HHS Public Access

Author manuscript

Obesity (Silver Spring). Author manuscript; available in PMC 2016 May 18.

Published in final edited form as:

Obesity (Silver Spring). 2015 November ; 23(11): 2223–2232. doi:10.1002/oby.21220.

The Ubiquitin Ligase Siah2 Regulates Obesity-induced Adipose Tissue Inflammation

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Abstract

Objective—Chronic, low-grade adipose tissue inflammation associated with adipocyte hypertrophy is an important link in the relationship between obesity and insulin resistance. Although ubiquitin ligases regulate inflammatory processes, the role of these enzymes in metabolically driven adipose tissue inflammation is relatively unexplored. Herein, we examined the effect of the ubiquitin ligase Siah2 on obesity-related adipose tissue inflammation.

Methods—Wild-type and Siah2KO mice were fed a low or high fat diet for 16 weeks. Indirect calorimetry, body composition, glucose and insulin tolerance were assayed along with glucose and insulin levels. Gene and protein expression, immunohistochemistry, adipocyte size distribution and lipolysis were also analyzed.

Results—Enlarged adipocytes in obese Siah2KO mice are not associated with obesity-induced insulin resistance. Proinflammatory gene expression, stress kinase signaling, fibrosis and crown-like structures are reduced in the Siah2KO adipose tissue and Siah2KO adipocytes are more responsive to insulin-dependent inhibition of lipolysis. Loss of Siah2 increases expression of PPARγ target genes involved in lipid metabolism and decreases expression of proinflammatory adipokines regulated by PPARγ.

Conclusions—Siah2 links adipocyte hypertrophy with adipocyte dysfunction and recruitment of proinflammatory immune cells to adipose tissue. Selective regulation of PPARγ activity is a Siah2-mediated mechanism contributing to obesity-induced adipose tissue inflammation.

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Disclosure: The authors declare no conflict of interest.

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Introduction

Obesity-associated insulin resistance is linked to dysregulation of lipid storage and chronic, low-grade inflammation of adipose tissue ([1]). As adipose tissue expands to accommodate the lipid storage demands of excess energy intake, adipocyte hypertrophy becomes a defining characteristic. When the capacity to expand is exceeded, inflammatory signaling in adipose tissue is activated ([2]). Adipocyte secretion of proinflammatory adipokines correlates with infiltration of M1-like macrophages and proinflammatory T lymphocytes ([1]), setting up an inflammatory state focused on removing necrotic adipocytes ([3]). Accompanied by increased release of fatty acids from adipose tissue, this leads to impaired insulin signaling in skeletal muscle and liver and systemic insulin resistance ([4]).

Adipose tissue inflammation is sustained by a positive feedback loop in which cytokines secreted by infiltrating macrophages activate stress kinase signaling pathways in adipocytes and macrophages that up-regulate proinflammatory genes via activator protein-1 (AP-1) and nuclear factor-κB (NF-κB) transcriptional activity ([5]). Although signaling events controlling NF-κB activation ([6]) are regulated by enzymes of the ubiquitin-proteasome system, involvement of the ubiquitin-proteasome system in obesity-induced adipose tissue inflammation is relatively unexplored.

Post-translational modification of proteins by ubiquitin, the major pathway controlling non-lysosomal intracellular protein degradation, begins with ubiquitin binding to the ubiquitin activating enzyme (E1), followed by transfer of ubiquitin from E1 to the targeted protein via ubiquitin conjugating enzymes (E2s) and ubiquitin ligases (E3s), which determine the specificity of ubiquitylation ([7]). Deletion of E3 ligases c-Cbl ([8]) or ITCH ([9]), an E3 ligase involved in T-cell differentiation, increases energy expenditure and prevents high fat-induced obesity. Our studies in 3T3-L1 adipocytes found the ubiquitin ligase mammalian homologue of seven-in-absentia-2 (Siah2) alters peroxisome proliferator-activated receptor γ (PPARγ) protein levels and selectively regulates PPARγ activity ([10]). Given the central role of PPARγ in forming and maintaining adipocytes, regulating insulin sensitivity, and inflammatory gene expression in adipocytes and macrophages ([11]), we hypothesized that Siah2 regulates obesity-induced changes in adipose tissue. In this study, we examined the adipose tissue phenotype in global Siah2-null mice challenged with chronic excess energy intake.

Methods

Experimental Animals

Siah2KO mice were generated and maintained as described ([12], [13]). All animal experiments were approved by the Pennington Biomedical Research Center Animal Care and Use Committee. The animals were housed with a 12-hr light-dark cycle at 24°C. At four weeks of age, wild-type and Siah2KO male mice were randomly assigned (n=10/group) to a defined 10% low fat or 45% high fat diet and were fed *ad libitum* for 4 months thereafter. Body weight was measured weekly and body composition was measured bi-weekly by NMR. Food intake, activity, and indirect calorimetry were measured at 12 weeks on each diet (TSE PhenoMaster). At the end of the study, the mice were euthanized between 8–11 AM. Adipose tissue was harvested for analysis of adipocyte size distribution and lipolysis

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from a separate cohort of wild-type and Siah2KO male mice maintained on low or high fat diets for 2 months.

Glucose and Insulin Tolerance Tests

For the glucose (GTT) and insulin (ITT) tolerance tests, the amount of glucose or insulin administered was normalized to fat-free mass ([14]), which did not vary significantly among groups (20.1 –/+ 0.13 gm) at 12 weeks on each diet. Mice were fasted 4 hours prior to administering 2 gm/kg fat-free mass of glucose/mouse (GTT) or 1U/kg lean mass insulin/ mouse (HumulinR) (ITT) by intraperitoneal injection.

Blood Chemistry and Lipolysis

Fasting serum glucose levels were measured by hexokinase activity assay (Sigma). Fasting insulin levels were assayed via ELISA (Crystal Chem). Serum nonesterified fatty acids (Abcam) and triglycerides (Eagle Diagnostics) levels were assayed according to manufacturers' instructions. For lipolysis assays, adipocytes were isolated by collagenase treatment from epididymal adipose tissue of HFD-fed mice ([15]). Insulin-mediated inhibition of lipolysis in the presence of isoproterenol (0.1 μM) was assessed by glycerol release (Zen-Bio).

Microarray Analysis

Epididymal adipose tissue RNA (RNA integrity number 8) was analyzed for gene expression on Illumina MouseRef-8v2.0 expression arrays. RNA from 8–10 animals/group was combined into 3 pooled samples/group. Raw gene expression signals were background adjusted and quantile normalized using GenomeStudio (V2011.1.Illumina Inc.). For each sample, probes were considered "expressed" if their detection p-value was <0.05. Probes that failed to reach a detection p-value <0.05 in any sample across the four treatments (wild-type high fat, low fat; Siah2KO high fat, low fat) were removed from analysis. Remaining probes were log transformed (base 2) and treatment specific fold-changes computed as log ratios. The statistical significance of differential expression was ascertained by regularized t-test, based on a Bayesian probabilistic framework ([16]). Genes with an absolute fold-change 1.5-fold and p 0.001 were considered significantly differentially expressed, unless otherwise noted. Biological pathway analysis was conducted on the gene expression signals using gene-set enrichment (GSEA) or over-representation based approaches (Ingenuity Pathway Analysis, Ingenuity). Statistical analyses were controlled for multiple testing via the false discovery rate (FDR) ([17]). The microarray dataset was submitted to Gene Expression Omnibus (GEO) data repository (GSE61839).

Quantitative PCR

Total RNA was purified from epididymal adipose tissue, (200 ng) reverse transcribed and real-time PCR performed with TaqMan chemistry as described ([10]). The results were normalized to *ubiquitin B* mRNA levels and analyzed by the $2^{-\quad CT}$ method with wild-type values used as the calibrator. The gene list is provided in Table S1.

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Preparation of Whole Cell Extracts and Immunoblotting

Adipose tissue was homogenized in a denaturing buffer and processed for immunoblotting or immunoprecipitation as described ([10]). Nitrocellulose membranes were incubated with antibodies (Table S2) for 1–2 hours at room temperature or overnight at 4°C.

Immunohistochemistry and Immunostaining

Epididymal adipose tissue was fixed in 10% formalin, then embedded in paraffin, sectioned onto slides and hematoxylin and eosin (H&E) stained. Adipose tissue collagen content was determined by trichrome staining and macrophage content by immunodetection using anti-Iba1 antibody.

Adipocyte Area and Size Distribution

H&E stained inguinal and epididymal adipose tissue were analyzed using Image J software programmed to measure the area of each adipocyte based on size and shape exclusion limits. The number of adipocytes counted/experimental condition ranged from 2,639–14,819. Epididymal adipocyte size distribution was determined as described ([18]) and analyzed on a Multisizer-3 (Beckman Coulter) using a 400-μm aperture with a dynamic linear range 12–320 μm and reported as the mean at 20 μm intervals from 22–240 μm.

Statistical Analysis

Normal distribution of glucose and insulin levels, food intake and body weight was assessed using the D'Agostino-Pearson omnibus normality test. Statistical significance for body weight, GTT and ITT was determined using ANOVA. Statistical significance for all other data was determined using an unpaired two-tailed *t* test. JMP Pro 10.0 (SAS Institute) and GraphPad Prism 5 software were used for statistical analyses. Variability was expressed as the mean –/+ SEM.

Results

Siah2 Regulates Energy Expenditure in Diet-Induced Obesity

To characterize the role of Siah2 in obesity, we conducted a feeding study with a 10% low fat (LFD) or 45% high-fat diet (HFD) diet over 4 months. Based on our studies in 3T3-L1 adipocytes showing Siah2 promotes adipogenesis ([10]), we anticipated loss of Siah2 *in vivo* would lead to reduced body weight and fat mass, and that Siah2KO mice would develop insulin resistance more readily than wild-type mice when fed a high fat diet. However, HFD Siah2KO mice body weight trended higher than wild-type and body weight for the LFD Siah2KO mice was significantly higher than wild-type mice until late in the study (Figure 1A). After 8 weeks, there was no significant difference in the percent fat mass (FM) within each diet group (Figure 1B), but percent fat free mass (FFM) was lower in the Siah2KO LFD mice at 4 and 12 weeks (Figure 1C). Food intake was unaffected by genotype (Figure 1D), although body weight positively correlated with food intake for both groups on the HFD (Figure S1A). Energy expenditure was measured after twelve weeks on each diet. Genotype had no effect on energy expenditure in the LFD fed mice. However, unlike wild-type, energy expenditure of the Siah2KO mice did not increase with HFD (Figure 1E) and

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loss of Siah2 had a significant effect on the relationship between energy expenditure and body weight or lean mass in the HFD-fed mice (Figure 1F). HFD Siah2KO mice activity was significantly lower than wild-type (Figure S1C). When the reduced activity of the HFD Siah2KO mice is considered, lower energy expenditure in the Siah2KO mice is largely accounted for by genotype, FFM and activity using analysis of covariance ([19]). However, the effect of genotype on energy expenditure remains significant, suggesting Siah2 also affects energy expenditure independent of body composition or activity (Figure 1F).

Obese Siah2KO Mice have Improved Insulin Sensitivity

To investigate the adipose tissue of the Siah2KO mice, we carried out H&E staining of the epididymal fat pad. Adipocytes from obese Siah2KO mice are hypertrophied, but more uniform in size and shape than obese wild-type adipocytes (Figure 2A). Adipocyte area measurements indicate Siah2KO adipocytes trend larger than wild-type adipocytes independent of diet or fat depot (Figure 2B). Analysis of adipocyte size distribution after two months on a low or high fat diet shows the shift toward larger adipocytes begins earlier in the Siah2KO mice than wild-type (Figure 2C), consistent with Siah2 affecting adipocyte size independent of diet, body weight or fat mass. However, circulating free fatty acid and triglyceride levels are significantly lower in the LFD and HFD-fed Siah2KO mice and triglyceride levels are lower in the obese Siah2KO mice compared to wild-type (Figure 2D). This suggests the hypertrophied Siah2KO adipocytes are less lipolytic. To test this, adipocytes were isolated from obese wild-type and Siah2KO mice and assayed for insulin-mediated inhibition of adrenergic-stimulated lipolysis. Siah2KO adipocytes are significantly more sensitive to insulin-mediated inhibition of lipolysis (Figure 2E).

The lower free fatty acid levels correspond to lower blood glucose levels in the Siah2KO mice and the glucose levels do not increase with obesity in the absence of Siah2 (Figure 3A). Fasting insulin levels trend lower in the lean Siah2KO mice compared to lean wild-type mice and increase with diet-induced obesity, but remain significantly lower than wild-type (Figure 3B). Although insulin levels in the Siah2KO mice increase with obesity, glucose (Figure 3C, D) and insulin tolerance (Figure 3E,F) tests show loss of Siah2 improves glucose tolerance and insulin sensitivity in the lean and obese mice. Notably, glucose and insulin tolerance in the obese Siah2KO mice is comparable to lean wild-type, indicating Siah2 contributes to the negative effects of obesity-induced adipocyte hypertrophy on glucose metabolism.

Siah2KO Mice have Reduced Adipose Tissue Inflammation

To begin understanding the mechanism underlying the effect of Siah2 on insulin sensitivity and adipocyte size, we conducted microarray analysis of Siah2KO and wild-type epididymal adipose tissue. The most significant differences in gene expression are related to inflammatory responses, hematological function, and immune cell trafficking ($p<0.02$) (Figure 4A). Specifically, pathways related to Toll-like receptor signaling, B- and T-cell receptor signaling and natural killer cell mediated cytotoxicity are significantly downregulated ($p<0.01$) in high-fat fed Siah2KO adipose, compared to wild-type samples (Table S3). Hierarchical clustering of the top fifty differentially expressed transcripts show

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down-regulation of a broad range of genes involved in pro-inflammatory responses (Figure 4B).

Adipose tissue expression of macrophage markers, including *F4/80*, *cd68*, *cd11c*, *cd11d*, *trem2 and Tyrobp* is reduced in the visceral adipose tissue of the obese Siah2KO mice (Figure 5A). Although pro-inflammatory markers are decreased (*F4/80, cd11c, cd11d*), expression of *Arg-1*, an indicator of anti-inflammatory macrophage is increased. Expression of pro-inflammatory cytokines, including *tnfa, ccl2, ccr2* (CCL2 receptor), *gdf3, saa3 and pai-1* are also significantly reduced in the Siah2KO adipose tissue (Figure 5B, full gene name given in Table S1). Two of these genes encode proteins strongly associated with inflammation and insulin resistance. Serum amyloid A3 (SAA3) is a proinflammatory and lipolytic cytokine secreted by macrophages and adipocytes that promotes macrophage recruitment ([20]). Plasminogen activator inhibitor-1 (serpine-1/PAI-1) is a serine protease inhibitor that is associated with obesity, inflammation and insulin resistance in rodents and humans ([21]). In contrast, adipsin, a complement factor secreted by adipocytes whose expression is increased by PPARγ agonists ([22]), but decreased in obesity ([23]) is expressed at higher levels in Siah2KO mice. Siah2-mediated changes in inflammatory markers also includes down-regulation of *tcirg1*, a T lymphocyte membrane ATPase that promotes pro-inflammatory T cell activation ([24]), and up-regulation of *fox3p*, a marker of the anti-inflammatory regulated T (T_{reg}) cells (Figure 5C).

Blunted proinflammatory responses to obesity in Siah2KO adipose tissue are further demonstrated by comparing inflammatory gene expression in the LFD and HFD-fed mice (Figure 5D). Although inflammatory marker gene expression is consistently reduced in the HFD-fed Siah2KO adipose tissue, gene expression in the LFD Siah2KO adipose tissue is either unchanged or increased, including *adam 8*, an extracellular matrix metallopeptidase upregulated with adipose tissue inflammation ([25]) and *col6a2,* a collagen VI subunit.

Reduced Fibrosis and Stress Kinase Activation in the Siah2KO Mice

The extracellular matrix surrounding adipocytes contributes to the relationship between adipocyte expandability and developing adipose tissue inflammation with obesity ([2]). The inflammatory response to adipocyte hypertrophy leads to excess collagen deposition and adipose tissue fibrosis, restricting the ability of the adipocytes to expand further ([26]). Khan *et al* showed collagens IV and VI are generally expressed at high levels in the visceral fat depot of db/db mice and are down-regulated by PPARγ activation or overexpression of adiponectin ([2]). Furthermore, we previously demonstrated that Siah2 has a promoting role for tissue fibrosis through collagens in wound healing ([27]). While the collagen IV subunit *col4a6* is up-regulated in Siah2KO adipose tissue, collagen VI subunit *col6a2* is reduced and *col6a3* is unchanged (Figure 6A). *Adam8* is two-fold lower in the Siah2KO visceral fat. Lumican, a proteoglycan promoting fibrosis that is negatively regulated by PPARγ, is reduced although decorin, another proteoglycan that regulates fibrosis ([2]), is unchanged (Figure 6A).

To determine if changes in expression of inflammatory markers and extracellular matrix components correlate with reduced fibrosis, trichrome staining of the Siah2KO epididymal adipose tissue was compared to wild-type (Figure 6B). The widespread collagen deposition

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present in wild-type is absent in the Siah2KO visceral fat. Consistent with reduced fibrosis, there are significantly fewer macrophages configured as crown-like structures surrounding adipocytes in the Siah2KO adipose tissue (Figure 6B, **Iba-1**).

Fibrotic changes with adipocyte expansion in obesity cause mechanical stress that activates stress kinase signaling pathways, including the Jun N-terminal kinase (JNK) and extracellular signal-regulated (ERK p42/44) MAPK pathways ([5]). Activation of JNK and NFκB by TNFα elicits proinflammatory changes in obese adipose tissue ([1]) and *in vitro* TNFα-mediated insulin resistance depends on activation of the ERK p42/44-MAPK pathway in adipocytes ([28]). Figure 6C demonstrates that activation of these key signaling pathways is attenuated in the obese Siah2KO animals.

Partial Activation of PPARγ in the Siah2KO Adipose Tissue

PPARγ transcriptional activity plays a pivotal role in regulating adipose tissue inflammation in response to chronic energy excess ([11], [29]). Our studies in 3T3-L1 adipocytes indicate Siah2 regulates expression of a subset of PPARγ target genes and affects ligand-dependent degradation of PPARγ proteins ([10]). Therefore, we assayed the expression of a subset of PPARγ target genes that were differentially regulated in our microarray analysis. Although classic markers of adipogenesis such as *pck-1* and *fabp4* are down-regulated in the Siah2KO epididymal fat, several genes encoding proteins involved in fatty acid transport and storage (*lpl*, *cd36, soat1*) and lipid synthesis (*fasn*, *lipin1*) are up-regulated (Figure 7A). While the increase in *lpl* expression is modest, expression of *fasn*, *cd36*, *lipin1,* and *soat1* is increased 2–5 fold. Activation of PPARγ is not associated with regulation of PPARγ1 or PPARγ2 gene expression in the Siah2KO adipose tissue (Figure 7B), but does correlate with increased PPARγ protein levels (Figure 7C, D, E) and decreased PPARγ ubiquitylation (Figure 7F,G). Although identified as a target of Siah2 *in vitro* ([30]), nuclear corepressor-1 (NCoR) levels are decreased and the levels of Siah2 target proteins, histone deacetylase-3 (HDAC3) ([31]) and prolyl-3-hydroxylase (PHD3) ([32]) are unaltered.

Discussion

The ubiquitin-proteasome system is well described as a critical regulator of immune responses ([6]). For example, TNFα-mediated activation of NF-κB is regulated by multiple ubiquitin ligases, including several TNF Receptor associated factors (TRAFs 2,5, and 6) as well as A20/TNFα-induced protein-3 ([6]) and ITCH, a JNK phosphorylation-dependent ligase that modulates TNFα signaling ([33]). Much less is known about how ubiquitin ligases affect the chronic inflammation that occurs in response to obesity. In this study, we report the relationship between obesity-related adipocyte hypertrophy, adipose tissue inflammation and insulin sensitivity is regulated by the ubiquitin ligase Siah2. Although Siah2KO adipocytes trend larger than wild-type, deletion of Siah2 protects mice with diet-induced obesity from adipose tissue inflammation and insulin sensitivity in the obese Siah2KO animals is comparable to lean wild-type. The striking decrease in pro-inflammatory cytokine and chemokine expression along with reduced fibrosis and crown-like structures and elevated levels of regulatory T cell markers indicates Siah2 is necessary for adipocyte hypertrophy to elicit the inflammatory changes in obese adipose tissue that lead to insulin

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resistance. Several mechanisms may underlie the effect of Siah2 on the relationship between adipocyte hypertrophy and insulin resistance.

Although a sustained influx of M1-like macrophages in adipose tissue restricts adipocyte expansion ([2], [34]), macrophage infiltration is essential for healthy adipose tissue remodeling in response to excess energy intake. As adipocytes expand, dysfunctional enlarged adipocytes are removed by macrophages and replaced by newly formed adipocytes ([35]). However, the early distribution toward larger adipocytes in the lean and obese Siah2KO mice and our *in vitro* studies showing loss of Siah2 impairs adipogenesis ([10]) argues against accelerated adipose tissue remodeling in the Siah2KO animals. Instead, the broad transcriptional response in markers of inflammation suggests Siah2 regulates the influx, production or activation of proinflammatory immune cells. Initial studies of Siah2 found Siah2 deletion enhances expansion of bone marrow-derived myeloid progenitor cells *in vitro* without altering the levels of myeloid cells *in vivo* ([12]). Our study found that Siah2 deficiency substantially reduces expression of *trem2* in adipose tissue, which encodes a protein found on monocyte-derived dendritic cells that promotes myeloid cell activation ([36]). The possibility that Siah2 is affecting inflammation via TREM-2 is supported by evidence that dendritic cells promote macrophage infiltration of adipose tissue ([37]).

Our data also show the absence of proinflammatory changes in the obese Siah2KO visceral adipose tissue corresponds to increased lipid storage capacity in the enlarged adipocytes. Isolated Siah2KO adipocytes are more sensitive to the anti-lipolytic effect of insulin, and this is reflected *in vivo* by reduced circulating fatty acids and triglyceride levels. The “lipid steal” hypothesis proposes that PPARγ activation in adipose tissue enhances the ability of adipocytes to store excess lipids in obesity ([11]). This protects other insulin-responsive tissues from reactive lipids, resulting in improved insulin sensitivity. Although markers of adipogenesis such as *fabp4*, *pck1* and *adiponectin* are not increased, PPARγ target genes involved in lipid uptake, lipid synthesis and lipid storage are up-regulated in the adipose tissue. Increased lipid storage in the hypertrophied adipocytes is consistent with our observation that genotype significantly contributes to decreased energy expenditure in the Siah2KO obese mice independent of body composition. Several adipokines influenced by PPARγ activation that impact adipose tissue inflammation are also regulated by Siah2. Higher adipsin levels and lower *mcp-1*, *saa3* and *pai-1* levels are associated with ligand activation of PPARγ ([11]), but unaltered expression of adiponectin and other PPARγ targets such as *pck-1* suggests that a role for PPARγ transcriptional activity in Siah2-mediated regulation of inflammation does not occur via classical PPARγ ligand binding. This is consistent with elevated PPARγ protein levels accompanied by only modest reductions in PPARγ ubiquitylation. PPARγ-mediated changes in lipid storage capacity can be regulated by TNFα ([1], [29]). TNFα inhibits PPARγ activity via multiple mechanisms ([29]), including caspase-dependent degradation of PPARγ proteins ([38], [39]). Moreover, Siah proteins are structurally similar to the TRAF ubiquitin ligases that regulate TNFα signaling ([40]). Therefore, reduced caspase cleavage of PPARγ proteins may also regulate PPARγ protein levels and activity in the Siah2KO animals.

The well-described role of Siah2 in regulating cellular responses to hypoxia ([32]) suggests Siah2 deletion inhibits hypoxia-related adipose tissue fibrosis and the subsequent influx of

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immune cells ([26]). This possibility is supported by our evidence that fibrosis is reduced in the adipose tissue of obese Siah2KO mice. A Siah2-mediated effect on hypoxia is unlikely to occur via a HIF-1α-dependent pathway since Siah2 deletion does not affect the protein levels of PHD3, the Siah2 target protein responsible for targeting HIF-1α for degradation. However, Siah2 also affects hypoxia responses via HIF-1α-independent pathways that involve MAPK signaling ([32]), which is reduced in the Siah2KO adipose tissue. A Siah2-dependent mechanism influencing the relationship between adipocyte hypertrophy and insulin sensitivity that indirectly affects inflammation is an attractive possibility given our results showing glucose tolerance and insulin sensitivity are also improved in the lean Siah2KO mice without consistent changes in inflammatory markers.

Future studies that take advantage of adipocyte-specific deletion of Siah2 are needed to fully address the mechanisms by which Siah2 is regulating adipose tissue inflammation, lipid metabolism and insulin sensitivity in adipose tissue. Nevertheless, our data clearly establishes a role for Siah2 in determining the inflammatory response to adipocyte hypertrophy and regulation of insulin sensitivity in obesity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding

This work is supported by the National Institutes of Health (NIDDK, 5R56DK89020, ZEF and NIDDK, 1R01DK099625, ZEF). This work used the Cell Biology and Bioimaging Core and the Genomics Core facilities at Pennington Biomedical Research Center that are supported in part by COBRE (NIH 8P20-GM103528) and NORC (NIH 2P30-DK072476) center grants from the National Institutes of Health. This work also used Core Services supported by grant DK097153 of NIH to the University of Michigan.

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What is already known about this subject?

- Obesity-related adipocyte hypertrophy in obesity is associated with adipose tissue inflammation
- Obesity-related insulin resistance has been linked to adipose tissue inflammation
- Enzymes of the ubiquitin-proteasome system are known to regulate inflammatory processes.

What does this study add?

- Obese Siah2 null mice have hypertrophied adipocytes, but reduced adipose tissue inflammation.
- Glucose and insulin tolerance is improved in the lean and obese Siah2 null mice.
- Siah2 regulates a subset of PPARγ target genes in adipose tissue that are involved in lipid metabolism and inflammatory responses.

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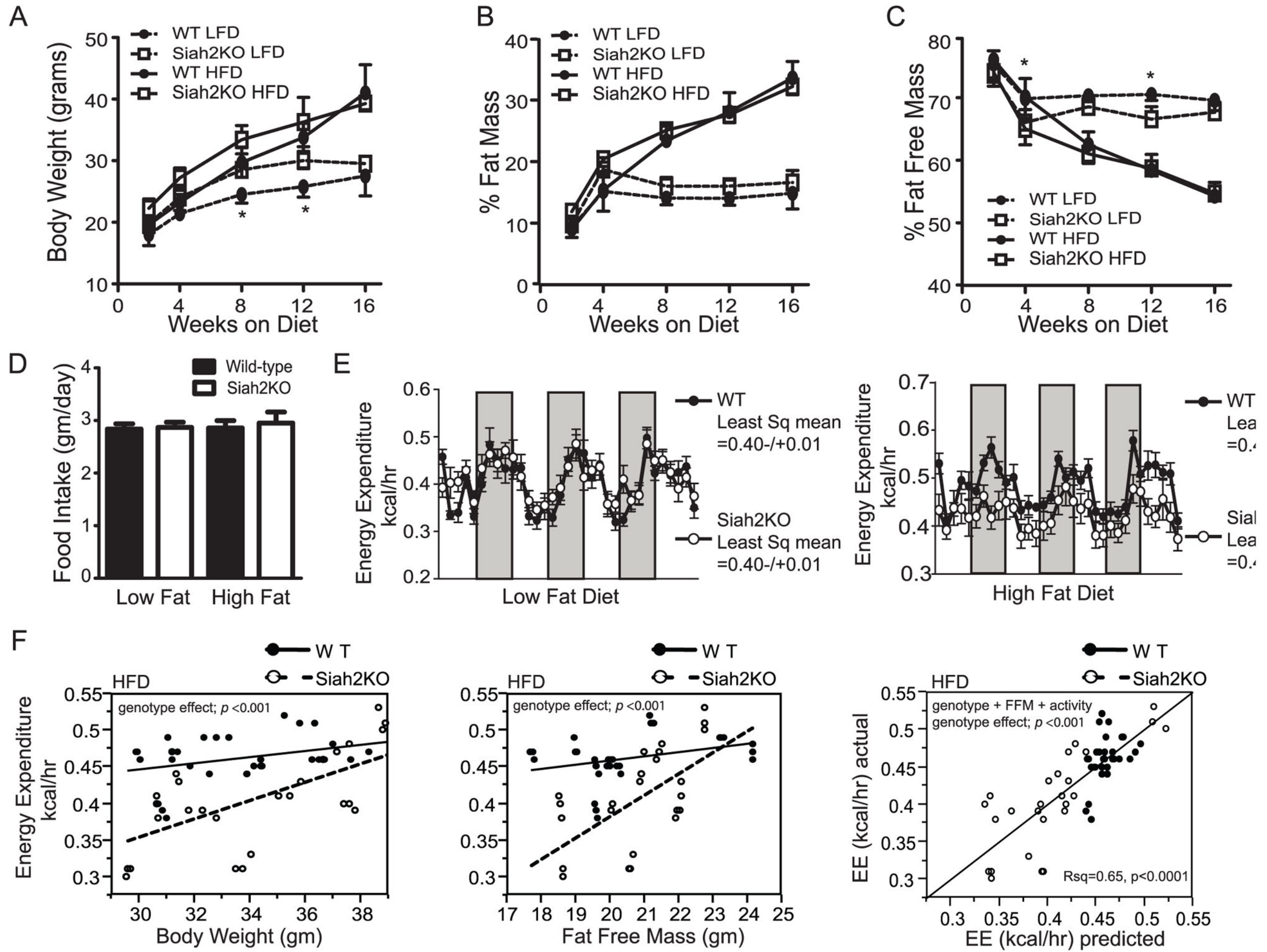

Figure 1. Siah2KO mice fed a high fat diet are obese with reduced energy expenditure compared to Wild-type

Body weight (**A**), percent fat mass (**B**) and percent fat free mass (**C**) were measured in the Wild-type (WT) and Siah2KO mice fed a defined low (LFD) or high (HFD) fat diet over sixteen weeks. Food intake (**D**) and energy expenditure (**E**) were measured at twelve weeks on each diet. Dark bars indicate 7P-7A. (**F**) Regression analysis of energy expenditure (kcal/hr) related to body weight or fat free mass (lean mass) was carried out using the daily energy expenditure of each animal over 3 consecutive days. Activity and RER were also measured (Figure S1). Statistical significance compared to wild-type; *, $p<0.05$.

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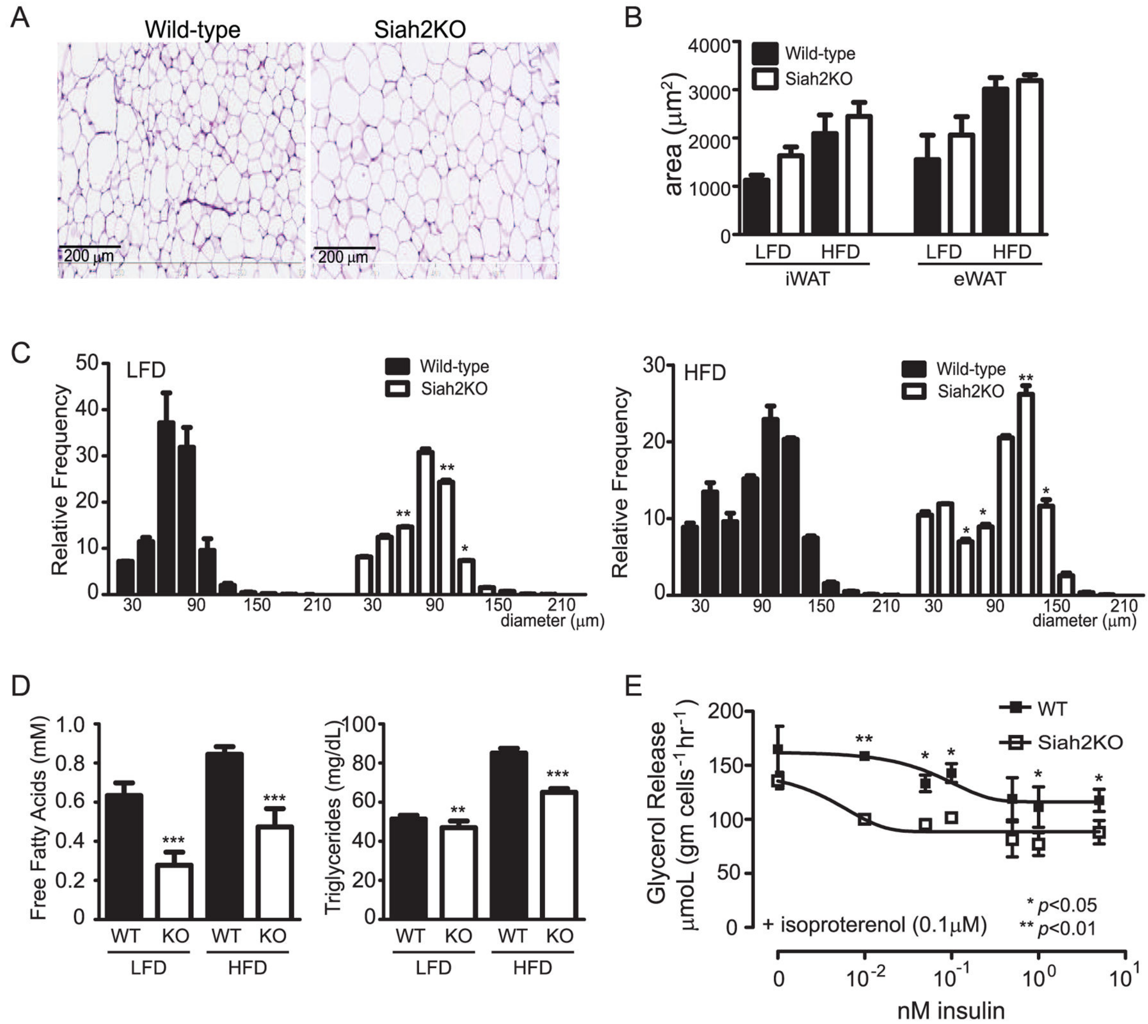

Figure 2. Siah2KO adipocytes are enlarged, but more insulin-sensitive than Wild-type

(**A**) H&E staining of HFD Wild-Type and Siah2KO epididymal fat. (**B**) Adipocyte size (area) quantitated using Image J. iWAT, inguinal adipose tissue, eWAT, epididymal adipose tissue. (**C**) Adipocyte size distribution of epididymal adipocytes at two months on LFD or HFD analyzed using Coulter counting. (**D**) Wild-type (WT) and Siah2KO (KO) serum free fatty acids and triglyceride levels. (**E**) Insulin-dependent inhibition of lipolysis in isolated HFD Wild-type and Siah2KO epididymal adipocytes. Statistical significance compared to Wild-type; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$. LFD, low fat diet; HFD, high fat diet

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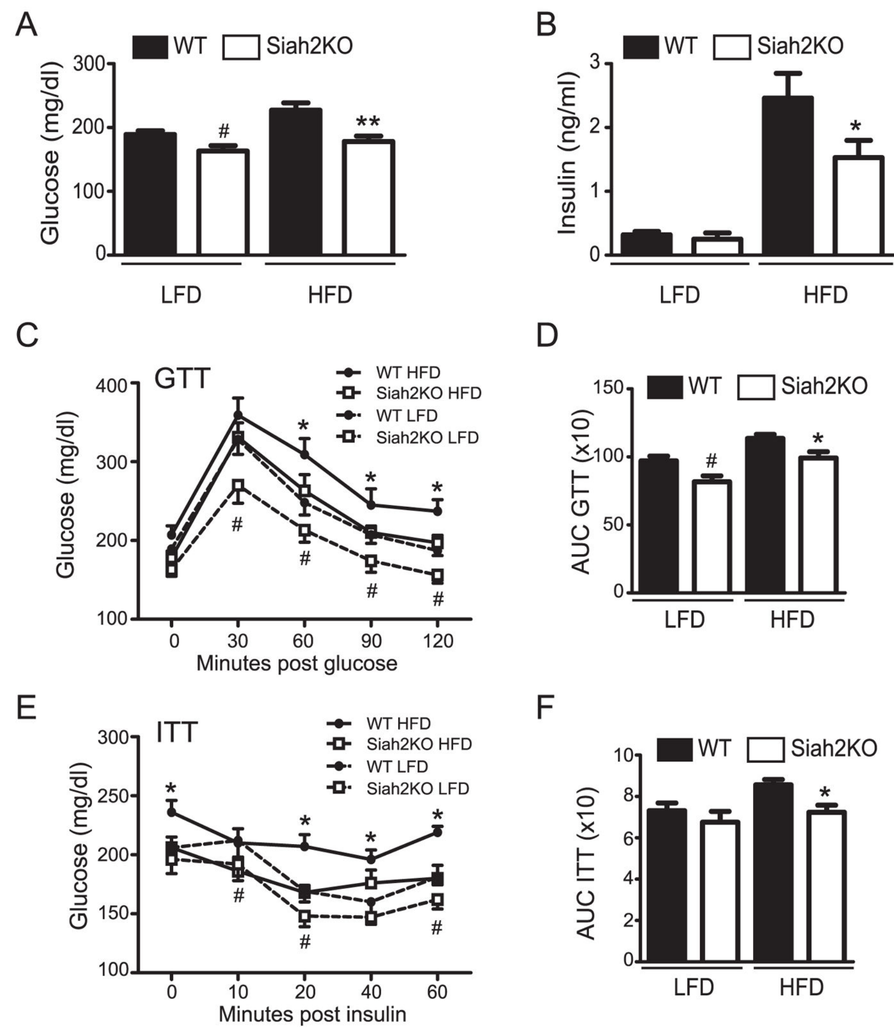

Figure 3. Carbohydrate metabolism is improved in the lean or obese Siah2KO mice

(**A**) Fasting blood glucose and (**B**) insulin levels assayed at 16 weeks on the LFD or HFD. (**C**) Glucose tolerance test and (D) GTT AUC and (**E**) insulin tolerance test and (**F**) ITT AUC at 12 weeks on each diet. Statistical significance compared to Wild-type; #, $p<0.05$ for low fat diet and *, $p<0.05$; **, $p<0.01$ for high fat diet.

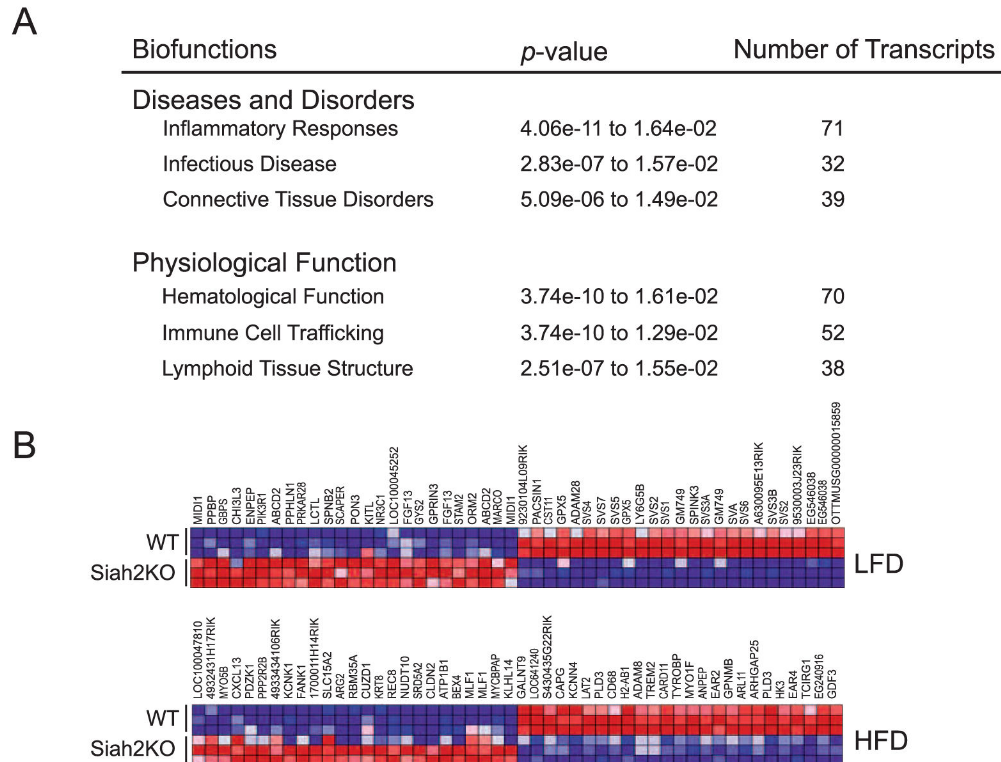

Figure 4. Siah2 controls adipose tissue inflammation

Wild-type (WT) and Siah2KO epididymal adipose tissue was evaluated using microarray analysis of gene expression. (**A**) Most significant biological functions affected by Siah2 determined by Ingenuity software analysis (**B**) Hierarchical clustering of the top 50 genes up or down-regulated by Siah2 in the low fat (LFD) or high fat (HFD) fed mice.

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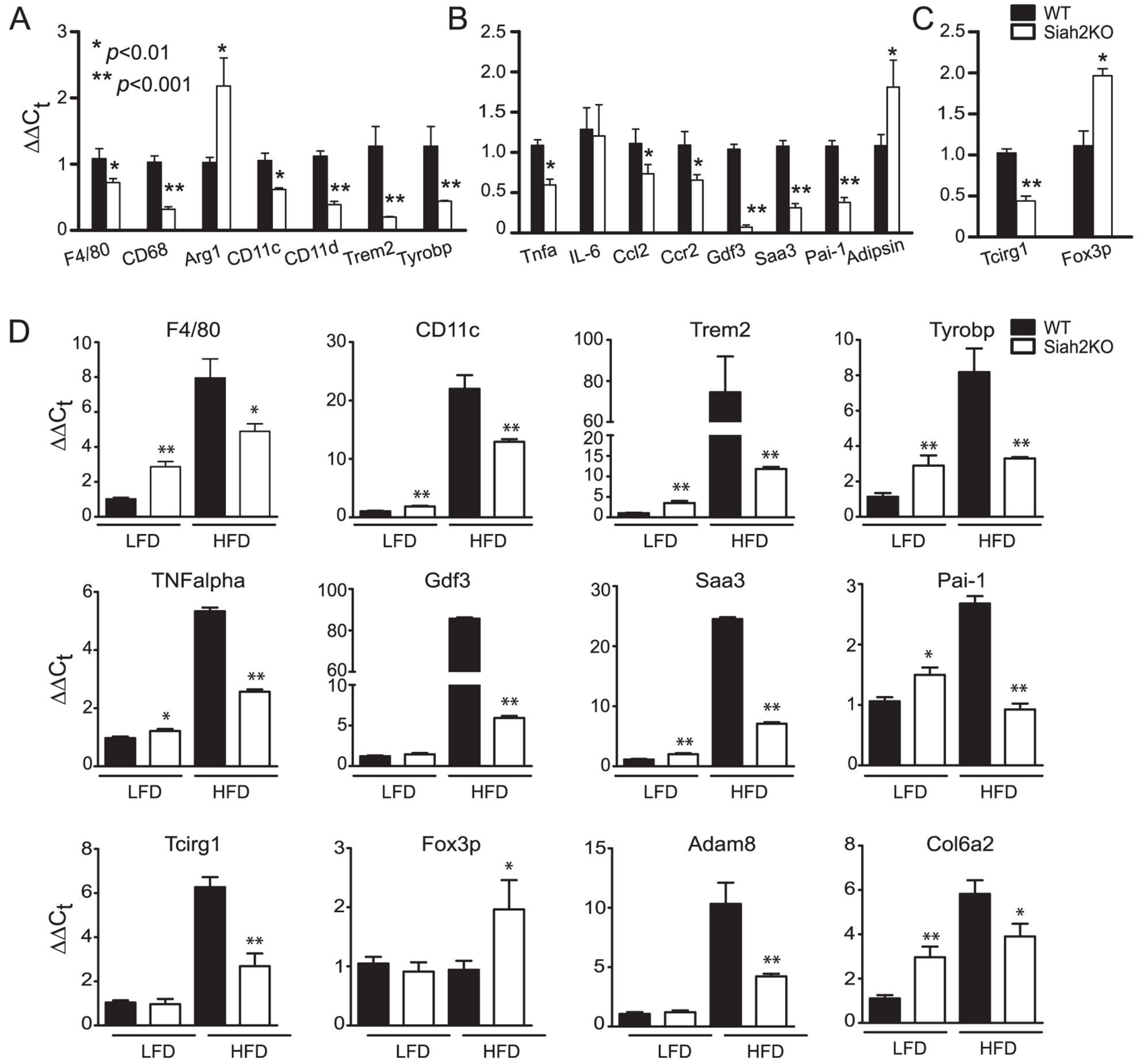

Figure 5. Siah2 regulates inflammatory gene expression in adipose tissue of high fat fed mice

Gene expression of markers of (**A**) inflammation, (**B**) cytokines and chemokines, and (**C**) T lymphocytes was assayed in the high-fat fed mice or (D) compared between the low fat and high fat fed mice using real-time qRT-PCR. Statistical significance compared to Wild-type; *, p<0.01; **, p <0.001.

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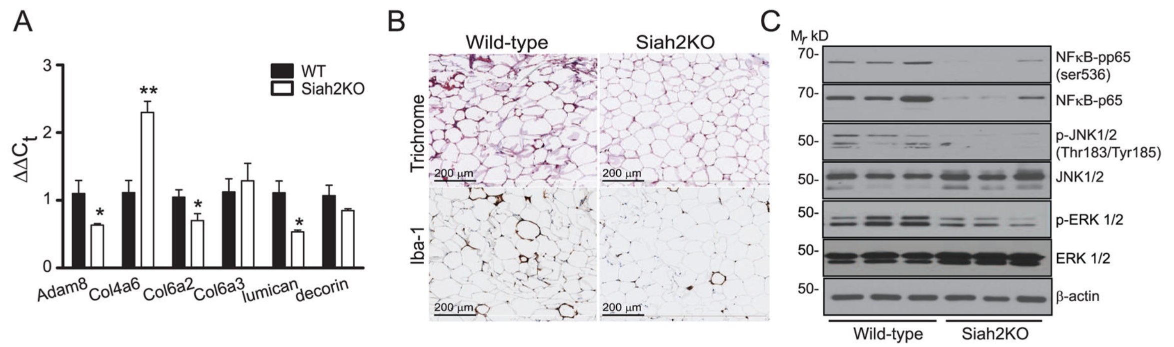

Figure 6. Siah2 deficiency decreases markers of adipocyte dysfunction in obesity

Indicators of adipocyte dysfunction were assayed in epididymal adipose tissue from high-fat fed mice. (A) Extracellular matrix components were determined using real-time qRT-PCR. Statistical significance compared to Wild-type; *, $p<0.05$; **, $p<0.01$. (B) Fibrosis was determined using trichrome staining of collagen and crown-like structures were visualized using anti-Iba-1 immunostaining of macrophages. (C) Stress kinase activation was determined via western blot analysis. β-actin is included as a loading control.

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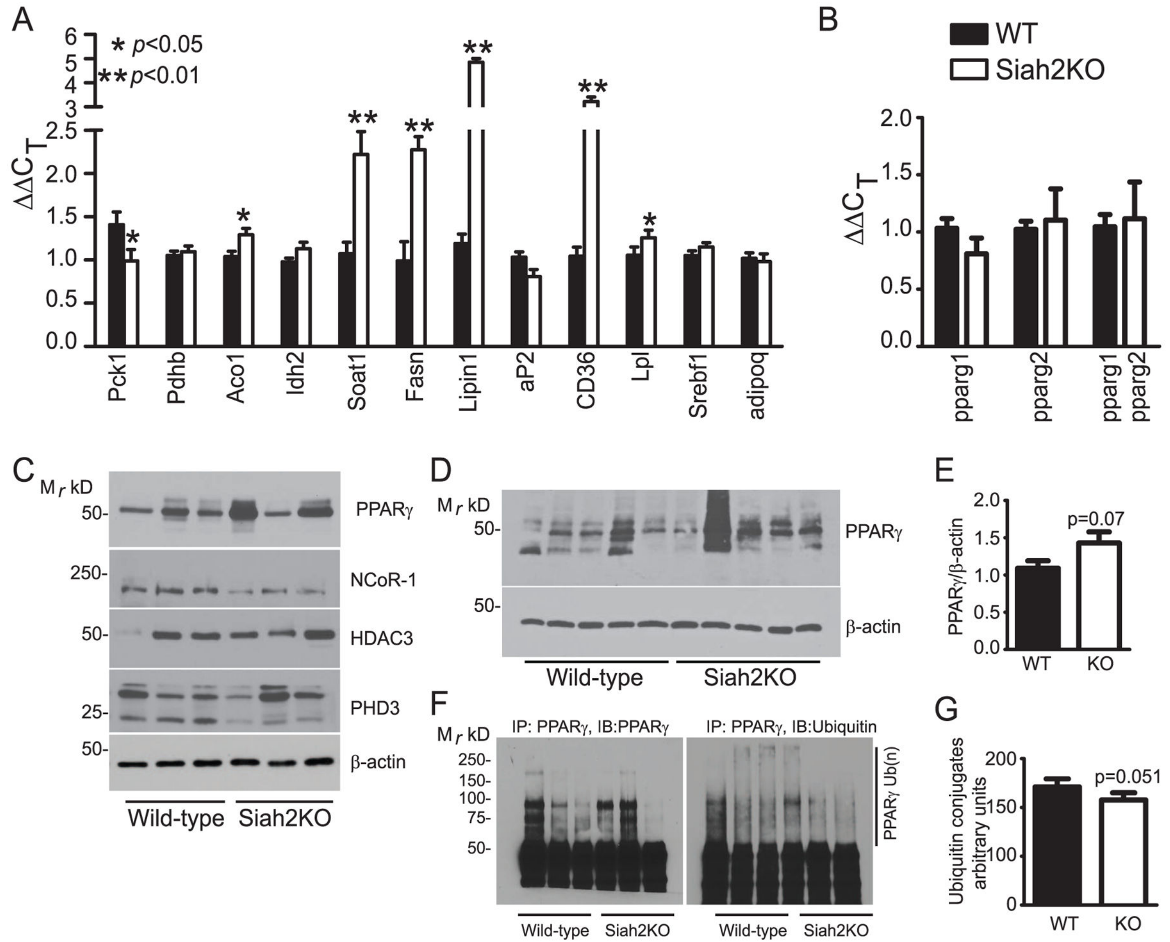

Figure 7. Loss of Siah2 selectively regulates PPARγ target genes involved in lipid metabolism and alters PPARγ protein levels

All measurements were carried out in epididymal adipose tissue from high-fat fed mice. (A) A subset of PPARγ target genes selected based on microarray analysis were analyzed via real-time qRT-PCR (B) Gene expression of PPARγ1, PPARγ2 or PPARγ1 and PPARγ2 combined. (C) Steady-state level of PPARγ proteins and selected Siah2 target proteins. (D) PPARγ protein levels from expanded number of mice. β-actin is included as a loading control in (C, D). (E) Quantification of PPARγ protein levels in (D). (F) Ubiquitin modification of PPARγ determined by western blot analysis of PPARγ (IP PPARγ, IB PPARγ) or ubiquitin (IP PPARγ, IB ubiquitin) after immunoprecipitation of PPARγ from Wild-type or Siah2KO epididymal adipose tissue. (G) Quantification of high molecular weight PPARγ ubiquitin conjugates. Statistical significance compared to Wild-type; *, $p<0.05$; **, $p<0.01$.