

Adipogenesis and insulin sensitivity in obesity are regulated by retinoid-related orphan receptor gamma

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Obesity is a well-known risk factor for the development of secondary complications such as type 2 diabetes. However, only a part of the obese population develops secondary metabolic disorders. Here, we identify the transcription factor retinoid-related orphan receptor gamma (ROR γ) as a negative regulator of adipocyte differentiation through expression of its newly identified target gene matrix metalloproteinase 3. *In vivo* differentiation of adipocyte progenitor cells from ROR γ -deficient mice is enhanced and obese ROR $\gamma^{-/-}$ mice show decreased adipocyte sizes. These small adipocytes are highly insulin sensitive, leading to an improved control of circulating free fatty acids. Ultimately, ROR $\gamma^{-/-}$ mice are protected from hyperglycemia and insulin resistance in the state of obesity. In adipose stromal-vascular fraction from obese human subjects, ROR γ expression is correlated with adipocyte size and negatively correlated with adipogenesis and insulin sensitivity. Taken together, our findings identify ROR γ as a factor, which controls adipogenesis as well as adipocyte size and modulates insulin sensitivity in obesity. ROR γ might therefore serve as a novel pharmaceutical target to treat obesity-associated insulin resistance.

INTRODUCTION

Obesity is a major risk factor for the development of type 2 diabetes and the metabolic syndrome. Even though there is a clear link between obesity and insulin sensitivity, the degree of insulin resistance in obese subjects can vary enormously (Desai et al, 2006), one possible reason being the variability of adipose tissue plasticity (Sethi & Vidal-Puig, 2007). Adipose tissue mass increases during obesity via two distinct mechanisms, increasing cell number (hyperplasia) and/or increasing

adipocyte size (hypertrophy) (Jo et al, 2009; Spalding et al, 2008). Hyperplasia occurs via *de novo* differentiation of preadipocytes, which reside in the stromal-vascular fraction (SVF) of adipose tissue (Gesta et al, 2007; Rodeheffer et al, 2008). The balance between hyperplasia and hypertrophy strongly influences the metabolic outcome of obesity. While smaller cells retain insulin sensitivity and normal function, large hypertrophic cells are insulin resistant and change their secretory profile towards pro-inflammatory adipocytokines (Roberts et al, 2009; Tilg & Moschen, 2006). As a result, lipolytic release of free fatty acids is enhanced in large adipocytes, leading to cytotoxic fatty acid accumulation in extra-adipose tissue (Unger, 2003). Shifting the balance towards hyperplasia replenishes the pool of small, functional adipocytes, thus ameliorating the metabolic consequences of obesity. A pharmacological example for this mechanism is the clinical use of glitazones activating PPAR γ (Crossno et al, 2006; Johnson et al, 2007), which leads to an increase in adipose tissue mass with a shift towards smaller, insulin sensitive adipocytes.

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An important regulator of the adipogenic process is the proteolytic remodelling of the extracellular matrix, as preadipocytes have to undergo cell shape transition during differentiation (Kubo et al, 2000). Several classes of metalloproteinases (MMPs) affect the adipogenic process (Lilla et al, 2002). For example, global inhibition of MMP activity inhibits differentiation of preadipocytes and adipose tissue development (Bouloumie et al, 2001; Lijnen et al, 2002). On the other hand, *Mmp3*-deficient mice show accelerated adipogenesis during mammary gland involution, suggesting a negative regulatory role for MMP3 in adipocyte differentiation (Alexander et al, 2001).

Retinoid-related orphan receptor gamma (ROR γ) is a nuclear receptor with an N-terminal (A/B) domain followed by the DNA-binding domain and a C-terminal ligand-binding domain. RORs bind as monomers to the consensus half-site PuGGTCA (Jetten, 2009). Recently, several ligands have been proposed to regulate ROR γ transcriptional activity (Solt et al, 2011; Wang et al, 2009). ROR γ has two isoforms: the main isoform is expressed in adipose tissue, liver, muscle and kidney, whereas expression of ROR γ t is restricted to immune cells. ROR γ t has been studied in the context of interleukin (IL)-17 producing T-helper cells (Zhang et al, 2008; Zhou et al, 2008); the role of ROR γ in other tissues, however, is less clear (Meissburger & Wolfrum, 2008). With regard to adipose tissue, single nucleotide polymorphisms of ROR γ have been linked to fat accumulation in cattle (Barendse et al, 2007). During *in vitro* adipogenesis, Ror γ expression is upregulated in late stages of differentiation (Austin et al, 1998). However, a functional role for ROR γ in adipose tissue plasticity has yet to be identified.

In this study, we investigated the mechanistic role of ROR γ in adipocyte differentiation and show that ROR γ is an important negative regulator of adipogenesis. By controlling fat cell development ROR γ influences the progression of obesity-associated insulin resistance in mice and humans.

RESULTS

ROR γ regulates adipogenesis

In order to identify candidate genes that regulate adipose tissue plasticity, we analysed transcription profiles of visceral adipose SVF from lean and obese patients. The highest upregulated gene in the state of obesity was the transcription factor Ror γ (Table S1 of Supporting information). Increased expression of Ror γ was confirmed by quantitative mRNA analysis in visceral adipose tissue of obese patients *versus* lean patients (mean values lean 7.9 ± 1.1 *vs.* obese 17.6 ± 2.9 , Fig S1A of Supporting information). As this factor is highly expressed in the preadipocyte-containing SVF, we analysed whether ROR γ plays a role in regulating adipose tissue plasticity.

During differentiation, mRNA and protein expression of Ror γ is high in confluent 3T3-L1 preadipocytes, whereas the isoform ROR γ t could not be detected (Figs 1A and 2A, data not shown). Upon induction of differentiation, Ror γ mRNA and protein levels exhibit a sharp decline suggesting that ROR γ might influence the process of differentiation (Figs 1A and 2A). To

follow up on this finding, we transiently expressed ROR γ in 3T3-L1 preadipocytes and analysed the effect on adipogenesis. ROR γ overexpression in 3T3-L1 cells decreases the amount of differentiated adipocytes as well as expression of the mature adipocyte marker adipocyte fatty acid binding protein (A-FABP; Fig 1B and C and Fig S1B of Supporting information). This diminished adipogenesis is retained, albeit to a lesser extent, when rosiglitazone is added, suggesting that ROR γ acts upstream of PPAR γ (Fig 1B and C). Conversely, siRNA mediated knockdown of ROR γ increases the adipogenic potential of 3T3-L1 preadipocytes (Fig 1D and E and Fig S1B of Supporting information). No changes were found in the mRNA expression of closely related family members Ror α and Ror β suggesting that the effect of ROR γ is not due to compensatory regulation of other family members (Fig S1C of Supporting information).

To further pinpoint at which stage ROR γ interferes with the differentiation process, we analysed expression of key regulatory adipogenic genes in a time course of differentiation. Transient overexpression of ROR γ increased endogenous protein levels approximately 3–4-fold and resulted in a decreased expression of key adipogenic proteins such as C/ebp α , C/ebp β , C/ebp δ and Ppar γ (Fig 2A). In contrast, protein expression of Pref1, a marker for undifferentiated preadipocytes, was not downregulated upon induction of differentiation in cells overexpressing ROR γ . These findings were corroborated by the fact that induction of adipogenic genes such as c-Jun, c-Fos and A-Fabp was repressed by ROR γ (Fig 2B–D). Taken together, these data demonstrate that ROR γ exerts its inhibitory effect during the initial steps of adipogenesis repressing early adipogenic genes.

To confirm that ROR γ acts upstream of PPAR γ , we measured PPAR γ transcriptional activity in differentiating cells transiently overexpressing ROR γ or control vector. PPAR γ activity in ROR γ transfected cells was suppressed in the first 2 days after induction of differentiation (Fig 2E), which is in line with our observation that overexpression of ROR γ led to a reduction in Ppar γ expression (Fig 2A). On the other hand, activity of the ROR γ promoter was not affected by the PPAR γ agonist rosiglitazone suggesting that PPAR γ does not regulate Ror γ expression (Fig S1D of Supporting information). As REV-ERB α is known to modulate transcription of PPAR γ (Wang & Lazar, 2008), we tested whether ROR γ could directly influence Ppar γ expression or whether the effect was due to reduced C/EBP activity, as would be expected from the lowered expression levels of these transcription factors (Fig 2A). As seen in Fig 2F, ROR γ did not influence PPAR γ promoter activity at early time points. However, induction of PPAR γ during differentiation was inhibited in cells transfected with ROR γ . Taken together, these findings demonstrate that ROR γ tightly regulates adipocyte differentiation by regulating early phase genes, which in turn leads to a reduction in PPAR γ expression and activity.

The effect of ROR γ on adipogenesis is mediated by MMP3

To identify new target genes of ROR γ that are involved in the repression of adipogenesis, we analysed transcription profiles of 3T3-L1 preadipocytes transiently overexpressing ROR γ (Table S2 of Supporting information). Genes upregulated by

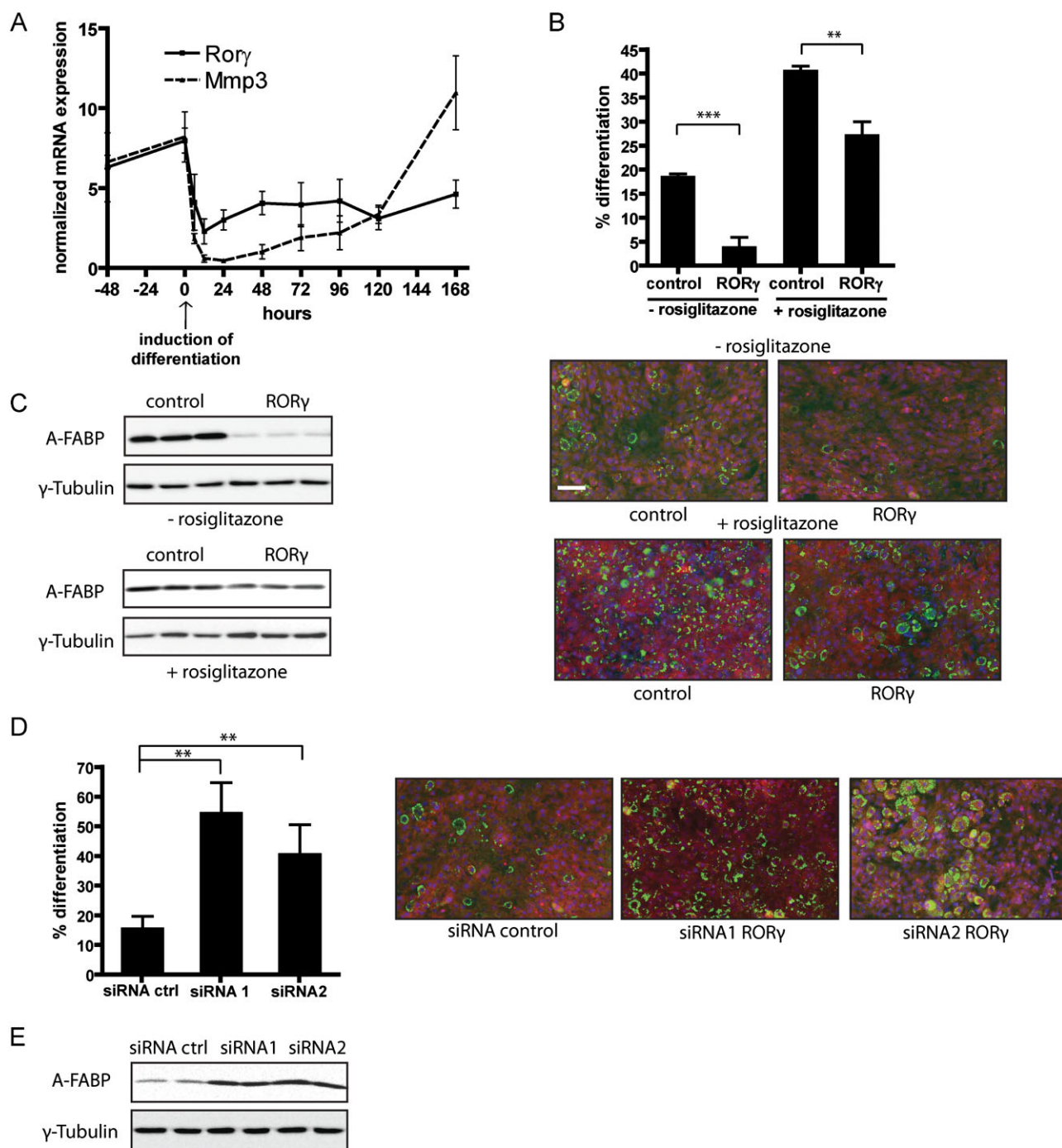


Figure 1. ROR γ inhibits adipogenesis.

- A.** mRNA expression profile of ROR γ and Mmp3 during 3T3-L1 adipogenesis. Expression was normalized to 36b4 ($n = 4$).
- B.** Fluorescent staining of 3T3-L1 adipocytes overexpressing ROR γ or empty vector at day 7 of differentiation with or without rosiglitazone. Automated image acquisition (blue: nuclei, red: cytosol, green: lipid droplets, 12 pictures per well, scale: 100 μ m) was followed by automated image quantification using CellProfiler ($n = 4$).
- C.** Whole cell extracts of differentiated 3T3-L1 adipocytes were blotted against the mature adipocyte marker A-FABP using γ -Tubulin as a loading control.
- D,E.** Fluorescent staining ($n = 4$) and A-FABP Western blot of differentiated 3T3-L1 cells transfected with control siRNA or siRNA against ROR γ 3 days before induction of differentiation. The results shown are representative of three independent differentiation experiments. Values represent means \pm SD, * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$.

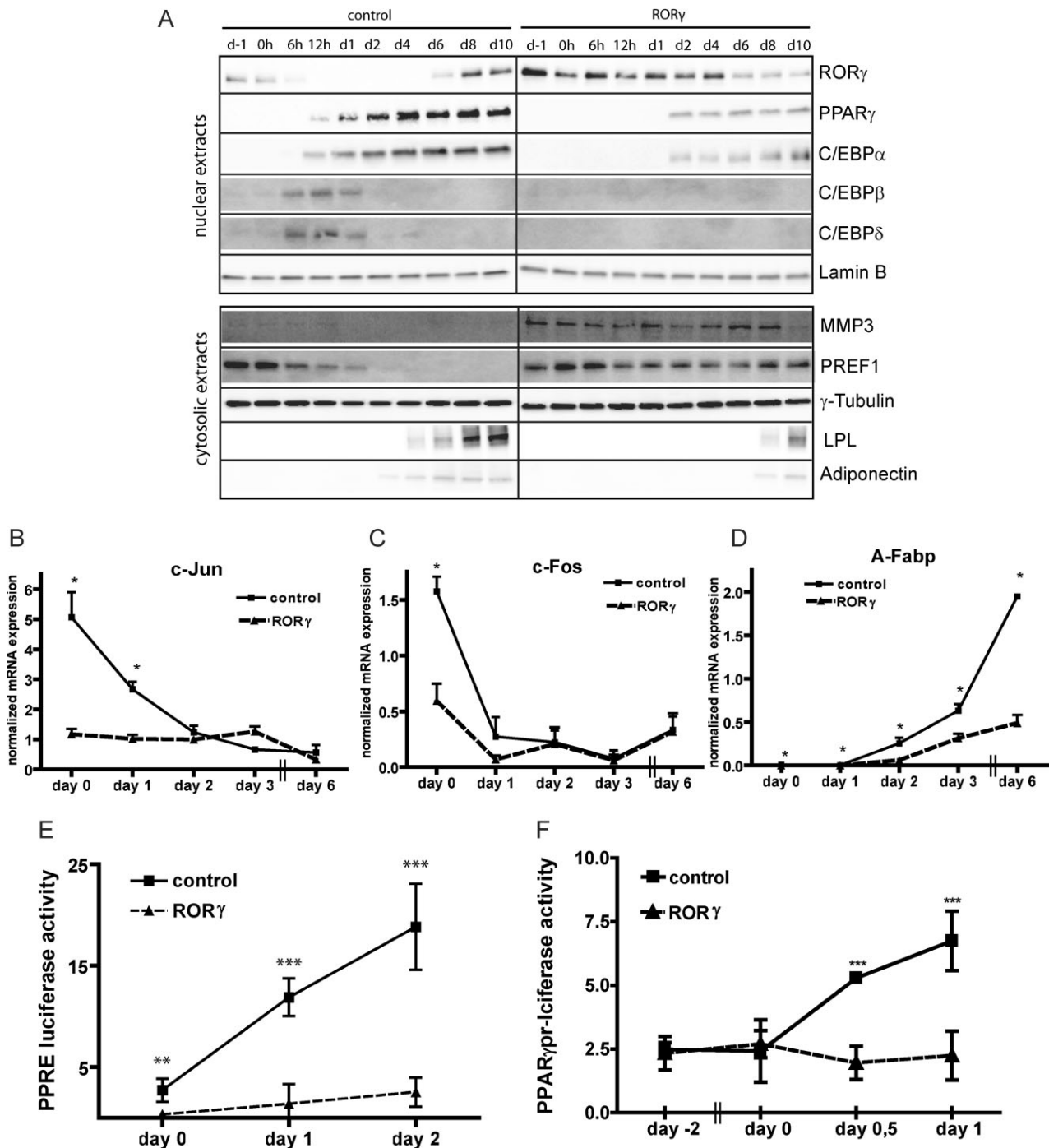


Figure 2. ROR γ inhibits expression and activity of differentiation genes.

- A.** Western blot of nuclear extracts (ROR γ , PPAR γ , C/EBP α , C/EBP β and C/EBP δ) or cytosolic extracts (MMP3, PREF-1, LPL and Adiponectin) during differentiation of 3T3-L1 cells overexpressing ROR γ or empty vector. Differentiation without rosiglitazone was induced at time point 0 h. Lamin B and γ -Tubulin were used as loading controls.
- B,C,D.** qPCR for the adipogenic genes c-Jun, c-Fos and A-Fabp during differentiation of 3T3-L1 cells overexpressing ROR γ or empty vector ($n = 4$).
- E.** Firefly luciferase activity of the PPAR γ response element (PPRE) normalized to renilla luciferase before, 1 and 2 days after induction of differentiation in 3T3-L1 cells overexpressing ROR γ or empty vector ($n = 4$).
- F.** Firefly luciferase activity of PPAR γ promoter normalized to renilla luciferase in 3T3-L1 cells overexpressing ROR γ or empty vector during differentiation at indicated time points ($n = 8$). The results shown are representative of three independent experiments. Values represent means \pm SD, * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$.

ROR γ and highly expressed in murine SVF were chosen for a functional siRNA screen to test their impact on adipogenesis (Fig 3A). Furthermore, we analysed whether transcription of these genes is regulated by ROR γ using a nuclear run-on assay (Fig 3B and Fig S1E of Supporting information). Whereas transcription of several identified genes was upregulated by ROR γ , only knockdown of MMP3 led to an increase in adipocyte differentiation (Fig 3A). We confirmed Mmp3 mRNA expression to be upregulated by transient or viral overexpression of ROR γ (Fig 3C). Interestingly, expression of Mmp3 mRNA and protein during early 3T3-L1 adipogenesis closely follows the expression profile of Ror γ with high expression in preadipocytes that is downregulated upon induction of differentiation (Figs 1A and 2A). To elucidate whether MMP3 is a direct target gene of ROR γ , we screened the MMP3 promoter for potential RREs. The promoter sequence contains two potential RREs: the first RRE (AGGTCA) matches the consensus half-site, whereas the second RRE has the half-core motif AGGTAA. Using different luciferase constructs, we could show that ROR γ -induced transcriptional activation of the MMP3 promoter depends on the first, but not on the second RRE and reaches levels similar to the known ROR γ target gene IL-17 (Fig 3D). These findings were corroborated by ChIP assays demonstrating binding of ROR γ to the first but not to the second RRE in the MMP3 promoter (Fig 3E).

To assess whether the effect of ROR γ on adipogenesis is mediated by MMP3, we added a MMP3 inhibitor during adipocyte differentiation. Similar to siRNA-mediated knockdown of MMP3 (Fig 3A), pharmacological inhibition of MMP3 enhances 3T3-L1 adipogenesis (Fig S2A of Supporting information). Moreover, inhibition of differentiation by ROR γ can be partially rescued with the MMP3 inhibitor (Fig 3F). Additionally, we could show that overexpression of MMP3 prevents the increased differentiation capacity caused by siRNA-mediated knockdown of ROR γ (Fig S2B of Supporting information). Conversely, siRNA-mediated knockdown of MMP3 rescues the inhibition of differentiation caused by ROR γ , whereas combined knockdown of MMP3 and ROR γ does not enhance differentiation compared to knockdown of ROR γ alone (Fig S2C of Supporting information). To analyse whether the effect of ROR γ on adipogenesis and Mmp3 expression is specific, we measured the effect of ROR α and a DNA binding-deficient mutant of ROR γ (ROR γ m) on Mmp3 expression and adipogenesis. We could show that ROR α inhibited differentiation as described previously (Ohoka et al, 2009) but did not modulate Mmp3 expression. A DNA binding-deficient ROR γ m, which does not bind to DNA because of the mutations C31A and C49A in the DNA binding domain (data not shown), influenced neither adipogenesis nor Mmp3 expression (Fig S2D and E of Supporting information). Taken together, these results show that MMP3 is a direct target of ROR γ and that inhibition of adipogenesis by ROR γ is at least partly mediated through induction of MMP3.

ROR γ is a key factor regulating preadipocyte differentiation *in vivo*

To assess whether the effect of ROR γ on adipogenesis could be recapitulated *in vivo*, we implanted murine adipose SVF

containing adipocyte precursor cells into mice (Kawaguchi et al, 1998, 1999). SVF from GFP-expressing mice resuspended in Matrigel and injected into the subcutaneous layer of the neck could be retrieved as a distinct pad 4–6 weeks after transplantation. Cells in these pads stained positive for GFP as well as for the adipocyte marker A-FABP and had a distinct adipocyte structure when acceptor mice were fed a high-fat diet (Fig 4A). Taken together, these results demonstrate that preadipocytes derived from a donor mouse can undergo adipogenesis *in vivo* without major host cell infiltration. To determine the effect of ROR γ on the process of adipogenesis *in vivo*, we injected GFP positive SVF of *wild-type* and Ror γ knockout animals into C57Bl/6 acceptor mice. After 4 weeks of high-fat diet excised pads with Ror γ ^{-/-} cells contained more mature adipocytes than pads with cells from control animals, demonstrating that the absence of ROR γ potentiates adipocyte differentiation (Fig 4A, right panel). Quantification of adipocyte numbers by automated image analysis demonstrated a 2.5-fold increase in *de novo* formed adipocytes in the absence of ROR γ (Fig 4B). In contrast, differentiation of SVF implanted into mice fed with chow diet was lower compared to high-fat diet feeding and did not differ between Ror γ ^{-/-} and *wild-type* SVF cells (Fig S3A of Supporting information).

Loss of ROR γ leads to increased formation of adipocytes in mouse models of obesity

We next examined whether the altered differentiation capacity of Ror γ ^{-/-} preadipocytes affects fat cell size within the adipose tissue. Fat cell sizes from adipose tissue sections were determined in obese Ror γ ^{-/-} and *wild-type* animals by automated image analysis. Whereas Ror γ ^{-/-} animals on a chow diet showed mildly decreased mean adipocyte sizes compared to their *wild-type* littermates (Fig S3B of Supporting information), this effect was potentiated in obese Ror γ ^{-/-} animals fed a high-fat diet for 8 weeks (DIO) or crossed into the leptin-deficient *ob/ob* background (Fig 4C). Analysis of the adipocyte size distribution revealed that in Ror γ ^{-/-} animals especially the number of small adipocytes (4000–8000 μm^2 in size) is increased (Fig 4D and E). To estimate the number of adipocytes, we also quantified whole fat volume using CT scanning and fat pad excision. Fat pad weights did not differ in mice fed a chow diet and were mildly decreased in obese Ror γ ^{-/-} mice (data not shown, Fig S3C and D of Supporting information). Based on the average adipocyte volume and the size of the fat pad, we quantified the increase in adipocyte number in the different models. Such calculations will only yield estimations since the cells are not perfectly round and sections are not cut in the middle of each cell. Nevertheless, we observed a slight increase in adipocyte numbers in male DIO subcutaneous and visceral adipose tissue in Ror γ ^{-/-} animals compared to *wild-type* littermates (6 and 11%). In female DIO and in male *ob/ob* subcutaneous and visceral adipose tissue, the calculated increase in adipocyte numbers was much more pronounced (average of 40%). To confirm that the observed changes in adipocyte size were caused by altered differentiation capacity rather than altered adipocyte function, functional assays were performed in 3T3-L1 adipocytes with siRNA-mediated

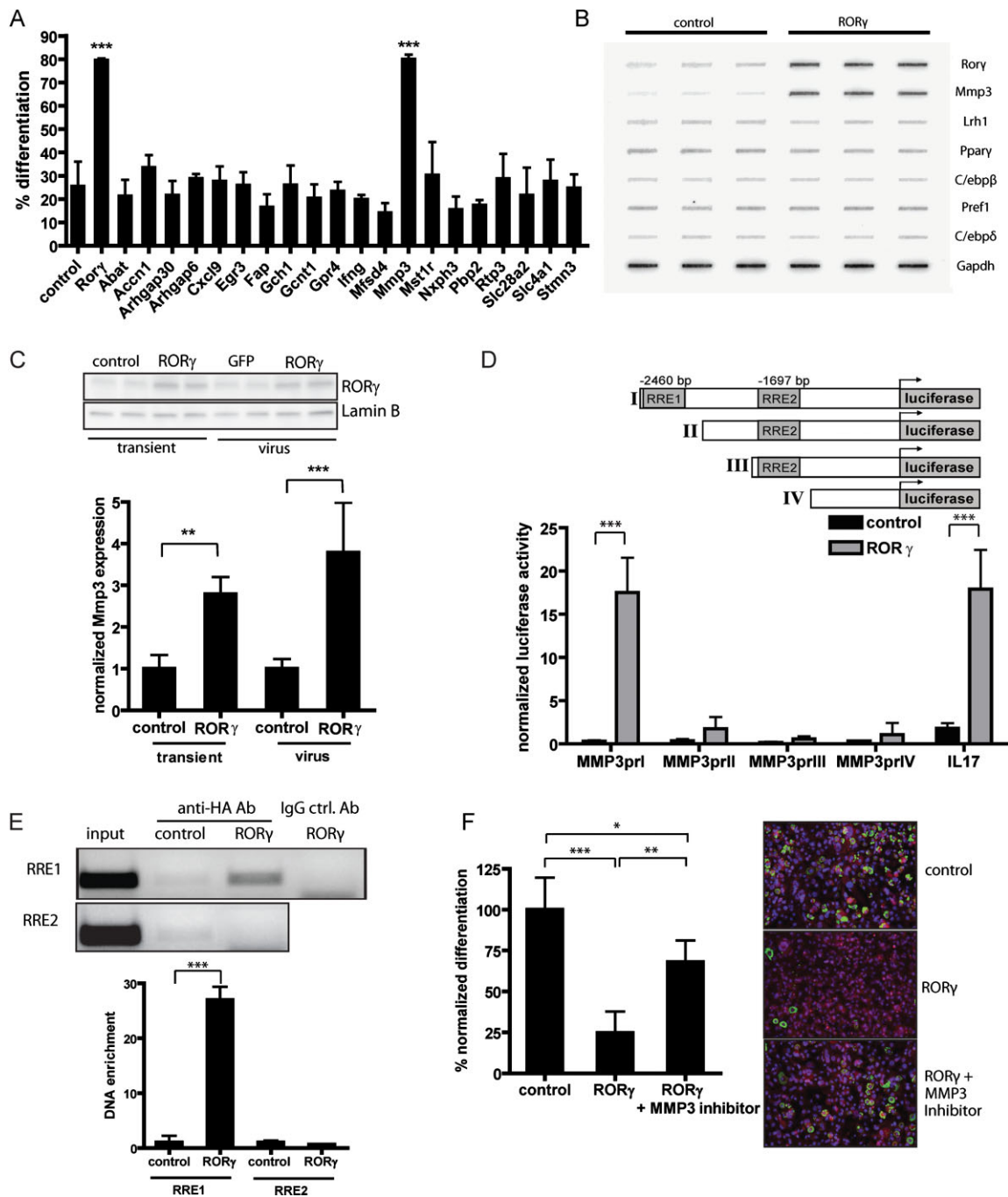


Figure 3. MMP3 is a target gene of ROR γ that inhibits adipogenesis.

- A.** Functional siRNA screen of potential ROR γ target genes identified by microarray analysis of 3T3-L1 cells overexpressing ROR γ . 3T3-L1 cells were transfected with siRNA pools, differentiated and fluorescently stained to assess the percentage of differentiated cells ($n = 4$).
- B.** Nuclear run-on assay for transcriptional activity of genes in undifferentiated 3T3-L1 cells infected with control or ROR γ -overexpressing lentivirus. 32P-labelled mRNA was hybridized to cDNA from the indicated genes on a slot blot and analysed by autoradiography.
- C.** Expression of Mmp3 mRNA in 3T3-L1 preadipocytes overexpressing ROR γ by transient transfection or viral infection.
- D.** MMP3 promoter constructs with potential ROR response elements (RREs) cloned into a luciferase reporter vector. Luciferase assay in 3T3-L1 preadipocytes overexpressing ROR γ or control vector 48 h after transfection normalized to renilla luciferase activity. Activation of IL17 promoter was used as a control ($n = 4$).
- E.** Chromatin immunoprecipitation of ROR γ in 3T3-L1 cells using anti-HA antibody 6 days after infection with ROR γ or control lentivirus. DNA was quantified by qPCR using specific primers for RRE1 and RRE2 and normalized to input ($n = 3$).
- F.** Fluorescent staining of differentiated 3T3-L1 adipocytes expressing ROR γ or control vector during differentiation treated with or without 20 μ M of MMP3 inhibitor 2 days before and after induction of differentiation (scale: 100 μ m). The results shown are representative of three independent experiments. Values represent means \pm SD, * $p \leq 0.05$ and ** $p \leq 0.01$, *** $p \leq 0.001$.

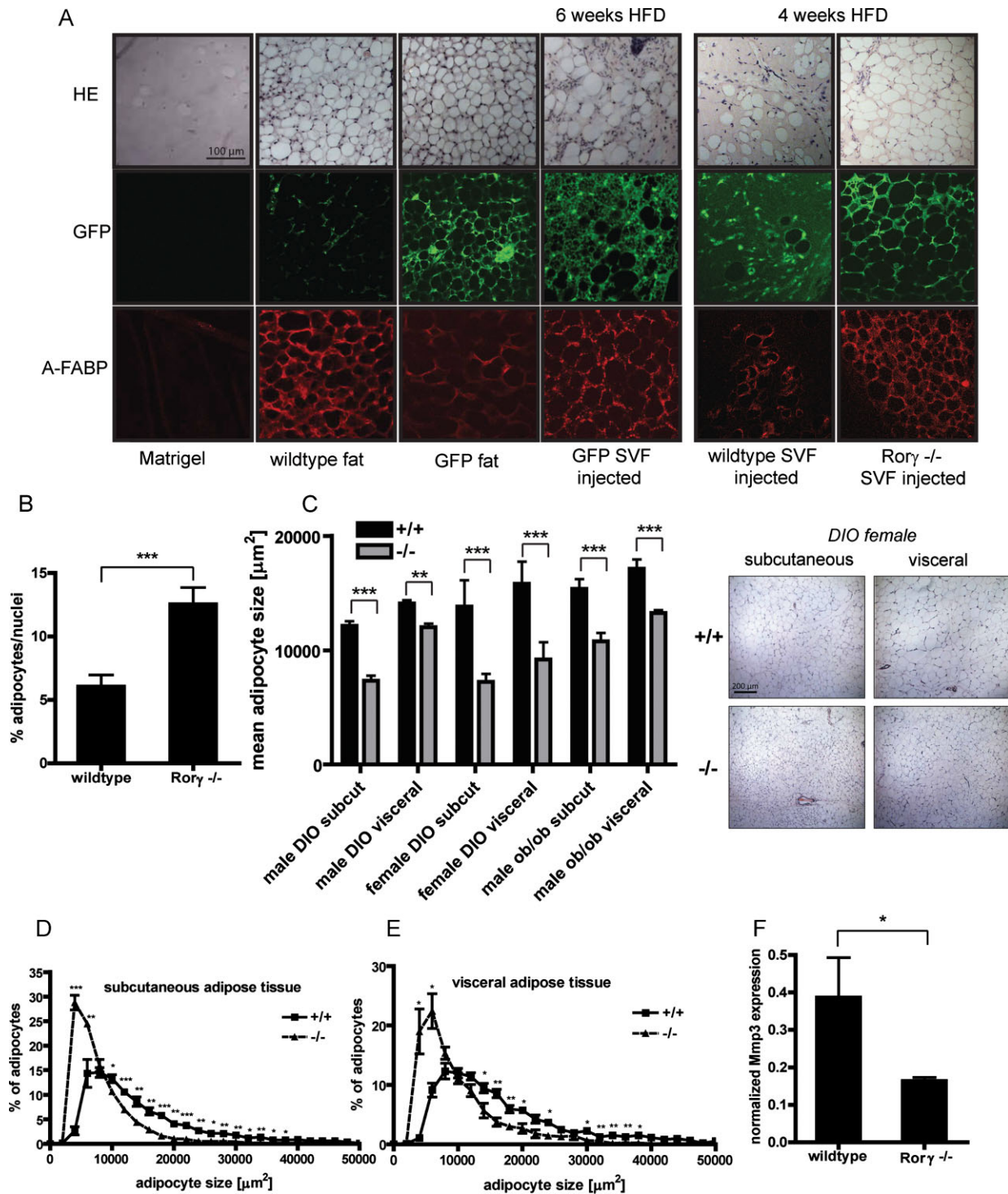


Figure 4. Loss of ROR γ leads to increased adipocyte formation *in vivo*.

- A.** Injection of SVF from different donor mice (as indicated) into the scapular subcutaneous region of C57Bl/6 mice. Fat pads were excised after 6 or 4 weeks of HFD feeding and stained as indicated.
- B.** Quantification of adipocyte differentiation in SVF transplants from *wild-type* and *Rory^{-/-}* mice fed a high-fat diet for 4 weeks ($n = 5$).
- C.** Mean adipocyte size of *Rory^{-/-}* and *wild-type* mice crossed into the *ob/ob* background or fed a high-fat diet for 8 weeks, insets are fat sections from representative samples of female DIO *wild-type* and *Rory^{-/-}* mice ($n = 7-10$).
- D,E.** Adipocyte size distributions from subcutaneous and visceral adipose tissue of female DIO *wild-type* and *Rory^{-/-}* mice ($n = 7$).
- F.** Mmp3 mRNA expression in visceral SVF of *Rory^{-/-}* and *wild-type* mice on chow diet ($n = 5$). Values represent means \pm SD, * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$.

knockdown or overexpression of ROR γ . Neither glucose uptake nor lipolysis were altered by ROR γ (Fig S3E–G of Supporting information, data not shown). Differences in adipocyte size were not due to altered energy balance as Ror $\gamma^{-/-}$ mice did not exhibit altered food intake or oxygen consumption in metabolic cage experiments after 7 weeks of high-fat diet (Fig S3H and I of Supporting information). As MMP3 is a direct target gene of ROR γ and at least partially responsible for the alteration of adipogenesis, we compared mRNA expression of Mmp3 in visceral SVF of 12-week-old Ror $\gamma^{-/-}$ and *wild-type* animals on a normal chow diet. Mmp3 expression was reduced 2.4-fold in the knockout animals (Fig 4F) suggesting that MMP3 is regulated by ROR γ *in vivo*.

Loss of ROR γ protects against obesity-induced insulin resistance

As Ror $\gamma^{-/-}$ mice show alterations in adipocyte size distribution, we examined the metabolic consequences of altered fat cell size in obese mouse models. Mean body weight was slightly reduced for male and female knockout animals on a high-fat diet (Fig 5A and Fig S4A of Supporting information), whereas chow fed animals did not show any changes in body weight (Fig S4B of Supporting information). In all models, Ror $\gamma^{-/-}$ mice were protected from obesity-induced hyperglycemia (Fig 5B), whereas no effect on glucose levels was observed in chow diet fed animals (Fig S4C of Supporting information). Decreased blood glucose levels in obese Ror $\gamma^{-/-}$ mice were accompanied by lower insulin levels in the fasted and in the fed state (Fig 5C and Fig S4D of Supporting information). In an insulin tolerance test, male Ror $\gamma^{-/-}$ animals showed improved insulin sensitivity compared to control mice after 8 weeks of high-fat diet (Fig 5D). Besides whole body insulin sensitivity, we also tested insulin sensitivity of isolated primary adipocytes from obese Ror $\gamma^{-/-}$ mice and control littermates. As expected from the observed differences in adipocyte size, insulin-mediated repression of lipolysis in obese Ror $\gamma^{-/-}$ animals was similar to *wild-type* chow fed animals. In contrast, adipocytes from obese *wild-type* mice were completely insulin-resistant (Fig 5E). In line with this, concentrations of circulating free fatty acids were significantly decreased in obese Ror $\gamma^{-/-}$ mice both in fasted and fed state (Fig 5F and Fig S4E of Supporting information). Triglyceride concentrations were mildly altered only in male high-fat diet animals (Fig S4F of Supporting information).

To analyse how Ror γ and Mmp3 expression is regulated in the course of obesity development and how this regulation is linked to fat cell size, we monitored body weight, adipocyte size and mRNA expression in visceral adipose tissue of mice in a time course of high-fat diet feeding for 24 weeks (Fig 5G). Interestingly, Ror γ expression initially dropped within the first weeks of high-fat diet feeding before expression increased during later stages of diet-induced obesity. Expression of the ROR γ target gene Mmp3 also increased at later stages of obesity. When looking at mean adipocyte size during the course of high-fat diet feeding, increased Ror γ expression at later stages paralleled accelerated adipocyte hypertrophy.

As ROR γ is expressed in muscle tissue as well as in fat, we analysed the ability of ROR γ to modulate glucose uptake in

muscle cells. Neither overexpression by viral infection with Ad-ROR γ , nor siRNA-mediated knockdown of ROR γ in differentiated C2C12 cells influenced basal or insulin-stimulated glucose uptake, whereas knockdown of insulin receptor (IR) completely abolished insulin-induced glucose uptake. These results suggest that the metabolic changes observed in Ror $\gamma^{-/-}$ mice are not due to altered muscle function (Fig S4G–J of Supporting information).

As ROR γ is implicated in immunological processes, we quantified different markers of inflammation associated with insulin resistance including TNF α , INF γ , IL-1 β , IL-2, IL-12 and IL-6 in serum of Ror $\gamma^{-/-}$ mice. To further address the inflammatory state of adipose tissue in obese *wild-type* and Ror $\gamma^{-/-}$ mice, we also measured fat-specific expression of the pro-inflammatory cytokines Tnf α and Il-6 as well as Cd68 and Emr1 as markers of macrophage infiltration. None of the inflammation markers linked to adipose tissue inflammation and macrophage infiltration was significantly altered in these mice (Fig S5A and B of Supporting information). Furthermore, we analysed expression of ROR γ in different subfractions of adipose SVF. As can be seen in Fig S5C of Supporting information, ROR γ is expressed both in CD4⁺ immune cells as well as in preadipocytes (Rodeheffer et al, 2008), albeit with a approximately fourfold higher expression in the latter population.

Taken together, our results show that adipose tissue of obese Ror $\gamma^{-/-}$ mice is composed of small, insulin-sensitive adipocytes with intact lipolytic control, which prevents systemic free fatty acid overload and diet-induced insulin resistance.

ROR γ expression in obese subjects predicts adipocyte size and insulin sensitivity

As we have shown that ablation of ROR γ serves as a protective mechanism against the development of obesity-associated insulin resistance in mice, we next analysed the link between Ror γ expression and insulin resistance in human obesity. To this end, we obtained subcutaneous fat biopsies from 21 obese patients (11 female and 10 male) with body mass indices (BMI) of 32–56 kg/m². As shown in previous studies, we observed a significant link between mean fat cell size and whole body insulin resistance determined by HOMA-index (Roberts et al, 2009; Fig S6A of Supporting information). To quantify the hyperplastic response of adipose tissue, we measured the differentiation potential of human SVF from subcutaneous adipose tissue biopsies using *ex vivo* differentiation assays, in which adipogenesis was quantified by high-throughput image analysis (Fig S6B of Supporting information). Interestingly, we found an inverse correlation between mean fat cell size and the differentiation potential of the corresponding SVF, which suggests that fat cell size is also partly determined by the ability to produce new fat cells (Fig S6C of Supporting information). Ror γ mRNA expression in SVF showed a positive correlation with adipocyte size and an inverse correlation with adipocyte differentiation potential (Fig 6A and B), demonstrating that Ror γ expression predicts adipocyte cell number in fat of obese patients similar to mice. Furthermore, expression of the ROR γ target gene Mmp3 in the SVF is correlated both with ROR γ

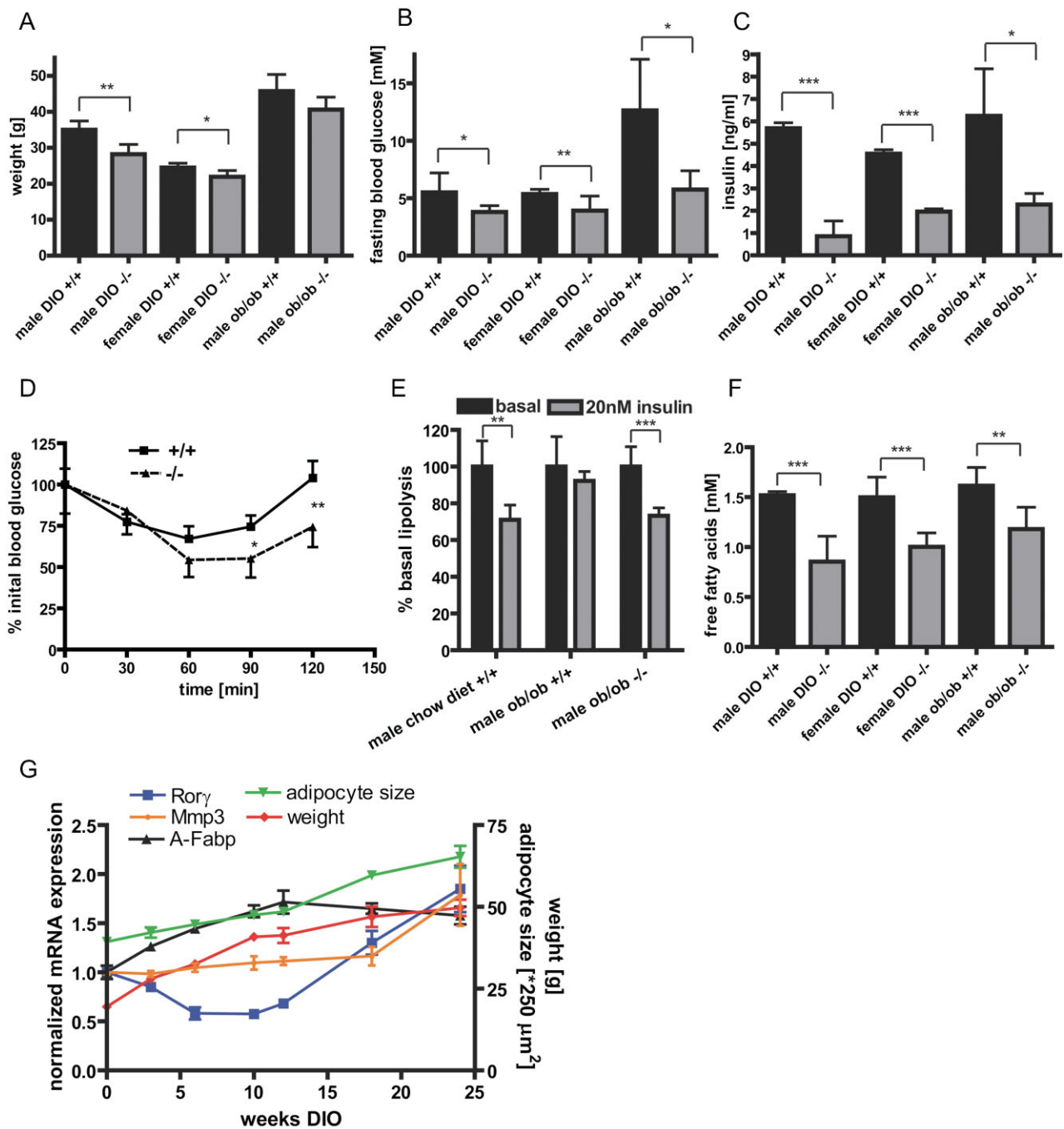


Figure 5. Loss of ROR γ protects against diet-induced insulin resistance in mice.

- A.** Body weight of $Ror\gamma^{-/-}$ and *wild-type* mice (12 weeks) crossed into the *ob/ob* background or fed a high-fat diet (DIO) for 8 weeks ($n = 7-10$).
- B,C.** Fasting blood glucose and serum insulin of *ob/ob* and high-fat diet $Ror\gamma^{-/-}$ and *wild-type* mice ($n = 7-10$).
- D.** Insulin tolerance test with male $Ror\gamma^{-/-}$ and *wild-type* mice after 8 weeks of high-fat diet injected intraperitoneal (i.p.) with 0.75 U/kg insulin ($n = 7$).
- E.** *In vitro* lipolysis of isolated adipocytes from 12-week-old *wild-type* mice on a chow diet, *ob/ob wild-type* and *ob/ob Ror $\gamma^{-/-}$* mice treated with or without insulin normalized to cell number and set to 100% for basal lipolysis ($n = 4$).
- F.** Fasting free fatty acid serum concentrations of *ob/ob* and high-fat diet $Ror\gamma^{-/-}$ and *wild-type* mice ($n = 7-10$).
- G.** Expression of *Ror γ* , *Mmp3* and *A-Fabp* in visceral adipose tissue of male C57Bl/6 mice during a time course of high-fat diet feeding. Body weight and mean adipocyte size of the animals was monitored.

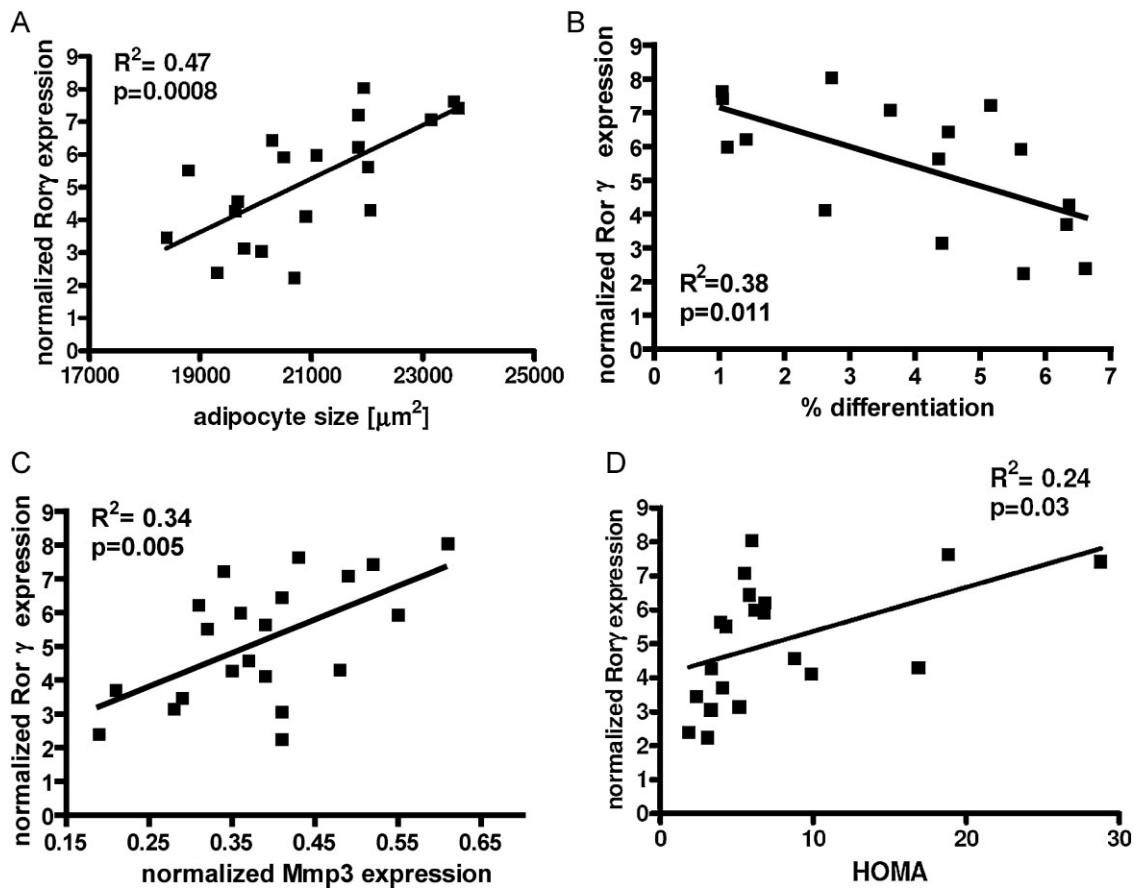


Figure 6. Expression of RORγ correlates with adipocyte size and insulin resistance in humans.

- A.** RORγ mRNA expression normalized to Gapdh in human subcutaneous SVF of obese subjects plotted against mean adipocyte cell size ($n = 20$).
B. RORγ mRNA expression in human subcutaneous SVF of obese subjects plotted against percent *in vitro* differentiated adipocytes of human subcutaneous SVF ($n = 16$).
C. RORγ mRNA expression plotted against Mmp3 expression in subcutaneous SVF of obese patients ($n = 21$).
D. RORγ expression in subcutaneous SVF plotted against HOMA index of obese subjects ($n = 20$). Values represent means \pm SD, * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$.

expression and with adipocyte size (Fig 6C and Fig S6D of Supporting information). RORγ mRNA expression in the SVF of obese subjects correlates with insulin sensitivity as measured by HOMA index (Fig 6D); however, RORγ mRNA expression does not show a significant correlation with BMI suggesting that RORγ is a predictor of insulin resistance independent of body weight in obese patients (Fig S6E of Supporting information).

DISCUSSION

Our results identify RORγ as a negative regulator of adipogenesis through expression of its target gene MMP3. The adipocyte size profile in obese RORγ knockout mice is shifted towards smaller cells and these mice remain insulin sensitive in adipose tissue as well as in the whole body. Since adipocyte precursor cells from RORγ knockout mice show a higher differentiation capacity in a physiological environment, it is intriguing to

speculate that RORγ knockout mice respond to nutrient overload with an increased hyperplastic response in adipose tissue, generating small, insulin-sensitive adipocytes by *de novo* differentiation. Indeed, it has been proposed that stimulation of adipogenesis improves whole body insulin sensitivity through reduced systemic free fatty acid overload and that this is one reason for the differences between metabolically healthy and metabolically unhealthy obese subjects (Lonn et al, 2010; O'Connell et al, 2010).

During high-fat diet feeding of mice, adipocyte size is only mildly affected in the early phase of weight gain, whereas adipocyte hypertrophy mainly occurs in late adiposity. This mirrors fat cell development in humans proposed by Spalding et al who suggested that adipocyte hyperplasia decreases with age (Spalding et al, 2008). Interestingly, RORγ expression in adipose tissue drops at the onset of high-fat diet feeding and increases later on concomitant with pronounced adipocyte hypertrophy. These results further hint at a role for RORγ in adipogenesis in response to nutritional overload.

To establish a direct link between ROR γ , adipocyte hyperplasia and insulin sensitivity, a conditional knockout of ROR γ in adipocyte precursor cells would be necessary. Unfortunately, no specific preadipocyte marker has been described to date that would allow recombination of ROR γ solely in adipocyte precursor cells. Furthermore, it is possible that other tissues and cell types also contribute to the observed phenotype. To this end, we tested the role of ROR γ in mature adipocytes and metabolic muscle function, as ROR γ is expressed in these cell types (Austin et al, 1998; Raichur et al, 2007). Neither adipocytes nor muscle cells showed altered glucose metabolism dependent on ROR γ . Moreover, improved insulin sensitivity in Ror γ knockout mice apparently does not depend on decreased expression of classical inflammatory cytokines. However, it is possible that secretion of the ROR γ target gene MMP3 from adipocytes and other cell types influences adipogenesis in a paracrine manner and/or exerts additional functions. Therefore, it would be interesting to explore whether expression of MMP3 is regulated by ROR γ in other cell types apart from adipocytes and preadipocytes and whether lack of MMP3 mirrors the metabolic phenotype of Ror γ knockout animals.

In obese, nondiabetic patients, Mmp3 expression is decreased in subcutaneous adipose tissue compared to lean subjects (Traurig et al, 2006), whereas we observe an increase of Ror γ and Mmp3 in the SVF of visceral adipose tissue from obese, diabetic patients. These discrepancies could be due to a fat depot-specific effect, as visceral adipose tissue is an independent determinant for the development of obesity-associated metabolic complications (Heilbronn et al, 2004; Ravussin & Smith, 2002), or due to differences in the degree of insulin resistance and further studies are needed to address this point. Within a cohort of obese patients, however, our data suggests that Ror γ expression is mainly associated with changes in insulin sensitivity.

The link between ROR γ and MMP3 regulating adipogenesis is intriguing as extracellular matrix remodelling by MMPs is an important aspect of adipocyte differentiation (Lilla et al, 2002). Whereas global inhibition of MMPs reduces differentiation (Chavey et al, 2003), Mmp3^{-/-} mice show an approximately 35% accelerated adipocyte differentiation during mammary gland involution (Alexander et al, 2001). Given the low specificity of MMPs for distinct extracellular matrix proteins, it will be a challenge to explore the exact nature of matrix reorganization by MMP3 leading to altered adipocyte differentiation in future studies.

In a time course of adipocyte differentiation, ROR γ and MMP3 are immediately repressed upon induction paralleled by down-regulation of other inhibitory markers such as Pref1 (Smas & Sul, 1993), which suggests an inhibitory role for ROR γ in early differentiation events. This is corroborated by the fact that ROR γ inhibits expression of early transcription factors such as C/ebp β and C/ebp δ leading to decreased expression of C/ebp α and Pparg and thus to reduced transcriptional activity of PPAR γ . In contrast to REV-ERB α , which directly modulates activity of the PPAR γ promoter (Wang & Lazar, 2008), ROR γ did not alter PPAR γ promoter activity in undifferentiated cells. As ROR γ -

mediated repression of adipogenesis cannot be reversed by the PPAR γ activator rosiglitazone, it can be presumed that ROR γ inhibits adipogenesis at an early stage through a PPAR γ -independent mechanism, which ultimately leads to a less pronounced induction of PPAR γ . ROR α has been previously described to inhibit adipogenesis by blocking the transcriptional activity of C/EBP β , thus reducing the induction of Pparg and C/ebp α expression (Ohoka et al, 2009). As ROR γ regulates genes preceding induction of C/EBP β and ROR α does not regulate Mmp3 expression it is likely that ROR γ and ROR α regulate adipogenesis through different pathways.

Mice with decreased ROR α (sg/sg) expression are resistant towards the development of diet-induced obesity (Lau et al, 2008). The mechanism for this phenotype is tightly linked to lipid mobilization as well as increased energy expenditure and also involves central regulation of food intake. These findings are quite different from ROR γ -deficient mice, which do not show major changes in triglyceride or cholesterol serum levels nor altered energy expenditure suggesting that ROR α and ROR γ control different metabolic aspects under high-fat diet feeding.

Loss of ROR γ leads to an increase in total adipocyte numbers in female DIO mice and in *ob/ob* mice, whereas this effect is less pronounced in male DIO mice. One possibility could be a sexual dimorphism phenotype, as regulation of adipogenesis includes gender specific aspects (Dieudonne et al, 2000). Further studies will be needed to address this point.

Given the fact that ROR γ in adipose tissue is linked to insulin sensitivity and that activity can be regulated by ligands, that is by hydroxylated cholesterol or the sulfonamide T0901317 (Kumar et al, 2009; Solt et al, 2011; Wang et al, 2009) ROR γ might be a novel target for the treatment of obesity-associated insulin resistance. Inhibition of ROR γ would mirror the effect of glitazones in clinical use, where activation of PPAR γ leads to improved insulin sensitivity partly through *de novo* adipocyte differentiation (Czoski-Murray et al, 2004; Zanchi et al, 2007).

Taken together, our studies reveal ROR γ as a novel factor in adipocyte differentiation through regulation of its target gene MMP3. ROR γ expression in adipose tissue is linked to adipocyte size, thereby influencing lipolytic control and insulin resistance in obesity.

MATERIALS AND METHODS

Animals

All mouse models were maintained in the C57Bl/6J background and kept on a 12-h/12-h light/dark cycle in a pathogen-free animal facility. Groups of mice were fed a high-fat diet (Provimi Kliba AG) containing 60% fat for 8 weeks. ROR γ global knockout mice (Sun et al, 2000) were a kind gift from D. Littmann.

Plasmids

ROR γ cDNA amplified from murine adipocyte cDNA was subcloned into pCR2.1 (Invitrogen) and subsequently into pcDNA3 (Invitrogen) for transfection or pCCL.sin.PPT.hPGK.IRES.GFP.PRE for lentiviral production. For adenoviral production HA-tagged ROR γ was cloned into pENTRcmv and recombined to pAdDEST (Invitrogen). MMP3 was

The paper explained

PROBLEM:

Obesity is a prevalent disease in western societies and poses a well-known risk for patients to develop secondary malignancies such as type 2 diabetes, hypertension and cardiovascular complications. Interestingly, only a part of obese subjects develops these problems, which is still largely unexplained, but could be due to differences in the development of adipose tissue. One hypothesis in this regard is that patients with small adipocytes are better at controlling the release of fatty acids from adipose tissue, thereby, protecting other tissues from toxic or harmful lipids.

RESULTS:

In a screen of obese and lean patients we identified the transcription factor ROR γ to be highly expressed in obesity. In addition, we could show that this factor controls adipose tissue development through a gene, which is responsible for reshaping

the extracellular environment, namely matrix metalloproteinase 3. If ROR γ is depleted from adipocyte precursor cells, these cells differentiate much more readily into adipocytes, both *in vitro* as well as *in vivo*. This enhanced potential to develop fat cells can lead to the buildup of small adipocytes that are highly insulin sensitive, and, thereby, much better at controlling lipid release. In mice, loss of ROR γ protects from the development of hyperglycemia, insulin resistance and ultimately type 2 diabetes. In obese human subjects, expression of this factor is inversely correlated with the ability to develop adipocytes and also with general insulin sensitivity, suggesting that ROR γ might indeed be an important factor for adipose tissue development in humans.

IMPACT:

Thus, ROR γ might serve as a new pharmaceutical target for the treatment of obesity-associated insulin resistance.

amplified from murine adipose tissue cDNA and cloned into the pcDNA3-V5/his-TOPO vector (Invitrogen). Different parts of the MMP3 promoter (Fig 3C) or 3 kb of the ROR γ promoter or 700 bp of the PPAR γ promoter (Wang & Lazar, 2008) were cloned from murine C57Bl/6J genomic DNA into pGL3. Single siRNAs or siRNA pools were purchased from Dharmacon. Primer sequences are available upon request.

Cell culture

3T3-L1 preadipocytes were cultured on collagen-coated plates in high-glucose DMEM (Invitrogen) supplemented with 10% foetal bovine serum and penicillin/streptomycin. For differentiation experiments, 3T3-L1 cells were seeded in 96-well plates at a density of $1.5 \times 10^4/\text{cm}^2$ and transfected the next day using Fugene (Roche) and overexpression vectors or siRNA according to the manufacturer's protocol. Adipogenesis was induced 3 days after transfection with $1 \mu\text{g}/\text{ml}$ insulin, $1 \mu\text{M}$ dexamethasone and $0.115 \text{ mg}/\text{ml}$ isobutylmethylxanthine, followed by 2 days of insulin medium ($1 \mu\text{g}/\text{ml}$) and 2 days of normal medium. Where indicated, the MMP3 inhibitor II (Merck) was added 2 days before and after induction of differentiation. For stable transfection, cells were kept on geneticin ($500 \mu\text{g}/\text{ml}$) for 2 weeks. C2C12 was cultured in high-glucose DMEM (Invitrogen) supplemented with 10% foetal bovine serum and penicillin/streptomycin. For differentiation experiments serum concentration was reduced to 1%.

Automated analysis of adipocyte differentiation

Differentiated cells were fixed with 5% formaldehyde prior to staining with BODIPY for lipid droplets, Hoechst for nuclei and Syto60 for cytosolic staining (Invitrogen). Twenty-five pictures per well were taken with an automated microscope imaging system (CellWorx/Operetta). Pictures were analysed using Cell Profiler or the Harmony software as described previously (Meissburger et al, 2011; Trajkovski et al, 2011).

Viral infection

Lentiviral stocks were prepared as described (Naldini et al, 1996). For functional assays differentiated 3T3-L1 cells were infected for 24 h with virus containing medium supplemented with polybrene ($8 \mu\text{g}/\text{ml}$) 2 days after induction of differentiation. Infection at this late time point did not alter differentiation capacity of the cells. Differentiation was continued until day 8. For adenoviral production HEK293A cells were infected with crude pAd-ROR γ virus lysate. Adenovirus was purified using a column system (Virapur). C2C12 cells were infected in media supplemented with 0.0001% poly-lysine for 24 h and glucose uptake was performed 3 days after infection.

Western blotting

Whole cell lysates, cytosolic extracts or nuclear extracts were prepared as described previously (Wolfrum et al, 2003). A-FABP was detected with an antibody raised against recombinant mouse A-FABP (N. Haunerland). C/EBP α,β,δ and PPAR γ (Cell Signaling) as well as ROR γ (Abcam) and ROR γ t (eBioscience) were detected according to the manufacturer's instructions. γ -Tubulin (Sigma) or Lamin B (Cell Signaling) were used as loading control.

Luciferase assay, chromatin immunoprecipitation

Luciferase assays were performed as described before (Wolfrum et al, 2003). For ChIP assays 3T3-L1 cells were infected with ROR γ or control lentivirus at 60% confluence. Days 6–8 after transfection chromatin immunoprecipitation was performed using the chromatin IP Assay Kit (LucernaChem) according to the manufacturer's protocol. Two putative RREs in the MMP3 promoter were tested: RRE1 (–2940 to –2928) TTCCAAGGTCA and RRE2 (–1727 to –1715) TTAAGTAGGTAA.

Nuclear run-on assay

The nuclear run on assay was performed in undifferentiated 3T3-L1 preadipocytes according to the method described by Reeder and

Roeder (Reeder & Roeder, 1972). In brief, nuclei were extracted 5 days after virus infection and transcription was allowed to finish in the presence of ^{32}P -ATP. cDNA for the genes of interest was immobilized on a slot blot, labelled mRNA was hybridized to the blot and analysed by autoradiography. Primer sequences are available upon request.

Real-time PCR

mRNA was isolated and transcribed into cDNA using the Multi-MACS cDNA kit (Miltényi). mRNA expression was assessed by real-time PCR using SybrGreen (Invitrogen) according to the manufacturer's protocol. mRNA expression was normalized to 36b4 or Gapdh. Primer sequences are available upon request. For expression of ROR γ and MMP3 in human samples TaqMan gene expression assays (Ambion) were used according to the manufacturer's protocol.

Microarrays

Total RNA was extracted using Trizol (Invitrogen). Genomic DNA was digested using the DNA-free kit (Ambion) and RNA was labelled with either Affimetrix labelling system (human samples) or with One-Colour RNA Spike-In Kit (Agilent) for mouse samples. Labelled cRNA was hybridized to human genome U133 arrays (Affimetrix) or 44k whole mouse genome arrays (Agilent). For the human analysis six patients per group were analysed. For identification of ROR γ target genes two independent experiments using transient overexpression of ROR γ were performed. Genes that were upregulated >twofold and show a high expression also in primary mouse SVF (internal microarray data) were selected for a functional siRNA differentiation screen. Data were submitted to the EMBL-EBI database and the accession numbers are E-MEXP-3313 for the human SVF array and E-MEXP-3312 for the ROR γ 3T3L1 cell array.

Glucose uptake and lipolysis

Glucose uptake and lipolysis was determined essentially as described previously (Wolfrum et al, 2003). Briefly, cells were preincubated with 10 nM (C2C12), 20 nM (primary adipocytes) or 50 nM (3T3-L1 adipocytes) insulin for 10 min. Primary adipocytes were spun through dinoyl-phthalate oil, washed and the aqueous phase was collected. Osmium-tetroxide fixed primary adipocytes (Mersmann & Macneil, 1986) were counted for normalization. Glycerol release in the supernatant was measured to assess lipolysis using glycerol reagent (Sigma) according to the manufacturer's protocol.

In vivo differentiation of SVF

SVF from subcutaneous and visceral fat was prepared as described before (Hansen et al, 1998). Briefly, fat tissue was dissected, minced and incubated in collagenase type II for 1 h at 37 °C. Pelleted stromal-vascular fraction was strained through a 40 μm net, erythrocytes were lysed and 10^6 cells were resuspended in 100 μl of Matrigel (BD). Cells were injected subcutaneously into a skin fold of the neck. The protocol for injection and differentiation was adapted from Kawaguchi et al (Kawaguchi et al, 1998, 1999). After 4–6 weeks Matrigel pads were excised and treated as described below for the determination of adipocyte size. From each pad pictures of three full sections were taken and adipocyte numbers as well as the number of nuclei were determined automatically with Cell Profiler.

Adipocyte 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 (Chen & Farese, 2002). Microscopic pictures were taken and cell size was analysed using Cell Profiler. Fat pads were either weighted after excision or measured with an animal CT-Scanner (LaTheta). At least 5000 adipocytes were measured per animal to determine adipocyte size.

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 using C.f.a.s. standard (Roche/Hitachi). Free fatty acids were measured with the NEAFA-HR kit (Wako). For serum ILs a micro-bead based assay system (Luminex) was used.

Ex vivo differentiation of human SVF

Differentiation of the preadipocytes was induced as described previously with minor modifications (Isakson et al, 2008). Complete SVF fraction was obtained from subcutaneous biopsies of obese patients and 5×10^4 cells were plated on collagen coated 96-well plates. Cells were induced with a differentiation cocktail consisting of 1 μM insulin, 10 μM dexamethasone, 0.5 mM IBMX, 100 nM pioglitazone, 33 μM biotin and 17 μM pathenonate in DMEM/F12 supplemented with 3% FBS, 2 mM glutamine and antibiotics. Two days post induction medium was changed to a medium containing only insulin and dexamethasone in DMEM/F12, 10% FBS with glutamine and antibiotics. Cells were analysed as described above at day 8 of differentiation. Differentiated cells were co-stained with adiponectin to verify differentiation.

Bioethics

All mouse work was carried out in Zürich and was approved by the Kantonale Veterinärämter Zürich. The human work on obese *versus* lean patients was performed in Bratislava and approved by the ethics committee of the Slovak Academy of Science. Informed consent was obtained from all patients, studied. The work on obese subjects was carried out at the University Hospital of Heidelberg, with approval from the ethics committee of the University Hospital. Informed consent was obtained from all patients, studied.

Statistical analysis

Results are given as mean \pm standard deviation. Statistical analyses were performed using a two-tailed Student's *t*-test. Linear regression was calculated using Graph Pad Prism.

Author contributions

NB and MG performed the *in vivo* differentiation experiments; DT performed the analysis on circulating inflammation markers; WL provided help with the CT scanning of mice; BC helped with the ChIP assays; BM and CW performed all other experiments (except for obtaining patient samples); JU and DG obtained the patient material of the obese *versus* lean subjects; ER, GR and PPN obtained patient material and characterized the obese patient cohort; CW, BM, PPN and GR wrote and edited the manuscript.

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Supporting information is available at EMBO Molecular Medicine online.

The authors declare that they have no conflict of interest.

For more information

Ensembl:

Rorc [ENSMUSG00000028150]

http://www.ensembl.org/Mus_musculus/Gene/Summary?g=ENSMUSG00000028150

Mmp3 [ENSMUSG00000043613]

http://www.ensembl.org/Mus_musculus/Gene/Summary?g=ENSMUSG00000043613

Uniprot:

P51450 (RORG_MOUSE)

<http://www.uniprot.org/uniprot/P51450>

P28862 (MMP3_MOUSE)

<http://www.uniprot.org/uniprot/P28862>

MGI:

RORC MGI:104856

http://www.informatics.jax.org/searches/accession_report.cgi?id=MGI:104856

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