Adipocyte SIRT1 controls systemic insulin sensitivity by modulating macrophages in adipose tissue

Xiaoyan Hui1,2, Mingliang Zhang3, Ping Gu1,2,4, Kuai Li5,6, Yuan Gao1,2, Donghai Wu5,6, Yu Wang1,7,* & Aimin Xu1,2,7,**

Abstract

Adipose tissue inflammation, characterized by augmented infiltration and altered polarization of macrophages, contributes to insulin resistance and its associated metabolic diseases. The NAD+-dependent deacytase SIRT1 serves as a guardian against metabolic disorders in multiple tissues. To dissect the roles of SIRT1 in adipose tissues, metabolic phenotypes of mice with selective ablation of SIRT1 in adipocytes and myeloid cells were monitored. Compared to myeloid-specific SIRT1 depletion, mice with adipocyte-selective deletion of SIRT1 are more susceptible to diet-induced insulin resistance. The phenotypic changes in adipocyte-selective SIRT1 knockout mice are associated with an increased number of adipose-resident macrophages and their polarization toward the pro-inflammatory M1 subtype. Mechanistically, SIRT1 in adipocytes modulates expression and secretion of several adipokines, including adiponectin, MCP-1, and interleukin 4, which in turn alters recruitment and polarization of the macrophages in adipose tissues. In adipocytes, SIRT1 deacytlates the transcription factor NFATc1 and thereby enhances the binding of NFATc1 to the Il4 gene promoter. These findings suggest that adipocyte SIRT1 controls systemic glucose homeostasis and insulin sensitivity via the crosstalk with adipose-resident macrophages.

Keywords insulin resistance; macrophage infiltration and polarization; metabolic inflammation; obesity; SIRT1

Introduction

Adipose tissue is now well recognized as an active endocrine organ and serves as a key player of systemic energy homeostasis. Dysfunction of adipose tissue plays a central role in pathogenesis of obesity and its related comorbidities [1]. Studies in the past decade have demonstrated the existence of a low-grade, chronic inflammation within obese adipose tissue [2] and macrophage infiltration is integral to these inflammatory changes [3]. The absolute number of adipose-resident macrophages is tightly linked to the degree of metabolic dysfunction in obese human. On the other hand, selective depletion of CD11c+ macrophages resulted in rapid normalization of insulin sensitivity in mice [4]. In addition to the increased number of macrophages recruited to local adipose tissues, adipose macrophages undergo a phenotypic switch from the alternatively activated, anti-inflammatory M2 state to the classically activated, pro-inflammatory M1 state during obesity [5,6]. Mice with disruption of PPARγ in myeloid cells had impaired alternative macrophage activation and were prone to diet-induced insulin resistance [7], while enhanced expression of macrophage PPARγ/β by Th2 cytokines facilitated activation of M2 macrophages [8]. However, the underlying mechanism that controls adipose tissue macrophage polarization under obese conditions remains poorly understood.

SIRTuins are a group of NAD+-dependent protein deacytases considered as key regulators of natural aging and other stress-related conditions [9,10]. SIRT1, the founding member of the family, plays a pivotal role in maintaining metabolic function and health. Activation of SIRT1 in mice by either small pharmacological molecules [11–13] or genetic manipulation [14,15] prevents high-fat diet (HFD)- or natural aging-associated metabolic dysfunctions. Adipose tissue appears to be an important action site of SIRT1. It has been reported that HFD led to the proteolytic cleavage of SIRT1 by caspase-1 selectively in adipose tissues, and adipocyte-selective...
ablation of SIRT1 resulted in exacerbated insulin resistance [16]. SIRT1 within adipose tissue exert its protective roles by multiple mechanisms. In mature adipocytes, SIRT1 promotes fat mobilization through repression of PPARγ [17]. In another study, SIRT1 was shown to deacetylate and activate PPARγ, thereby facilitating the browning of white adipose tissue in mice [18]. In addition, adipose-selective overexpression of a dominant-negative SIRT1 caused exacerbation of hyperglycemia, dyslipidemia, ectopic lipid deposition, and insulin resistance, possibly through the abnormal epigenetic modifications and chromatin remodeling [19]. On the other hand, SIRT1 in macrophage protects mice from obesity-induced insulin resistance by antagonizing the actions of key molecules in inflammatory pathways [20].

Considering the dynamic interplay between adipocytes and macrophages within adipose tissue during the pathogenesis of obesity, we generated adipocyte and myeloid cell-specific SIRT1 knockout mice and compared the metabolic phenotypes of these two types of mice in the context of dietary obesity. We found that during the onset of obesity, SIRT1 deficiency in adipocytes, but not in myeloid cells, accelerated peripheral insulin resistance via modulation of both macrophage infiltration and polarization, which was independent of adiposity. Furthermore, we have discovered regulation of IL-4 production by SIRT1 as a possible mechanism whereby adipocyte SIRT1 modulates macrophage polarization.

Results

Adipocyte-specific inactivation of SIRT1 accelerates HFD-induced peripheral insulin resistance independent of adiposity

To elucidate the physiological functions of SIRT1 in adipose tissue, adipose-specific (AKO) and myeloid cell-specific (MKO) Sirt1 knockout mice were generated as described in Materials and Methods (Fig EV1A). Tissue-selective inactivation of SIRT1 was achieved by deletion of exon 4 which encodes a 51-amino acid catalytic domain of SIRT1. PCR results showed the genomic deletion in AKO mice was evident in mature adipocytes but not stromal vascular fraction (SVF) of the white adipose depots and peritoneal macrophages (Fig EV1B). Consistently, Western blot analysis demonstrated a truncated form of SIRT1 in mature adipocytes isolated from epididymal fat (Fig EV1C) of AKO mice, while it remained intact in SVF, peritoneal macrophages, and other tissues (Fig EV1C). Likewise, mature adipocytes, but not peritoneal macrophages isolated from AKO mice, exhibited hyperacetylation of p53, a well-established substrate of SIRT1 [21] (Fig EV1D and E), further confirming the adipocyte-specific inactivation of SIRT1 in AKO mice. In contrast, macrophage-specific truncation of SIRT1 and hyperacetylation of p53 were evident in MKO mice, and no obvious difference was observed in p53 acetylation in mature adipocytes between MKO and wild-type (WT) mice (Fig EV1C–E).

When fed a standard chow diet (SCD), no obvious difference was observed in body weight and fat composition among WT, AKO, and MKO mice (Fig EV2A and B). These mice did not show significant differences in glucose utilization and insulin levels until they were 30 weeks old, when mild glucose intolerance began to develop in AKO mice (Fig EV2C–E). On the other hand, MKO mice remained glucose tolerant and had comparable levels of insulin to WT mice under normal chow diet (Fig EV2C–E).

When the mice were challenged with HFD, there was no significant difference in body weight, food intake, body composition, oxygen consumption, and respiratory exchange ratio (RER) among the three groups (Fig EV3). However, AKO mice exhibited more severe glucose intolerance and hyperinsulinemia after as early as 4 weeks of HFD, and such metabolic changes were progressively deteriorated after prolonged HFD (Fig 1A–C). In contrast, glucose tolerance and insulin levels were comparable between MKO and WT mice 4 weeks post-HFD, and a mild glucose intolerance in MKO mice was observed only after 16 weeks of HFD. Likewise, AKO mice were more insulin resistant after 4 weeks of HFD, while there was no obvious difference in insulin sensitivity between MKO mice and WT mice at this stage (Fig 1D and E). These data suggested that AKO mice, but not MKO mice, manifested early onset of HFD-induced insulin resistance.

To further validate the above findings, insulin signaling in peripheral tissues of these mice was assessed. Compared to WT mice, insulin-evoked phosphorylation of Akt and its downstream target glycogen synthase kinase β (GSK3β) was significantly blunted in both liver and skeletal muscles of AKO mice (Fig 1F and G), whereas such changes were not obvious in MKO mice. To more accurately measure the tissue-specific actions of insulin in vivo, hyperinsulinemic/euglycemic clamp was performed. The glucose infusion rate (GIR), as quantified by the amount of exogenous glucose required to maintain euglycemia, was substantially declined in AKO mice (Fig 1H). The insulin-stimulated suppression of hepatic glucose production (Fig 1I and J) and glucose uptake in soleus muscle (Fig 1K) was also markedly attenuated in AKO mice when compared to the control group, demonstrating that adipocyte-specific disruption of SIRT1 triggers insulin resistance in both liver and skeletal muscles. But no significant difference on insulin-stimulated glucose uptake was observed in epididymal adipose tissue (Fig 1L).

Deletion of SIRT1 in adipocytes exacerbates HFD-induced macrophage infiltration and polarization toward the pro-inflammatory phenotype in adipose tissues

Since systemic inflammation contributes to insulin resistance, a panel of inflammatory markers were measured. Serum levels of TNF-α, IL-1β, and adipocyte fatty acid binding protein (A-FABP) remained comparable in 8-week-old lean mice. However, these inflammatory markers were markedly elevated in obese AKO mice (Fig 2A–C). Both high molecular weight (HMW) and hexameric forms of adiponectin in HFD-fed AKO mice were significantly lower than their littermate controls (Fig 2E). In contrast, serum leptin level remained unchanged between WT and AKO mice under both SCD and HFD conditions (Fig 2D).

Adipose tissue inflammation, characterized by increased infiltration and polarization of macrophages to the pro-inflammatory M1 phenotype, is an important player of obesity-induced chronic systemic inflammation and insulin resistance [3]. We next investigated whether SIRT1 deletion in adipocytes leads to alterations in adipose tissue inflammation. Immunohistochemical staining with anti-F4/80 (a cell surface marker of macrophages) showed that the
number of infiltrating macrophages in the adipose tissue of AKO mice was obviously higher than WT mice after 4 weeks of HFD (Fig 2F). Likewise, obese AKO mice exhibited much higher expression levels of F4/80 and several markers for pro-inflammatory M1 macrophages (Tnfa, Il6, Mcp1, and Cd11c) (Fig 2G and H).

On the other hand, the adipose tissue expression of the
anti-inflammatory M2 macrophage markers, including arginase 1 (Arg1), macrophage galactose lectin 1 (Mgl1), Mgl2, and macrophage mannose receptor 2 (Mrc2), was significantly dampened in AKO mice (Fig 2H). Consistently, flow cytometry analysis of the SVF also revealed elevated recruitment of F4/80+ cells in adipose tissue of obese AKO mice (Fig 2I and Appendix Fig S1). This change was accompanied by increased composition of M1 macrophages (CD11c\textsuperscript{high}CD206\textsuperscript{low} cells) and decreased composition of M2 macrophages (CD11c\textsuperscript{low}CD206\textsuperscript{high}) as compared to the WT mice (Fig 2J and Appendix Fig S1), suggesting that adipocyte SIRT1 deficiency
enhances the polarization of adipose macrophages to the pro-inflammatory M1 subset.

As CD3⁺ T lymphocytes have been shown to be an important contributor to macrophage recruitment and adipose tissue inflammation [22], we next compared the composition of CD3⁺ T cells in adipose tissues of AKO mice and control littersmates. Both the total number of infiltrating CD3⁺ T lymphocytes and the percentage of CD3⁺ CD8⁺ cytotoxic T lymphocytes and CD3⁺ CD4⁺ T helper cells were largely the same in epididymal fats of WT and AKO mice (Appendix Fig S2).

**SIRT1 in adipocytes controls macrophage recruitment and polarization by modulating the production of several adipokines**

It has been reported that adipocyte insulin sensitivity regulates macrophage infiltration and inflammation [23]. Therefore, we examined whether insulin responses in adipocytes were altered upon deletion of SIRT1. As shown in Fig EV4, no differences between WT and SIRT1-deficient adipocytes were observed in both insulin-stimulated glucose uptake and Akt phosphorylation, indicating that SIRT1 does not directly regulate adipocyte insulin sensitivity. We next investigated the direct effects of adipocyte-expressed SIRT1 in mediating the cross talk between adipocytes and macrophages using an indirect co-culture system. Migration of peritoneal macrophages was significantly augmented when co-cultured with SIRT1-deficient adipocytes (Fig 3A and B). Furthermore, when incubated in conditioned medium from SIRT1-deficient adipocytes, macrophages displayed higher expression of M1 macrophage markers, accompanied by significant downregulation of M2 macrophage markers as compared to those incubated in conditioned medium of control adipocytes (Fig 3C), implicating the existence of SIRT1-dependent, adipocyte-derived factors that mediate the alterations in macrophage migration and polarization. Indeed, in mature adipocytes SIRT1 deficiency resulted in a marked elevation in both expression and secretion of MCP-1 (Fig 3D and E), a chemokine responsible for macrophage recruitment and polarization. In contrast, production of adipocyte-derived Th2 cytokines interleukin 4 (IL-4) and IL-13 were reported to promote the polarization of adipose-resident macrophages toward the anti-inflammatory M2 subtype [8]. By realtime PCR analysis, we found that the transcriptional level of Il4, but not Il13, was significantly reduced in SIRT1-deficient adipocytes (Fig 4A). Moreover, the protein concentration of IL-4 in the conditioned medium of SIRT1-deficient adipocytes was much lower than that in WT adipocytes (Fig 4B). Consistently, mature adipocytes fractionated from adipose tissues of AKO mice exhibited much less amount of IL-4 protein (Fig 4C), while in SVF, IL-4 protein was similar between AKO and WT mice. Flow cytometry analysis for the SVF of epididymal adipose tissue showed comparable level of eosinophils and neutrophils between obese WT and AKO mice (Fig EV5).

To determine whether downregulation of IL-4 in adipocytes contributes to enhanced macrophage polarization toward the pro-inflammatory M1 phenotype in adipose tissues of AKO mice, recombinant IL-4 protein was supplemented into the conditioned medium of SIRT1-deficient adipocytes to evaluate its impact on macrophage phenotypes. This analysis showed that replenishment of IL-4 partially rectified M1 macrophage polarization caused by conditioned medium derived from SIRT1-deficient adipocytes (Fig 4D and E).

**SIRT1 induces Il4 transcription through nuclear factor of activated T cells, cytoplasmic 1 (NFATc1)**

To further investigate the role of SIRT1 activation in controlling IL-4 production, we evaluated the effects of resveratrol on both transcription and secretion of IL-4 in adipocytes. In both 3T3-L1 adipocytes and primary adipocytes from WT mice, resveratrol significantly increased both the mRNA level of Il4 and its protein concentration in the conditioned medium (Fig 5A and B, and Appendix Fig S3). However, such a stimulatory effect of resveratrol was abolished in SIRT1-deficient adipocytes.

NFATc1 is a principal factor that transactivates Il4 gene through direct binding to the multiple cis elements on both human and mouse Il4 promoter [26,27]. We next tested whether SIRT1-induced Il4 expression is mediated by NFATc1 in adipocytes. ChIP assay with anti-NFATc1 antibody demonstrated that resveratrol treatment led to a strong increase in the recruitment of NFATc1 to its binding sites on the mouse Il4 promoter, whereas both basal and resveratrol-induced binding of NFATc1 to the Il4 promoter was abolished in SIRT1-deficient adipocytes (Fig 5C).

We then investigated whether NFATc1 is a direct target of SIRT1 in adipocytes. Immunoprecipitation analysis showed that SIRT1 was physically associated with NFATc1 in epididymal adipose tissue (Fig 5D). To further pin down the interactive domain between SIRT1 and NFATc1, plasmids encoding NFATc1 were co-transfected with 3xFLAG-tagged full-length or truncated forms of SIRT1, which lack either the C-terminus (1-499aa) or C-terminus plus catalytic domain is essential for its interaction with NFATc1. We found that NFATc1 was co-immunoprecipitated with full-length SIRT1, and the interaction was lost when the C-terminus of SIRT1 (500-747aa) was deleted (Fig 5E), suggesting that this domain is essential for its interaction with NFATc1. Furthermore, we tested whether the acetylation level of NFATc1 was increased upon SIRT1 deletion. To this end, mature adipocytes from obese WT or AKO mice were isolated and endogenous NFATc1 was immunoprecipitated using anti-NFATc1 antibody demonstrated that resveratrol treatment led to a strong increase in the recruitment of NFATc1 to its binding sites on the mouse Il4 promoter, whereas both basal and resveratrol-induced binding of NFATc1 to the Il4 promoter was abolished in SIRT1-deficient adipocytes (Fig 5F).

**Discussion**

Although SIRT1 in both adipocytes and macrophages has been shown to protect against obesity-induced metabolic dysregulation [16,20,28], its relative contribution in these two cell types to the onset and progression of the disease remains unknown. Although aP2 gene is found expressed in both adipocytes and macrophages, the SIRT1fl/fl; aP2-Cre mice exhibited the exclusive deletion of...
SIRT1 in mature adipocytes, but not in macrophages, which is consistent with the previous report [28]. In addition, as a pan-adipocyte marker, aP2 is expressed in both white and brown adipocytes. Therefore, deletion of SIRT1 in brown adipose tissue (BAT) is also expected. A previous study by Qiang et al. [18] showed that whole-body knockout of Sirt1 did not influence expression of brown markers in BAT, but only led to lower levels of brown markers in subcutaneous WAT after overnight cold exposure at 4°C. Consistent with their finding, no significant difference on energy expenditure was detected between WT and AKO mice in our CLAMS study which was performed under ambient temperature.

As an important risk factor for a cluster of metabolic diseases, HFD-induced obesity is well known to evoke a series of biological events including inflammation and insulin resistance. Previous studies provide evidence that SIRT1 serves as a protective player to antagonize metabolic deterioration in multiple cell types [14] although such effects were not evident in muscle [29,30]. In line with these findings, our current study demonstrated that deletion of SIRT1 in adipocytes or macrophages further exacerbates obesity-induced metabolic dysregulation compared to the WT mice, suggesting SIRT1 in these two cell types safeguards metabolic homeostasis upon overnutrition. In addition, our study showed that selective deletion of SIRT1 in adipocytes, but not in macrophages, accelerated the onset of dietary obesity-induced systemic insulin resistance and glucose intolerance without obvious changes in adiposity. On the other hand, more severe insulin resistance and metabolic dysregulation in MKO mice were observed only at the late stage of obesity.

Figure 3. SIRT1 deficiency in adipocytes promotes macrophage migration and polarization to the M1 phenotype.
A, B Peritoneal macrophages were co-cultured with adipocytes derived from WT or AKO mice or without adipocytes (basal) for 24 h, followed by staining of migrated cells. (A) Representative images of migrated macrophages. (B) Quantification of migrated macrophages. The experiments were run in triplicate, and ten fields from each chamber were counted and averaged. Data are expressed as means ± SEM from three independent repeats with one-way ANOVA and Tukey test. *P < 0.05. Scale bar: 200 μm.
C Bone marrow-derived macrophages were cultured in blank medium (basal), or conditional medium (CM) derived from WT or AKO adipocytes for 48 h. The expression levels of M1 (left panel) and M2 (right panel) markers were examined by real-time PCR. Data are expressed as means ± SEM (n = 6) from three independent repeats with one-way ANOVA and Tukey test. *P < 0.05, **P < 0.01.
D, E mRNA (D) and protein concentration (E) of Mcp1 and adiponectin in SVF-derived adipocytes from AKO and WT mice. Data are expressed as means ± SEM (n = 6) from three independent repeats with two-sided paired Student’s t-test. *P < 0.05, **P < 0.01.
F Peritoneal macrophages were co-cultured with adipocytes derived from WT or AKO mice with or without treatment with RS-102895 (1 μM) or adiponectin (10 μg/ml) for 24 h. Migrated macrophages were quantified. The experiments were run in triplicate, and ten fields from each chamber were counted and averaged. Data are expressed as means ± SEM from three independent repeats with one-way ANOVA and Tukey test. *P < 0.05, **P < 0.01.
These findings suggest that adipocyte SIRT1 plays a more prominent role in maintaining glucose/lipid homeostasis during the early onset of obesity, whereas macrophage SIRT1 alleviates aggravation of these metabolic disorders only at a relatively late stage. This finding coincides with the study by Oh et al [31] who adopted in vivo monocyte/macrophage tracking method to demonstrate that the extent of macrophage accumulation in adipose tissues is mainly determined by the recipient mice, regardless of the source of donor monocytes.

Augmented infiltration of macrophages is a hallmark of adipose tissue inflammation, which in turn contributes to systemic inflammation and peripheral insulin resistance in obesity and aging [3]. Although a number of chemokines and extracellular factors have been identified as triggering factors for infiltration and activation of adipose-resident macrophages [24,25], the mechanisms underlying augmented production of these macrophage attractants in hypertrophic adipocytes remain poorly understood. Notably, our results demonstrated that ablation of SIRT1 in adipocyte, but not in macrophage itself, led to exacerbated infiltration of adipose-resident macrophages at the early stage of dietary obesity, and such a change is attributed at least in part to increased expression of MCP1 and reduced production of adiponectin. This conclusion was further supported by our in vitro co-culture experiment, which showed that conditioned medium from SIRT1-deficient adipocytes is much more potent in promoting macrophage migration than that from wild-type adipocytes, and these changes can be rectified by neutralization of MCP1 or supplementation of adiponectin. Indeed, MCP1 and adiponectin have been reported as positive and negative modulators of macrophage recruitment in adipose tissues, respectively. As a major member in the C-C chemokine family, MCP1 is the most potent chemotactic factor for monocyte/macrophage [32]. Mice with targeted ablation of Mcp1 or its receptor Ccr2 display dampened macrophage infiltration in adipose tissue without any obvious change in body weight [24,25], whereas overexpression of MCP-1 causes the opposite effects [33]. In contrast, transgenic ob/ob mice with a modest elevation of adiponectin are almost completely protected from macrophage infiltration in adipose tissues despite of the massive obesity [34]. The inhibitory effect of adiponectin on macrophage migration might be achieved at least partially by its indirect actions on expression of several pro-inflammatory cytokines/chemokines including MCP-1, IL-6, and IL-8 [35]. SIRT1 modulates the production of a panel of cytokines and adipokines through multiple mechanisms. In particular, SIRT1 deacetylates NF-κB and thus inhibits NF-κB binding to its target gene promoters including Mcp1 [36]. The expression and secretion of adiponectin is also under tight control of SIRT1 at both transcriptional and posttranslational levels. SIRT1 increases transcription of the adiponectin gene in adipocytes by enhancing the complex formation between Foxo1 and C/EBPα [37], but inhibits the secretion of HMW adiponectin by inhibition of endoplasmic reticulum oxidoreductase Ero1-Lα [38]. Therefore, reduced SIRT1 activity in adipocytes, which has been observed in both obesity and aging [16,19], may serve as a driving

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**Figure 4.** SIRT1 ablation in adipocytes augments the macrophage polarization toward the M1 phenotype by reducing IL-4 production.

A, B The mRNA expression levels of Il4 and Il13 determined by real-time PCR (A) and concentrations of IL-4 in the conditioned medium (CM) of SVF-derived adipocytes (B). Data are expressed as means ± SEM (n = 8) from three independent repeats with two-sided Student’s t-test. *P < 0.05, **P < 0.01.

C IL-4 levels in SVF and adipocyte fraction of epididymal (epi) and subcutaneous (sub) adipose tissues. Data are expressed as means ± SEM (n = 8) from three independent repeats with two-way ANOVA. *P < 0.05.

D, E Bone marrow-derived macrophages were cultured in blank (basal) or CM from adipocytes with (WT) or without SIRT1 (AKO) and supplemented with recombinant IL-4 (10 pg/ml). M1 and M2 markers were quantified by real-time PCR. Data are expressed as means ± SEM (n = 6) from three independent repeats with one-way ANOVA and Tukey test. *P < 0.05, **P < 0.01.
force for infiltration and activation of adipose-resident macrophages. Indeed, an inverse association between SIRT1 expression in adipose tissue and macrophage infiltration has been reported in rats and human [39].

Apart from increases in total number of macrophages, adipose-resident macrophages also exhibit a biased polarization from the anti-inflammatory M2 phenotype to the pro-inflammatory M1 phenotype [5–7]. However, the molecular switches that control polarization of adipose-resident macrophages remain poorly defined. Wu et al [40] demonstrated that eosinophils in white adipose tissues of mice induce polarization of M2 macrophages through an IL-4/IL-13-dependent pathway. Furthermore, activation of natural killer T cells enhances polarization of alternatively activated M2 macrophage which is mediated via the IL-4/STAT6 signaling axis in obese adipose tissue [41]. In addition to the above-mentioned adipose tissue-resident immune cells, adipocytes per se might also be a possible source of Th2 cytokines that are responsible for M2 macrophage polarization in adipose tissues [8]. In this connection, our current study demonstrated that ablation of SIRT1 in adipocytes, but not in macrophages, led to shifted ratio of M1–M2 in adipose tissues. Although the precise mechanism by which adipocyte SIRT1 controls polarization of adipose-resident macrophages remains obscure, we provide both in vitro and in vivo evidence that IL-4 is a downstream target of SIRT1 in adipocytes. Our in vitro study using 3T3-L1 adipocytes and SVF-derived adipocytes showed that activation of SIRT1 increased mRNA and protein levels of IL-4 in a SIRT1-dependent manner. In vivo, IL-4 protein was only found to be reduced in mature adipocyte fractions of AKO epididymal adipose, but not the SVFs. However, it is important to note that compared to adipocytes, IL-4 protein is expressed at a much higher level in eosinophils and other immune cells. The contribution of adipocytes to adipose tissue-resident IL-4 remains unclear at this stage, although adipocytes are the predominant type (~80–90%) of cells in adipose tissues. The possibility also exists that AKO mice express higher levels of chemotactic factors which indirectly recruit the appropriate immune cells for secretion of IL-4 into the adipose tissue.

Figure 5. SIRT1 induces Il4 transcription through NFATc1.

A, B Primary adipocytes were treated with resveratrol (RSV, 50 μM) or DMSO for 48 h. mRNA level of Il4 was determined by real-time PCR (A) and IL-4 protein concentration in CM (B). Data are expressed as means ± SEM (n = 6) from three independent repeats with two-way ANOVA. *P < 0.05, **P < 0.01.

C Chip analysis in primary adipocytes using an antibody against NFATc1. Mouse non-immune IgG was used as the negative control. The relative binding of NFATc1 on the p1 and p2 cis elements of the mouse Il4 was quantified by real-time PCR. Data are expressed as means ± SEM (n = 6) from three independent repeats with two-way ANOVA. *P < 0.05.

D Epididymal fat lysates of WT mice were immunoprecipitated with anti-SIRT1 antibody (IP), followed by Western blot analysis with anti-SIRT1 or anti-NFATc1 antibody.

E Upper panel: schematic diagram of domain organization of SIRT1. Lower panel: cell lysates from HEK293T co-transfected with plasmids encoding NFATc1 and various forms of FLAG-SIRT1 were immunoprecipitated using the anti-FLAG M2 agarose beads, and blotted with anti-FLAG or anti-NFATc1 antibody. Δ500–747aa and Δ244–747aa: truncated forms of SIRT1.

F Cell lysates of primary adipocytes from AKO and WT mice were immunoprecipitated with anti-NFATc1 antibody (IP), followed by Western blotting for acetylated lysine (Ac-Lys) and NFATc1.

Source data are available online for this figure.
Although the precise mechanism whereby SIRT1 induces IL-4 expression in adipocytes has yet to be determined, our result demonstrated that Nfatc1, a key transcriptional activator of the Il4 gene by binding to several highly conserved cis elements (namely P elements that share a purine-rich core motif) in the proximal region of Il4 promoter [26,27], is the direct target of SIRT1. In adipocytes, SIRT1 deacetylates Nfatc1, thereby enhancing the binding of NFATc1 to the Il4 promoter for further transactivation.

In addition to the classical Th2 cytokine IL-4/IL-13, adiponectin has also been reported to polarize macrophages toward the anti-inflammatory M2 phenotype in peritoneal macrophages and Kupffer cells via an IL-4/STAT6-dependent mechanism [42–44]. In our study, supplementation of adiponectin failed to rectify the defective anti-inflammatory M2-polarizing ability in SIRT1-deficient adipocytes (data not shown), which can be possibly explained by the lack of IL-4 in these adipocytes. Furthermore, whether or not the biased polarization of adipose tissue macrophages in AKO mice is partially attributed by the impaired proliferation of M2 macrophages elicited by adiponectin warrants future investigation [45].

Several other types of immune cells are also populated in adipose tissue [46]. In particular, CD8+ T-lymphocyte infiltration were reported to precede the accumulation of macrophages [22]. In our study, although we also observed an increase in CD8+ T-cell population in adipose tissue after 4 weeks of HFD feeding, no significant differences were observed between WT and AKO mice. Thus, it is unlikely that SIRT1 deletion in adipocytes affects macrophage infiltration and function via CD8+ T cells. However, at present we cannot exclude the possible contribution of other types of immune cells to the altered macrophage infiltration/polarization in AKO mice.

In summary, our present study uncovered a novel role of SIRT1 within adipocytes in protection against the onset and progression of obesity-related insulin resistance and metabolic dysregulation by blocking infiltration of macrophages and by promoting the polarization of anti-inflammatory M2 macrophages, thereby reducing inflammation in adipose tissues and bloodstream. These findings further highlight the importance of cross talk between adipocytes and macrophages in maintaining adipose tissue homeostasis and whole-body metabolic health. Given the fact that adipose SIRT1 undergoes proteolytic degradation upon prolonged HFD feeding and aging, it is likely that decreased SIRT1 activity in adipocytes is an important contributor to low-grade systemic inflammation and metabolic disorders. Therapeutic interventions that either prevent SIRT1 degradation or promote its activity in adipocytes might represent a promising strategy to combat obesity or aging-related chronic inflammation and to maintain metabolic health.

Materials and Methods

Animal experiments

Mice with floxed SIRT1 allele exon 4 (SIRT1fl/fl) [47] and mice expressing Cre recombinase driven by ap2 (ap2-Cre) or LysM-Cre or LysM-Cre promoter (LysM-Cre) were obtained from Jackson Laboratory (Bar Harbor, ME, USA). SIRT1fl/fl was mated with ap2-Cre or LysM-Cre after being backcrossed for 10 generations into the C57BL/6j background. SIRT1fl/fl mice were mated with SIRT1fl/fl ap2-Cre(+/−) or SIRT1fl/fl LysM-Cre(+/−) to generate AKO and MKO mice. Cre-negative littermates were used as WT controls. Four-week-old male mice with the same genotypes were randomized to fed ad libitum with either a standard laboratory chow diet (LabDiet 5053, LabDiet, Purina Mills, Richmond, IN, USA) or a HFD (D12451, Research Diet, New Brunswick, NJ, USA) containing 48.95% fat, 20% protein, and 30.15% carbohydrate. Animals were kept under 12-h light–dark cycles at 22–24°C. For measurement of fed insulin, the serum samples of mice were collected from tail tip at 10:00 AM and subjected to ELISA. For glucose tolerance test, the mice were fasted for 14 h (from 20:00 to 10:00 AM). After that, the mice were intraperitoneally (IP) injected with D-glucose at 1 g/kg body weight. The glucose level was monitored at the indicated time points. For insulin-induced phosphorylation of Akt and GSK3β, mice were fasted for 6 h followed by IP injection of insulin (0.75 IU insulin/kg body weight). The mice were sacrificed 15 min later for tissue collection. All animal experiments were conducted in accordance with the Guidelines of HKU Animal Care and Use Committee (CULATR 2844-12).

Hyperinsulinemic/euglycemic clamp

Hyperinsulinemic/euglycemic clamp was performed as described [48]. Mice were catheterized for 4 days before the experiment. Mice fasted for 6 h were given a 5-μCi bolus of [3-H] glucose at t = −90 min, followed by infusion with [3-H] glucose 0.05 μCi/min for 90 min. Basal glucose production was determined from blood samples at t = −10 and 0 min, respectively. The clamp began at t = 0 with a continuous infusion of human insulin (10 μU/kg/min, Humulin R; Eli Lilly) after a bolus. The [3-H] glucose infusion was increased to 0.1 μCi/min for the remaining part of the experiment. Euglycemia (115–125 mg/dl) was maintained by measuring blood glucose every 10 min from t = 0 min. A 10-μCi bolus of 2[14C] deoxyglucose (2[14C]DG) was given at t = 75 min. Blood samples were taken every 10 min from t = 90–120 min to determine [3-H] glucose and 2[14C]DG levels in plasma. At t = 120 min, mice were sacrificed and the soleus, gastrocnemius, superficial vastus lateralis, epididymal fat, liver were isolated, and the radioactivity was determined by scintillation.

Indirect calorimetry

Whole-body oxygen consumption was measured using an indirect calorimetry system with automatic temperature and light controls (Columbus Instruments). Mice had access ad libitum to chow and water in the respiration chambers, acclimated for 24 h before data were recorded for a 48-h period with temperature at 24°C and light on between 07:00 and 19:00.

Western blot analysis

Proteins were extracted from tissues by homogenizing in a RIPA buffer (0.5% NP-40, 0.1% sodium deoxycholate, 150 mM NaCl, 50 mM Tris–HCl, pH 7.4) containing protease inhibitors. The homogenate was centrifuged at 4°C for 15 min at 12,000 g, and the liquid phase was collected. Fifty microgram of tissue lysates was loaded in each lane onto denaturing polyacrylamide gels, transferred onto nylon membranes, probed with rabbit polyclonal antibodies.
recognizing SIRT1 (EMD Millipore, 07-131), acetylated (EMD Millipore, 06-758) and total p53 (Cell Signaling Technology, #9282), phosphor (S473) [Antibody and Immunohistochemistry Service center (AIS) at HKU] and total Akt (R&D systems, MAB2055), phosphor (S9)-GSK3β (Cell Signaling Technology, #9336), or mouse monoclonal anti-total GSK3β (Cell Signaling Technology, #9832) followed by incubation with HRP-conjugated anti-rabbit IgG and visualization of the proteins using enhanced chemiluminescence.

Histological, immunohistochemical, and biochemical analyses

Paraffin-embedded adipose sections were immunostained with rat monoclonal anti-F4/80 antibody (Bio-Rad, MCA497R, Clone CI:A3-1) and developed by SIGMAFAST™ DAB (Sigma). Immunoassay kits for insulin (AIS, HKU), A-FABP (Biovendor), leptin (Biovendor), TNF-α (R&D), IL-1β (R&D) were used for measurement of serum levels of these hormones or inflammatory factors, respectively.

Quantitative PCR (qPCR)

Total RNA in adipose tissues was extracted using the RNeasy Lipid Tissue Mini Kit (Qiagen) and reverse-transcribed into first-strand cDNA using the ImProm-II reverse transcription system (Promega, Madison, WI). Quantitative real-time PCR was performed as described [49]. The primer sequences are listed in Appendix Table S1.

Isolation of mature adipocytes and SVF in adipose tissue

The primary adipocytes were isolated as described [50] with minor modifications. Briefly, fat pad was digested in 0.1% (w/v) type I collagenase (Invitrogen) for 1 h at 37°C with gentle shaking. The digestion mixture was passed through a 100-μm cell strainer (BD Biosciences) and centrifuged at 800 g for 10 min. The upper floating layer containing the mature adipocytes and the pelleted SVF was collected and washed twice before further analysis.

Flow cytometry

1 × 10⁵ freshly isolated stromal vascular cells were stained on ice for 30 min in dark with rat monoclonal anti-CD206 Alexa Fluor 647 (Biolegend, C0682C, clone C0682C), rat monoclonal anti-F4/80 PE (Biolegend, 123119, clone BM8), Armenian Hamster anti-CD11c FITC (Biolegend, 117305, clone N418) for macrophages, rat anti-Cd11b PE (BD Pharmingen, 557397, clone M1/70), rat anti-Siglec-F-AF647 (BD Pharmingen, 562680, clone E-50-2440) for eosinophils, and rat anti-Ly6G FITC (BD Pharmingen, 551460, clone 1A8) and rat anti-Cd11b PE for neutrophils. Their isotype control antibodies were used as controls. After washing, the cells were fixed in 300 μl of 1% formalin and analyzed on BD LSRFortessa Cell Analyzer (BD). For analysis of T-lymphocyte subtypes, the cells were stained with CD3/CD4/CD8 cocktail or isotype cocktail (BD Pharmingen) per FACS tube. The data were analyzed using FlowJo software.

Co-culture of adipocytes and macrophages

3T3-L1 preadipocytes were cultured in Dulbecco’s modified Eagle’s medium (DMEM), high glucose (Invitrogen) supplemented with 10% fetal bovine serum (FBS), followed by induction for differentiation as described [51]. For the macrophage migration assay, thiglycolate-elicited peritoneal macrophages were isolated and seeded onto the upper chamber of 5-μm Boyden wells (Nunc, Denmark), with differentiated adipocytes at the lower chamber. The cells were co-cultured for 48 h. The unmigrated cells were removed by gently scraping the top side of the well with a wet cotton swab. The macrophages transmigrated to the lower side were visualized by HE staining. Alternatively, SVEs from subcutaneous adipose tissue of WT and AKO adipose tissue were isolated and differentiated to mature adipocytes. At the end of day 8, mature adipocytes were incubated in fresh medium (DMEM + 10%FBS). Forty-eight hours later, the condition medium were collected, filtered through 0.45-μm filter, and subjected to co-culture study or stored at −80°C for future use. For macrophage polarization, bone marrow-derived macrophages were differentiated in RPMI1640 plus 20% L929 conditioned medium for 7 days. On day 8, differentiated macrophages were incubated in the conditioned medium derived from the adipocytes for 48 h and the macrophages were harvested for gene expression analysis.

Insulin-stimulated glucose uptake and Akt phosphorylation

Stromal vascular fraction from epididymal adipose tissue of WT and AKO mice were isolated and differentiated to mature adipocytes. Insulin-stimulated glucose uptake was performed as described [52]. Briefly, after overnight fasting, cells were changed to glucose-free KRH buffer (121 mM NaCl, 4.9 mM KCl, 1.2 mM MgSO4, 0.33 mM CaCl2, 12 mM HEPES, pH 7.4) and stimulated with 100 nM insulin or vehicle control for 13 min. Glucose uptake was initiated by addition of 2-deoxyglucose (Sigma) to a final concentration of 100 μM and 0.5 μCi 1-deoxy-D-2-[1,2-3H(N)]glucose (PerkinElmer) per well in glucose-free KRH buffer. After a 15-min incubation at 37°C, cells were washed twice with ice-cold KRH buffer plus 25 mM glucose and lysed in 10% SDS and counted for radioactivity in Ultima Gold scintillation cocktail (PerkinElmer) using an LS6500 Multi-Purpose Scintillation Counter (Beckman Coulter). Non-carrier-mediated uptake was determined in the presence of 5 μM cytochalasin B (Sigma-Aldrich) and deducted from the totals, which were normalized by protein concentrations. Alternatively, overnight-starved cells were stimulated with 10 nM insulin or vehicle control and harvested in RIPA lysis buffer 15 min later. Phospho-Akt (S473) and total Akt were examined by Western blotting.

Chromatin immunoprecipitation (ChiP)

ChiP was carried out as described [53] with minor modifications. Briefly, in vitro differentiated adipocytes were cross-linked with 1% formaldehyde and the adipocyte lysates were sonicated on ice to shear chromatin DNA to around 500–2,000 base pairs. Fifty microgram of lysates was incubated with 1 μg of mouse monoclonal antibody against NFATc1 (clone 7A6, Thermo Fisher) or mouse pre-immune IgG at 4°C overnight, followed by immunoprecipitation with Protein G beads pre-saturated with BSA and salmon sperm DNA. After several rounds of washing, the precipitated DNA–protein complex was eluted by 100 mM NaHCO3, 1% SDS. Fifty microgram of lysates was used as input. The DNA was extracted and resolved in de-ionized H2O. The relative amount of
target DNA fragment was quantified by real-time PCR. The primer sequences used are listed in Appendix Table S1. Each sample was normalized to their respective input.

**Immunoprecipitation**

Five hundred microgram of adipocyte lysates were incubated with 1 μg of anti-NFATc1 (Thermo Fisher, MA-03-024, clone 7A6) or anti-SIRT1 (EMD Millipore, 07-131), or equal amount of mouse or rabbit non-immune IgG at 4°C overnight with rotation. Twenty microliter of protein G beads (1:1 slurry, GE Healthcare) were added to the mixture and incubated for another 1 h at room temperature. Alternatively, 3xFLAG-tagged human full-length SIRT1 was constructed as described [54] and the truncated forms of SIRT1 (1–243aa, 1–237aa, 1–199aa) were generated using primers in Appendix Table S1. HEK293T cells were co-transfected with plasmids encoding human-NFATc1 (Addgene) and different forms of FLAG-human SIRT1 or empty vector; 48 h post-transfection, cells were harvested and lysed in RIPA lysis buffer and 500 μg of cell lysates was incubated with 20 μl of anti-FLAG M2 beads (Sigma-Aldrich) at 4°C overnight with rotation. The protein G beads or M2 beads were washed five times with lysis buffer and eluted with 100 mM glycine, pH 2.5. The eluate was neutralized with 1/20 volume of 100 mM Tris–Cl, pH 9.0, and subjected to Western blotting using following antibodies: rabbit polyclonal anti-acetylated-lysine (Cell Signaling Technology, #9441), rabbit polyclonal anti-SIRT1 (EMD Merck, 07-131), mouse monoclonal anti-NFATc1, or rabbit monoclonal anti-FLAG (Cell Signaling Technology, #14793).

**Statistical analysis**

Experiments were performed routinely using six to eight mice per group with values presented as means ± SEM. All analyses were performed with Statistical Package for Social Sciences version 14.0 (SPSS, Chicago, IL, USA). Sample sizes of animal studies were predetermined sample size. Comparisons with P < 0.05 were considered statistically significant.

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**Author contributions**

XH designed the study, carried out the research, analyzed, interpreted the results, and wrote the manuscript. MZ, PG, KL, and YG conducted the experiments; DW reviewed the manuscript; and YW and AX designed the study and wrote and edited the manuscript.

**Conflict of interest**

The authors declare that they have no conflict of interest.

**References**


