which showed an increase of adipocytes number [28]. The observation that genistein disrupts adipogenic program through the inhibition of C/ebpα and Pparγ expression [31], as well as the described down regulation of Pparγ expression by E2 in mouse pre-osteoblast-like cell lines, supports our finding that a loss of Shp leads to a reduction of Pparγ expression during differentiation.

Furthermore, it has also been reported that Shp inhibits the transcriptional activity of ERα und ERß, via the AF-2 domain, by competing for binding of co-activators [32]. These findings suggest that Shp inhibits estrogen signaling by Lrh1 mediated suppression of estrogen synthesis and by directly inhibiting ER transcriptional activity. Interestingly, it was shown that Shp expression is rapidly induced within 2 h following treatment with ethynylestradiol or the ERα-elective compound propyl pyrazole triol (PPT) and that ERα directly induces Shp promoter activity [33]. This high sensitivity of Shp to the induction by E2 suggests that Shp expression could be regulated by natural fluctuations in estrogen levels.

Our findings on adipocyte size, in vivo, most likely reflect alterations in adipogenesis, as both Shp and Lrh1 are not expressed in the mature adipocyte and thus will not impact adipocyte function. Alterations in adipogenesis, coupled with a normal metabolism would lead to shift in the

balance between adipocyte hypertrophy vs. adipocyte hyperplasia. As reduced adipocyte hyperplasia might be an underlying factor of impaired metabolic control under challenged conditions [16] it is possible that changes in the Shp/Lrh1 network might contribute to the development of insulin resistance in obesity. This is supported by our correlative data demonstrating that Shp levels are reduced in obese patients and that a further reduction in Shp is seen in obese/diabetic patients.

Recent work in which Shp expression was induced in mature adipocytes showed an effect of this factor on weight gain and diet induced obesity [34]. Similarly, it was shown that whole body ablation of Shp leads to altered brown adipose function tissue thus affecting whole body energy homeostasis [35]. As we were interested to elucidate the effect of Shp and Lrh1 on adipogenesis our aim was to ablate expression of this factor in pre-adipocytes. Given the fact that no specific marker for pre-adipocytes is known, precluding the use of a genetic knock-out, we used an adenoviral approach targeting the complete SVF in adipose tissue. It has to be noted that this approach will also target other cells in the SVF besides pre-adipocyte.

5. CONCLUSIONS

We could show here, that Shp and Lrh1 are part of a transcriptional network modulating estrogen signaling, which in turn influences the process of adipogenesis. In that respect, Shp activates and Lrh1 inhibits the process of adipogenesis. As it is known that increased adipocyte size causes adipocyte insulin resistance deregulation of Shp and Lrh1 in obesity might contribute to altered adipocyte size and ultimately changes in metabolic control.

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CONFLICT OF INTEREST

None declared.

APPENDIX A. SUPPORTING INFORMATION

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.molmet.2013.03.003.

REFERENCES

[1] Ogden, C.L., Carroll, M.D., Curtin, L.R., McDowell, M.A., Tabak, C.J., and Flegal, K.M., 2006. Prevalence of overweight and obesity in the United States, 1999–2004. Journal of the American Medical Association 295:1549–1555.

[2] Rosen, E.D., and Spiegelman, B.M., 2006. Adipocytes as regulators of energy balance and glucose homeostasis. Nature 444:847–853.

[3] Rosen, E.D., and MacDougald, O.A., 2006. Adipocyte differentiation from the inside out. Nature Reviews Molecular Cell Biology 7:885–896.

[4] Pedersen, S.B., Borglum, J.D., Eriksen, E.F., and Richelsen, B., 1991. Nuclear estradiol binding in rat adipocytes. Regional variations and regulatory influences of hormones. Biochimica et Biophysica Acta 1093:80–86.

[5] Heine, P.A., Taylor, J.A., Iwamoto, G.A., Lubahn, D.B., and Cooke, P.S., 2000. Increased adipose tissue in male and female estrogen receptor-alpha knockout mice. Proceedings of the National Academy of Sciences USA 97:12729–12734.

[6] Jones, M.E., Thorburn, A.W., Britt, K.L., Hewitt, K.N., Wreford, N.G., Proietto, J., et al., 2000. Aromatase-deficient (ArKO) mice have a phenotype of increased adiposity. Proceedings of the National Academy of Sciences USA 97:12735–12740.

[7] Okazaki, R., Inoue, D., Shibata, M., Saika, M., Kido, S., Ooka, H., et al., 2002. Estrogen promotes early osteoblast differentiation and inhibits adipocyte differentiation in mouse bone marrow stromal cell lines that express estrogen receptor (ER) alpha or beta. Endocrinology 143:2349–2356.

[8] Gambacciani, M., Ciaponi, M., Cappagli, B., Piaggesi, L., De Simone, L., Orlandi, R., et al., 1997. Body weight, body fat distribution, and hormonal replacement therapy in early postmenopausal women. Journal of Clinical Endocrinology & Metabolism 82:414–417.

[9] Fayard, E., Auwerx, J., and Schoonjans, K., 2004. LRH-1: an orphan nuclear receptor involved in development, metabolism and steroidogenesis. Trends in Cell Biology 14:250–260.

[10] Clyne, C.D., Kovacic, A., Speed, C.J., Zhou, J., Pezzi, V., and Simpson, E.R., 2004. Regulation of aromatase expression by the nuclear receptor LRH-1 in adipose tissue. Molecular and Cellular Endocrinology 215:39–44.

[11] Clyne, C.D., Speed, C.J., Zhou, J., and Simpson, E.R., 2002. Liver receptor homologue-1 (LRH-1) regulates expression of aromatase in preadipocytes. Journal of Biological Chemistry 277:20591–20597.

[12] Lu, T.T., Makishima, M., Repa, J.J., Schoonjans, K., Kerr, T.A., Auwerx, J., et al., 2000. Molecular basis for feedback regulation of bile acid synthesis by nuclear receptors. Molecular Cell 6:507–515.

[13] Goodwin, B., Jones, S.A., Price, R.R., Watson, M.A., McKee, D.D., Moore, L.B., et al., 2000. A regulatory cascade of the nuclear receptors FXR, SHP-1, and LRH-1 represses bile acid biosynthesis. Molecular Cell 6:517–526.

[14] Nishizawa, H., Yamagata, K., Shimomura, I., Takahashi, M., Kuriyama, H., Kishida, K., et al., 2002. Small heterodimer partner, an orphan nuclear receptor, augments peroxisome proliferator-activated receptor gamma transactivation. Journal of Biological Chemistry 277:1586–1592.

[15] Nishigori, H., Tomura, H., Tonooka, N., Kanamori, M., Yamada, S., Sho, K., et al., 2001. Mutations in the small heterodimer partner gene are associated with mild obesity in Japanese subjects. Proceedings of the National Academy of Sciences USA 98:575–580.

[16] Meissburger, B., Ukropec, J., Roeder, E., Beaton, N., Geiger, M., Teupser, D., et al., 2011. Adipogenesis and insulin sensitivity in obesity are regulated by retinoid-related orphan receptor gamma. EMBO Molecular Medicine 3:637–651.

[17] Trajkovski, M., Hausser, J., Soutschek, J., Bhat, B., Akin, A., Zavolan, M., et al., 2011. MicroRNAs 103 and 107 regulate insulin sensitivity. Nature 474:649–653.

[18] Volle, D.H., Duggavathi, R., Magnier, B.C., Houten, S.M., Cummins, C.L., Lobaccaro, J.M., et al., 2007. The small heterodimer partner is a gonadal gatekeeper of sexual maturation in male mice. Genes & Development 21:303–315.

[19] Hung, C.C., Farooqi, I.S., Ong, K., Luan, J., Keogh, J.M., Pembrey, M., et al., 2003. Contribution of variants in the small heterodimer partner gene to birthweight, adiposity, and insulin levels: mutational analysis and association studies in multiple populations. Diabetes 52:1288–1291.

[20] Meissburger, B., Stachorski, L., Roder, E., Rudofsky, G., and Wolfrum, C., 2011. Tissue inhibitor of matrix metalloproteinase 1 (TIMP1) controls adipogenesis in obesity in mice and in humans. Diabetologia 54:1468–1479.

[21] Dutta, U., and Pant, K., 2008. Aromatase inhibitors: past, present and future in breast cancer therapy. Medical Oncology 25:113–124.

[22] Li, Y., Choi, M., Suino, K., Kovach, A., Daugherty, J., Kliewer, S.A., et al., 2005. Structural and biochemical basis for selective repression of the orphan nuclear

receptor liver receptor homolog 1 by small heterodimer partner. Proceedings of the National Academy of Sciences USA 102:9505–9510.

[23] Kovacic, A., Speed, C.J., Simpson, E.R., and Clyne, C.D., 2004. Inhibition of aromatase transcription via promoter II by short heterodimer partner in human preadipocytes. Molecular Endocrinology 18:252–259.

[24] Kim, J., Min, G., and Kemper, B., 2001. Chromatin assembly enhances binding to the CYP2B1 phenobarbital-responsive unit (PBRU) of nuclear factor-1, which binds simultaneously with constitutive androstane receptor (CAR)/retinoid X receptor (RXR) and enhances CAR/RXR-mediated activation of the PBRU. Journal of Biological Chemistry 276:7559–7567.

[25] Kim, H.I., Koh, Y.K., Kim, T.H., Kwon, S.K., Im, S.S., Choi, H.S., et al., 2007. Transcriptional activation of SHP by PPAR-gamma in liver. Biochemical and Biophysical Research Communications 360:301–306.

[26] Song, G., Park, K., and Wang, L., 2009. Gene expression profiling reveals a diverse array of pathways inhibited by nuclear receptor SHP during adipogenesis. International Journal of Clinical and Experimental Pathology 2:275–285.

[27] Fujimori, K., Aritake, K., and Urade, Y., 2007. A novel pathway to enhance adipocyte differentiation of 3T3-L1 cells by up-regulation of lipocalin-type prostaglandin D synthase mediated by liver X receptor-activated sterol regulatory element-binding protein-1c. Journal of Biological Chemistry 282:18458–18466.

[28] Dang, Z.C., van Bezooijen, R.L., Karperien, M., Papapoulos, S.E., and Lowik, C. W., 2002. Exposure of KS483 cells to estrogen enhances osteogenesis and inhibits adipogenesis. Journal of Bone and Mineral Research 17:394–405.

[29] Liao, Q.C., Li, Y.L., Qin, Y.F., Quarles, L.D., Xu, K.K., Li, R., et al., 2008. Inhibition of adipocyte differentiation by phytoestrogen genistein through a potential downregulation of extracellular signal-regulated kinases 1/2 activity. Journal of Cellular Biochemistry 104:1853–1864.

[30] Heim, M., Frank, O., Kampmann, G., Sochocky, N., Pennimpede, T., Fuchs, P., et al., 2004. The phytoestrogen genistein enhances osteogenesis and represses adipogenic differentiation of human primary bone marrow stromal cells. Endocrinology 145:848–859.

[31] Harmon, A.W., Patel, Y.M., and Harp, J.B., 2002. Genistein inhibits CCAAT/enhancer-binding protein beta (C/EBPbeta) activity and 3T3-L1 adipogenesis by increasing C/EBP homologous protein expression. Biochemical Journal 367:203–208.

[32] Johansson, L., Thomsen, J.S., Damdimopoulos, A.E., Spyrou, G., Gustafsson, J. A., and Treuter, E., 1999. The orphan nuclear receptor SHP inhibits agonist-dependent transcriptional activity of estrogen receptors ERalpha and ERbeta. Journal of Biological Chemistry 274:345–353.

[33] Lai, K., Harnish, D.C., and Evans, M.J., 2003. Estrogen receptor alpha regulates expression of the orphan receptor small heterodimer partner. Journal of Biological Chemistry 278:36418–36429.

[34] Tabbi-Anneni, I., Cooksey, R., Gunda, V., Liu, S., Mueller, A., Song, G., et al., 2010. Overexpression of nuclear receptor SHP in adipose tissues affects diet-induced obesity and adaptive thermogenesis. American Journal of Physiology—Endocrinology and Metabolism 298:E961–E970.

[35] Wang, L., Liu, J., Saha, P., Huang, J., Chan, L., Spiegelman, B., et al., 2005. The orphan nuclear receptor SHP regulates PGC-1alpha expression and energy production in brown adipocytes. Cell Metabolism 2:227–238.

