Inhibition of *Sam68* triggers adipose tissue browning

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Abstract

Obesity is associated with insulin resistance and type 2 diabetes; molecular mechanisms that promote energy expenditure can be utilized for effective therapy. Src-associated in mitosis of 68 kDa (*Sam68*) is potentially significant, because knockout (KO) of *Sam68* leads to markedly reduced adiposity. In the present study, we sought to determine the mechanism by which *Sam68* regulates adiposity and energy homeostasis. We first found that *Sam68* KO mice have a significantly reduced body weight as compared to controls, and the difference is explained entirely by decreased adiposity. Interestingly, these effects were not mediated by a difference in food intake; rather, they were associated with enhanced physical activity. When they were fed a high-fat diet, *Sam68* KO mice gained much less body weight and fat mass than their WT littermates did, and they displayed an improved glucose and insulin tolerance. In *Sam68* KO mice, the brown adipose tissue (BAT), inguinal, and epididymal depots were smaller, and their adipocytes were less hypertrophied as compared to their WT littermates. The BAT of *Sam68* KO mice exhibited reduced lipid stores and expressed higher levels of *Ucp1* and key thermogenic and fatty acid oxidation genes. Similarly, depots of inguinal and epididymal white adipose tissue (WAT) in *Sam68* KO mice appeared browner, their multilocular *Ucp1*-positive cells were much more abundant, and the expression of *Ucp1*, *Cidea*, *Prdm16*, and *Ppargc1a* genes was greater as compared to WT controls, which suggests that the loss of *Sam68* also promotes WAT browning. Furthermore, in all of the fat

Key Words

- insulin
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depots of the *Sam68* KO mice, the expression of M2 macrophage markers was up-regulated, and that of M1 markers was down-regulated. Thus, *Sam68* plays a crucial role in controlling thermogenesis and may be targeted to combat obesity and associated disorders.

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Introduction

Obesity, which is characterized by abnormal or excessive fat accumulation resulting from energy intake exceeding expenditure, has become a global epidemic and is associated with an array of medical conditions, including insulin resistance, type 2 diabetes, and cardiovascular disease (Bornfeldt & Tabas 2011). In the body, white adipose tissue (WAT) is a major lipid depot that contains unilocular white adipocytes for storing vast amounts of nutrients as lipids. Brown adipose tissue (BAT), on the other hand, is a key site of energy expenditure via heat production (thermogenesis). The brown adipocytes display multilocular lipid droplet structures and express uncoupling protein 1 (*Ucp1*), which, when activated, short circuits the electrochemical gradient of ATP production and generates heat (Cypess *et al.* 2009). Because heat production is an important component of energy expenditure, targeting the molecular and cellular mechanisms that control thermogenesis could be an effective strategy for preventing and treating obesity.

Recently, important advancements have been made in the identification of clusters of *Ucp1*-expressing adipocytes with thermogenic capacity in WAT (Vitali *et al.* 2012, Wu *et al.* 2012). These cells, which are called beige or brite fat cells, are defined by their multilocular lipid droplet morphology, high mitochondrial content, and their expression of a core set of brown fat-specific genes (e.g., *Ucp1*, *Cidea*, and *Pgc1a*). The accumulation of beige adipocytes in WAT is referred to as 'browning', and it includes the induction of *Ucp1* expression and a gene program that gives rise to uncoupled respiration and heat production. Thus, both brown and beige adipocytes are specialized in energy expenditure through thermogenesis (Saito *et al.* 2009); and evidence suggests that their activities contribute to weight loss and the improvement of diabetes in animals, and they correlate with leanness in humans (Feldmann *et al.* 2009, Harms & Seale 2013). At present, a number of genes that activate brown adipocytes and/or drive the induction of beige cells have been reported (Wang *et al.* 2013, Cohen *et al.* 2014); however, many of them have not yet been validated (Nedergaard &

Cannon 2014). Identifying novel factors that induce the white-to-brown transition will not only contribute to a better understanding of the comprehensive regulation of energy homeostasis but also provide potential molecular targets for intervening in obesity and its metabolic consequences.

In addition to environmental temperature and nutrient intake, inflammation is increasingly being recognized as a significant regulator of energy homeostasis (Rao *et al.* 2014). In fact, obesity is now considered a state of chronic low-grade systemic inflammation (Solinas 2012). In obese animals, fat tissues have been shown to display a substantial degree of inflammation (Shoelson *et al.* 2006), secrete inflammatory cytokines (Hotamisligil *et al.* 1993), and harbor various immune cells (Osborn & Olefsky 2012). More recently, studies have shown that adipose tissue macrophages derived from lean mice express markers of M2, or 'alternative activated', macrophages. In contrast, obese states are concomitant with M1, or 'classically activated', macrophages (Lumeng *et al.* 2007). Interestingly, M2 macrophages in the adipose tissues have been shown to secrete catecholamines to induce thermogenic gene expression in BAT and to induce lipolysis in WAT (Nguyen *et al.* 2011, Qiu *et al.* 2014), whereas M1 macrophage polarization is pro-inflammatory (Eguchi *et al.* 2013).

Src-associated in mitosis of 68 kDa (*Sam68*), also known as KH domain containing, RNA binding, signal transduction associated 1 (*Khdrbs1*), is the prototype of the STAR (signal transducer and activator of RNA) family of RNA-binding proteins (Richard 2010, Sette 2010). It is involved in a number of cellular processes, including RNA processing (Bielli *et al.* 2011, Iijima *et al.* 2011), growth factor receptor and kinase signaling (Huot *et al.* 2009, Ramakrishnan & Baltimore 2011), transcription (Zhou *et al.* 2009, Fu *et al.* 2013), cell cycle regulation, and apoptosis (Lukong & Richard 2003, Bielli *et al.* 2014). Most strikingly, *Sam68*-null (*Sam68* knockout (KO)) mice exhibit a markedly reduced adiposity (Huot *et al.* 2012), but the underlying mechanism for this is not completely

understood. In the present study, we demonstrated that the loss of *Sam68* in mice enhances the adipose thermogenic program and triggers WAT browning; these in turn contribute to their lean phenotype, resistance to the induction of obesity, and improved glucose metabolism.

Materials and methods

Animals

Sam68^{+/-} mice on C57BL background were provided by Dr Stéphane Richard (McGill University, Montreal, QC, Canada; Richard *et al.* 2005) and were bred to obtain *Sam68*^{-/-} (*Sam68* KO) mice and WT littermate controls. All breeding, maintenance, and experimental procedures were approved by the Institutional Animal Care and Use Committee of Northwestern University and were conducted at the University's Center for Comparative Medicine. Mice were maintained on a 12 h light:12 h darkness cycle and were allowed to feed and drink water *ad libitum*. Genotypes were determined by PCR of tail DNA using the primers listed in the [Supplementary Table S1](#), see section on [supplementary data](#) given at the end of this article. For all of the experiments in the present report, male mice were used. For diet-induced obesity studies, mice were fed with a high-fat diet (HFD; 42 kcal % fat with high sucrose, 88137, Harlan Teklad, Harlan Laboratories, Inc. IN, USA) for 12 weeks.

Nuclear magnetic resonance analysis of body compositions

Body compositions were analyzed with Echo MRI Whole Body Composition Analyzer (Echo Medical Systems, Echo MRI Whole Body Composition Analyzer, Houston, TX, USA) in the Comprehensive Metabolic Core of Northwestern University following standard operation procedures.

Open field test of general activity

The open field arena (56×56 cm) was used to assess ambulation and anxiety levels in the Behavioral Phenotyping Core of Northwestern University. Briefly, each mouse is placed in the center of the arena, and its ambulation activity is collected by the LimeLight Software (Wilmette, IL, USA) for 20 min. The software provides the total distance traveled as well as the percentage of time/distance within different parts of the arena. An anxious mouse will spend more of its time along the perimeter of the arena. A hyperactive mouse will have a large ambulation score.

Glucose and insulin tolerance tests

For the glucose tolerance test (GTT), mice were fasted overnight. Glucose (2 g/kg) was administered intraperitoneally, and then blood glucose levels were measured at 0, 15, 30, 60, and 120 min with a blood glucose meter (Bayer Contour, Whippany, NJ, USA). For the insulin tolerance test (ITT), mice were fasted for 6 h. Insulin (0.7 U/kg) was administered intraperitoneally, and blood glucose was measured at 0, 15, 30, 60, and 120 min.

Histology

Fat tissues were fixed in 10% neutral buffered formalin and embedded in paraffin. Five micrometer sections were cut, stained with hematoxylin and eosin (H&E), and photographed under a light microscope. Immunohistochemical staining was performed with anti-*Ucp1* antibodies (Abcam, Inc., Cambridge, MA, USA), as described previously by the Rosen laboratory (Kong *et al.* 2014).

Western blotting

Fat tissues were collected and immediately frozen in liquid nitrogen. Proteins were extracted with RIPA buffer (Cell Signaling Technology, Inc., Boston, MA, USA) supplemented with complete protease inhibitor cocktail (Roche). For western blotting analyses, protein samples were subjected to SDS-PAGE under reducing conditions, transferred, and blotted with antibodies to IRS1 (Millipore, Temecula, CA, USA), phospho-IRS1 (Tyr612) (Abcam, Inc.), AKT, and phospho-AKT (Ser473) (Cell Signaling Technology, Inc.), and HSP90 (Santa Cruz Biotechnology, Inc.).

Quantitative real-time RT-PCR

RNA extraction and quantitative real-time RT-PCR (qRT-PCR) were performed as described previously (Tang *et al.* 2009). Primer sequences are listed in [Supplementary Table S1](#).

Statistical analysis

All values are reported as means±s.e.m. Unpaired two-tailed Student's *t*-tests were used for comparisons between two groups. Two-way ANOVAs with repeated measures were used for comparing two factors at multiple levels in the time course studies. A *P* value of <0.05 was considered statistically significant.

Results

Loss of *Sam68* protects against diet-induced obesity

We initiated the present investigation by measuring body weight and composition in *Sam68* KO mice and in their WT littermates. *Sam68* KO mice displayed significantly reduced body weight and fat mass, and the difference was explained entirely by decreased adiposity, seeing as their lean mass was normal (Fig. 1A and B). Further analyses showed that BAT, inguinal, and epididymal depots were smaller in the *Sam68* KO mice (Fig. 1C), and their adipocytes showed less hypertrophy than those of control mice did, and there were no weight changes in the liver or spleen (Supplementary Figure S1, see section on supplementary data given at the end of this article). The effect on adiposity was not mediated by food intake, which did not differ between the two genotypes (Fig. 1D); rather, it was associated with enhanced physical activity (Fig. 1E). Taken together, these data suggest that the *Sam68* KO mice were probably leaner because of a significant increase in physical activity that was not related to feeding behavior. Next, we chose *Sam68* KO mice and WT controls that were matched for body weights and fed them with an HFD. Notably, the increases in body

weight and fat mass were significantly attenuated in *Sam68* KO mice as compared to the WT controls (Fig. 2A and B).

Loss of *Sam68* results in improved insulin sensitivity

Diet-induced obesity is frequently associated with glucose imbalance and progressive metabolic dysfunction. Notably, *Sam68* KO mice on HFD showed improved glucose and insulin tolerance to a degree that was consistent with their suppressed body weight (Fig. 2C and D). In addition, we assessed the effect of *Sam68* on insulin signaling and found that the insulin-induced phosphorylation of IRS1 (Tyr612) and AKT (Ser473) was augmented in the WAT of *Sam68*^{-/-} mice (Fig. 2E). These findings indicate that the *Sam68* KO animals were protected against HFD-induced glucose intolerance and insulin resistance.

Sam68 is required for normal thermogenesis in BAT

Adipose tissue is an important organ that contributes to glucose homeostasis. BAT has been confirmed recently to play a crucial role in glucose metabolism (Stanford *et al.* 2013); thus, we evaluated whether *Sam68* expression is required for BAT structure and function. The *Sam68* KO

Figure 1

Loss of *Sam68* decreases adiposity in mice on regular chow. (A) Body weight of WT mice and *Sam68* KO mice ($n=6-10/\text{group}$, $*P<0.05$ and $**P<0.01$). (B) Body composition, fat mass (left panel), and lean mass (right panel) as assessed by nuclear magnetic resonance (NMR) in 6-month-old mice ($n=6-10/\text{group}$, $*P<0.05$). (C) Gross morphology of 6-month-old WT and

Sam68 KO mice and BAT, inguinal, and epididymal fat pads. (D) Food intake measured daily for 3 days in 4-month-old mice and averaged ($n=6/\text{group}$; NS, not significant). (E) Representative (left and middle panels) and quantification (right panel) of general activity of 6-month-old WT and *Sam68* KO mice by open field test ($n=4/\text{group}$, $*P<0.05$).

Figure 2

Loss of *Sam68* attenuates diet-induced obesity (DIO) and improves insulin sensitivity. Male 8-week-old WT and *Sam68* KO mice were first matched with body weight and then fed an HFD for 12 weeks, at which point body composition, GTT, ITT, and insulin signaling in adipose tissues were assessed. (A) Serial measurements of body weight ($n=6/\text{group}$, $*P<0.05$

and $**P<0.01$). (B) Body composition by NMR (left panel, fat mass and right panel, lean mass; $n=6/\text{group}$, $**P<0.01$ and NS, not significant). (C) GTT and (D) ITT ($n=6$, $*P<0.05$). (E) Western blotting analyses of WAT fat depots from WT and *Sam68* KO mice 30 min after i.p. injection of insulin.

mice had smaller brown adipose depots (Fig. 1C) and reduced lipid stores of BAT at room temperature (Fig. 3A). BAT from *Sam68* KO mice maintained at 24 °C expressed higher levels of *Ucp1* than did BAT from control mice (Fig. 3B and Supplementary Figure S2, see section on supplementary data given at the end of this article). This phenotypic change was accompanied by an increased expression of key thermogenic (e.g., *Ucp1*, *Prdm16*, *Dio2*, *Cidea*, and *Elovl3*) as well as fatty acid oxidation (e.g., *Cidec* and *Cpt1b*) genes, but it did not result in a concomitant increase in mitochondrial (e.g., *β 3ar* and *Cox8b*) or adipose tissue marker (e.g., *Fabp4* and *Pparg*) genes (Fig. 3C). Thus, genetic inactivation of *Sam68* leads to enhanced BAT structure, augmented *Ucp1* activation, and elevated thermogenic gene expression.

Ablation of *Sam68* mice enhances the browning signature of WAT

Interestingly, depots of inguinal and epididymal WAT of *Sam68* KO mice appeared browner than their WT counterparts did (Figs 1C and 4A). Additionally, the weight of various fat depots was lighter in the KO mice

as compared to control littermates (data not shown). Furthermore, multilocular *Ucp1*-positive cells were much more abundant, and UCP1 total protein levels were higher in the iWAT and eWAT of *Sam68* KO mice than they were in WT control mice (Fig. 4B and Supplementary Figure S2). Consistently, the expression of *Ucp1*, *Cidea*, *Prdm16*, and *Pparg1a* was increased in the WAT of the *Sam68* KO mice (Fig. 4C and D). Taken together, these results suggest that the deletion of *Sam68* promotes adipose browning.

Sam68 KO mice favor M2 macrophage anti-inflammatory phenotypes

Obesity initiates a state of low-grade inflammation and fibrosis that ultimately predisposes to the progression to insulin resistance and type 2 diabetes (Shoelson *et al.* 2006, Sun *et al.* 2011, Osborn & Olefsky 2012). In particular, a high degree of pro-inflammatory macrophages infiltrates into the adipose tissue during obesity. Thus, we looked at the expression of macrophage markers in different fat depots. As was expected, the mRNA levels of M2 markers (e.g., *Arg1* and *Il10*) were up-regulated, whereas those of M1 markers (e.g., *Ccr2*, *Il1 β* , and *Il6*) were decreased in all

Figure 3

Sam68 is required for normal thermogenesis in BAT. BAT was isolated from male 10-month-old WT and *Sam68* KO mice. (A) H&E staining of paraffin-embedded sections. Original magnification: 100×. (B) Immunohistochemical detection of *Ucp1* (brown). Original

magnification: 100×. (C) QRT-PCR quantification of mRNA expression. Data are normalized to the mRNA level of ribosomal protein 36b4 ($n=5$ /group, * $P<0.05$).

fat depots (Fig. 5). These data suggest that the loss of *Sam68* also leads to macrophages switching so that they augment thermogenic gene expression in BAT and promote browning in WAT.

Discussion

In the present study, we showed for the first time that the ablation of *Sam68* in mice leads to an increase in adipose thermogenic gene expression and WAT browning, which reveals a critical role for *Sam68* in the control of thermogenesis and energy expenditure. We further demonstrated that *Sam68* KO mice were resistant to diet-induced obesity and display improved insulin sensitivity. Thus, *Sam68* provides a novel link between energy homeostasis and obesity.

Sam68 is the prototype of the STAR family of RNA-binding proteins. It has been shown to modulate various cellular signaling and to link that signaling to RNA processing and gene expression (Vogel & Richard 2012). *Sam68* is expressed abundantly in most tissues (Zhou J & Qin G, unpublished observations), and its structural characteristics permit multiple types of post-translational modifications; this is compatible with its involvement in many cellular functions in different tissue types (Lukong & Richard 2003, Zhou *et al.* 2009, Iijima *et al.* 2011,

Ramakrishnan & Baltimore 2011) and in the diverse phenotypes of *Sam68*-deficient mice (Vogel & Richard 2012). *Sam68*-null mice appear to be grossly normal and to have a normal life span; however, they display a higher rate of perinatal death, male infertility, female-delayed mammary gland development, attenuated age-related osteoporosis, and reduced capacity for motor coordination (Vogel & Richard 2012). Most strikingly, these mice demonstrate reduced adiposity and increased energy expenditure, which have been associated with decreased adipose differentiation (Huot *et al.* 2012); however, the underlying mechanism for this remains incompletely understood. In the present study, we characterized the fat tissues in *Sam68* KO mice and revealed a previously unrecognized function of *Sam68* that suppresses browning and thermogenesis. Recently, much has been learned about the types of adipocytes and the core gene program of thermogenesis (van der Lans *et al.* 2013). The present data identify *Sam68* as a crucial upstream mechanism in the process, and they are also consistent with the notion that energy expenditure through thermogenesis plays a significant role in body weight control.

The present results suggest that *Sam68* suppresses thermogenesis, at least in part, by controlling physical activity and by repressing M2 anti-inflammatory cytokine expression. Although physical activity is known to be a robust means of increasing thermogenesis and energy

Figure 4

Ablation of *Sam68* enhances the browning signature of WAT. Inguinal and epididymal WATs were isolated from male 10-month-old WT and *Sam68* KO mice. (A and B) H&E staining (A) and immunohistochemical detection of *Ucp1* (brown) (B) in paraffin-embedded sections. Original magnification:

100×. (C and D) QRT-PCR quantification of mRNA expression in inguinal WAT (C) and epididymal WAT (D). Data are normalized to the 36b4 mRNA ($n=5/\text{group}$, $*P<0.05$).

expenditure, the important role of anti-inflammatory mediators has been recognized only recently. For example, the M2 macrophages in the adipose tissues have been shown to participate in thermogenesis via the secretion of catecholamines (Nguyen *et al.* 2011, Qiu *et al.* 2014). We found a significantly up-regulated M2 macrophage signature as well as a down-regulated M1 macrophage signature in the adipose tissues of *Sam68* KO mice. Interestingly, *Sam68*

has recently been shown to promote NF- κ B activation by TNF α and to modulate the NF- κ B selection of target promoters in mouse embryonic fibroblasts and activated T cells respectively (Ramakrishnan & Baltimore 2011, Fu *et al.* 2013). Because NF- κ B is an important regulator of macrophage polarization (Porta *et al.* 2009), it is conceivable that NF- κ B might be involved in the alteration of the M2–M1 balance in *Sam68* KO mice. Taken together, the present data

Figure 5

Sam68 KO mice favor M2 macrophage anti-inflammatory phenotypes. RNAs were isolated from the adipose tissues of male 10-month-old WT and *Sam68* KO mice. QRT-PCR was performed, and the values were normalized

to 36b4 ($n=5$, $*P<0.05$) for assessment of the inflammatory gene expression in BAT (A), inguinal WAT (B), and epididymal WAT (C).

suggest that *Sam68* acts to coordinate the activities of multiple tissues to suppress heat generation. Furthermore, the role of *Sam68* in the gene expression program may be cell type- and biological context-dependent. Whereas the levels of the adipose genes *Ppar γ* and *Fabp4* are unaltered in the adipose tissues of *Sam68* KO mice, their expressions are down-regulated in 3T3-L1 cells after the knockdown of *Sam68* and culture in adipose differentiation conditions (Huot *et al.* 2012). Ongoing studies are currently under way to dissect the relative contributions from brown fat cells, beige cells, and macrophages as well as the molecular underpinnings of how *Sam68* regulates mRNA expression in these different cell types.

In summary, the present study demonstrates that *Sam68* plays a crucial role in adipose thermogenesis, WAT browning, and body weight control. Thus, targeting *Sam68* in relevant tissues may provide a potential strategy for combating obesity and associated metabolic disorders.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JOE-14-0727>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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