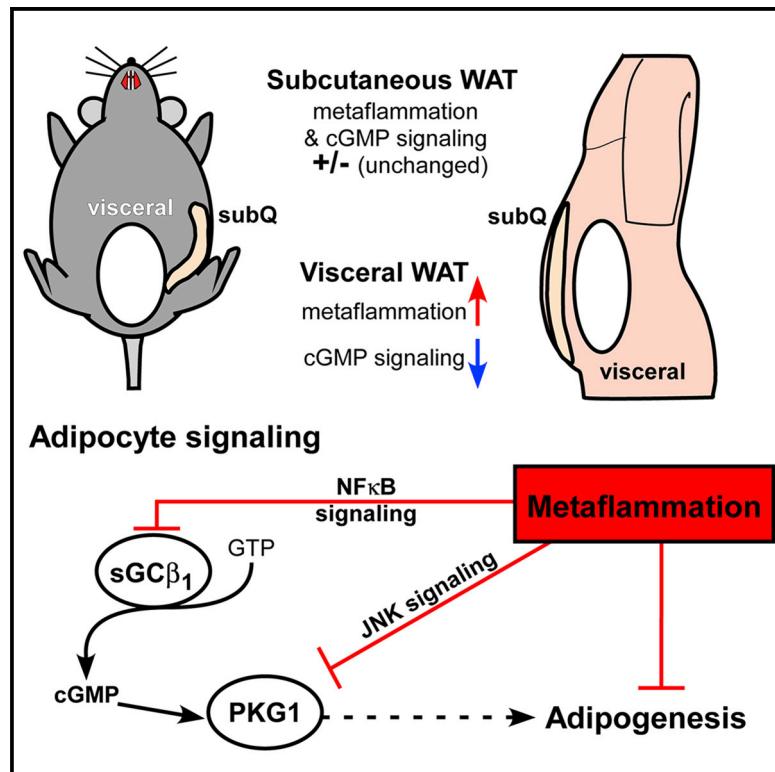


# Interplay between Obesity-Induced Inflammation and cGMP Signaling in White Adipose Tissue

## Graphical Abstract



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## In Brief

Sanyal et al. report that the dysregulation of cGMP signaling in adipose tissue of obese mice is proportional to the degree of metaflammation through distinct pathways. Since targeting cGMP signaling ameliorates obesity and associated co-morbidities, knowledge gained from this study suggests avenues for therapy.

## Highlights

- Metaflammation impedes cGMP signaling in visceral, but not subcutaneous, WAT
- cGMP signaling dysregulation corresponds to the degree of WAT inflammation
- NF-κB and JNK signaling differentially regulate cGMP signaling component expression
- Status of metaflammation and cGMP signaling are alike in murine and human obesity

# Interplay between Obesity-Induced Inflammation and cGMP Signaling in White Adipose Tissue

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## SUMMARY

Current worldwide figures suggest that obesity is pandemic. Understanding the underlying molecular mechanisms would help develop viable anti-obesity therapies. Here, we assess the influence of obesity-induced inflammation on white adipocyte cyclic guanosine monophosphate (cGMP) signaling, which is beneficial for adipocyte differentiation and thermogenesis. We find that murine gonadal and not inguinal fat is prone to obesity-induced inflammation. Correspondingly, the cGMP cascade is dysregulated in gonadal but not in inguinal fat of obese mice. Analysis of two independent human cohorts reveals a defective cGMP pathway only in visceral fat of obese subjects. Congruently, cGMP signaling is dysregulated in tumor necrosis factor  $\alpha$  (TNF- $\alpha$ )-treated primary white adipocytes. TNF- $\alpha$ -mediated suppression of sGC $\beta_1$  is mediated via NF- $\kappa$ B, whereas PKG is repressed by JNK signaling. Additionally, TNF- $\alpha$ -activated JNK signaling suppresses PPAR $\gamma$  and aP2. Taken together, the intensity of obesity-induced inflammation dictates the amplitude of cGMP signaling dysregulation in white adipocytes through distinct pathways.

## INTRODUCTION

The prevalence of obesity has dramatically increased over the past few decades (Ng et al., 2014). The World Health Organization (WHO) estimates that 1.9 billion human adults are overweight, of whom 600 million are obese (World Health Organization, 2014). Obesity, characterized by unhealthy expansion of the adipose tissue (AT) (Rutkowski et al., 2015), is associated with pathophysiological changes in AT (Sun et al., 2011; Özcan

et al., 2004) that lead to chronic inflammation (Gutierrez et al., 2009; Castoldi et al., 2016; Dalmas et al., 2011), which is also known as metaflammation (Gregor and Hotamisligil, 2011). Obesity alters the AT immune cell profile from an anti- to pro-inflammatory state (Lumeng et al., 2007a, 2008; Weisberg et al., 2003; Lee and Lee, 2014; Lumeng and Saltiel, 2011). Pro-inflammatory cytokines (Lee and Lee, 2014), such as tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ), released by AT cells, have been shown to play a major role in the development of insulin resistance and diabetes (Hotamisligil and Spiegelman, 1994). Systemic insulin resistance eventually perturbs metabolic homeostasis and ultimately contributes to obesity-associated co-morbidities (de Luca and Olefsky, 2008; Gutierrez et al., 2009; Jung and Choi, 2014).

Cyclic guanosine monophosphate (cGMP) is a second messenger involved in the regulation of the cardiovascular system (Tsai and Kass, 2009; Handa et al., 2011), as well as AT function (Haas et al., 2009; Mitschke et al., 2013; Ayala et al., 2007; Hoffmann et al., 2015). cGMP is produced by two types of guanylate cyclases (GCs) (Koesling et al., 1991):  $\alpha$ - $\beta$  heterodimeric soluble GC (sGC), when stimulated by nitric oxide (NO) (Koesling et al., 1991; McDonald and Murad, 1996; Poulos, 2006); and membrane-bound particulate GC (pGC), upon extracellular natriuretic peptide activation (Miyashita et al., 2009; Waldman et al., 1984). In turn, cGMP can activate cyclic nucleotide gated ion channels (Pifferi et al., 2006) and the cGMP-dependent protein kinase (PKG) (Butt et al., 1994; Francis et al., 2010), which has been identified as the major mediator of cGMP effects in adipocytes (Haas et al., 2009; Mitschke et al., 2013; Withers et al., 2014). Phosphodiesterases (PDEs) like PDE5A degrade cGMP to GMP (Francis et al., 2010), thereby regulating cGMP signaling activation. Although, the cGMP-PKG cascade enhances differentiation and thermogenesis (energy expenditure by heat generation) in adipocytes (Haas et al., 2009; Hoffmann et al., 2015; Mitschke et al., 2013; Bordicchia et al., 2012), not much is known about its regulation during obesity. Therefore, examining cGMP signaling modulation in obesity is important and might have therapeutic implications.



Here, we assess the impact of genetic and diet-induced obesity (DIO) on cGMP signaling in white adipose tissue (WAT). We demonstrate that sGC-PKG1-mediated cGMP signaling is diminished in gonadal or visceral WAT (WATg), but not in inguinal or subcutaneous WAT (WATi), of mice. Interestingly, sGC $\beta_1$  and PKG1 mRNA expression is reduced in omental or visceral AT (VAT), but not in subcutaneous AT (SAT), of obese humans. In parallel, we find that murine WATg displays strong characteristics of obesity-induced inflammation, whereas WATi is minimally inflamed. Treatment of primary murine white adipocytes (WAs) with TNF- $\alpha$  represses the sGC-PKG1 pathway, as well as expression of major adipogenic regulators (peroxisome proliferator-activated receptor gamma [PPAR $\gamma$ ] and adipocyte protein 2 [aP2]). Subsequently, we identify that TNF- $\alpha$ -activated nuclear factor  $\kappa$ B (NF- $\kappa$ B) signaling suppresses sGC $\beta_1$  expression, while PKG1, PPAR $\gamma$ , and aP2 are downregulated by c-Jun N-terminal kinase (JNK) signaling.

## RESULTS

### Obesity Downregulates cGMP Signaling in WATg, but Not in WATi, of Mice

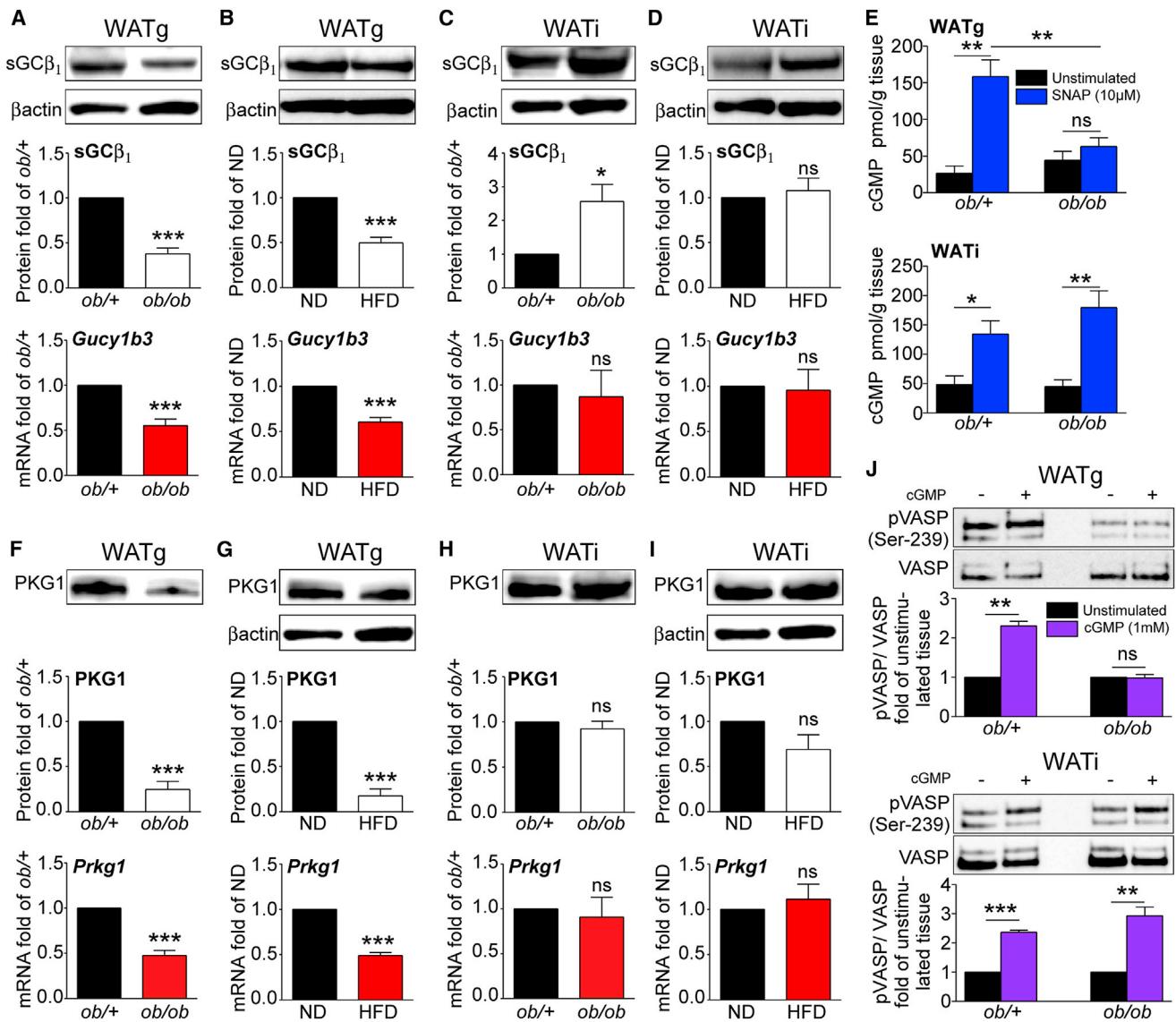
To understand the impact of obesity on the cGMP signaling pathway in WAT, we analyzed WATg and WATi of leptin-deficient genetically obese (*ob/ob*) and lean (*ob/+*) mice for expression of the major cGMP signaling components sGC $\beta_1$  and PKG1. To exclude artifacts resulting from leptin deficiency, we corroborated our observations by analyzing mice fed a high-fat diet (HFD) compared to controls fed a normal diet (ND). The *ob/ob* mice were significantly heavier than *ob/+* mice (whole body weight as well as WAT and liver weights; Figure S1A and S1B). sGC $\beta_1$  protein content in WATg was significantly reduced in *ob/ob* and HFD mice compared to their respective controls (Figures 1A and 1B, top). In addition, *Gucy1b3* (sGC $\beta_1$ ) mRNA expression was lower in WATg from *ob/ob* and HFD mice (Figures 1A and 1B, bottom). Conversely, sGC $\beta_1$  protein expression in WATi was significantly increased in *ob/ob* but unaltered in HFD mice (Figures 1C and 1D, top). *Gucy1b3* expression was not significantly altered in WATi of genetically or diet-induced obese mice compared to corresponding lean controls (Figures 1C and 1D, bottom). Expression of sGC $\alpha_1$  (*Gucy1a3*), the other component of sGC, was also significantly reduced in WATg but unaltered in WATi from *ob/ob* mice (Figures S1C). To study the functional consequence of sGC repression, we analyzed cGMP production upon acute stimulation with the nitric oxide (NO) donor (SNAP), which was significantly diminished in WATg explants from *ob/ob* compared to *ob/+* mice (Figure 1E, top). In contrast, SNAP-stimulated cGMP levels were not significantly different between WATi from *ob/+* and *ob/ob* mice (Figure 1E, bottom). Interestingly, expression of PKG1, the major target of cGMP in adipocytes, was reduced in WATg of *ob/ob* and HFD-fed mice compared to their respective controls (Figures 1F and 1G) but was unchanged in WATi (Figures 1H and 1I). Fittingly, cGMP-induced phosphorylation of vasodilator-stimulated phosphoprotein (VASP), a major PKG target in adipocytes (Jennissen et al., 2012), at serine-239 was repressed only in WATg, but not in WATi, of *ob/ob* mice (Figure 1J). In a separate cohort of DIO mice (body and tissue weights in Figures S1D and S1E),

*Gucy1b3* and *Prkg1* expression was significantly reduced in adipocytes as well as the stromal vascular fraction (SVF) of WATg from HFD mice but unchanged in WATi (Figures S1F and S1G). Altogether, we observe that sGC-PKG1-mediated cGMP signaling is dysregulated in WATg, but surprisingly not in WATi, of obese mice.

### Different Degrees of Metaflammation in WATg and WATi in Obesity

Unhealthy expansion of adipose tissue during obesity leads to metaflammation (Gregor and Hotamisligil, 2011; Lee and Lee, 2014; Lumeng et al., 2007a; Lumeng and Saltiel, 2011). To explain the discrepancies in sGC and PKG1 expression patterns between the WAT depots, we assessed the inflammatory profiles of WATg and WATi in lean and obese mice. Expression of pro-inflammatory genes like *Tnfa*, interleukin 6 (*Il6*), interleukin 1 $\beta$  (*Il1b*), and chemokine (C-C motif) ligand 3 (*Ccl3*) and 7 (*Ccl7*) was significantly increased in WATg from *ob/ob* compared to *ob/+* mice (Figure 2A). In contrast, expression of *Il6* and *Ccl7* was unchanged in WATi from *ob/ob* and *ob/+* mice, while *Tnfa*, *Il1b*, and *Ccl3* were repressed (Figure 2B). *Ccl2* was upregulated in WATi, but to a lesser extent than in WATg from *ob/ob* mice. F4/80 staining in histological sections demonstrated a visibly greater AT macrophage (ATM) content in WATg than in WATi from *ob/ob* mice (Figure 2C). Flow cytometric analysis (gating strategy in Figure S2A) of WAT from *ob/+* and *ob/ob* mice revealed that ATM (CD45 $^+$  F4/80 $^+$  CD11b $^+$  Ly6C $^-$ ) content was significantly higher in WATg from *ob/ob* mice than *ob/+* mice (Figures 2D and 2E). However, macrophage counts only moderately increased in WATi (Figures 2D and 2E). The proportion of pro-inflammatory ATMs (CD45 $^+$  F4/80 $^+$  CD11b $^+$  Ly6C $^-$  CD11c $^+$ ; Lumeng et al., 2007a; Morris et al., 2011) significantly increased only in WATg, but not WATi, of *ob/ob* mice when compared to *ob/+* mice (Figures 2F and S2B). On the contrary, anti-inflammatory ATM proportion (CD45 $^+$  F4/80 $^+$  CD11b $^+$  Ly6C $^-$  CD11c $^-$  CD206 $^+$  CD301 $^+$ ; Morris et al., 2011) was diminished in WATg of *ob/ob* mice when compared to *ob/+* mice but unaltered in WATi (Figure 2G). Nonetheless, their content increased in both WATg and WATi of *ob/ob* mice compared to *ob/+* mice (Figure S2C). These observations indicated that WATg from *ob/ob* mice has a greater macrophage content and is more inflamed than WATi.

In DIO, *Tnfa* expression was higher in WATg, but not in WATi, from HFD mice compared to ND mice (Figures 2H and 2I). Although *Il6* and *Ccl2* were mildly upregulated in WATi of HFD mice, overall, their expression was greater in WATg. Inflammatory markers were highly upregulated in the SVF and adipocyte compartments of WATg, but not in WATi, in HFD mice (Figures S3A–S3D). Adipocyte and SVF biomarker gene expression was determined to ascertain purity of the samples analyzed (Figures S3E–S3J). *Ccl2* and *Ccl3* mRNA were moderately elevated in WATi, while their expression in both SVF and adipocytes from WATg was higher in HFD mice (Figures S3A–S3D). *Adgre1* (F4/80) expression was greatly increased in WATg of HFD mice but only moderately increased in WATi (Figures 2H and 2I). Mirroring mRNA expression, TNF- $\alpha$ , IL-6, IL-1 $\beta$ , monocyte chemoattractant protein 1 (MCP-1), MCP-3, and macrophage inflammatory protein 1 $\alpha$  (MIP-1 $\alpha$ ) content was significantly higher in WATg of

**Figure 1.** Obesity Represses sGC-PKG1 Signaling Only in WATg of Obese Mice

(A and B) Representative western blots (WBs) and densitometric quantification of sGC $\beta_1$  protein (top) and *Gucy1b3* mRNA expression (bottom) in WATg from (A) *ob/ob* mice compared to *ob/+* mice ( $n = 6-10$ ; t test) and (B) mice fed a high-fat diet (HFD) compared to a normal diet (ND) ( $n = 4-8$ ; t test).

(C and D) Representative WB and densitometric quantification of sGC $\beta_1$  protein (top) and mRNA (*Gucy1b3*, bottom) expression in WATi from (C) *ob/ob* ( $n = 5-9$ ) and (D) HFD-fed ( $n = 4-7$ ) mice compared to respective controls (t test).

(E) cGMP content in unstimulated and 10  $\mu$ M SNAP-stimulated WATg (top) and WATi (bottom) explants of *ob/+* and *ob/ob* mice ( $n = 5-7$ ; t test).

(F and G) Representative WB and densitometric quantification of PKG1 protein (top) and *Prkg1* mRNA (bottom) expression in WATg of (F) *ob/ob* mice compared to *ob/+* ( $n = 6$ ; t test; refer to [A] for loading control) and (G) HFD-fed compared to ND-fed mice ( $n = 4-8$ ; t test).

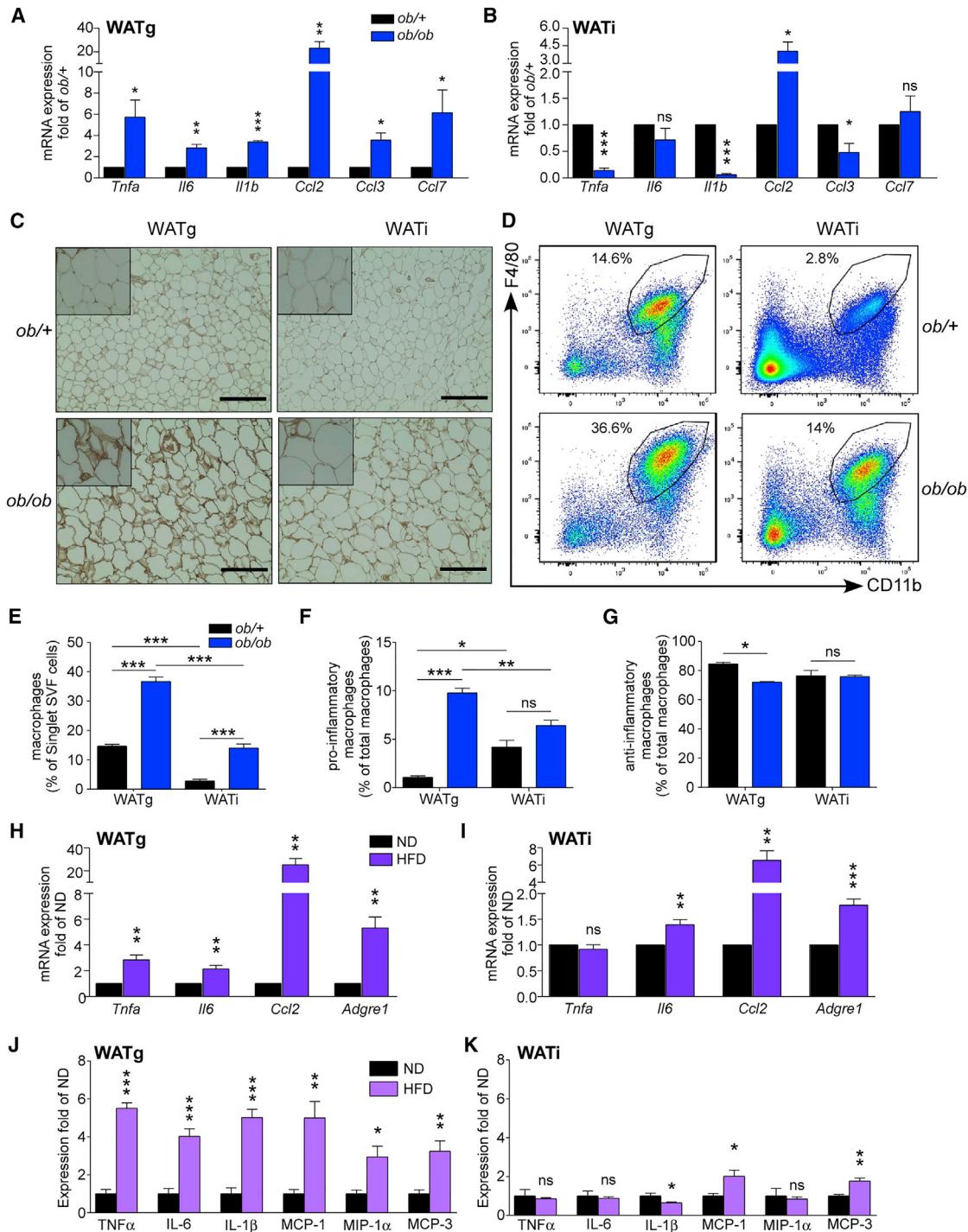
(H and I) WB quantification of PKG1 protein (top) and *Prkg1* mRNA (bottom) in WATi of (H) *ob/ob* compared to *ob/+* mice ( $n = 10$ ; t test; refer to [C] for loading control) and (I) HFD-fed compared to ND-fed mice ( $n = 3-6$ ; t test).

(J) Representative WB of phospho-VASP (Ser239) and VASP and densitometric quantification of Phospho-VASP/VASP in WATg (top) and WATi (bottom) of *ob/+* and *ob/ob* mice treated without (unstimulated) or with 1 mM 8-Br cGMP (cGMP) (fold of respective unstimulated tissues;  $n = 3$ ; t test).

Data are presented as mean  $\pm$  SEM. ns, not significant; \*p < 0.05, \*\*p < 0.01, and \*\*\*p < 0.001. See also Figure S1.

HFD mice than in WATg of ND mice (Figures 2J and S3K). Conversely, TNF- $\alpha$  and IL-6 were unchanged, while MCP-1 and MCP-3 were modestly upregulated and IL-1 $\beta$  was reduced in WATi of HFD mice (Figures 2K and S3L). The serum level of MCP-3 was significantly higher in HFD mice, while IL-6 elevation

was not statistically significant (Figure S3M). TNF- $\alpha$ , IL-1 $\beta$ , MCP-1, and MIP-1 $\alpha$  content in circulation was unaffected by DIO (Figure S3M). Together, these data suggest that although obesity results in expansion of WAT in mice, metaflammation is pronounced only in WATg.



**Figure 2. Pro-inflammatory Phenotype in WATg, but Not WATi, of Obese Mice**

(A and B) Pro-inflammatory gene expression in (A) WATg ( $n = 5\text{--}9$ ) and (B) WATi ( $n = 5\text{--}9$ ) of ob/ob compared to ob/+ mice (t test).

(C) Representative paraffin sections of WATg and WATi from ob/+ and ob/ob mice stained for F4/80 observed under 20 $\times$  magnification. Insets represent sections under 40 $\times$  magnification. Bars indicate 200  $\mu\text{m}$ .

(D) Representative FACS plots of CD45 $^+$  Ly6C $^-$  singlet SVF cells gated for F4/80 $^+$  CD11b $^+$  macrophages in WATg and WATi of ob/+ and ob/ob mice.

(E) Percent content of CD45 $^+$  F4/80 $^+$  CD11b $^+$  Ly6C $^-$  macrophages in singlet SVF cells in WATg and WATi of ob/+ and ob/ob mice ( $n = 3\text{--}4$ ; one-way ANOVA followed by Bonferroni multiple column post-test).

(F) CD11c $^+$  pro-inflammatory macrophage proportion of total macrophage content in WATg and WATi of ob/ob and ob/+ mice ( $n = 3\text{--}4$ ; one-way ANOVA followed by Bonferroni multiple column post-test).

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### TNF- $\alpha$ Impairs cGMP Signaling and Adipogenic Differentiation in Primary Murine White Adipocytes

To determine whether metaflammation dysregulates cGMP signaling in adipocytes, we treated primary murine WAs with TNF- $\alpha$ . Chronic TNF- $\alpha$  treatment of preadipocytes during differentiation (Figure S4A) impaired the sGC-PKG1 pathway. Expression of sGC $\beta_1$  protein and mRNA was significantly repressed in TNF- $\alpha$ -treated cells (Figure 3A). In addition, *Gucy1a3* mRNA expression was greatly reduced (Figure S4B), while PDE5A (protein and mRNA) was unaltered upon chronic TNF- $\alpha$  treatment (Figure S4C). Accordingly, DEA/NO-stimulated cGMP production was significantly lower in TNF- $\alpha$ -treated cells (Figure 3B). PKG1 expression was also significantly downregulated at the protein and mRNA levels after TNF- $\alpha$  treatment (Figure 3C). Diminished PKG1 expression translated into reduced cGMP-PKG-dependent VASP phosphorylation in TNF- $\alpha$ -treated cells (Figure 3D). Treatment of WAs with TNF- $\alpha$  not only dysregulated the cGMP cascade but also curtailed adipocyte differentiation (Cawthorn and Sethi, 2008; Zhang et al., 1996; Sethi and Hotamisligil, 1999; Ruan et al., 2002). As indicated by reduced oil red O staining (Figure 3E) and tri-glyceride (TG) content (Figure 3F), lipid content was significantly lower in TNF- $\alpha$ -treated cells than in untreated WAs. This TNF- $\alpha$ -induced inability of preadipocytes to accumulate lipids was corroborated by a drastic repression of the adipogenic marker PPAR $\gamma$  and aP2 (Figures 3G and 3H) and mRNA expression (Figure S4B).

Next, we tested whether TNF- $\alpha$  regulated cGMP signaling in differentiated WAs. Mature WAs treated with TNF- $\alpha$  for 4 days (Figure S4D) demonstrated a significant reduction in sGC $\beta_1$  as well as PKG1 protein and mRNA expression (Figures 3I, 3J, and S4E). Although TNF- $\alpha$  treatment did not visibly alter lipid content in differentiated WAs (Figures 3K and 3L), PPAR $\gamma$  and aP2 expression was significantly diminished (Figures S4F–S4H). Apart from TNF- $\alpha$ , *Gucy1b3* and *Prkg1* were also repressed by IL-1 $\beta$  and interferon  $\gamma$  (IFN- $\gamma$ ) treatment of differentiated WAs (Figure S4I). However, only IL-1 $\beta$  had a negative impact on adipogenic marker expression (Figure S4I). This demonstrated that metaflammation inhibits sGC-PKG1-mediated cGMP signaling as well as adipogenic markers in differentiating and mature WAs. Notably, despite the differences in inflammation, adipogenic marker expression was diminished in both WATg and WATi from obese mice (Figures S4J–S4N), suggesting that along with metaflammation, other obesity-induced stresses adversely affected AT function.

### TNF- $\alpha$ Inhibits *Gucy1b3* via NF- $\kappa$ B and *Prkg1* via JNK Signaling

We next determined whether repression of sGC $\beta_1$  and PKG1 was a direct result of TNF- $\alpha$ -induced inflammation or an indirect

consequence of reduced adipogenesis. We found that acute TNF- $\alpha$ -treatment (6 hr) of differentiated WAs significantly downregulated *Gucy1b3*, *Prkg1*, *Pparg*, and *Fabp4* expression (Figure 4A). This suggested a direct involvement of TNF- $\alpha$ -stimulated pathways in repressing these genes. To identify the mechanisms responsible for these TNF- $\alpha$ -mediated effects, we pharmacologically inhibited two major TNF- $\alpha$ -activated pathways, NF- $\kappa$ B and JNK signaling. TNF- $\alpha$ -repressed *Gucy1b3* expression was restored to control levels in cells co-treated with the NF- $\kappa$ B signaling inhibitor MG132 (10  $\mu$ M), Bay11-7085 (10  $\mu$ M), or cardamonin (10  $\mu$ M) (Figure 4B). On the other hand, co-treatment with these inhibitors did not rescue TNF- $\alpha$ -induced repression of *Prkg1*, *Pparg*, or *Fabp4* expression (Figure 4B). Likewise, small hairpin RNA (shRNA)-mediated p65 knockdown improved TNF- $\alpha$ -mediated *Gucy1b3* repression (Figure 4C). However, shRNA-mediated knockdown of p65 had no effect on TNF- $\alpha$ -mediated *Prkg1*, *Pparg*, and *Fabp4* suppression (Figures 4D–4F). Conversely, pharmacological inhibition of JNK signaling by SP600125 or TCS JNK 60 (10  $\mu$ M each) ameliorated TNF- $\alpha$ -induced repression of *Prkg1*, *Pparg*, and *Fabp4*, but not *Gucy1b3* (Figure 4G). In agreement, shRNA-mediated cJun knockdown rescued TNF- $\alpha$ -mediated inhibition of *Prkg1*, *Pparg*, and *Fabp4* expression, but not *Gucy1b3* (Figures 4H–4K). To confirm these in vitro effects, WATg explants from ND and HFD mice were treated with Bay 11-7085 or TCS JNK 60 (10  $\mu$ M; 6 hr). *Gucy1b3* expression, lowered in HFD mice, was rescued only by ex vivo Bay 11-7085 treatment (Figure S5A), while expression of *Prkg1*, *Pparg*, and *Fabp4* was restored only by TCS JNK 60 (Figures S5B–S5D).

The efficacy of pharmacological agents used was determined by analyzing NF- $\kappa$ B-p65 (Ser-536) and cJun (Ser-63) phosphorylation, which are increased by TNF- $\alpha$  (Figures S5E and S5F). Knockdown efficiency and target specificity of the shRNAs were also confirmed (Figures S5G and S5H).

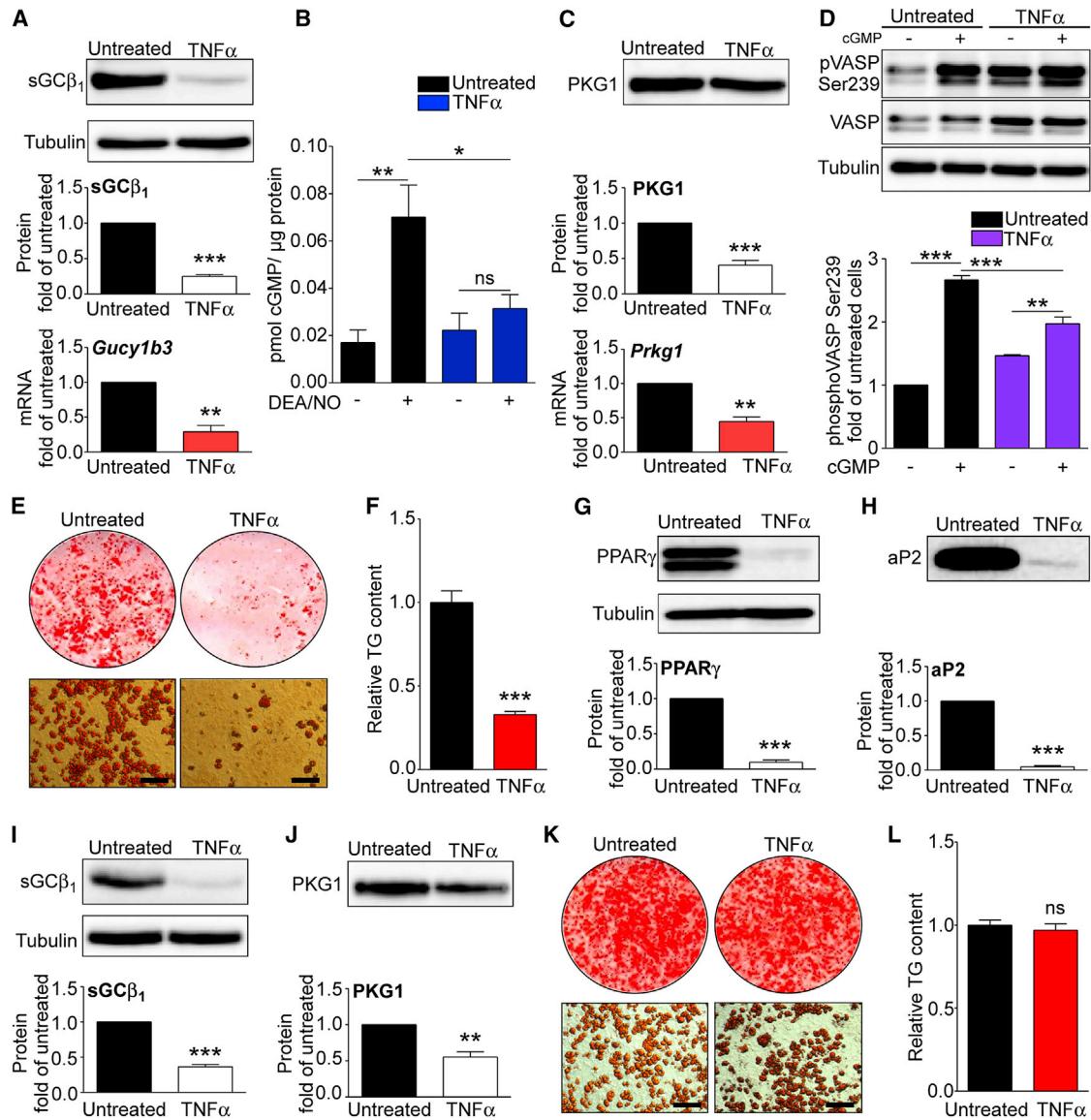
We next assessed whether reduced WA differentiation was caused by diminished sGC-PKG1 signaling in TNF- $\alpha$ -treated cells. Through lentiviral shRNA-mediated knockdown of sGC $\beta_1$  (Figures S6A and S6B), we mimicked the obesity-induced reduction in sGC-PKG1-mediated cGMP signaling in vivo (WATg) and examined its consequences on adipocyte differentiation and inflammation. Silencing sGC $\beta_1$  significantly diminished basal as well as TNF- $\alpha$ -induced *Tnfa* and *Nos2* expression (Figure S6C). As expected (Hoffmann et al., 2015), knocking down sGC $\beta_1$  severely compromised PPAR $\gamma$  and aP2 expression (Figures S6D and S6E). Nonetheless, *Pparg* and *Fabp4* were further repressed in acute TNF- $\alpha$ -treated sGC $\beta_1$  knocked-down cells (Figure S6F). Other adipogenic markers (*Fasn*, *Plin1*, *Lpl*, *Cd36*, and *Slc2a4*) were downregulated in sGC $\beta_1$  knocked-down cells (Figure S6G). Interestingly, *Plin1*, *Lpl*, *Cd36*, and *Slc2a4* were further repressed upon acute TNF- $\alpha$  treatment (Figure S6G). These observations highlighted

(G) CD11c $^-$  CD206 $^+$  CD301 $^+$  anti-inflammatory macrophage proportion of total macrophages in WATg and WATi of *ob/+* and *ob/ob* mice (n = 3–4; one-way ANOVA followed by Bonferroni multiple column post-test).

(H and I) Pro-inflammatory gene (mRNA) expression in (H) WATg and (I) WATi of HFD compared to ND mice (n = 5–8; t test).

(J and K) TNF- $\alpha$ , IL-6, IL-1 $\beta$ , MCP-1, MIP-1 $\alpha$ , and MCP-3 protein expression in (J) WATg and (K) WATi from HFD-fed mice and fold of expression in ND-fed mice (n = 5–8; t test).

Data are presented as mean  $\pm$  SEM. ns, not significant; \*p < 0.05, \*\*p < 0.01, and \*\*\*p < 0.001. See also Figures S2 and S3.

**Figure 3. TNF- $\alpha$  Suppresses sGC-PKG1 Signaling and Differentiation In Vitro**

(A) Representative sGC $\beta_1$  WB, densitometric quantification (top), and mRNA expression of Gucy1b3 (bottom) in differentiating WAs treated with TNF- $\alpha$  (20 ng/mL), compared to untreated cells ( $n = 5$ ; t test).

(B) cGMP content (picomoles per microgram protein) in unstimulated and 50  $\mu$ M DEA/NO-stimulated cells following chronic TNF- $\alpha$  treatment during differentiation ( $n = 5$ ; t test).

(C) Representative PKG1 WB, densitometric quantification (top; refer to [A] for loading control) and Prkg1 mRNA (bottom) expression in differentiating WAs treated with TNF- $\alpha$ , compared to untreated cells ( $n = 5$ ; t test).

(D) Representative WB and densitometric quantification of pVASP-Ser239 in unstimulated and 8-Br cGMP (200  $\mu$ M) stimulated WAs following chronic TNF- $\alpha$  treatment (fold of unstimulated control [untreated] cells;  $n = 3$ ; one-way ANOVA followed by Bonferroni multiple column test).

(E) Oil red O staining of untreated and chronically TNF- $\alpha$ -treated differentiated adipocytes. The top panel depicts the well and the bottom panel shows microscopic images of the stained cells.

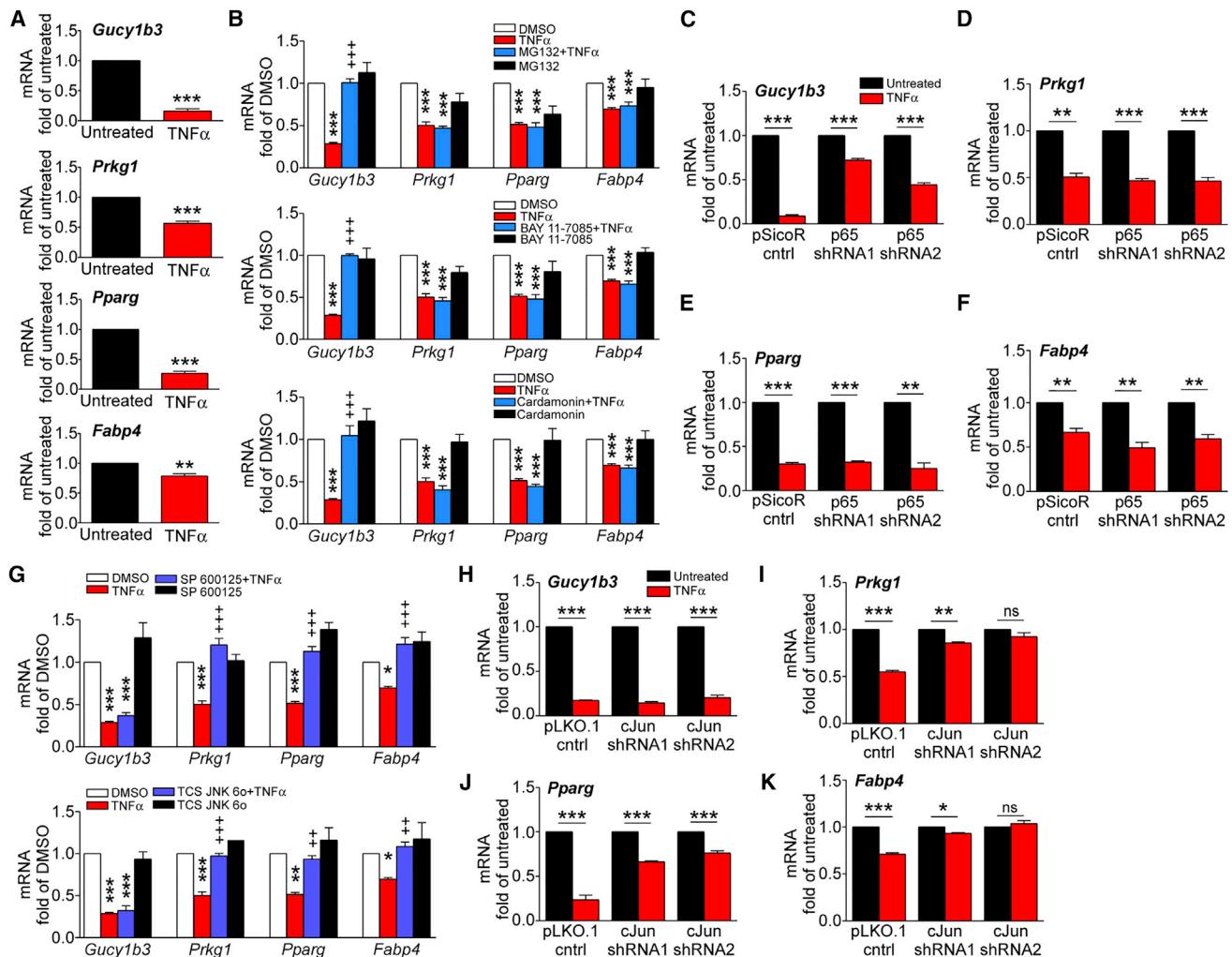
(F) Quantification of triglyceride content in adipocytes treated chronically with TNF- $\alpha$  as fold change of untreated WAs ( $n = 4$ ; t test).

(G and H) Representative WB and quantification of (G) PPAR $\gamma$  and (H) aP2 (refer to [G] for loading control) protein content in WAs treated chronically with TNF- $\alpha$  (fold of untreated cells;  $n = 5$ ; t test).

(I and J) Representative WB and quantification of (I) sGC $\beta_1$  ( $n = 4$ ) and (J) PKG1 ( $N = 3$ ; refer to [I] for loading control) protein content in mature WAs treated for 4 days with TNF- $\alpha$  (fold change of untreated cells; t test).

(K and L) Oil red O staining (K) and TG content (L) in TNF- $\alpha$ -treated mature WAs (fold of untreated cells;  $n = 4$ ; t test). Scale bars, 200  $\mu$ m.

Data are presented as mean  $\pm$  SEM. ns, not significant; \* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.001$ . See also Figure S4.



**Figure 4. sGC $\beta_1$  Expression Is Inhibited by NF- $\kappa$ B, while PKG1, PPAR $\gamma$ , and aP2 Expression Are Down-Regulated by JNK Signaling**

(A) *Gucy1b3*, *Prkg1*, *Pparg*, and *Fabp4* mRNA expression in mature WAs treated acutely (6 hr) with TNF- $\alpha$  (fold change of untreated cells; n = 5; t test).

(B) mRNA expression of *Gucy1b3*, *Prkg1*, *Pparg*, and *Fabp4* in mature WAs acutely co-treated with TNF- $\alpha$  and NF- $\kappa$ B signaling blockers MG132 (top), Bay 11-7085 (middle), and cardamomine (bottom) (fold of DMSO; n = 3; one-way ANOVA followed by Bonferroni multiple column test; \*, compared to DMSO; +, compared to TNF- $\alpha$ ).

(C–F) *Gucy1b3* (C), *Prkg1* (D), *Pparg* (E), and *Fabp4* (F) mRNA expression in pSicoR control and p65 shRNA (sh1 and sh2) transduced WAs followed by acute TNF- $\alpha$  treatment (fold of respective untreated cells; n = 4; t test).

(G) *Gucy1b3*, *Prkg1*, *Pparg*, and *Fabp4* mRNA expression in mature WAs acutely co-treated with TNF- $\alpha$  and JNK signaling blockers (10  $\mu$ M each) SP600125 (top) and TCS JNK 60 (bottom) (fold of DMSO; n = 3; one-way ANOVA followed by Bonferroni multiple column test).

(H–K) *Gucy1b3* (H), *Prkg1* (I), *Pparg* (J), and *Fabp4* (K) mRNA expression in pLKO.1 control and cJun shRNA (sh1 and sh2) transduced WAs treated acutely with TNF- $\alpha$  (fold of respective untreated cells; n = 3; t test).

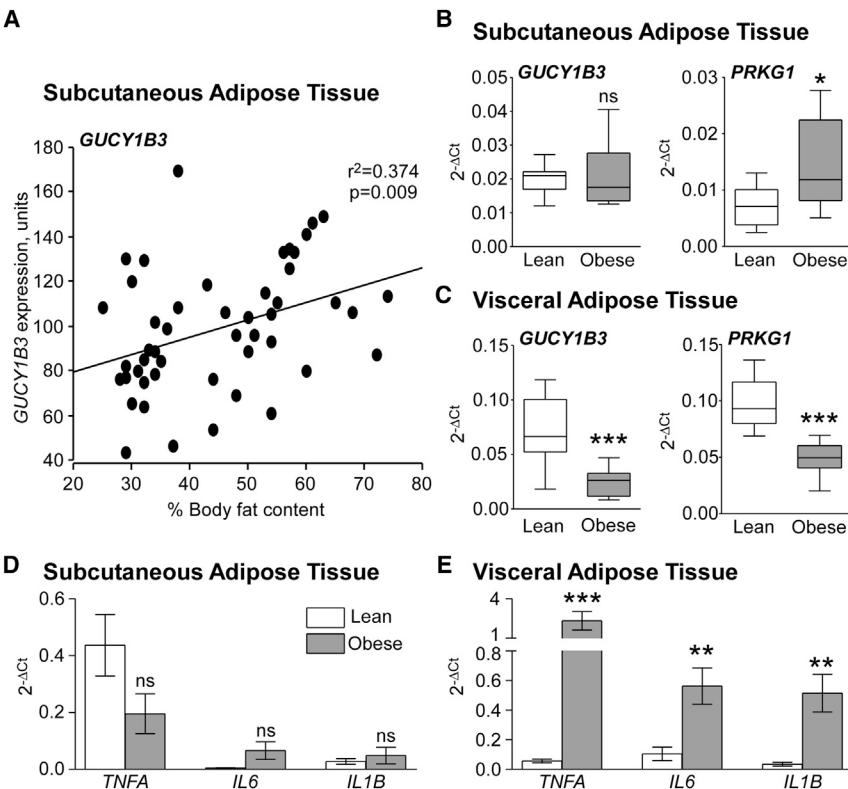
Data are presented as mean  $\pm$  SEM. ns, not significant; \*p < 0.05; \*\*/++, p < 0.01; and \*\*\*/+++, p < 0.001. See also Figures S5 and S6.

the pro-adipogenic and anti-inflammatory importance of cGMP signaling in adipocytes.

#### GUCY1B3 and PRKG1 Expression Is Repressed in Visceral, but Not Subcutaneous, Adipose Tissue of Obese Humans

To corroborate our observations in mice, we examined whether the expression of sGC $\beta_1$  and PKG1 was altered in SAT and omental or VAT from lean (BMI  $\leq$  25 kg/m $^2$ ) and obese (BMI  $\geq$  30 kg/m $^2$ ) humans. *GUCY1B3* mRNA expres-

sion in SAT positively correlated with body fat content of individuals from cohort 1 (Figure 5A), while, it was unchanged in SAT from lean and obese individuals (Figure 5B, left) from a second, independent cohort (cohort 2). Although, *PRKG1* expression was higher in SAT (Figure 5B, right), expression of both *GUCY1B3* and *PRKG1* was significantly reduced in VAT from obese patients of cohort 2 (Figure 5C). In the same samples, inflammatory marker gene expression was unaltered in SAT but significantly enhanced in VAT of obese patients (Figures 5D and 5E). This suggested that like in



**Figure 5. Obesity-Induced Disparity in cGMP Signaling Component and Pro-inflammatory Marker Expression in Human WAT**

(A) *GUCY1B3* expression in subcutaneous AT of human cohort 1 correlated to percent body fat content ( $n = 49$ ; linear regression).

(B and C) *GUCY1B3* and *PRKG1* mRNA expression in (B) subcutaneous and (C) visceral AT of lean ( $BMI \leq 25$ ) and obese ( $BMI \geq 30$ ) human subjects from cohort 2. Data are presented as box and whiskers; whiskers indicate 5<sup>th</sup>–95<sup>th</sup> percentile ( $n = 10$ –16; t test).

(D and E) *TNFA*, *IL6*, and *IL1B* mRNA expression in (D) subcutaneous and (E) visceral AT from lean and obese subjects from cohort 2 ( $n = 10$ –16; t test). Data are presented as mean  $\pm$  SEM; ns, not significant; \* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.001$ .

mice, human VAT is prone to metaflammation and cGMP signaling dysregulation.

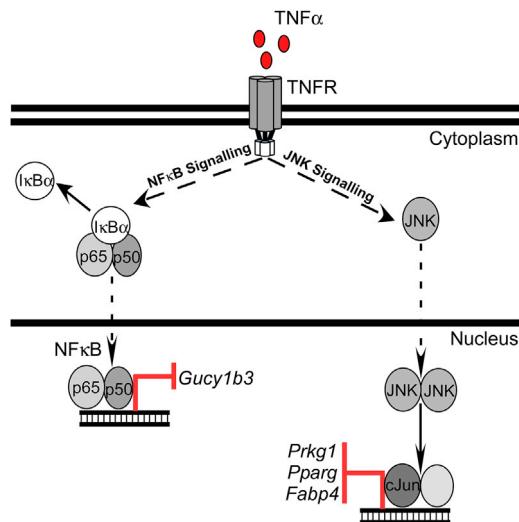
## DISCUSSION

In this study, we assessed the impact of obesity-associated metaflammation on cGMP signaling in WAT. Previously, we and others have shown that cGMP signaling enhances differentiation and activates a thermogenic program in murine as well as human adipocytes (Bordicchia et al., 2012; Haas et al., 2009; Hoffmann et al., 2015; Mitschke et al., 2013). We report that major components of the cGMP cascade, sGC $\beta_1$  and PKG1, are downregulated in WATg, but not in WATi, of obese mice (both in adipocytes and SVF). Consequently, cGMP production by sGC upon NO stimulation is significantly lower in WATg from *ob/ob* mice than in control animals (*ob/+*). Conversely, sGC function remains intact in obese WATi. Similarly, PKG1-mediated VASP phosphorylation is dampened in WATg, but not in WATi, of *ob/ob* mice. These observations suggest that sGC-PKG1-mediated cGMP signaling is severely impaired only in WATg from obese mice. Additionally, reduced *Gucy1b3* and *Prkg1* mRNA levels imply that metabolic stress-induced cellular pathways inhibit their expression at the transcriptional level. Notably, like in mice, *GUCY1B3* and *PRKG1* expression is diminished only in VAT, but not SAT, of obese humans.

Our results demonstrate a discrepancy in the expression and function of cGMP signaling components in subcutaneous and visceral AT from obese mice and humans. Earlier reports show that obesity leads to mechanical and endoplasmic reticu-

lum (ER)-stress-induced unfolded protein response (UPR) (Kawasaki et al., 2012; Özcan et al., 2004; Kusminski and Scherer, 2012), dyslipidemia (Gutierrez et al., 2009), AT hypoxia (Hosogai et al., 2007; Ye et al., 2007; Pasarica et al., 2009), lipotoxicity (Unger and Scherer, 2010; Ertunc and Hotamisligil, 2016), and oxidative stress (Furukawa et al., 2004; Kusminski and Scherer, 2012). These processes, in concert with increased pro-in-

flammatory immune cell presence (Lee and Lee, 2014; Lumeng et al., 2007a; Weisberg et al., 2003; Huh et al., 2014; Nishimura et al., 2009; Talukdar et al., 2012; Wensveen et al., 2015), disturb AT microenvironment (Sun et al., 2011), impede AT function (leading to ectopic lipid deposition) (Carobbio et al., 2011; Unger and Scherer, 2010), enhance metaflammation (Gregor and Hotamisligil, 2011; Gutierrez et al., 2009), and eventually result in metabolic dysregulation (de Luca and Olefsky, 2008; Gutierrez et al., 2009; Jung and Choi, 2014). Targeting inflammation or inhibiting pro-inflammatory cytokines, such as TNF- $\alpha$ , is known to be metabolically beneficial (Aouadi et al., 2013; Hotamisligil et al., 1993; Kusminski et al., 2016). Interestingly, we observe heightened expression of pro-inflammatory markers only in WATg, but not WATi, of obese mice. Macrophages, the major mediators of metaflammation-induced AT dysfunction (Aouadi et al., 2013; Boutens and Stienstra, 2016; Kamei et al., 2006), accumulate in obese WAT (Weisberg et al., 2003). This results from higher influx of blood monocytes into AT (Weisberg et al., 2006; Oh et al., 2012; Lumeng et al., 2007b; Kamei et al., 2006) as well as in situ proliferation (Amano et al., 2014). ATMs also undergo a phenotypic switch from an anti-inflammatory to a pro-inflammatory state (Lumeng et al., 2007a; Castoldi et al., 2016; Lumeng et al., 2008). In *ob/ob* mice, we observe a robust increase in WATg macrophage content, whereas WATi AT content increases marginally. Interestingly, the proportion of pro-inflammatory ATMs is greater in WATg than WATi of *ob/ob* mice. This suggests that although white AT content increases in murine obesity, only WATg accumulates pro-inflammatory macrophages and is hence vulnerable to metaflammation.



**Figure 6. TNF- $\alpha$ -Mediated Repression of cGMP Signaling in White Adipocytes**

TNF- $\alpha$  downregulates sGC $\beta_1$  expression through activation of NF- $\kappa$ B signaling, whereas TNF- $\alpha$ -actuated JNK signaling represses PKG1, PPAR $\gamma$ , and aP2 expression.

Similarly, we observe that VAT is greatly inflamed in obese humans, while SAT does not demonstrate traits of severe metaflammation. Others have also demonstrated a disparity in AT macrophage content of obese humans, with higher numbers in VAT than SAT (Cancello et al., 2006; O'Rourke et al., 2009). Moreover, in humans, VAT mass is a dominant risk factor for the development of obesity-associated metabolic abnormalities (Harman-Boehm et al., 2007; Tchkonia et al., 2013). Thus, murine and human VAT is at a greater risk of obesity-induced inflammation and its consequences than SAT.

Based on the observations thus far, we hypothesize that the degree of AT inflammation corresponds to the extent of cGMP signaling disruption. Mimicking metaflammation by TNF- $\alpha$  treatment inhibits the cGMP cascade in differentiating and mature adipocytes. sGC expression and NO-stimulated cGMP production is diminished in TNF- $\alpha$ -treated cells during differentiation. Likewise, reduced PKG1 expression in TNF- $\alpha$ -treated WAs results in diminished cGMP-stimulated VASP phosphorylation. Apart from impeding the cGMP cascade, TNF- $\alpha$  also suppresses adipocyte differentiation and adipogenic gene expression (Cawthon and Sethi, 2008; Zhang et al., 1996). In congruence, lipid accumulation in differentiating WAs is severely blunted, with a concomitant repression of PPAR $\gamma$  and aP2. Although TNF- $\alpha$  does not alter lipid content in differentiated adipocytes, PPAR $\gamma$  and aP2 expression is significantly reduced. These detrimental effects on cGMP signaling and adipogenesis are not exclusive to TNF- $\alpha$ . IL-1 $\beta$  and IFN- $\gamma$  treatment also downregulates sGC $\beta_1$ , PKG1, and adipogenic marker expression in WAs. Thus, metaflammation plays a leading role in dysregulating cGMP signaling in WAs. Interestingly, several essential adipogenic genes are downregulated in both WATg and WATi of obese mice, suggesting that in concert with inflammation, other obesity-associated mechanisms, such as ER-stress-induced

UPR, hypoxia, and others, hamper lipid synthesis in and the storage capacity of WATi. Therefore, even though obesity-induced inflammation is a major player, especially in WATg, it is not solely responsible for the inhibition of WAT adipogenesis and the resulting lipid spillover.

We next investigate whether inflammation directly and simultaneously represses cGMP signaling and adipogenesis. Since acute TNF- $\alpha$  treatment reduces *Gucy1b3* and *Prkg1* expression, we predict that TNF- $\alpha$ -activated cellular pathways directly impede cGMP signaling. A positive feed-forward effect of dysregulated cGMP signaling on inflammatory signaling is not probable, as knocking down sGC $\beta_1$  does not aggravate pro-inflammatory gene expression. In parallel, *Pparg*, *Fabp4* and several other adipogenic genes are repressed by acute TNF- $\alpha$  exposure. Although genetic ablation of cGMP signaling components result in reduced adipogenic gene expression (Haas et al., 2009; Hoffmann et al., 2015; Mitschke et al., 2013), TNF- $\alpha$  treatment can independently further downregulate *Pparg*, *Fabp4*, *Plin1*, *Lpl*, *Cd36*, and *Slc2a4*. This suggests that metaflammation diminishes adipogenesis by directly inhibiting adipogenic gene expression, as well as indirectly, through disruption of the cGMP cascade.

We also identify the cellular mechanisms responsible for TNF- $\alpha$ -induced repression of sGC $\beta_1$ , PKG1, PPAR $\gamma$ , and aP2. Pharmacological and genetic inhibition of NF- $\kappa$ B signaling specifically restores TNF- $\alpha$ -driven *Gucy1b3* repression. Conversely, TNF- $\alpha$ -induced *Prkg1* suppression is ameliorated by impeding the JNK cascade. TNF- $\alpha$  is known to represses *Pparg* and *Fabp4* expression in 3T3-L1 adipocytes (Ruan et al., 2002; Zhang et al., 1996) through inhibition of CCAAT/enhancer-binding protein  $\delta$  (C/EBP $\delta$ ) expression (Kudo et al., 2004). In ST2 cells, TNF- $\alpha$  has been shown to inhibit PPAR $\gamma$  function through NF- $\kappa$ B signaling (Suzawa et al., 2014). However, we demonstrate that TNF- $\alpha$ -activated JNK, and not NF- $\kappa$ B signaling, diminishes *Pparg* and *Fabp4* expression in primary murine WAs. Our observations suggest that NF- $\kappa$ B and JNK signaling have distinct effects on cGMP signaling and adipogenesis (Figure 6).

Multiple reports demonstrate that activating cGMP signaling improves insulin sensitivity (Ayala et al., 2007), WATg inflammation (Handa et al., 2011), and adipogenic marker expression and induces thermogenesis in obese mice (Hoffmann et al., 2015). We conclude that the extent of metaflammation in WAT dictates the degree of cGMP signaling dysfunction through specific pathways. Given that targeting cGMP signaling (Pfeifer and Hoffmann, 2015) is metabolically beneficial, our findings highlight the importance of inflammatory mechanisms governing cGMP signaling dysfunction. Combining inhibition of inflammation and enhancing cGMP signaling, could be a potential anti-obesity therapeutic avenue.

## EXPERIMENTAL PROCEDURES

### Animal Studies

Breeding pairs of *ob/+* mice were purchased from Jackson Laboratory. Male 16-week-old *ob/+* and *ob/ob* mice were used. Six-week-old C57BL/6J mice, assessed for the impact of DIO on WAT cGMP signaling, were fed an HFD (D12492; Ssniff) or ND (D12450B; Ssniff) for 18 weeks. They were part of a previously published cohort identifying the metabolic effects of sGC $\beta_1$  stimulation (by Bay 41-4583) in established obesity (Hoffmann et al., 2015). These studies

were permitted by the Landesamt für Natur, Umwelt und Verbraucherschutz (LANUV) Nordrhein-Westfalen, Germany. Additional details can be found in [Supplemental Experimental Procedures](#).

#### Human Samples

Subcutaneous AT gene expression of *GUCY1B3* was investigated using data from a previously published global microarray of 56 women (cohort 1) (Arner et al., 2012). The cohort comprised of 30 healthy obese ( $BMI > 30 \text{ kg/m}^2$ ) and 26 healthy non-obese ( $BMI < 30 \text{ kg/m}^2$ ) individuals. This cohort was part of a study approved by the Karolinska University Hospital ethics committee. For cohort 2, VAT and SAT samples were obtained from the AT bank at the University of Leipzig, Germany. Samples were extracted from consenting patients undergoing surgery. Phenotypic characterization of the AT donors was performed as previously described (Klöting et al., 2010). Samples were collected from informed consenting subjects. These samples were part of a larger study approved by the University of Leipzig ethics committee. Detailed patient information from both cohorts can be found in [Supplemental Experimental Procedures](#).

#### Isolation and In Vitro Culture of Primary Murine Adipocytes

The SVF was isolated as previously described (Mitschke et al., 2013). SVF isolation and WA culture are detailed in [Supplemental Experimental Procedures](#).

#### Western Blot

Frozen tissues and cells were lysed in ice-cold lysis buffer (Singh et al., 2009). Antibodies used and other details are available in [Supplemental Experimental Procedures](#).

#### Quantitative Real-Time PCR

mRNA expression was determined as previously described (Haas et al., 2009). Primer sequences and other details are available in [Supplemental Experimental Procedures](#).

#### cGMP Measurement

cGMP content in WAT explants and adipocytes (WAs) was determined using the Monoclonal Anti-cGMP EIA Kit (New East Biosciences) following the manufacturer's instructions. Details are available in [Supplemental Experimental Procedures](#).

#### FACS Analysis

SVF isolation, staining, and fluorescence-activated cell sorting (FACS) was performed as previously described (Zhang et al., 2008). Details are available in [Supplemental Experimental Procedures](#).

#### Luminex Assay

Luminex assay was performed per manufacturer instructions using ProcartaPlex multiplex immunoassay kit (eBioscience). Additional information is available in [Supplemental Experimental Procedures](#).

#### shRNA Transduction of WAs

Lentiviral particles were generated in the laboratory using purchased shRNA plasmids as previously described (Pfeifer et al., 2001). 2 days after transduction, cells were put through 48 hr of puromycin selection. Details are available in [Supplemental Experimental Procedures](#).

#### Statistical Analysis

Data are presented as mean  $\pm$  SEM. Single comparisons were made using a two-tailed t test. Multiple comparisons were performed by one-way ANOVA followed by a Bonferroni multiple comparison post-test. A comparison with p value  $\leq 0.05$  was considered statistically significant. Statistical analyses were performed using GraphPad Prism 5.0 software.

#### SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and six figures and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2016.12.028>.

#### AUTHOR CONTRIBUTIONS

A. Sanyal designed, planned, and performed the experiments, analyzed the data, and wrote the manuscript. J.N. handled the HFD mice and generated shRNA lentiviral vectors. L.S.H. planned the experiments and reviewed the manuscript. A.C.-K. performed and quantified WB on WATi samples and reviewed the manuscript. A.E. and P.A. provided the data for human cohort 1 and reviewed the manuscript. M.B. provided human AT samples for cohort 2 and reviewed the manuscript. A. Schlitzer analyzed the acquired FACS data. A.P. supervised all experiments and wrote and reviewed the manuscript.

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