SRA gene knockout protects against diet-induced obesity and improves glucose tolerance

The running title: SRA deficiency protects against diet-induced obesity

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Key words: adipose tissue biology, diet-induced obesity, glucose homeostasis

Capsule

Background: The non-coding RNA, steroid receptor RNA activator (SRA), promotes adipocyte differentiation in vitro.

Results: SRA gene knockout protects against obesity and improves glucose tolerance in mice fed a high fat diet.

Conclusion: SRA is an important regulator of adiposity in diet-induced obesity (DIO).

Significance: This is the first report that the Sra1 gene plays an important role in adipose tissue biology in vivo.

Abstract

We have recently shown that the non-coding RNA, steroid receptor RNA activator (SRA), functions as a transcriptional coactivator of PPARγ and promotes adipocyte differentiation in vitro. To assess SRA function in vivo, we have generated a whole mouse Sra1 gene knockout (SRA-/-). Here, we show that the Sra1 gene is an important regulator of adipose tissue mass and function. SRA is expressed at a higher level in adipose tissue than other organs in wild type mice. SRA-/- mice are resistant to high fat diet-induced obesity, with decreased fat mass and increased lean content. This lean phenotype of SRA-/- mice is associated with decreased expression of a subset of adipocyte marker genes and reduced plasma TNFα levels. The SRA-/- mice are more insulin sensitive, as evidenced by reduced fasting insulin, and lower blood glucoses in response to IP glucose and insulin. In addition, the livers of SRA-/- mice have fewer lipid droplets after high fat diet feeding, and the expression of lipogenesis-associated genes is decreased. To our knowledge, these data are the first to indicate a functional role for SRA in adipose tissue biology and glucose homeostasis in vivo.

Introduction

Obesity results from an imbalance between energy intake and expenditure, in
which the excess energy is stored as triglyceride in white adipose tissue (WAT) with both increased fat cell size (hypertrophy) and number (hyperplasia) (1). Obesity induces a state of chronic, low grade inflammation in fat that is accompanied by the local secretion of cytokines and chemokines attenuating insulin action (2,3). Recent genetic, cellular and biochemical studies have identified a molecular network controlling the differentiation of WAT that involves a transcriptional cascade and epigenetic program beginning with CCAAT/enhancer-binding protein beta (C/EBPβ) and delta (C/EBPδ) and the glucocorticoid receptor (GR), which induce C/EBPα and peroxisome proliferator activated receptor gamma (PPARγ), the major transcriptional regulators of adipose differentiation (4-9). In addition, adipogenesis is regulated positively by a number of growth factors and hormones including insulin, insulin-like growth factor 1 and the bone morphogenetic proteins, and negatively by Wnt signaling (10,11).

Long non-coding RNAs (lncRNAs) are non-coding transcripts that have recently emerged as important regulators in diverse biological processes (12,13) including stem cell pluripotency, embryogenesis and cellular differentiation. However, the function of lncRNAs in adipogenesis remains to be elucidated. The steroid receptor RNA activator SRA was initially characterized as an lncRNA that functions as an RNA coactivator to enhance steroid receptor-dependent gene expression (14). Our and other subsequent studies demonstrated that SRA also functions as an RNA coactivator for non-steroid nuclear receptors (15,16) and the myogenic differentiation factor MyoD (17). The Sra1 gene also produces an alternative transcript that encodes a protein denoted SRAP (18,19) although the function of SRAP is largely unknown. While SRA has been implicated to play a role in myogenesis (17,20), steroidogenesis (21), breast tumorigenesis (22-24) and cardiomyopathy (25), the lack of a loss-of-function mouse model has limited our understanding of the in vivo biology of SRA.

We have recently shown that SRA promotes adipocyte differentiation and improves insulin-stimulated glucose uptake in adipocytes in vitro through multiple mechanisms, such as coactivating the transcriptional activity of PPARγ, promoting S-phase entry during mitotic clonal expansion, increasing phosphorylation of Akt/protein kinase B and forkhead box protein O1 (FOXO1) in response to insulin, and inhibiting expression of adipocyte-related inflammatory genes (26). To assess SRA function in vivo, we have generated a whole mouse Sra1 gene knockout (SRA-/-). In this study, we show that SRA is expressed at higher levels in adipose tissue than other organs. SRA-/- mice are resistant to diet-induced obesity with reduced fat content, decreased expression of subsets of adipocyte genes and inflammation genes, and improved insulin sensitivity. These data indicate an important role for SRA in adipose tissue biology in vivo.

EXPERIMENTAL PROCEDURES

Generation of Sra1 gene knockout (SRA-/-) mice. Three mutant ES cell clones generated by gene targeting for mouse Sra1 were obtained from the European Conditional Mouse Mutagenesis Program (EUCOMM) (http://www.knockoutmouse.org/about/eucomm). These targeted ES cell clones were generated in the C57BL/6N cell line JM8.N4 and are heterozygous for wild type Sra1 and for Sra1tm1a(EUCOMM)Hmgu. For purposes of brevity, mice homozygous for the tm1a allele are referred to as SRA-/-, and heterozygotes for wild type Sra1 are referred to as SRA+/-.

The targeting cassette was inserted at position 36828917 of Chromosome 18 upstream of Sra1 exon 3 (the targeting vector is shown in Figure 2A). The cassette is composed of an FRT site followed by a lacZ sequence and a loxP site. The first loxP site is followed by a neomycin vector, a second FRT site and a second loxP site. A third loxP site is inserted downstream.
of the targeted exon 3. The critical exon 3 is thus flanked by loxP sites. Although a "conditional ready" (floxed) allele can be created by flp recombinase expression, in the present study the FRT-flanked cassette is left in place, resulting in a functional Sra1 global knockout. Before injection, the ES cells were confirmed to contain the Sra1 mutant gene by long range PCR (LR-PCR). In addition, chromosome counting was performed to make sure that the receiving ES cells have at least 50% normal chromosome count. Finally, the ES cell clone HEPD0528_3_F03 with the best LR-PCR signal and more than 80% normal chromosome count was micro-injected into blastocysts of albino C57BL/6 mice (Jackson Laboratory Stock number 000058) to generate ES cell-mouse chimeras. Twenty-four male chimeras were bred with albino C57BL/6 female mice. Germ line transmission was evaluated by coat color and PCR. Genotyping was determined by LoxP3 site-spanning PCR (Forward, 5’ TCCAAGTCTTTCCAGGAAAATG; Reverse, 5’ ACAGAGCTTGTTTGTCTCTTC), LacZ PCR (Forward, 5’ TTCACGTGCGCCGTCTTTTACAACTGC GTGA; Reverse, 5’ ATGTGACGCGATAACACAACCGTCGGA TTCT) and long range PCR (primers used were as suggested by EUCOMM at http://www.eummcr.org/products/es-cells.php). Thus, we generated functional Sra1 gene global knockout mice (ablation of both SRA RNA and SRAP expression), denoted as heterozygous SRA+/− and homozygous SRA−/− (SRAKO) hereafter. The global SRAKO was further confirmed by RT-qPCR for SRA RNA and immunoblot for SRAP (antibody against SRAP was from Bethyl Laboratory, Inc., Cat# A300-742A). F0 germ-line transmitted males were backcrossed with C57BL/6J mice, the resulting F1 SRA+/− mice were intercrossed to derive SRA+/+ (wild type, WT), SRA+/− and SRA−/− mice used for phenotype analysis and metabolic studies. Only male mice were studied for the phenotype of SRAKO. Genotyping was carried out by PCR analysis of genomic DNA extracted from the tails of progenies. Animals were housed on a 12-h-light and 12-h-dark cycle in the Unit for Laboratory Animal Medicine at the University of Michigan, with free access to water and a standard mouse diet – normal chow (9% fat; Lab diet) or a high fat diet (HFD) (60% fat; Cat# D12492, Research Diets, Inc. New Brunswick, NJ). All procedures were approved by the Institutional Animal Care and Use Committee of the University of Michigan.

**Glucose and insulin tolerance tests.** For glucose tolerance tests (GTT), mice were subjected to fasting overnight (16 h) and D-glucose (2 g/kg of body weight) was injected intraperitoneally. Blood glucose was monitored at 0, 15, 30, 60, 90, 120 and 180 min after glucose injection. For insulin tolerance tests (ITT), mice were subjected to fasting for 6 h and human insulin (1 IU/kg of body weight) was injected intraperitoneally. Blood glucose was monitored at 0, 15, 30, 60 and 120 min after insulin injection.

**Food intake.** Food intake of normal chow was determined by measuring the remaining food weight weekly for singly housed mice for 6 weeks, and calculated from a 42 d average. At the end point of HFD feeding, the remaining weight of food provided was determined daily in metabolic cages for singly housed mice. Daily food consumption was calculated from a 3 d average.

**Energy expenditure and respiratory quotient.** SRA−/− or WT littermate control mice post-HFD were placed in metabolic cages. The University of Michigan Metabolic Phenotyping Core measured oxygen consumption (VO₂), carbon dioxide production (VCO₂) and spontaneous motor activity during 3 consecutive days using the Comprehensive Laboratory Animal Monitoring System (Columbus Instruments), an integrated open-circuit calorimeter equipped with an optical beam activity monitoring system. Values presented are...
normalized to lean body mass (LBM) plus 20% fat mass (LBM+0.2FM) to account for the fact that the body composition differs between SRA-/- and WT mice (27). The respiratory quotient was calculated by dividing the carbon dioxide production by the oxygen consumption. We used the mean values for the light and dark cycles to analyze statistical significance.

Body composition. Body fat, lean body mass and fluid content in SRA-/- mice and WT littermate controls after HFD experiments were measured using nuclear magnetic resonance analysis in The University of Michigan Metabolic Phenotyping Core.

Histology. Tissues were fixed in 10% buffered formalin, and histology was assessed by hematoxylin and eosin (H&E) staining at the University of Michigan Cancer Center Research Histology Laboratory.

Gene expression analysis. Isolated mouse tissues were rinsed in phosphate buffered saline (PBS), frozen in liquid nitrogen and stored at -80°C until extraction. Total RNAs from WAT, brown adipose tissue (BAT), liver and skeletal muscle were isolated using Trizol reagent followed by treatment with DNaseI (Qiagen). RT-qPCR analysis of gene expression was as described previously (21,26). Primers are listed in Table 1. To determine whether SRA expression is induced by high fat diet feeding, samples of inguinal WAT from male C57BL/6J mice were provided by Dr. Cliff Rosen (Maine Medical Center Research Institute, Scarborough ME). The mice had been fed the standard chow diet or HFD ad libitum from 3 to 15 weeks of age. The mice became obese on the HFD with 40% body fat compared to 18% body fat with the normal chow diet, at which point inguinal WAT was isolated for RNA purification as described (28) and subjected to RT-qPCR.

Stromal vascular fraction (SVF) and adipocyte isolation. We digested excised epididimal white adipose tissue (eWAT) from 8 week old male C57BL/6J mice in PBS containing 1% bovine serum albumin (BSA) and 1 mg ml⁻¹ type II collagenase for 30 min at 37 °C with gentle agitation. The cell suspension was filtered through a 100-µm filter and then centrifuged at 700g for 5 min to separate floating adipocytes from the SVF pellet. Floating adipocytes were then washed twice with PBS containing 1% BSA. The pellets representing the SVF were collected after each wash.

Blood chemistry analysis. Blood samples collected from the tail vein were used to measure blood glucose by OneTouch Ultra Glucometer. Plasma from mice fasted for 6 h was isolated from whole blood collected into heparinized tubes. Plasma insulin, leptin, TNFα and IL6 concentrations were measured using luminex technology in a multianalyte panel plate purchased from Millipore in the Chemistry Laboratory of University of Michigan Diabetes Research Center. Plasma adiponectin levels were determined using a mouse adiponectin radioimmunoassay kit from Millipore (Cat # MADP-60HK). Serum triglyceride (TG) and serum and liver non-esterified fatty acid (NEFA) levels were measured using TG (Pointe Scientific Inc. Cat#T7532-120) and NEFA assay kits (Wako Pure Chemical Industries, Ltd). Liver TG was extracted by chloroform: methanol 2:1, evaporated to dryness and hydrolyzed by KOH. After addition of MgCl₂, the glycerol liberated from TG was assayed by glycerol reagent (Sigma Cat# F6428) for TG determination.

In vivo analysis of insulin-stimulated phosphorylation of Akt in HFD-fed mice. Mice after two week HFD feeding were fasted 16 hrs, then injected with PBS or human insulin (3 units/kg body weight) via the tail vein. Five minutes later, eWAT, liver and gastrocnemius muscles were dissected, frozen in liquid nitrogen, and stored at -80°C. Tissues were homogenized in ice cold T-PER Tissue Protein extraction Reagent (Pierce #78510) containing phosphatase inhibitor cocktail (Thermo Scientific,
Cat#1862495) and protease inhibitor cocktail (Roche, Cat#11836170001). The extracts were subjected to gel electrophoresis and immunoblotted with antibodies directed against Phospho-Akt (Ser473) (Cat# 9271) or Akt (Cat# 9272) obtained from Cell Signaling Technology (Danvers, MA).

**Statistical analyses.**

All values are presented as the mean ± S.D. and were analyzed by two-tailed Student's t test. Statistical significance is defined as P <0.05, and the term “trend” is used to describe comparisons for which 0.05 ≤ P ≤ 0.10.

**RESULTS**

**SRA is highly expressed in adipose tissues.** We have shown recently that SRA expression is induced during 3T3-L1 cell adipocyte differentiation in vitro (26). To study SRA function in vivo, we first examined the expression levels of SRA in several murine tissues, and showed that SRA is expressed at the highest levels in WAT, followed by BAT (Figure 1A). Expression of SRA was significantly higher in the adipocyte fraction (AD) than in the stromal-vascular fraction (SV) of WAT from lean mice (Figure 1B). In addition, SRA expression was significantly induced in WAT from HFD-induced obese mice compared to control mice fed normal chow (Figure 1C). Consistent with our previous findings that SRA promotes adipogenesis in cell culture (26), these observations suggested that SRA may also play an important role in adipose tissue biology in vivo.

**Generation of Sra1 gene knockout (SRAKO) mice.** To directly investigate the role of SRA in adipose tissue in vivo, we have generated SRAKO mice and have characterized them with respect to energy and glucose homeostasis. The targeting vector includes a lacZ/neo cassette with transcription termination signals flanked by FRT sites (Figure 2A). The cassette was not excised, which is predicted to result in loss of SRA expression and hence a global functional gene knockout (29).

Five males and four females of F0 germ line transmitted mice were obtained. After backcrossing with C57BL/6J mice, the resulting F1 heterozygous (SRA+/-) mice were intercrossed to derive wild type control (SRA+/+), heterozygous (SRA+/-) and homozygous (SRA-/-) mice, which were confirmed by PCR genotyping (Figure 2B). The SRA+/- and SRA-/- mice appeared normal. The genotypes of 233 F1 pups obtained at weaning stage included 50 SRA+/+, 120 SRA+/- and 63 SRA-/- mice, which nearly matched the expected 1:2:1 Mendelian ratio.

SRA RNA expression was assessed in WAT, BAT, liver and skeletal muscle by RT-qPCR (Figure. 2C, upper panel). SRA RNA expression was reduced ~50% in SRA+/- mice and was absent in SRA-/- mice. Similar results were obtained for SRA protein (SRAP), assessed by Western blot (Figure 2C, lower panel). These results confirm successful knockout of SRA and SRAP expression.

**SRA-/- mice are protected against diet-induced obesity (DIO) and have decreased expression of adipocyte genes.** The SRA-/- mice were without significant body weight changes at the age of weaning (3 wks) and during postweaning 3-6 wks when fed a normal chow diet (Figure 3A). At age 6 wks the SRA-/- and WT littermate control (SRA+/+) mice were placed on a high fat diet (HFD). After 14 wks on this diet, SRA-/- mice weighed 6 grams less than the WT controls, indicating the SRA-/- mice are resistant to diet-induced obesity (Figure 3A). Characterization of body composition revealed that the differences in total body weight were due to reduced % fat mass, with increased % lean mass in SRA-/- mice (Figure 3B). As expected from these results, the SRA-/- mice had reduced epididymal white fat (eWAT) and subcutaneous white fat (sWAT) mass (Figure 3C). Interestingly, SRA-/- mice also had reduced liver mass (Figure 3D). The reduced fat mass in SRA-/-
mice was associated with small adipocytes in eWAT compared to WT mice (Figure 3E).

We have recently shown that SRA promotes adipocyte differentiation in vitro at least in part through coactivating the transcriptional activity of PPARγ, the master regulator of adipoogenesis (26). Consistent with this, in addition to having a decreased amount of WAT, the SRA-/- mice also had reduced expression or a trend toward reduced expression of the adipocyte genes fatty acid binding protein 4 (Fabp4), and adiponectin (Adipoq) in the sWAT (Figure 3G) and eWAT (Figure 3F). In contrast, SRA-/- mice had a trend toward increased eWAT expression of phosphoenolpyruvate carboxykinase 1 (Pck1), an enzyme that regulates the balance between free fatty acids and triglycerides in adipose tissue (Figure 3F).

In addition, SRA-/- mice had reduced eWAT expression of the inflammation genes tumor necrosis factor alpha (Tnf) and chemokine (C-C motif) ligand 2 (Ccl2) (also known as MCP-1), and a trend to reduced interleukin 6 (Il6) (Figure 3F). This is important because diet-induced obesity is known to be a state of increased inflammation (30). Steroid nuclear receptor coactivators (SRCs), which include Ncoa1 (SRC1), Ncoa2 (SRC2) and Ncoa3 (SRC3), are implicated in the control metabolism. SRC1-/- mice are prone to, but SRC2-/- and SRC-3-/- are protected against, diet-induced obesity (31,32). As shown in Figure 3F, the expression of all three SRC genes is unchanged in the SRA-/- mice, suggesting that the expression of these genes is neither dependent on SRA nor altered in an attempt to compensate for the loss of SRA.

In addition to the classical and constitutive BAT located in the interscapular region of mice, recent studies have revealed a recruitable BAT that resides within WAT, that has alternatively been called beige, brite or inducible BAT (33-36). Beige adipocytes are most abundant in the inguinal WAT, a major subcutaneous fat depot in rodents (37). Brown and beige adipocytes express high levels of Ucp1 and other thermogenic genes (34,36), and therefore have the potential to counteract obesity and type 2 diabetes. Since increased activities of brown and beige adipocytes have been linked to obesity resistance in many mouse models (38), we assessed the expression of beige adipocyte genes in the sWAT of SRA-/- mice under HFD-feeding. Overall, we did not find strong evidence of sWAT browning. The sWAT of SRA-/- mice showed decreased expression of the brown adipocyte marker PR domain-containing protein 16 (Prdm16), and no change in the expression of Ucp1 or CD137, a beige adipocyte cell surface marker (36) (Figure 3G). However, SRA-/- mice under HFD-feeding did have increased sWAT expression of Fndc5 (Irisin), that has recently been shown to stimulate Ucp-1 expression in white adipocytes in culture and increase energy expenditure in mice (39). In addition, there was a trend of increased expression of Bmp8b, which has the capacity to increase energy dissipation in BAT (40), and of beige adipocyte marker Tbx1 (36,41), but not of Tbx15.

Furthermore, SRA-/- mice also had reduced intrascapular BAT mass with reduced brown adipocyte size (Figure 4A, B). The expression levels of several BAT genes within the SRA-/- BAT were similar to those in WT mice (Figure 4C).

**Energy expenditure is unchanged in SRA-/- mice.** Since adiposity is determined by the balance of calorie intake and utilization, we investigated parameters of energy homeostasis in SRA-/- and WT control mice. In considering the contributions of both lean body mass (LBM) and fat mass (FM), we normalized oxygen consumption and carbon dioxide production to LBM plus 20% FM (LBM+0.2FM), as this has been shown to be a more accurate way to account for variations in FM than simple normalization to LBM or total body weight (27). Upon this analysis, oxygen consumption and respiratory quotient (RQ) were not significantly changed in SRA-/- mice (Figure 5A, B). Furthermore, the absolute food intake of SRA-/- mice from a...
42-day average was unaltered on a normal chow diet (data not shown) and during HFD feeding (Figure 5A, B). In addition, the total locomotor activity was also unchanged between two groups (Figure 5A, B) during HFD feeding. These experiments suggest that the lean phenotype of SRA-/- mice does not result from changes of caloric intake and energy expenditure. However, as will be discussed below, it is possible that changes in these parameters were too subtle to detect.

**SRA-/- mice have improved insulin sensitivity and decreased liver fat.**

Consistent with the reduced fat mass with HFD feeding, SRA-/- mice had significantly reduced insulin levels with no change in fasting blood glucose compared to the WT control mice (Figure 6A). In addition, there was a trend toward lower plasma leptin, and no change in plasma adiponectin, triglyceride (TG) or free fatty acid levels. Furthermore, HFD-fed SRA-/- mice had improved glucose tolerance following intraperitoneal glucose challenge relative to HFD-fed WT mice (Figure 6B), as well as more marked hypoglycemia following insulin challenge (Figure 6C). Consistent with this increased insulin sensitivity, HFD-fed SRA-/- mice had increased Akt phosphorylation in WAT, liver and gastrocnemius muscle following insulin challenge, relative to HFD-fed WT mice (Figure 6D).

The proinflammatory cytokine TNFα is produced in adipose tissue and is elevated in obesity and correlates with insulin resistance (42). SRA-/- mice had reduced plasma TNFα levels, but unchanged levels of IL6, another inflammatory cytokine correlated with insulin resistance (43) (Figure 6E).

In humans, as a hallmark of the metabolic syndrome, fatty liver is tightly associated with insulin resistance and type 2 diabetes (44). SRA is well expressed in the liver (Figure 1). Interestingly, SRA-/- mice had a 30% reduction in liver weight compared to WT controls after HFD feeding (Figure 3D). Strikingly, in contrast to the large lipid droplets that were apparent by H&E staining in the livers from WT mice, lipid droplets were greatly reduced in the livers of SRA-/- mice (Figure 7A). Further investigation revealed that liver triglyceride and free fatty acid levels were 61% and 54% lower in SRA-/- mice than WT controls with HFD feeding, respectively (Figure 7B and C). To further investigate this protection from hepatosteatosis, we analyzed the expression of key lipogenic and metabolic genes in the livers of HFD-fed SRA-/- and WT mice. As shown in Figure 7D, the expression of Ppara, Pparg, Fabp4, and hormone-sensitive lipase (Lipe) was significantly reduced in the livers of SRA-/- mice, with a trend toward reduced expression of stearoyl-Coenzyme A desaturase 1 (Scd1) (p=0.051). These data suggest that the decreased fatty liver in SRA-/- mice may contribute to the improved insulin sensitivity. However, the expression of several genes involved in hepatic gluconeogenesis and glycolysis was not significantly changed.

**DISCUSSION**

We have previously shown that overexpression of the lncRNA SRA in ST2 mesenchymal precursor cells promotes their differentiation into adipocytes, and that knockdown of endogenous SRA inhibits 3T3-L1 cell differentiation into adipocytes (26). Here we demonstrate an important role for SRA in adipocyte biology in mice in vivo. To our knowledge, this is the first report for a lncRNA playing a role in the regulation of fat mass in vivo. We found that SRA is expressed at a much higher level in adipose tissue than other mouse organs. Also, SRA is induced in WAT in mice under HFD feeding. Global knockout of SRA protects against diet-induced obesity and improves whole body glucose homeostasis. These results are consistent with *in vitro* data showing that SRA can function as a PPARγ coactivator. We cannot rule out the potential coactivation by SRA of other transcription factors that may play roles in adipogenesis. For example, SRA can promote GR-mediated transactivation (14),
and recently GR has been shown to play an important role regulating early adipogenesis (7). In addition, our microarray data reveal hundreds of SRA-responsive genes in adipocyte cell lines in culture, including genes related to the cell cycle, insulin and TNFα signaling (26). While many of these SRA-mediated changes are likely indirect, a substantial fraction may be direct given that SRA is a transcriptional coregulator. Thus, the contribution of SRA to adipogenesis in vivo may be complex and through multiple pathways.

The mechanism of transcriptional coactivation by SRA is not resolved, but SRA exists in a ribonucleoprotein complex and may serve as a scaffold to recruit coactivator proteins to target genes. In addition, IncRNAs can bind to DNA to form a RNA:DNA:DNA triplex (45) that has been proposed to recruit IncRNA binding proteins to DNA (46).

The Sra1 gene is unusual in that it expresses both a IncRNA (SRA) and a protein (SRAP) by alternative splicing (18,19). Based upon cell culture experiments with the non-coding SRA RNA (26), it is most likely that this IncRNA exerts the major adipogenic effects observed here. However, since the mouse knockout abolishes expression of both SRA and SRAP, we cannot exclude a possible role of SRAP in adipocytes.

SRA-/- mice under HFD feeding demonstrate reduced adiposity with decreased percent body fat and increased percent lean mass (Figure 3). This observation is further evidenced by reduced epididymal and subcutaneous WAT mass, reduced BAT mass, and decreased expression of several WAT marker genes (Figure 3). Recent studies indicate that “brown-like” cells exist in subcutaneous WAT, called beige cells or brite cells (34,36,47,48). The activities of both classical brown and beige fat cells reduce obesity in mice and correlate with leanness in human (38,49). However, SRA-/- mice did not have a generalized increase in beige cell markers within WAT at the end of HFD-feeding. Overall, these data suggest that the reduced adiposity in these mice is not due to increased activity of BAT and beige fat cells.

Our data indicate that SRAKO in mice improves whole body insulin sensitivity when fed a HFD (Figure 6B,C). This effect also is manifest at a molecular level, since the SRA-/- mice show increased phosphorylation of Akt in WAT, liver and muscle in response to acute insulin challenge (Figure 6D). Since these mice have decreased fat mass, the simplest explanation for the improved insulin sensitivity is a direct effect of loss of SRA on adipogenesis and adipocyte function, probably at least in part through decreased PPARγ function. However, these mice have a global loss of SRA, and therefore other tissues may contribute to the improved whole body insulin sensitivity. For example, SRA is expressed at significant levels in liver, and the SRAKO mice have decreased hepatic steatosis. Whether the loss of hepatocyte SRA is a factor in this remains to be determined.

Furthermore, it is well recognized that chronic tissue inflammation is an important cause of obesity-induced insulin resistance (3,50). One example of evidence for this relationship is that the cytokine TNFα is elevated in the adipose tissue of obese rodents and inhibition of this cytokine improves glucose tolerance and insulin sensitivity (42). Importantly, compared to WT control mice, SRA-/- mice have significantly reduced expression of a subset of inflammation genes including Tnf (TNFα) and Ccl2 (MCP-1) in WAT (Figure 3F) and decreased plasma TNFα levels (Figure 6E). These results suggest that reduced inflammatory signaling may contribute significantly to the improved insulin sensitivity in SRA-/- mice. Since SRA is expressed at a relatively high level in mouse spleen (Figure 1), as well as in monocytes derived from mouse spleen and murine macrophage J774A.1 cells (data not shown), SRAKO may have direct anti-inflammatory effects on the immune system.
It is at first glance puzzling that SRA-/- mice are protected from diet-induced obesity, yet there is no detectable change in food intake or energy expenditure. Perhaps they are inefficient at absorbing food from the gut, which could be an effect of SRAKO in the mouse itself or a secondary effect on the gut microbiome (51,52). It also is possible that changes in energy expenditure or food intake went undetected because they are too subtle. For example, it has been calculated that for mice to gain an excess of 10 g over 25 weeks (a rate of change almost identical to that observed in this study), an energy imbalance of only ~3% is required (27). Detecting a 2.5% change in energy intake with 80% power would require a sample size of 350 mice (53), and detecting the expected changes would be even more daunting if the underlying basis is multifactorial.

In summary, the current studies, which are the first report of an in vivo SRA knockout, demonstrate an important role for SRA in the regulation of adipose tissue mass, fatty liver, glucose homeostasis and metabolism-related gene expressions in vivo. Future studies using tissue-specific knockouts will help clarify the underlying mechanisms regarding whole animal insulin sensitivity and will shed further light on the biological functions of SRA as a potential target to control obesity and type 2 diabetes.

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References


Figure legends

**Figure 1. SRA is highly expressed in adipose tissue in vivo.** A, Tissue expression was analyzed by RT-qPCR for mouse SRA. Total RNA was isolated from each tissue of age 9-week C57BL/6J mice. The locations of adipose depots analyzed were intrascapular BAT and epididymal WAT. Data were normalized to peptidylprolyl isomerase A (Ppia) mRNA and expressed relative to levels in whole brain, n = 6. B, Total RNA was isolated from the stromal vascular fraction (SV) or adipocytes (AD) of epididymal WAT from 8 week old male C57BL/6J mice. SRA RNA expression was determined by RT-qPCR. Expression of SRA was normalized to TATA box binding protein (Tbp) because it is expressed at similar levels in both fractions, and relative to levels in SV set at 1, n = 10. C, Male C57BL/6J mice were fed normal chow or HFD from 3 to 15 weeks of age. Total RNA was then isolated from inguinal WAT. Expression of SRA was analyzed by RT-qPCR and normalized to Ppia mRNA. Data are expressed as fold change relative to the SRA level of normal chow mice, n = 4–5 mice per group. Data are presented as mean ± S.D. Statistical significance in B and C was evaluated with Student’s t test: ** p < 0.01, * p < 0.05.

**Figure 2. Generation and validation of SRA gene knockout (SRAKO).** A, Strategy of Sra1 gene targeting of the mouse genome to produce the Sra1tm1a(EUCOMM)Hmgu knockout allele (SRA-). PCR primer locations used for genotyping are shown schematically. B, Genotyping SRA+/+, SRA+/- and SRA-/- mice by PCR using primers shown schematically above. Primers a and b span the LoxP3 site (LoxP3 PCR), c and d span the LacZ site (LacZ PCR), and GF3 and LAR3 span the Sra1 gene 5’ genomic DNA and target vector by long range PCR (LR-PCR). Note that the primer a and b PCR product is larger for WT than for the targeted allele, because the construct has a 119 bp deletion internal to the primer sites; C, Upper panel, validation of SRA global KO by RT-qPCR, data were normalized to Ppia mRNA and expressed relative to the level in SRA+/+, n = 4 mice at age of 8 weeks; Lower panel, SRAP content determined by immunoblot in WAT, liver and muscle; N.S., non-specific band used as a loading control in the same blot with anti-SRAP.

**Figure 3. SRAKO protects from diet-induced obesity and inhibits adipogenic, lipogenic and inflammatory gene expression in white adipose tissue (WAT).** A, SRAKO inhibits high fat diet (HFD)-induced obesity (DIO). Male mice body weights were measured from 3-19 weeks of age. HFD was started at 6 weeks of age. SRA+/+ n = 6, and SRA-/- n = 7; B, Body composition was determined by nuclear magnetic resonance after DIO; C, D, Reduced epididymal WAT (eWAT), subcutaneous WAT (sWAT) and liver mass in SRA-/- mice after DIO. E, Histology of eWAT in H&E stained sections. Scale bar, 200 µm. F, RT-qPCR analysis of mRNA expression in eWAT at the end of DIO. G, RT-qPCR analysis of mRNA expression in subcutaneous WAT at the end of DIO. F and G, Data were normalized to 60S ribosomal protein L10E (Rplp0) mRNA, *p<0.05 and **p<0.01, n= 6-7. SRC, steroid receptor coactivators.

**Figure 4. SRAKO reduces brown fat mass and results in smaller brown adipocytes.** A, SRAKO reduced intrascapular BAT mass after DIO; B, Histology of BAT in H&E stained sections. Scale bar, 200 µm. C, Brown adipocyte mRNA expression was determined by RT-qPCR. Data were normalized to Rplp0 mRNA, n=6-7.

**Figure 5: Energy expenditure profile in WT and SRA-/- mice upon DIO.** Oxygen consumption, respiratory quotient (RQ), total activity and food intake were determined over three days in SRA-/- and WT mice at the end of 14 weeks of HFD feeding, n=6–7 mice per group. The data in panel A represent the average dark and light cycle measurements during the third...
measurement day. The data in panel B represent the profiles across all three days. LBM, lean body mass; FM, fat mass.

**Figure 6. SRAKO improves DIO-induced insulin resistance.** A, Plasma hormone levels in SRA-/- mice were analyzed and compared to WT controls. All data in this figure were measured at the end of DIO. Blood glucose, plasma triglyceride (TG) and free fatty acid (NEFA) levels were measured at fasting conditions (6 hrs), n=6-7. B, Glucose tolerance test (GTT) at the end of DIO. Mice were fasted overnight (16 hrs), and a standard glucose tolerance test was performed; C, Insulin tolerance test (ITT) at the end point of DIO. Mice were fasted for 6 hrs during the day, and a standard insulin tolerance test was performed, n=6-7 mice. D, SRAKO enhances insulin-stimulated phosphorylation of Akt under HFD-feeding. HFD-fed SRA-/- and WT control mice were fasted overnight (16 hrs). Five minutes after PBS or insulin injection via tail vein, epididymal WAT, liver and gastrocnemius muscles were dissected. The tissue lysates were immunoblotted for pAkt (Ser473) and total Akt. E, Plasma TNFα and IL6 levels were analyzed as described for panel A. *p<0.05, **p<0.01.

**Figure 7. SRAKO inhibits the formation of fatty liver under HFD.** A, H&E sections of liver at the end point of DIO; B and C, Liver TG and NEFA levels were analyzed, respectively, n=6-7. D, RT-qPCR analysis of expression of liver genes involved in lipogenesis, gluconeogenesis and glycolysis. Data were normalized to Ppia mRNA and relative to the expression of each gene in WT mice, n=6-7. *p<0.05 and **p<0.01.
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Figure 1

A. Relative SRA expression across various organs (Brain, Lung, Liver, Heart, Kidney, Testis, Muscle, Brown Fat, White Fat, Spleen).

B. Relative SRA expression for SV and AD conditions.

C. Relative SRA expression for Normal Chow and HFD conditions.
Figure 2

A  WT
Sra1
Chr 18; 36,826,928 - 36,829,935 (3008 bp)

Sra1^m1a(EUCOMM)Hmg

targeted gene

(JM8.N4: C57BL/6N)

B

SRA

WT LoxP3 PCR

Tg LoxP3 PCR

LacZ PCR

LR-PCR

C

SRA

1  SRA^{+/+} (WT)

2  SRA^{+-}

3  SRA^{-/-}

Relative RNA expression

WAT  BAT  Liver  Muscle

IB: SRAP

N.S.
Figure 3

A) Body weight (g) vs. age (wk) for WT+/+ and SRA-/-. 

B) Body composition (%), with WT and SRA-/-. 

C) WAT Mass (g) for eWAT and sWAT. 

D) Liver Mass (g) for WT and SRA-/-. 

E) eWAT images for WT and SRA-/-. 

F) eWAT mRNA expression for Adipogenic and lipogenic, Inflammatory, SRC, and Glyceroneogenic pathways. 

G) sWAT mRNA expression for Pparg, Cebpα, Fabp4, Adipoq, Cd36, Lipe, Tnf, Il6, Ccl2, Ncoa1, Ncoa2, Ncoa3, Pck1, Ucp1, CD137, Bmp8b, Fndc5, Tbx1, Tbx15. 

Legend: *p < 0.05, **p < 0.01, ***p < 0.001.
Figure 5

A

VO2 (ml/kgLBM+0.2FM/hr) vs. Dark/Light for WT and SRA -/-.

RQ (VCO2/VO2) vs. Dark/Light for WT and SRA -/-.

Total Activity (counts/hr) vs. Dark/Light for WT and SRA -/-.

Food Intake (g/hr) vs. Dark/Light for WT and SRA -/-.

B

VO2 (ml/kgLBM+0.2 FM/hr) vs. 6 pm/6 am for WT and SRA -/-.

RQ (VCO2/VO2) vs. 6 pm/6 am for WT and SRA -/-.

Total Activity (counts/hr) vs. 6 pm/6 am for WT and SRA -/-.

Food Intake (g/hr) vs. 6 pm/6 am for WT and SRA -/-.
Figure 6
Figure 7

A

Liver

WT

SRA-/-

B

Liver Triglyceride (mg per g)

WT

SRA-/-

C

Liver NEFA (µmol/g)

WT

SRA-/-

D

Relative mRNA expression

lipogenic

Gluconeogenic & Glycolytic

Ppara, Pparg, Fabp4, Lipe, Srebf1, Scd1, Pck1, G6pc, Slc2a2, Pgc1a

* * *
SRA gene knockout protects against diet-induced obesity and improves glucose tolerance
Shannon Liu, Liang Sheng, Hongzhi Miao, Thomas Saunders, Ormond MacDougald, Ronald Koenig and Bin Xu

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