博士論文

論文題目　Roles of CDK5 regulatory subunit associated protein 1-like 1 in obesity and type 2 diabetes

（肥満・２型糖尿病における CDKAL1 の役割の検討）

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Contents

Abbreviation

AUC	**area under curve**
BAT	**brown adipose tissue**
BMI	**body mass index**
Dio2	**type II iodothyronine deiodinase**
eWAT	**epididymal white adipose tissues**
Fabp4	**fatty acid binding protein 4, also called aP2**
Fasn	**fatty acid synthase**
HFD	**high fat diet**
OGTT	**oral glucose tolerance test**
Pparg	**peroxisome proliferator-activated receptor γ**
Scd1	**stearoyl-CoA desaturase-1**
scWAT	**subcutaneous white adipose tissue**
SKM	**skeletal muscle**
SVF	**stromal vascular fraction**
Ucp1	**uncoupling Protein 1**
Wisp2	**WNT1 inducible signaling pathway protein 2**

Abstract

Cdk5 regulatory subunit associated protein 1-like 1 (Cdkal1) is one of the disease susceptible genes identified by genome-wide association studies for type 2 diabetes and obesity. Although Cdkal1 was recently shown to work as a tRNA ^{Lys} modifying enzyme and to be crucial in accurate translation and insulin secretion from the pancreatic β cells, little is known about its role in adipocytes. Unpublished data from our laboratory shows that in 3T3-L1 adipocytes, Cdkal1 inhibits adipocyte differentiation and lipid accumulation by suppressing expression of PPARγ, possibly through activation of the WNT signal pathway. In order to reveal the roles of Cdkal1 in adipose tissue *in vivo*, I generated adipose-tissue-specific Cdkal1 transgenic (Tg) mice under the control of the aP2 promoter. Cdkal1 Tg mice exhibited smaller mass of subcutaneous white adipose tissue (scWAT), less lipid droplet accumulation in brown adipose tissue (BAT) and scWAT, reduced triglyceride content of BAT, and an improved glucose profile and insulin sensitivity in an oral glucose tolerance test (OGTT) and an insulin tolerance test (ITT) under a high fat diet (HFD). Modification of tRNA ^{Lys} appeared to be unchanged in either adipose tissues of the Cdkal1 Tg mice or the Cdkal1 overexpressing 3T3-L1 cells. Expression of Cdkal1 was significantly decreased in BAT and a tendency to low

in scWAT of *db/db* obese and diabetic model mice. Examination of human adipose tissues revealed an inverse correlation of *CDKAL1* with body mass index (BMI) and waist circumference. Together, our data indicated that Cdkal1 in adipose tissue may potentially be involved in the development of diabetes and obesity.

Key words: Cdkal1, adipose tissue, obesity, T2D

Introduction

Type 2 diabetes (T2D) and obesity are thought to develop by a combination of environmental and genetic factors. The prevalence of diabetes is 9% in adult population now (1), and its prevalence continues to increase in many countries. Numbers of novel genetic susceptibility variants of T2D were identified by genome-wide association studies (GWAS) (2-5), and Cdk5 regulatory subunit associated protein 1-like 1 (*CDKAL1*) was one of such genes. The genetic variants of *CDKAL1* are demonstrated to be a reproducible susceptible gene in various ethnicities, such as Asians, Caucasians and Africans (25-31). Cdkal1 protein is reported to be an endoplasmic reticulum (ER) - resident protein (6, 7). *Cdkal1* mRNA is expressed in many tissues, prominently in skeletal muscle, pancreatic islets, and immune cells $CD19^+$ cell and $CD4^+$ cell (8-9). Several *Cdkal1* isoforms are available on the Ensemble Genome Browser. Only isoform 1 is expressed in human islets and pancreatic MIN6 cells (7, 8). Recently, Cdkal1 has been reported to be a mammalian tRNA methylthiotransferase that modifies $tRNA^{Lys}$ (6, 32). Pancreatic β cell-specific Cdkal1 knock out mice displayed an impaired insulin secretion and high blood glucose levels. In pancreatic β cells of Cdkal1 knockout mice, inaccurate and inefficient translation of Lys codon affects the translation of glucose-stimulated proinsulin synthesis, and leads to ER stress (6, 7), which eventually causes

diabetes. Moreover, deletion of Cdkal1 also was shown to affect mitochondrial ATP generation and high fat diet inducted fat accumulation (9, 10). In addition to tRNA methylthiotransferase, a splicing variant of *CDKAL1* termed *CDKAL1-v1*, which is a non-coding transcript, was reported to modulate cellular Cdkal1 protein level through competitive binding to a Cdkal1-target miRNA 494 (17). Although the function of Cdkal1 in pancreatic β cells was well characterized, little is known about its role in adipose tissue, which is also crucially relevant to the pathogenesis of T2D.

Recently, common variant at *CDKAL1* was observed to be associated with BMI in East Asian populations (12). Meanwhile, a Meta-analysis of East Asians reported three novel loci in or near the genes associated with BMI, including *CDKAL1* (13). Unpublished data from our laboratory showed that, Cdkal1 is expressed in adipocytes and its expression is regulated during adipocyte differentiation. Expression of Cdkal1 is increased during differentiation of 3T3-L1 adipocytes, and decreased in hypertrophic 3T3-L1 adipocytes after a long culture similarly to expression of adiponectin. Functionally, overexpression of Cdkal1 in 3T3-L1 inhibits adipocyte differentiation and lipid accumulation by suppressing expression of the critical master regulator PPARγ, possibly through activation of the WNT pathway, which is one of the well-characterized

pathways that suppress PPARγ expression and adipogenesis (34-40). Meanwhile, suppression of Cdkal1 in 3T3-L1 resulted in enhanced adipogenesis and more accumulation of the lipid. In this study, I generated adipose tissue specific Cdkal1 Tg mice and investigated the roles of Cdkal1 *in vivo*.

Materials and Methods

Cells culture

3T3-L1, plat E, and HEK293T cell culture were performed as described in (14). Briefly, cells were maintained in DMEM, supplemented with 10% FBS, 1% penicillin and streptomycin. For inducing adipocyte differentiation, two days after confluence, 3T3-L1 cells were treated with the standard hormone cocktail, dexamethasone (1 μM), IBMX (0.5 mM), and insulin (5 μg/ml) (DMI). 3T3-L1 cells were treated with DMI for 48 hours. The cells were then incubated in medium supplemented with insulin alone every two days.

Western blot

Cells were rinsed twice with PBS and lysed with lysis buffer on ice (1% N-P40, 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 25×proteinase inhibitor cocktail). The samples were scraped off the dish and the cell suspension was gently transferred into a pre-cooled micro-centrifuge tube, and subjected to DNA sonication. The same amount of protein was loaded on 10% SDS-PAGE gel, then transferred to a PVDF membrane. The membranes were blocked with 5% skim milk-TBS for one hour at room temperature and then reacted with primary antibody at 4°C overnight, followed by TBST washing

buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween-20). On the following day, after a brief washing, the membrane was incubated with HRP-conjugated secondary antibodies for one hour at room temperature. Then membranes were washed for thirty minutes in TBST washing buffer. HRP-conjugated antibodies were detected with ECL western blotting detection reagents (Amersham, RPN2106) according to the manufacturer's instructions. Primary antibodies were anti-Cdkal1 (Abcam, ab68045), anti-Flag (Sigma, F1804-5MG) and anti-actin (Santa Cruz, sc-1616).

RNA interference

The HEK 293T cells were transfected with either control siRNA (Silencer Select negative control) or CDKAL1 (NM_017774.3) (Silencer select s29718) (17) by using Lipofectamine ™ RNAiMAX (Invitrogen). Induction of differentiation was started two days after confluence, as described in the method for differentiation of 3T3-L1 cells.

Retrovirus expression system

Retrovirus expression was performed as described in (14). The Cdkal1 coding sequence from cDNA was prepared from mice adipose tissue and cloned into retroviral pMXs-puro vectors. FLAG-tagged plasmid was also generated at the N-terminus or C-

terminus. Plat E cells were transfected with the plasmids using Lipofectamine ™ 2000 (Invitrogen). For infection, 3T3-L1 cells or primary preadipocytes were incubated in the conditioned medium with viruses. Infected cells were selected in 4 μg/ml puromycin.

Oil Red O staining (lipid stain)

Lipid droplets in the cells were stained with Oil Red O as described in reference (14). The cells were washed with PBS, fixed in neutral formalin solution. 60% isopropanol was used to rinse the fixed cells, and the cells were stained with freshly made oil red o working solution (60% isopropanol) and incubate for one hour at RT. After washed by water, the cells were then air dried.

RNA analysis

Total RNA was isolated using TRIzol reagent (Invitrogen) as described in (14), reverse transcription was performed using 0.5 μg of total RNA with cDNA reverse transcription kits (Applied Biosystems, 4375222). QuantStudio 7 Flex System (Applied Biosystems) was used for real-time quantitative PCR. Expression level of genes measured were normalized to 36B4. The sequences of primers used in this study are listed in Table 1.

Adipose tissue triglyceride measurement

BAT was isolated from Cdkal1 Tg mice and WT mice. Less than 100 mg BAT was homogenized in 1ml homoginizing buffer (25mM Tris-HCl, 10mM EGTA, 10mM EDTA, 10mM $NaVO_4$, 100mM NaF, and 10 mM $Na_4P_2O_7/10H_2O$, pH7.4). 100μl of homogenized lysate was mixed thoroughly with 100μl chloroform and methanol (2:1) mixture, followed by a centrifuge at 14000 rpm for 10 min. Lower phase was transferred to a new tube, and was incubated at 55◦C for 1 hour. Precipitated triglyceride was dissolute in 1% Triton X-100/ethanol solution. After overnight incubation at RT, the concentration of triglyceride was measured on the following day with a kit (#432-40201, Wako) according to the manufacturer's instructions.

Quantification of lipid droplet size distribution of adipocyte

Paraffin sections of BAT and scWAT from Cdkal1 Tg mice and WT mice were stained with H&E, and images were acquired and analyzed by image J (NIH). For each section, four images were taken, total number of 600~2000 cells were analyzed for each mouse.

$tRNA^{Lys}$ modification index

Measurement of ms^2 modification index in total RNA isolated from 3T3-L1 cells,

HEK293T cells, and mice adipose tissues was performed by using a method established by Xie et al. (Figure 1A and reference 15). In this method, firstly I designed primers of reverse 1 (r1), reverse 2 (r2) and forward for reverse transcription (RT). R1 was designed to the middle of this tRNA, including the 37A position, whereas r2 was designed to the region downstream (3') of 37A (Figure 1). So if tRNA is fully modified at 37A, the Watson-Crick pairing between 37A and T would be prevented, resulting in the amount of cDNA generated by r2 to be less than generated by r1. By contrast, if tRNA is not modified, like in knock out or knock down conditions, the Watson-Crick pairing between 37A and T would not be prevented, so the amount of cDNA generated by r2 and r1 would be similar. The amount of cDNA is quantified by qPCR.

The method was performed as described in (15). Briefly, total RNA was adjusted to 50 ng/μl in water. 2 μl (100 ng) of RNA was digested by 2 μl of DNase (Promega, RQ1 RNase-Free DNase Kit) in a 20 μl reaction at 37°C for 30 min. After the DNase treatment, reverse transcription was performed with a kit (Applied Biosystems, #4375222) using reverse primer r1 (CCTGGACCCTCAGATTAAAA) or reverse primer r2 (GAACAGGGACTTGAACCCTG). SYBR green-based real-time PCR was performed using, 2 μl of cDNA, forward primer (GTCGGTAGAGCATCAGATCC),

and reverse primer r1 in Applied Biosystems Quant Studio 7 Flex Real-time PCR system (Applied Biosystems). Relative modification index was calculated as described in (15). (Figure 1B, 1C). The threshold cycle numbers (referred to as CTr1 or CTr2) were in the real-time qPCR was generated by primer r1 or primer r2. The difference between CTr1 and CTr2 was used to calculate the relative modification rate.

Primary culture

As described previously (16), the stromal vascular fraction (SVF) was isolated from Cdkal1 transgenic mice and the wild-type mice aged 6-8 weeks, digested the tissue with Collagenases D with a concentration of 1.5 U/ml (Roche, 70334223) and Dispase II with a concentration of 2.4 U/ml (Roche, 11466200). The digested mixture was plated in collagen-coated plates (BD Biosciences) and induced to differentiation when they came to 95-100% confluence with induction medium (supplemented with Dexamethasone, final conc. 2 μg/ml; Isobutyl-1-methylxanthine (IBMX), final conc. 0.5 mM; Insulin, final conc. 5 μg/ml; 3,3',5-Triiodo-L-thyronine (T3), final conc. 1 nM; Indomethacin, final conc. 125 μM) (day 0). After 48 hours (day 2) and 96 hours (day 4), medium was replaced to maintenance medium supplemented only with T3 (1 nM), insulin (5 μg/ml) and rosiglitazone (0.5 μM). Six days after induction, the mature

adipocytes were collected (Figure 2).

Animals

C57BL/6J wild type (WT) mice and *db/db* mice were obtained from Charles River Japan Inc. Animal rooms are maintained on a 12-hour light-dark cycle and were given normal chow (CLEA Rodent Diet CE-2). C-2 consists of 50% carbohydrates, 4.8% fat, 25.1% (wt/wt) protein, 4.2 fiber, 6.7% ash and 9.3% water. A high-fat diet (CLEA High Fat Diet 32), consisting of 29.4% carbohydrates, 32% fat, 25.5% (wt/wt) protein, 2.9 fiber, 4% ash and 6.2% water. Animal care and experimentations were approved by the Animal Care and Use Committee of The University of Tokyo (Approval Number 医-P12-111). Male mice were used for the experiments in this study.

Adipose tissue-specific Cdkal1 transgenic mice were generated on the C57BL/6J background. The mouse Cdkal1 cDNA was cloned into a plasmid containing the 5.4 kb aP2 promoter/enhancer with N-terminal FLAG tag (Figure 6). Purified linear construct was microinjected into fertilized embryos via standard pronuclear injection techniques. Founder (F0) mice carrying the Cdkal1 transgene were identified by PCR with primers listed in table 1 and further confirmed by southern blot. Subsequent offspring F1 mice were obtained by mating F0 mice with C57BL/6J mice. All offspring appeared grossly

normal. Measurement of BW and tissue weight, HE staining, gene expression level and GTT, ITT were performed in multiple groups of both lines 52 and 66. The data shown here are representative results.

Southern blot

Genomic DNA was extracted from mouse tail and digested with EcoR I at 37°C overnight, followed by loading on an agarose gel. After overnight running, digested DNA fragments were transferred to Hybond-N membrane (Amersham, #RPN303N). Then the membrane was exposed to UV radiation to permanently fix the transferred DNA to the membrane. The membrane was then placed on a similar sized roll hybridization tube. The tube was rotated in an oven at 65°C overnight, hybridized with southern probe which was labelled with (32P) using a Random Primer DNA Labeling Kit (Takara). Then the hybridized membrane was washed with SSC washing buffer, and visualized on X-ray film by autoradiography. The copy numbers of individual samples were calculated by Image J 64 software (NIH).

Oral glucose tolerance test (OGTT)

Mice were fasted for 18 hours, followed by administration of oral gavage glucose with a

working concentration of 1.5 g/kg. Blood glucose was detected by glucometer (Sanwa Kagaku Kenkyusho) at time points of 0, 15, 30, 60, and 120 min. Serum of individual mice was collected for insulin measurements at time points 0, 15, and 30 min to assess the glucose stimulated insulin secretion.

Insulin tolerance test (ITT)

Mice were fasted for 4 hours, followed by administration of intraperitoneal injection of insulin with a working concentration of 0.75 U/kg. Blood glucose was detected by glucometer (Sanwa Kagaku Kenkyusho) at time points 0, 20, 40, 60, 80, 100, and 120 min.

Human adipose tissue study

Subcutaneous adipose tissue (~1cm^3) was collected from 102 patients undergoing a gastric surgery in the University of Tokyo Hospital (71 male and 31 female subjects, the average of age was 67.5±12.5 years old, BMI was 22.3±3.0, waist was 82.6±9.0 cm). The protocol used in this study was approved by the ethics committee of the University of Tokyo and written informed consent was obtained from all the participants.

Statistical analysis

Data are presented as mean ± SD and differences were assessed using unpaired two-tailed t-tests. The null hypothesis is rejected at $p<0.05$. Statistical significance is displayed as *$P<0.05$ or **$p<0.01$.

Results

1. Generation of the Cdkal1 transgenic mice

To investigate the role of Cdkal1 *in vivo*, I generated Cdkal1 transgenic mice. First, I made retroviral expressing vectors for Cdkal1 and Cdkal1 with FLAG tag at either N-terminus or C- terminus. I confirmed that the N-terminal FLAG-tagged Cdkal1 worked equally well as the untagged Cdkal1 in terms of the effect on adipocyte differentiation, but not the C-terminal FLAG-tagged Cdkal1. Retroviral expression of both untagged and N-terminal FLAG-tagged Cdkal1 in 3T3-L1 adipocytes resulted in reduced lipid accumulation as judged by oil red o staining (Figure 3) and reduced expression of the adipogenic master regulator PPARγ (*Pparg*) and its target gene aP2 (*Fabp4*) (Figure 4). I suspected that because Cdkal1 has a hydrophobic domain close to its C terminus. Previous study revealed that deletion of the domain resulted in change in intracellular localization of Cdkal1 from ER to cytoplasm (6, 7). Therefore we speculate that the FLAG tag at the C-terminus might affect the function of this domain and cause change in intracellular localization of Cdkal1. The expression of N-terminal FLAG-tagged Cdkal1 was readily detected by Western blot using anti-FLAG and anti-Cdkal1 antibodies (Figure 5). Based on these results, I generated the construct for Cdkal1

transgenic mice. I inserted Cdkal1 mouse cDNA sequence with N-terminal FLAG tag into a transgenic cassette driven by the aP2 promoter (figure 6), which expressed in mature adipocyte specifically, as well as expressed in macrophages.

For generation of Cdkal1 transgenic mice, purified linear construct was microinjected into fertilized embryos via standard pronuclear injection techniques. The Founder Zero (F0) mice were additionally confirmed by Southern blot after PCR screening. As Figure 7 shows, the transgene creates a 2.4 kb fragment while the genome gives a 4.3 kb band using an aP2 promoter probe.

At the mRNA levels, selective expression of the Cdkal1 transgene was confirmed in brown adipose tissue (BAT) as well as subcutaneous and epididymal white adipose tissues (scWAT and eWAT), but not in liver and spleen (Figure 8). Modestly increased expression levels of *Cdkal1* were also observed in heart and skeletal muscle (SKM), which was consistent with results of a previously published paper investigating tissue expression pattern of a transgene under the control of the aP2 promoter (20). Based on the expression levels of the transgene, Line 52 and Line 66 were selected for multiplying and further investigation (Figure 9).

To detect protein expression of Cdkal1 in both wild type and Cdkal1 transgenic mice, I performed Western blotting using the anti-Cdkal1 antibody and the anti-FLAG antibody. Cdkal1 antibody detected endogenous Cdkal1 in wild type mice and enhanced Cdkal1 in transgenic mice in scWAT. FLAG-tagged Cdkal1 bands were only observed in the transgenic mice but not in the wild type mice, as expected (Figure 10). The bands of Cdkal1 expressed in adipose tissue of the transgenic mice were not located exactly at the same height as Cdkal1 expressed in 3T3-L1 adipocytes. This could be due to difference between in vivo and in vitro in the degree of modifications of Cdkal1 such as phosphorylation.

2. Characterization of the Cdkal1 transgenic mice on a normal chow

As shown in body weight curves (Figure 11), there was no apparent difference between Cdkal1 transgenic mice and the control mice in body weight during the observation period. Consistent with the body weight, no apparent difference in the tissue weight of scWAT, eWAT and BAT of the two genotypes was observed, as displayed in Figure 12. In histological analysis, as shown in the sections of H&E staining, I did not observe any significant difference between the two genotypes (Figure 13). *Cdkal1* gene expression

was increased in all adipose tissues but I did not observe significant changes in expression of other genes, including *Pparg*, *Fabp4*, and the WNT pathway target gene, *Wisp2* (Figure 14). I also conducted a glucose tolerance test but there was no statistically significant difference under this condition (Figure 15).

Overall, I did not find an overt or clear change in Cdkal1 transgenic mice compared to littermates in adulthood. There is a possibility that the effect of the transgene differs between adult and post-natal mice. Consequently, I looked at the tissues in the neonates. Both scWAT and BAT are known to rapidly develop during this period (21, 22). The results of tissue weight at Post-natal day 14 are shown in Figure 16. There was no significant difference detected between Cdkal1 transgenic neonates and the wild type neonates in body weight and mass of adipose tissues or liver tissue.

3. Characterization of Cdkal1 transgenic mice on a high fat diet (HFD)

Considering these results, I further questioned whether Cdkal1 may have an effect on the metabolism under chronic conditions. To do this, I challenged the animals with a HFD. Figure 17 showed the representative body weight curves of Line 66 and Line 52 under a HFD. Although the difference in body weight did not reach statistical

significance, the body weight of Cdkal1 transgenic mice showed a moderate tendency to be lower compared to the wild type mice. I did not observe any difference in food intake during the observation period (Figure 18).

Among the tissues I tested, the weight of scWAT of Cdkal1 transgenic mice was significantly lower than the wild type mice, and the BAT of transgenic mice had a slight tendency to be smaller (Figure 19). Histological analyses of H&E staining revealed that BAT and scWAT of Cdkal1 transgenic mice had less lipid droplet accumulation compared to wild type mice in both Line 52 and Line 66 (Figure 20 to 23). Tissue weight and histological analysis were also performed in eWAT, but no obvious difference was present (data not shown here). Consistent with histological analysis data, triglyceride content in BAT was reduced in Cdkal1 Tg mice (Figure 24). In gene expression studies, I observed increased levels of *Cdkal1*in BAT, scWAT and eWAT (Figures 25-27). I observed a small trend that *Fabp4* in scWAT and type II iodothyronine deiodinase (*Dio2*) in BAT were lower in Cdkal1 transgenic mice compared to wild type mice, but the differences of those genes did not reach statistical significance. Expression of other genes such as genes involved in lipid metabolism, inflammation and adipokines, ER stress relative genes *Chop* and *Bip,* as well as

adiponectin gene *adipoq* were also examined, but there was no significant change as far as I tested.

Next, I conducted metabolic studies, oral glucose tolerance test (OGTT) and insulin tolerance test (ITT) (Figure 28-30). The glucose levels of Cdkal1 transgenic animals were significantly lower than the wild type animals during the OGTT. Area under the curve was also significantly lower in Cdkal1 transgenic mice. There was a trend that Cdkal1 transgenic mice had lower insulin levels and the insulin resistance index, suggesting that Cdkal1 transgenic mice were more insulin sensitive. Consistent with the OGTT results, ITT experiments showed lower glucose levels in Cdkal1 transgenic mice.

4. Comparison of adipocyte differentiation and gene expression in primary adipocytes.

The results that expression of *Pparg* and *Wisp2* in adipose tissue of Cdkal1 transgenic mice did not show significant change, prompted us to examine the effect of Cdkal1 transgene in *ex vivo* adipocyte differentiation of preadipocytes isolated from adipose tissue of Cdkal1 transgenic mice. As reported previously (16), I digested the fat pads isolated from Cdkal1 transgenic and the wild type mice with collagenase, took the

stromal vascular fraction, plated them and differentiated them in culture dishes (Figure 2). I observed up to 10-fold overexpression of the *Cdkal1* transgene after differentiation (day 6), while the extent of the transgene overexpression is quite modest before differentiation (day 0) (Figure 31A). The time-dependency of the transgene expression is expected because the aP2 gene (*Fabp4*) promoter I used for the transgene expression is supposed to be activated in the latter phase of differentiation. In this condition, we did not observe significant difference in expression levels of *Pparg*, *Fabp4*, and *Wisp2* and other genes such as fatty acid synthase (*Fasn*) on day 6 (Figure 31A).There was also no difference in lipid accumulation, judged by Oil red O staining, between cells isolated from Cdkal1 transgenic mice and the wild type mice (Figure 31B). I next examined the effect of Cdkal1 overexpression in the primary adipocytes via retroviral infection that I used for the 3T3-L1 experiments. There was more than 100-fold increase of *Cdkal1* expression in Cdkal1-overexpressing cells compared to the control cells on both day 0 and day 6. Different from the aforementioned results of the primary cells from Cdkal1 transgenic mice, I observed significantly reduced expression of *Pparg* and *Fabp4*, and elevated expression of *Wisp2* at day 6 (Figure 32A) and reduced lipid accumulation as shown in oil red o staining (Figure 32B). These results were consistent with the effects of Cdkal1 overexpression in 3T3-L1 adipocytes. Based on these results, I speculated

that the discrepancy between the results of the primary cell experiments and those of the 3T3-L1 experiments may be due to the difference in the timing and/or the magnitude of the induction of Cdkal1 expression in the two methods.

5. $tRNA^{Lys}$ modification index in Cdkal1-expressing 3T3-L1 cells and Cdkal1 transgenic mice.

Tomizawa's group reported Cdkal1 is a methylthiotransferase that modifies $tRNA^{Lys}$. They have also developed a novel and simple method of quantitative PCR (qPCR) to measure $tRNA^{Lys}$ modification (15). To investigate whether modification of $tRNA^{Lys}$ was involved in the action of Cdkal1 I observed *in vitro* and *in vivo* experiments, I next performed this method on Cdkal1-overexpressing 3T3-L1 adipocytes and adipose tissues of Cdkal1 transgenic mice. As expected, I observed 80% reduction of $tRNA^{Lys}$ modification index in Cdkal1-knockdown HEK 293T cells compared to the control, which was consistent with the published result (Figure 33). There was no change in the index in Cdkal1-overexpressing HEK293T cells (Figure 33). Similarly, neither Cdkal1-overexpressing 3T3-L1 cells nor adipose tissues from Cdkal1 transgenic mice showed significant difference in the modification index (Figure 34). These data suggest that the effects of Cdkal1 in adipocytes may be independent of its action on the modification of

tRNA ^{Lys}.

6. CDKAL1 expression were lower in BAT and scWAT of *db/db* mice compared to the control mice.

To further investigate involvement of Cdkal1 in obesity and diabetes, I examined expression levels of Cdkal1 in *db/db* mice—the genetic mouse model for obesity and diabetes that lacks a functional leptin receptor. As shown in Figure 35, *db/db* mice had a much heavier body weight and a higher glucose level compared to control mice. In a gene expression level, RT-PCR analyses on adipose tissues confirmed that inflammatory cytokine genes such as *Tnfα* and *Ccl2* were increased and expression of the adiponectin gene (*Adipoq*) and *Ucp1* were decreased (Figure 36). Next, I detected the Cdkal1 expression level in *db/db* mice in both mRNA level and protein levels. Remarkably, the *Cdkal1* mRNA expression level was significantly lower in BAT compared to the wild-type mice (Figure 37). Consistent with the mRNA levels, the western blot data showed that CDKAL1 protein levels were also extremely lower in BAT and scWAT of *db/db* mice compared to the control mice (Figure 38). These data suggest that Cdkal1 may be involved in pathophysiology of *db/db* mice.

7. *CDKAL1* had an inverse correlation with BMI and waist circumference in human adipose tissue.

Finally, I investigated *CDKAL1* expression levels in subcutaneous adipose tissue of patients who underwent gastric surgery. As shown in Figure 39, *CDKAL1* exhibited a significant inverse correlation with BMI and waist circumference in human adipose tissue.

Discussion

In this study, I generated Cdkal1 transgenic mice, and found that Cdkal1 transgenic mice had a lower scWAT mass, less lipid droplet accumulation in BAT and scWAT, reduced BAT triglyceride content, and improved insulin resistance under a HFD. In adipose tissues of *db/db* mice, Cdkal1 expression was decreased in BAT and scWAT compared to control mice. *CDKAL1* also had an inverse correlation with BMI and waist circumference in human adipose tissue. These findings indicate that Cdkal1 may have a functional role in the regulation of lipid accumulation in adipose tissue and development of diabetes and obesity. In addition, in 3T3-L1 cell line, unpublished data from our laboratory showed Cdkal1 inhibits adipogenesis and lipid accumulation during adipocyte differentiation possibly via activation of the WNT pathway. For the regulation of Cdkal1, numbers of GWAS studies demonstrated that *CDKAL1* is one of the most reproducible susceptible risk genes for obesity and T2D. The SNPs of the CDKAL1 gene were located in the intron. There is no clear evidence that CDKAL1 expression is regulated by the SNPs. In our human data, there was a mild tendency of gene expression changed by the SNP we examined, but it did not reach statistical significance. In publication, Prof. Tomizawa's group argued that the disease-associated SNPs were more

profoundly correlated with expression of one of the splicing variants of CDKAL1-v1 (11). It would be interesting to examine the function of this variants as well.

Imaizumi, *et al* (10) provided the first report describing the impaired insulin secretion and ATP generation in pancreatic β cells of Cdkal1 KO mice. Okamura T, *et al*. demonstrated that global Cdkal1-deficent mice exhibited a transient and moderate reduction of body weight on a HFD and complex age-dependent alteration in insulin sensitivity. The knockout mice had impaired insulin sensitivity at an early stage, but enhanced insulin sensitivity at a later stage of life (9). In supplementary data, they also showed Cdkal1 transgenic mice with chicken β actin promoter displayed an increased body weight, which is opposite to what I observed (9). This is possibly due to the difference in the promoter to drive the Cdkal1 transgene (adipose tissue-selective vs whole body), especially considering ubiquitous expression and the functional roles of Cdkal1 in other tissues than adipose tissue, such as those in pancreatic β cells.

Cdkal1 has been reported to be a mammalian tRNA methylthiotransferase that modifies $tRNA^{Lys}$ in pancreatic β cells (23, 33). TRMT10A, another tRNA methyltransferase homolog gene in this family, enriched in pancreatic islets, was reported to be associated

with juvenile onset diabetes in humans (24). Tomizawa's group demonstrated that deficiency of Cdkal1 in pancreatic β cells resulted in a decrease in insulin secretion and impaired blood glucose levels. In Cdkal1-deficient β cells, misreading of Lys codon affects the efficient and accurate translation of glucose-stimulated proinsulin synthesis, and leads to ER stress (6, 7). Therefore, I investigated whether Cdkal1 might have a similar function in adipose tissue by using qPCR to measure $tRNA^{Lys}$ modification index as was reported recently (15). In both Cdkal1-overexpressing HEK 293T cells and Cdkal1 transgenic mice, no significant difference was observed. These data suggested that the action of Cdkal1 suppressing adipocyte differentiation may probably be independent of the action on modification of $tRNA^{Lys}$.

Our data indicated that there was no significant difference in expression levels of various genes such as *Pparg*, *Fabp4*, and *Wisp2* in adipose tissues from Cdkal1 transgenic mice and the wild type mice. I obtained similar negative results in the experiments using primary adipocytes isolated from scWAT of Cdkal1 transgenic mice. Retroviral expression of Cdkal1 in the primary adipocytes, however, resulted in significant decrease of expression levels of *Fabp4* and *Pparg*, and increased expression of *Wisp2* at day 6, and decreased lipid droplet formation, which were consistent with the

results of our 3T3-L1 experiments. In contrast to the retroviral gene delivery in *in vitro* experiments, the expression of the Cdkal1 transgene in the transgenic mice was driven by the -5.4kb aP2 (*Fabp4*) promoter. This promoter is activated in the latter phase of adipocyte differentiation. The magnitude of overexpression is also different by two methods. A possible explanation for the discrepancy between the results of the transgenic mice and those of the 3T3-L1 experiments could be resulted from the difference in the timing as well as the magnitude of the induction of Cdkal1 expression. Nevertheless, more prominent effect of Cdkal1 overexpression on insulin sensitivity could be due to other mechanisms than the action of Cdkal1 on *Pparg* expression, the WNT pathway and adipogenesis that I observed *in vitro* experiments. Although I did not observe any obvious phenotype in muscle and liver in experiments such as gene expression studies and histological examination, more detailed investigation of these tissues and whole body glucose metabolism such as clamp studies may give us a clue as to how Cdkal1 overexpression improves systemic insulin sensitivity.

Another unexplored question is the possible depot-selectivity of Cdkal1's action. Both scWAT and eWAT belong to white adipose tissue, only scWAT, but not eWAT, had lower tissue weight, less lipid droplet accumulation in Cdkal1 Tg mice compared to wild type

mice. This could be due to lower Cdkal1 expression levels in eWAT than scWAT (Fig. 14). In general, there are several differences between scWAT and eWAT. For example, scWAT is known to be susceptible to browning than eWAT in a cold challenge or stimulation by β adrenergic agonists (19, 41). In the development, scWAT is formed earlier than eWAT (21, 22). It has been established that accumulation of visceral adipose tissue is more closely associated than subcutaneous adipose tissue with metabolic syndrome and related diseases such as atherosclerosis (42-45). Further investigation is required to reveal a differential role of Cdkal1 on scWAT and eWAT.

It is also intriguing to examine whether similar downregulation of *Cdkal1* expression in adipose tissues—as in seen in the *db/db* mouse—would be observed in more physiological mouse models such as diet-induced obese mice.

In summary, I demonstrated that overexpression of Cdkal1 in adipose tissue have an impact on the mass and lipid accumulation of adipose tissues and systemic insulin sensitivity, and that the expression levels of Cdkal1 in adipose tissue is associated with adiposity in mice and human subjects. Cdkal1 in adipocytes may play a role in the development of diabetes and obesity.

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I would like to take this opportunity to express my sincere gratitude to my supervisor, Prof. Takashi Kadowaki. His encouragement, guidance, and support were innumerably valuable in the course of my Ph.D. study.

I would also like to thank Dr. Toshimasa Yamauchi and Dr. Hironori Waki, whose generous assistance and valuable discussion helped me very much throughout the production of this research. Additionally, all of other past and present members in Kadowaki lab are also acknowledged for their continuous support and warm-hearted help.

I would also like to express my gratitude to my parents, my husband and my 20-month son, for their moral support and warm encouragements in my every day and professional life.

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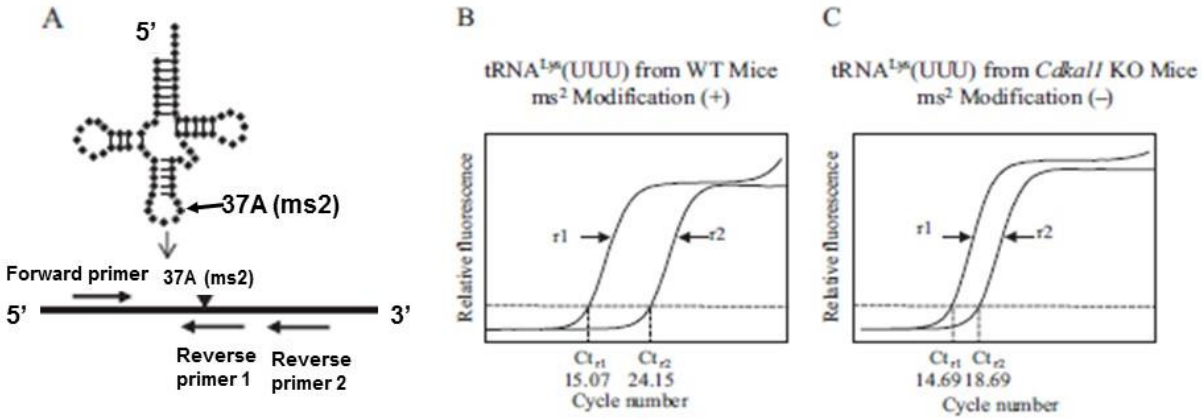
Figures



Xie P, Wei FY, Hirata S, et al. *Clin Chem*, 59 (11):1604-12,2013. (15)

Figure 1. Schematic diagram of the qPCR method for measuring ms^2 modification in tRNA^{Lys} index.

Reverse primer 1 (r1) and reverse primer 2 (r2) were used separately for reverse transcription (RT) pairing with forward primer. R1 was designed to the middle of this tRNA, including the 37A position, whereas r2 was designed to the region downstream (3') of 37A (A). qPCR data of tRNA^{Lys} from wild type (WT) mice, the Ct values of r1 and r2 are 15.07 and 24.15, separately (B). qPCR data of tRNA^{Lys} from *Cdkal1* KO mice, the Ct values of r1 and r2 are 14.69 and 18.69, separately (C).

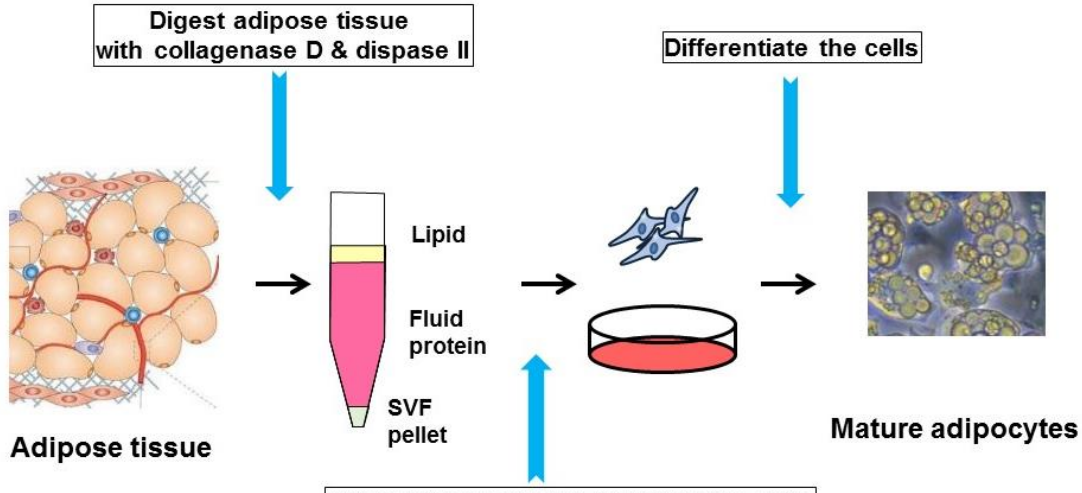


Image modified from Fig. 2 of Ouchi N et al, Nat Rev Immunol. 2011 Feb;11(2):85-972011

Figure 2. Flow chart of Isolation of primary adipocytes from adipose tissue. scWAT isolated from per 5 mice (6-8 weeks old) were mixed, and digested by collagenase D and dispase II. Followed by centrifuging (700g, 10min), the pallet (stromal vascular fraction, SVF) was dissolved, filtered by a cell strainer (50-70μm diameter), then plated into dishes, and induced to differentiate.

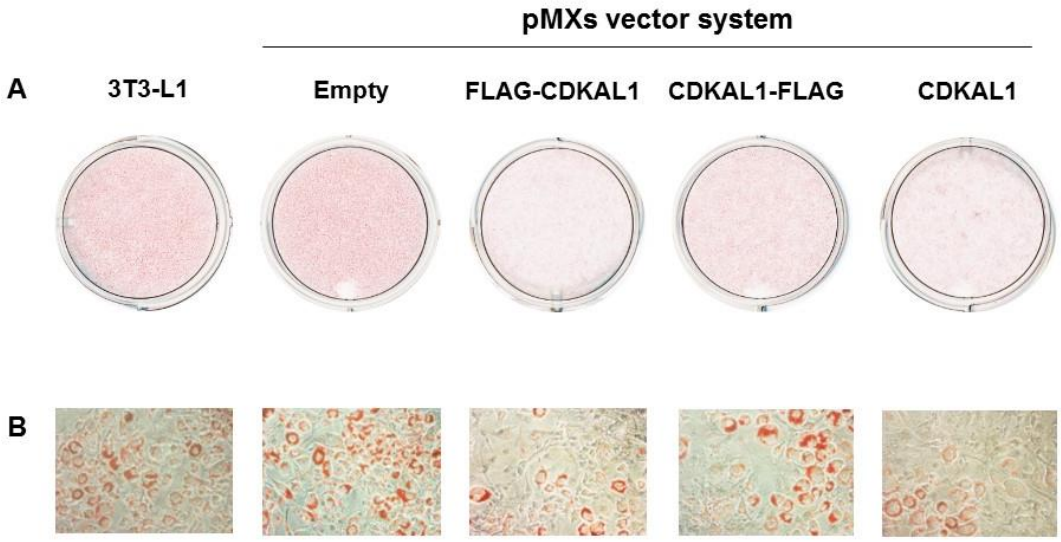


Figure 3. N-terminal FLAG-tagged CDKAL1 works equally well as untagged CDKAL1 in terms of the effect on adipocyte differentiation judged by ORO staining.

(Top panel) Oil red O staining of 3T3-L1 adipocyte, empty (3T3-L1 cells infected with pMXs empty vector), FLAG-Cdkal1 (Cdkal1-overexpressing 3T3-L1 cells with FLAG tag at N-terminus), CDKAL1-FLAG (Cdkal1-overexpressing 3T3-L1 cells with FLAG tag at C-terminus) and CDKAL1 (Cdkal1-overexpressing 3T3-L1 cells without FLAG tag), on day 7 after induced to differentiation (A). (Bottom panel) Oil red O stained cells of (A) were observed under microscope, separately (B).

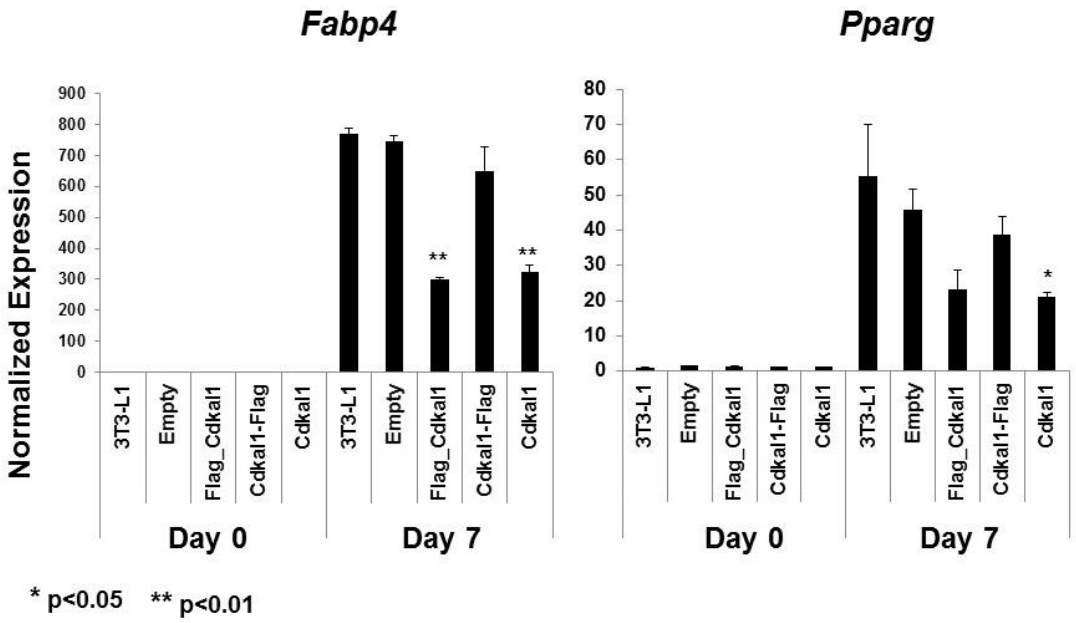
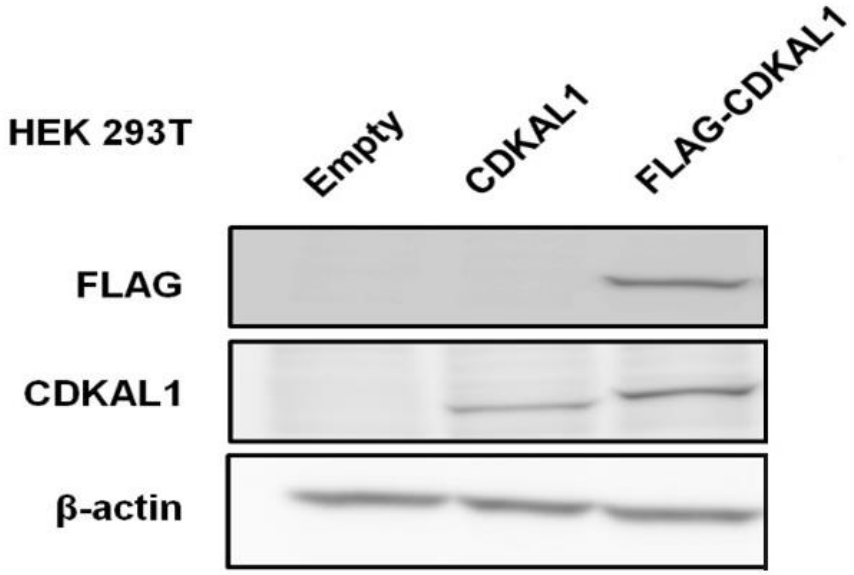


Figure 4. N-terminal FLAG-tagged CDKAL1 works equally well as untagged CDKAL1 in terms of the effect on adipocyte differentiation judged by mRNA expression.

Fabp4 (left chat) and *Pparg* (right chat) transcriptional expression were analyzed by real-time qPCR at before (day 0) and 7 days after induced to differentiation of 3T3-L1 cells and Cdkal1-overexpressing 3T3-L1 cells with FLAG tag at eight N-terminus (FLAG-CDKAL1) or C-terminus (CDKAL1-FLAG) and without FLAG tag (CDKAL1).



Figures 5. The expression of N-terminal FLAG-tagged CDKAL1 (FLAG-CDKAL1) in HEK 293T cells was detected by Western blot using anti-FLAG and anti-CDKAL1 antibodies.

FLAG and CDKAL1 protein expression in control, CDKAL1 and N-terminal FLAG-tagged CDKAL1 (FLAG-CDKAL1) overexpressed HEK 293T cells determined by western bolt.

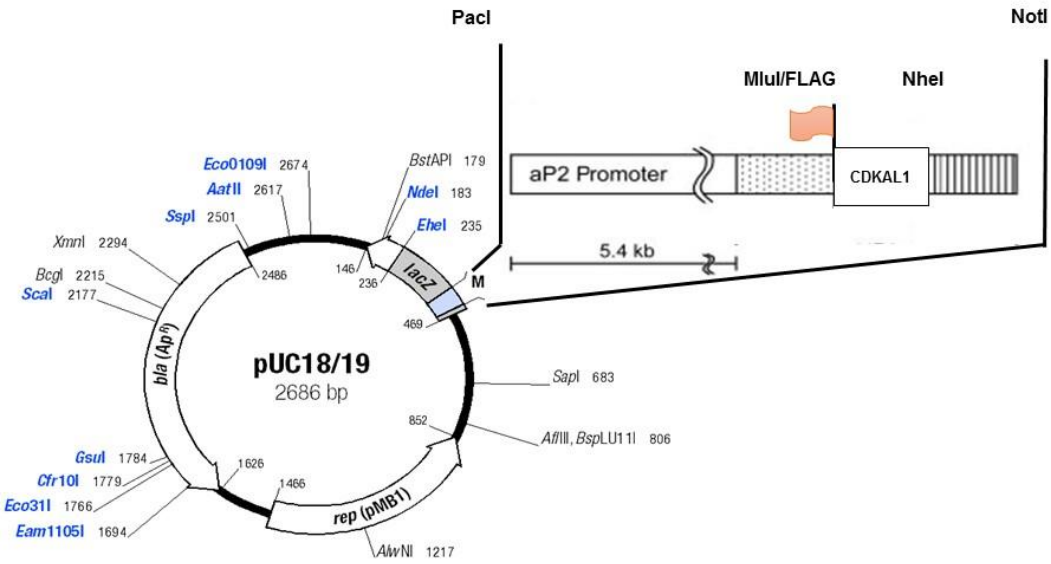


Figure 6. Diagram of the design of aP2-Cdkal1 construct with FLAG tagged at N-terminus.

Full-length cDNA (2.7kb) of Cdkal1 was created from the total RNA of eWAT isolated from C57BL/6J by reverse transcription. Tagged with N-terminal FLAG-tag, Cdkal1 cDNA was inserted into pUC19 vector (2686 bp) with the aP2 promoter (5.4kb).

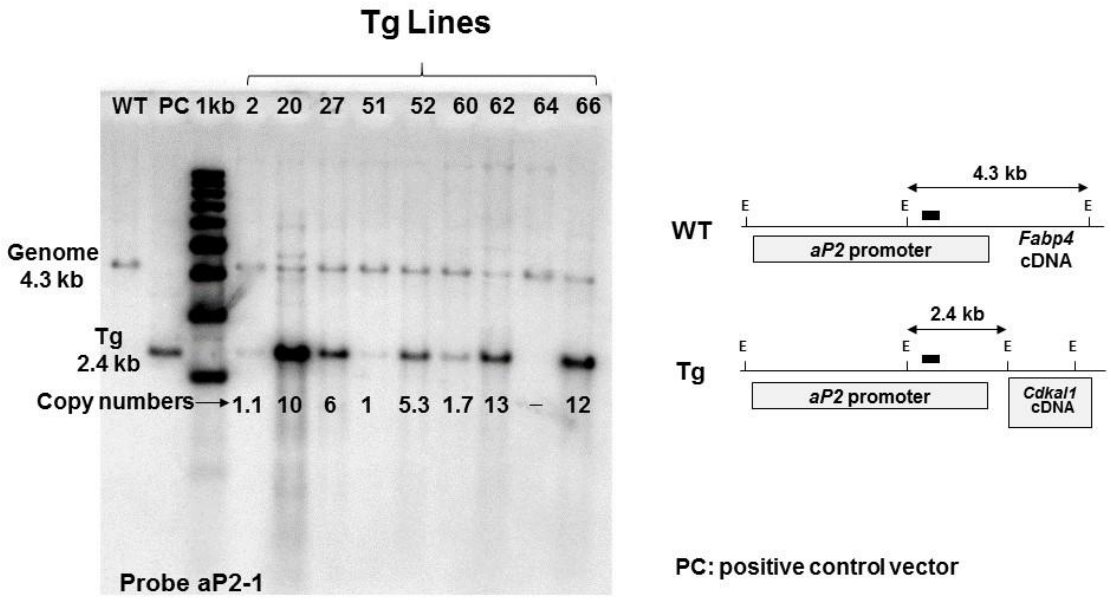


Figure 7. Southern blot of Founder Zero of aP2-Cdkal1 transgenic mice.
The right diagram shows the map for the EcoR I restriction enzyme. The transgene creates a 2.4 kb fragment while the genome gives a 4.3 kb band, when a Southern blot is performed using the aP2 promoter probe, which is shown by the black box. The left panel shows the actual results. Nine PCR screening positive F0 mice (#2, 20, 27, 51, 52, 60, 62, 64 and 66) were applied for southern. The numbers shown below the bands are estimated copy numbers.

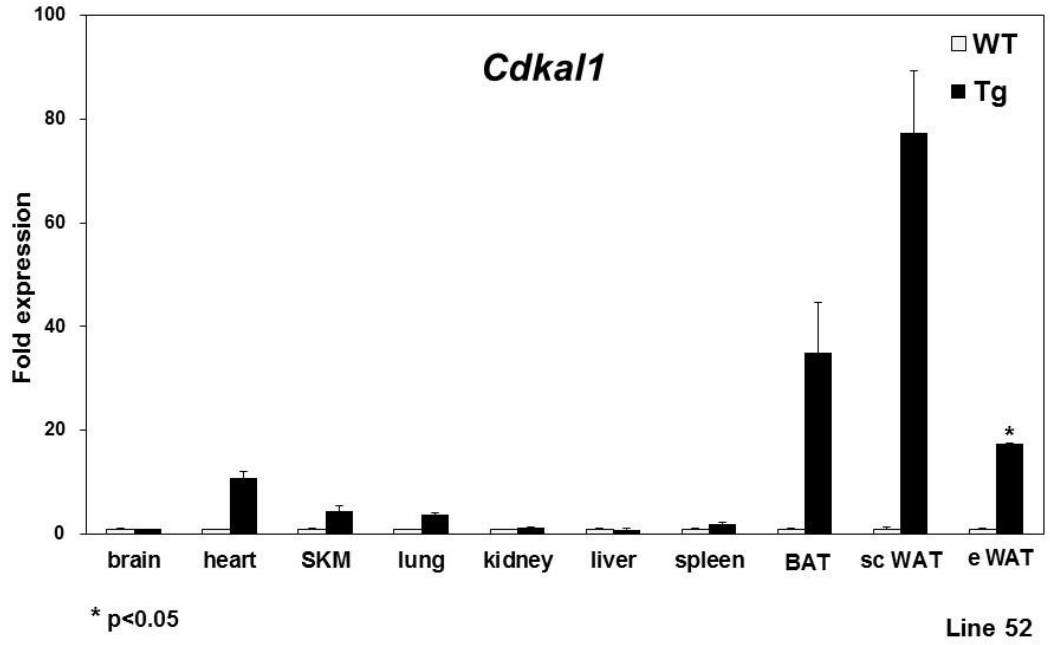


Figure 8. Selective mRNA expression of the CDKAL1 transgene was confirmed in brown and white adipose tissue, but not in liver, spleen or heart tissue.
Real-time PCR analysis of Cdkal1 gene expression in a series of tissues from Cdkal1 transgenic mice and littermate was confirmed, using the expression of 36B4 as an internal control. The Cdkal1/36B4 ratio for the brain was set as a baseline value, to which all transcript levels were normalized. N=2 each group, 5 weeks old. SKM: skeletal muscle; BAT: brown adipose tissue; scWAT: subcutaneous white adipose tissue; eWAT: epididymal white adipose tissues.

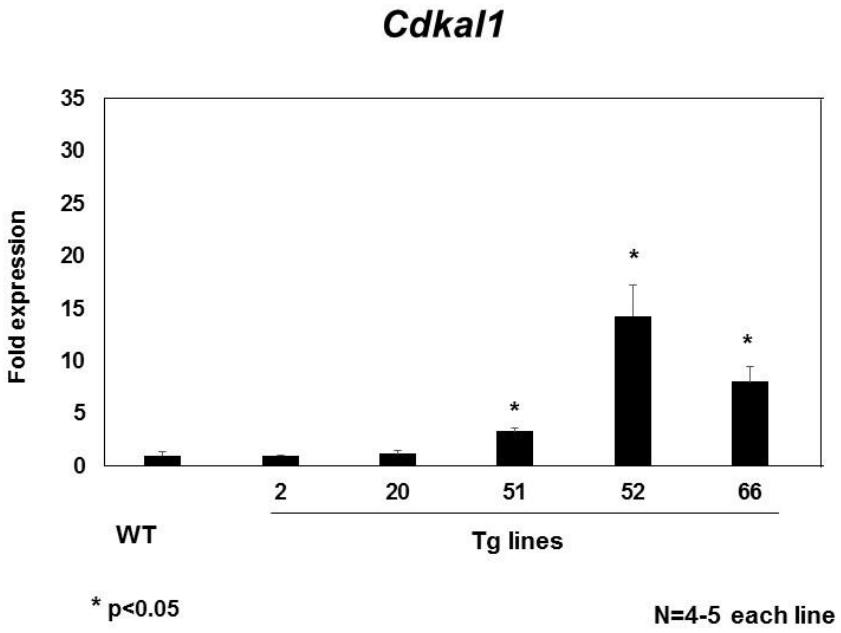


Figure 9. mRNA expression of Cdkal1 transgene in BAT from multiple transgenic mouse lines.

Real-time PCR analysis of Cdkal1 gene expression in various transgenic mouse lines was confirmed, using the expression of 36B4 as an internal control. The Cdkal1/36B4 ratio for the wild-type mouse was set as a baseline value, to which all transcript levels were normalized. N=4-5 each line, 9~11 weeks old.

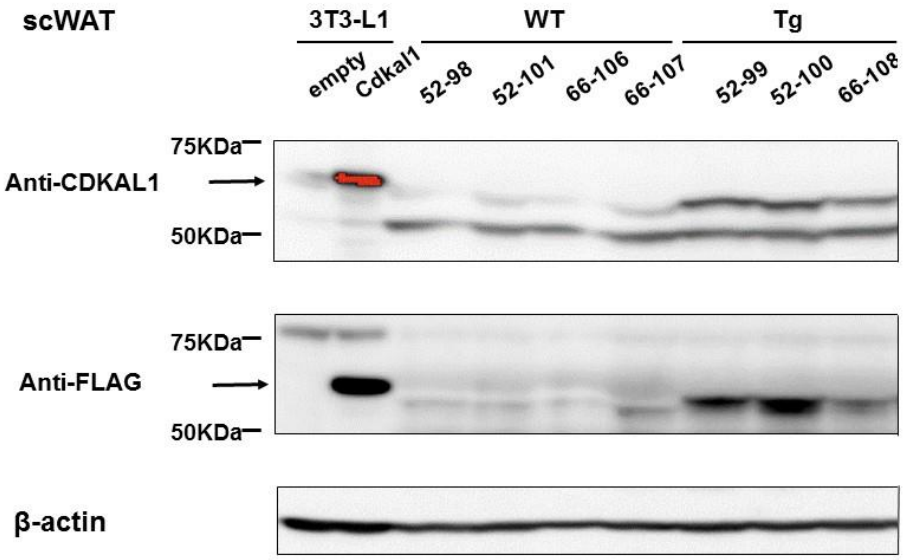


Figure 10. FLAG-tag bands and enhanced CDKAL1 bands were observed by Western bolt in Cdkal1 transgenic mice.

CDKAL1 (65KDa) and FLAG-tag (55KDa) protein level expression in scWAT of the wild type and Cdkal1 transgenic mice were determined by western blot with anti-CDKAL1 antibody and anti-FLAG antibody. “52-98”, “52-101”, “52-99” and “52-100” were the mice ID of line 52; “66-106”, “66-107” and “66-108” were the mice ID of line 66.Both wild type mice and transgenic mice were sacrificed at 5 weeks old. Exposure time 10 sec.

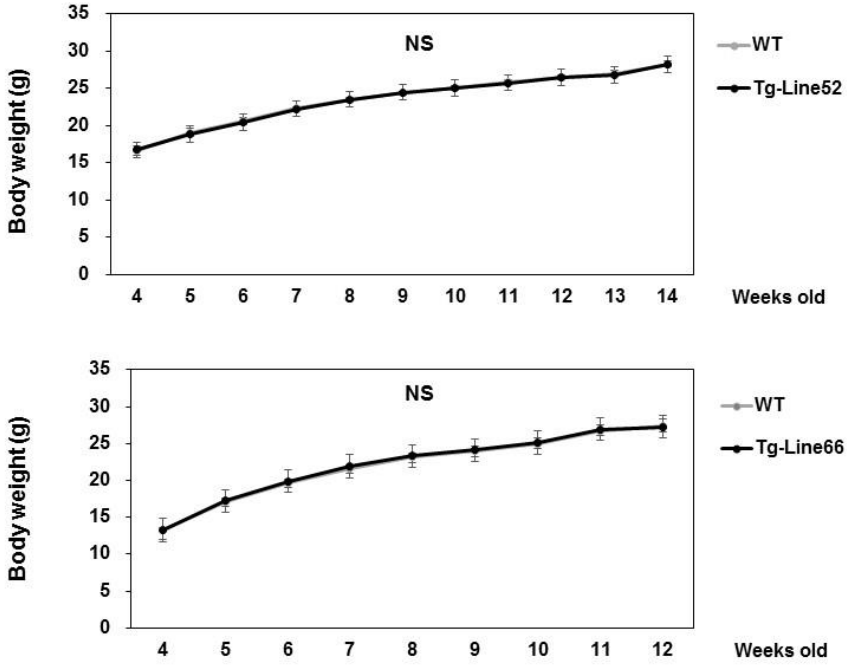


Figure 11. Body weight curves of Cdkal1 transgenic animals for lines 52 and 66.
Body weight curves of Cdkal1 transgenic mice (black line) and littermate (gray line) for lines 52 (top panel, N=7-8) and 66 (bottom panel, N=6-7) on a normal chow.

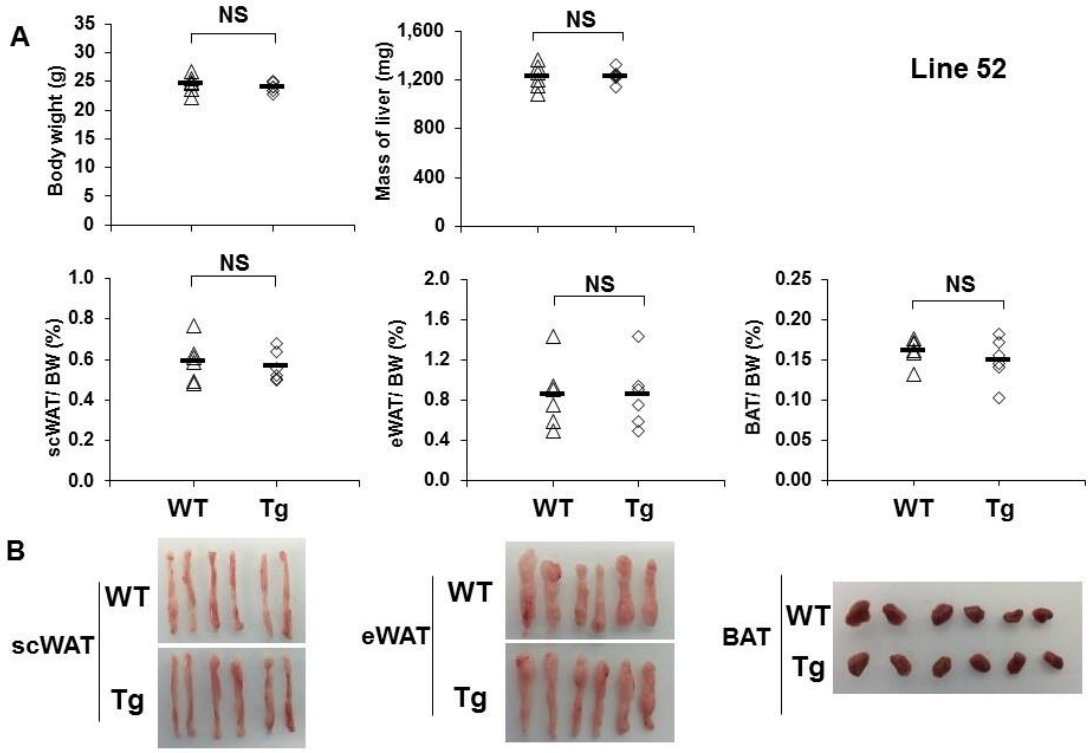


Figure 12. Body weight and the tissue weight of Cdkal1 transgenic mice and control mice.

Body weight (top panel, left), mass of liver (top panel, right), scWAT (middle panel, left), eWAT (middle panel, central) and BAT (middle panel, right) of 16 weeks old the wild-type (triangle spots) and Cdkal1 transgenic (diamond spots) mice on a normal chow (A). Representative gross of scWAT (left), eWAT (middle) and BAT (right) from the wild-type and Cdkal1 transgenic mice of (B). N=6 each group, 16 weeks old. BAT: brown adipose tissue; scWAT: subcutaneous white adipose tissue; eWAT: epididymal white adipose tissues.

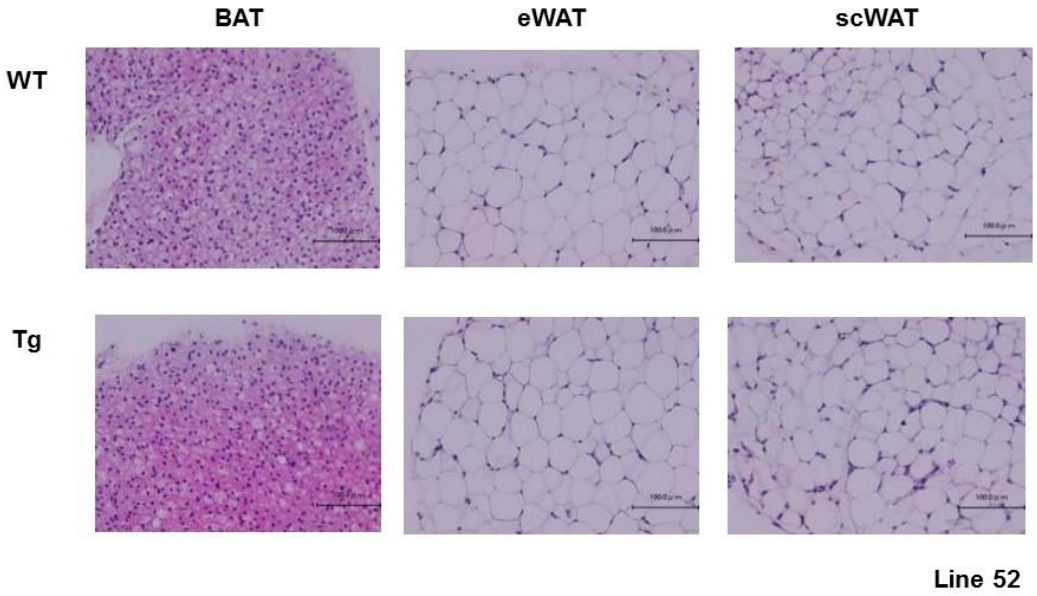


Figure 13. H&E staining of BAT, eWAT and scWAT showed the similar phenotypes between transgenic mice and wild type mice.

H&E staining of BAT, gWAT and scWAT from the wild type and Cdkal1 transgenic mice of 16 weeks old on a normal chow. BAT: brown adipose tissue; scWAT: subcutaneous white adipose tissue; eWAT: epididymal white adipose tissues.

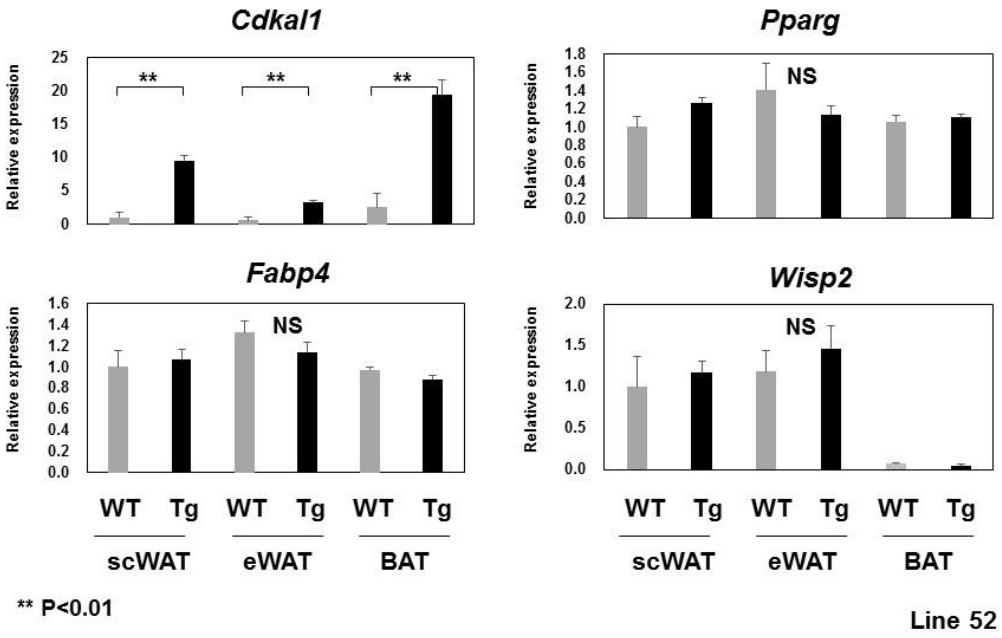


Figure 14. Gene expression profile of Cdkal1 transgenic mice compared to the wild type mice.

Real-time PCR analysis of gene expression of Cdkal1, *Pparg*, *Fabp4* and *Wisp2* in BAT, eWAT and scWAT from Cdkal1 transgenic and the wild type mice (N=6 each group). 16 weeks old. Data are presented as mean SEM. *p< 0.05. BAT: brown adipose tissue; sc WAT: subcutaneous white adipose tissue; eWAT: epididymal white adipose tissues.

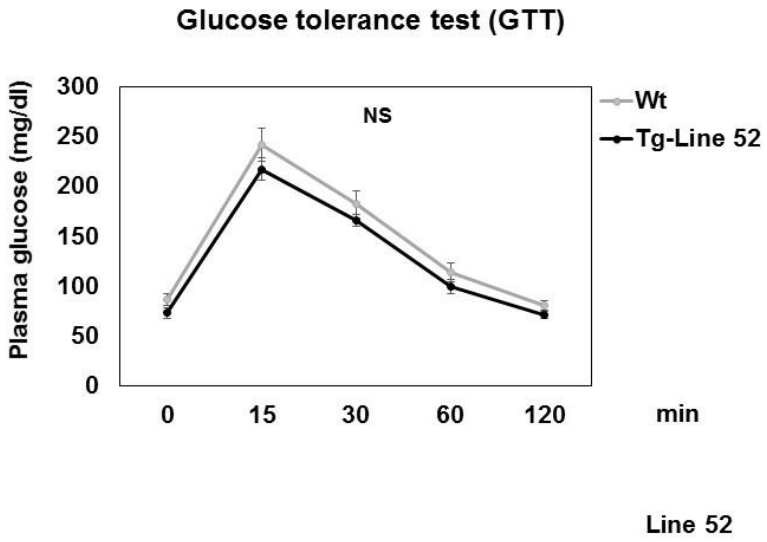


Figure 15. Significant difference was not detected between the genotypes in glucose tolerance test (GTT).

Plasma glucose level during GTT at 15 weeks old on a normal chow (N=6 each group).

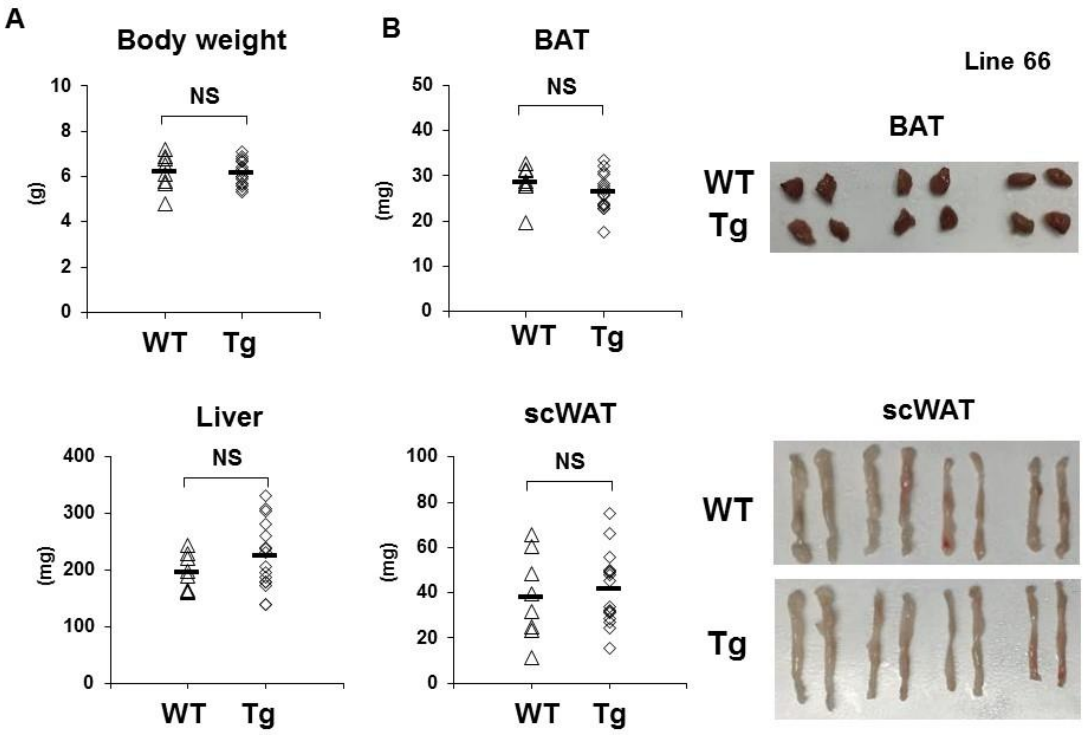


Figure 16. Body weight and tissue weight at neonates of postnatal 14 days
Body weight (top panel, left), mass of liver (bottom panel, left) in mice post-natal day 14 the wild-type (triangle spots) and Cdkal1 transgenic (diamond spots) mice (A). scWAT (bottom panel, middle), and BAT (top panel, middle), gross of scWAT (bottom panel, right) and BAT (top panel, right) from the wild-type and Cdkal1 transgenic mice (B). N=WT8: Tg18, postnatal 14 days. BAT: brown adipose tissue; scWAT: subcutaneous white adipose tissue; eWAT: epididymal white adipose tissues.

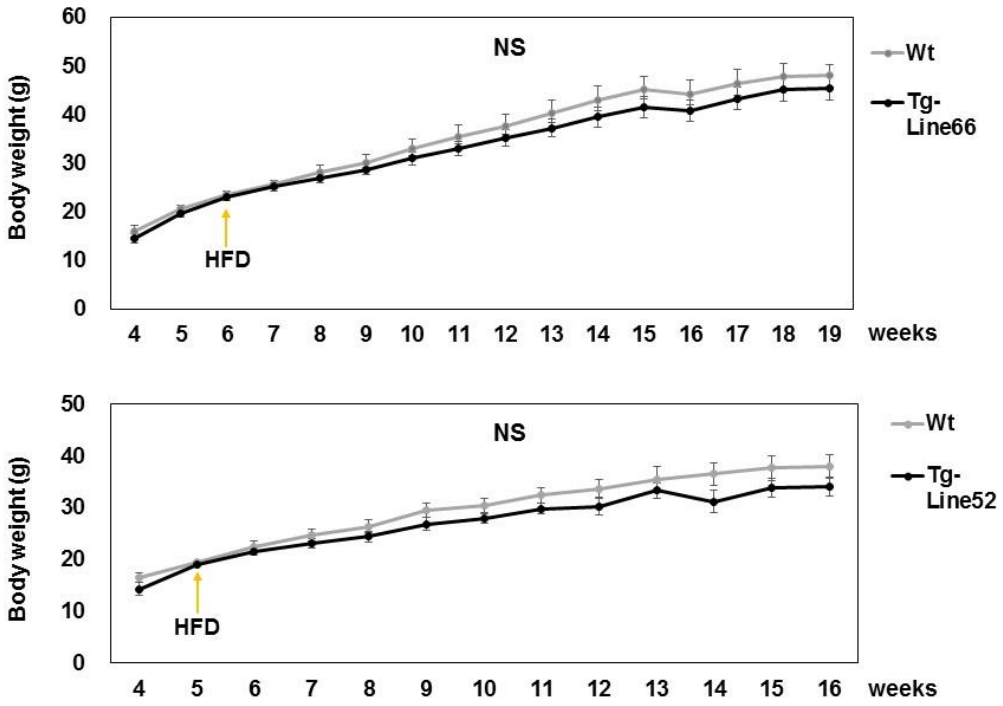


Figure 17. Body weight curves of Cdkal1 transgenic and the wild type animals for lines 52 and 66 on a HFD.

Body weight curves from Cdkal1 transgenic mice (black line) and littermate (gray line) for lines 66 (top panel, N=5-8) and 52 (bottom panel, N=6 each group) on a high fat diet from 6 weeks old throughout 19 weeks old (line 66) or from 5 weeks old throughout 16 weeks old (line 52).

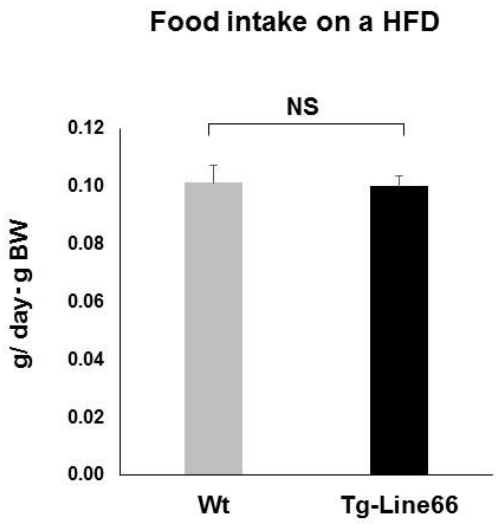


Figure 18. Food intake of Cdkal1 transgenic and the wild type mice on a HFD.
The mice were acclimated to individual cages. Food consummation was measured daily at the same time for consecutive 7 days. The amount consumed is calculated normalized by body weight (N=6 each group, 8 weeks old).

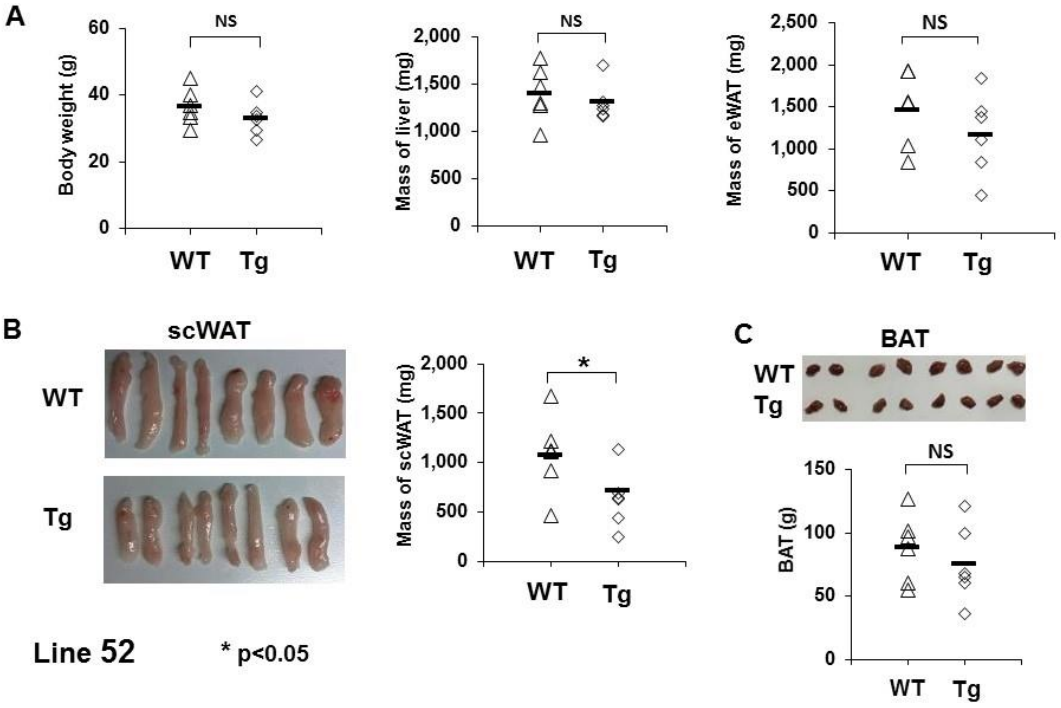


Figure 19. Body weight and the tissue weight of transgenic mice and littermate on a HFD.

Body weight (top panel, left), mass of liver (top panel, middle) and eWAT (top panel, right) from Cdkal1 transgenic mice and the wild type mice of 16 weeks old mice on a HFD (A). Representative gross (bottom panel, left) and mass of scWAT (bottom panel, middle) from the wild-type and Cdkal1 transgenic mice of (B). Representative Gross (right, middle panel) and mass of BAT (right, bottom panel) from mice of (C). N=6 each group, 16 weeks old (HFD for 12 weeks). BAT: brown adipose tissue; sc WAT: subcutaneous white adipose tissue; eWAT: epididymal white adipose tissues.

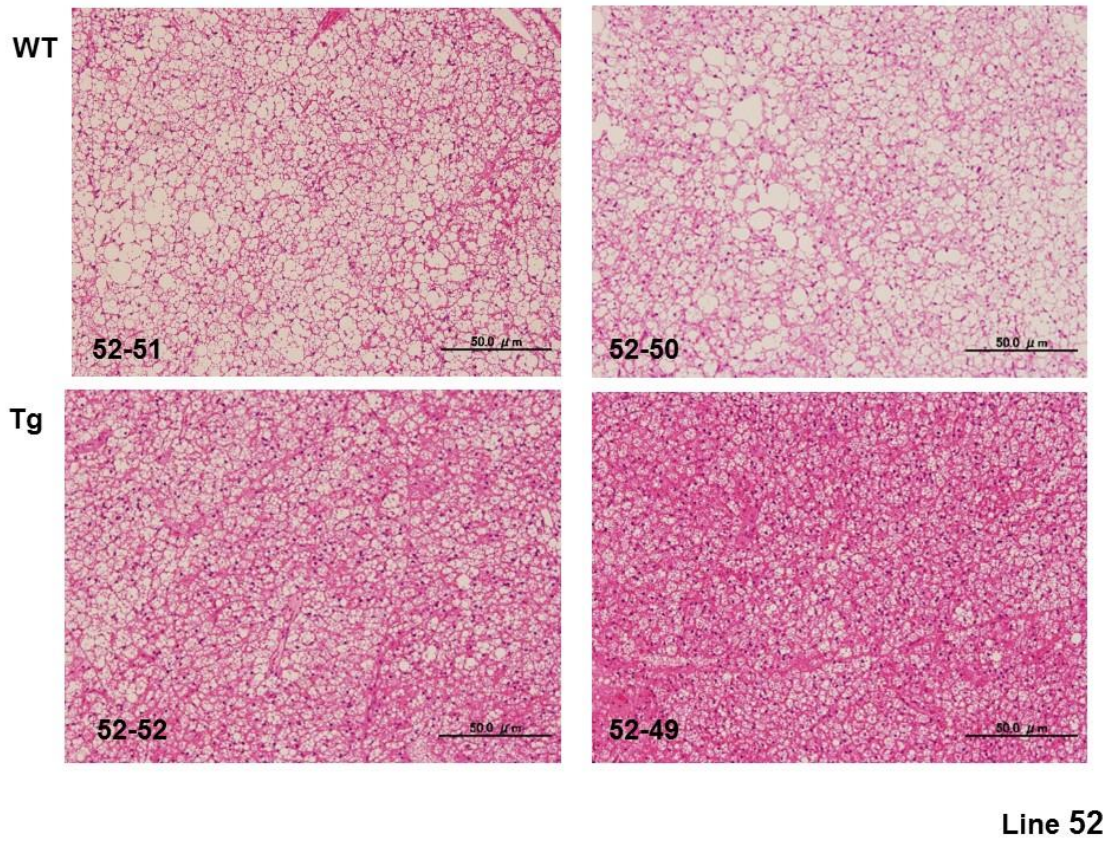


Figure 20. A low lipid droplets accumulation in BAT of the Cdkal1 transgenic mice in histology analysis.

Representative H&E staining of BAT from the wild type and Cdkal1 transgenic mice on a HFD. “52-49”, “52-50”, “52-51” and “52-52” were the mice ID of line 52, of which “52-51”, “52-52” were wild type mice, and “52-52” , “52-49” were transgenic mice. All of the mice were 16 weeks old (HFD for 12 weeks).

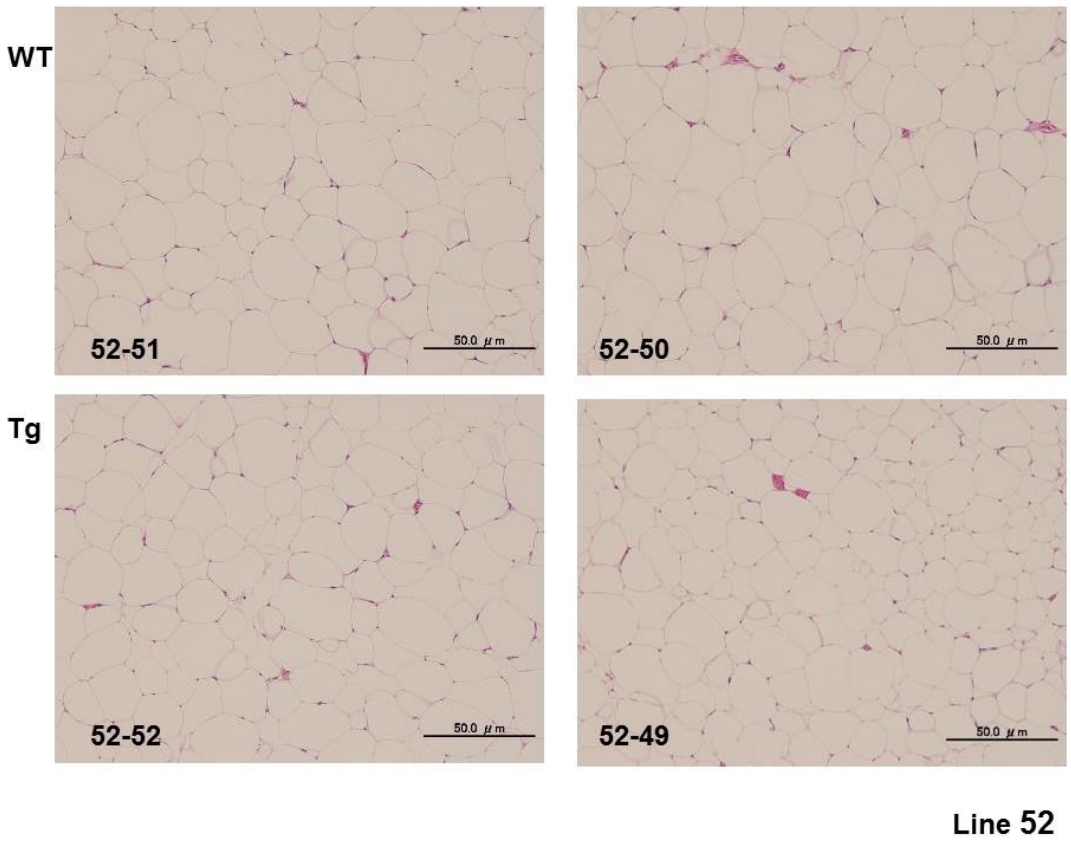


Figure 21. A smaller lipid droplets were displayed in scWAT of the Cdkal1 transgenic mice in histology analysis.

H&E staining of scWAT from the wild-type and Cdkal1 transgenic mice of 16 weeks old on a HFD for 12 weeks. “52-49”, “52-50”, “52-51”, and “52-52” were the mice ID of line52. All of the mice were 16 weeks old (HFD for 12 weeks).

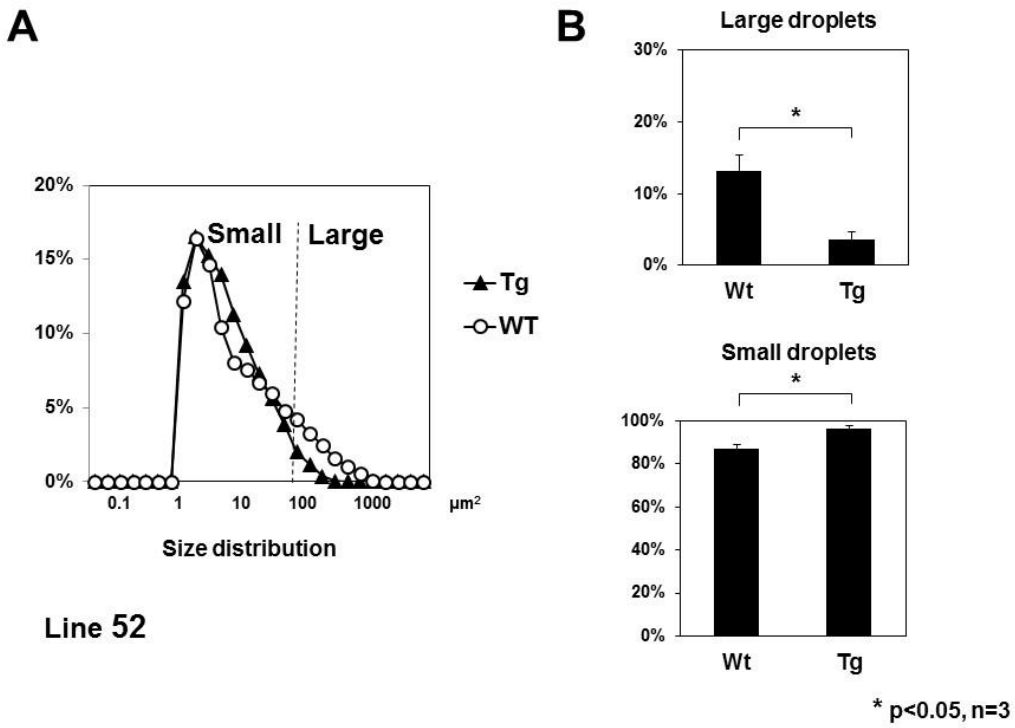


Figure 22. Histograms of lipid droplet size distribution of BAT.

H&E staining images were analyzed by image J (NIH). For each section, four images were taken, total number of 600~2000 cells were analyzed for each mouse, three mice in each group (A). The adipocytes were classified into large size (≥ 72μm2) and small size (<72μm2). Data are presented as mean SEM. *p< 0.05 (B). 16 weeks old (HFD for 12 weeks).

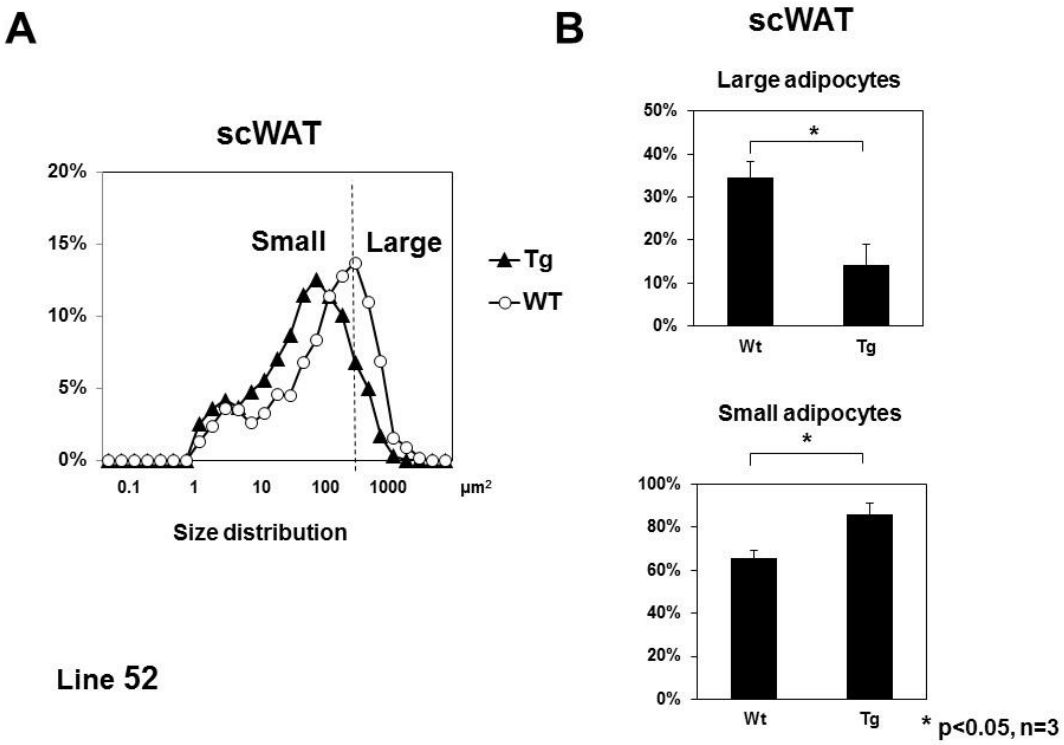


Figure 23. Histograms of lipid droplet size distribution of scWAT.

H&E staining images were analyzed by image J (NIH). For each section, four images were taken, total number of 600~2000 cells were analyzed for each mouse, three mice in each group (A). The adipocytes were classified into large size ($\leq 453\mu m^2$) and small size ($>453\mu m^2$). Data are presented as mean SEM. *p< 0.05 (B). 16 weeks old (HFD for 12 weeks).

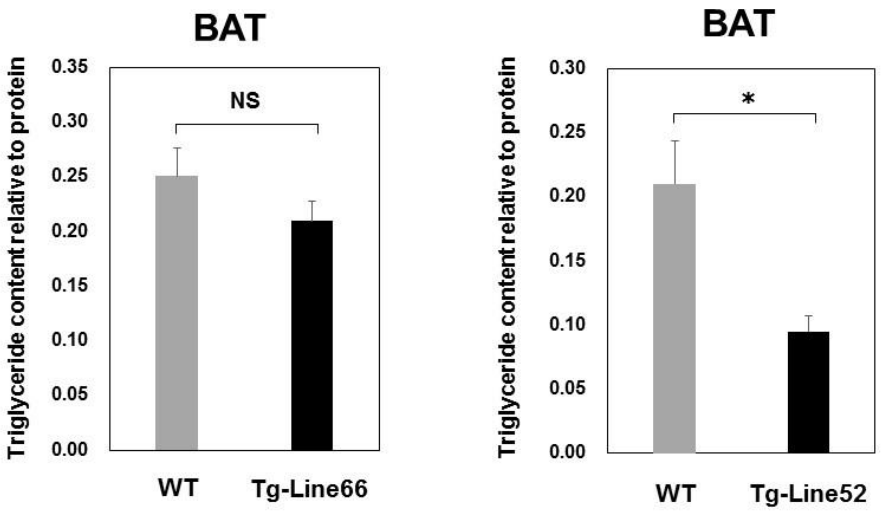


Figure 24. Triglyceride content of BAT was reduced in Tg mice on a HFD.
BAT was isolated from Cdkal1 Tg mice and WT mice. Less than 100 mg BAT was applied for measuring. Finally triglyceride content relative to protein level was calculated. Line 66 (left chat) N=5~8, 19 weeks old (HFD for 14 weeks). Line 52 (right chat) N=6, 16 weeks old (HFD for 12 weeks).

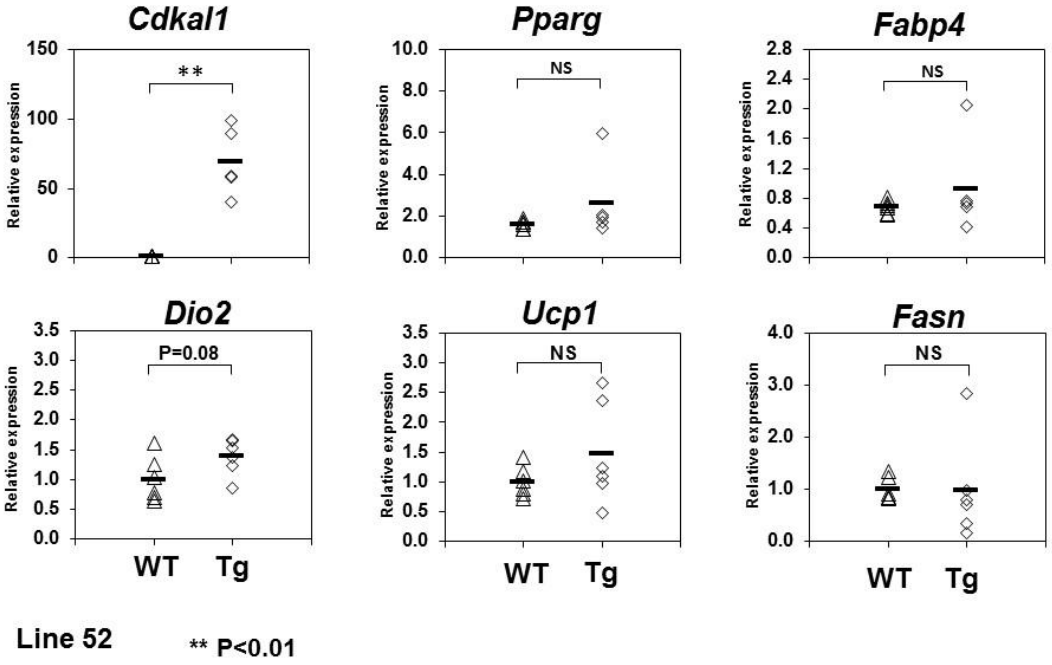


Figure 25. Comparison of mRNA expression in BAT between WT and Tg mice on a HFD (by qPCR).

Real-time PCR analysis of gene expression of Cdkal1, *Pparg*, *Fabp4* and UCP1 in BAT from Cdkal1 transgenic and the wild type mice, N=6 each group, 16 weeks old (HFD for 12 weeks). Data are presented as mean SEM. *p< 0.05.

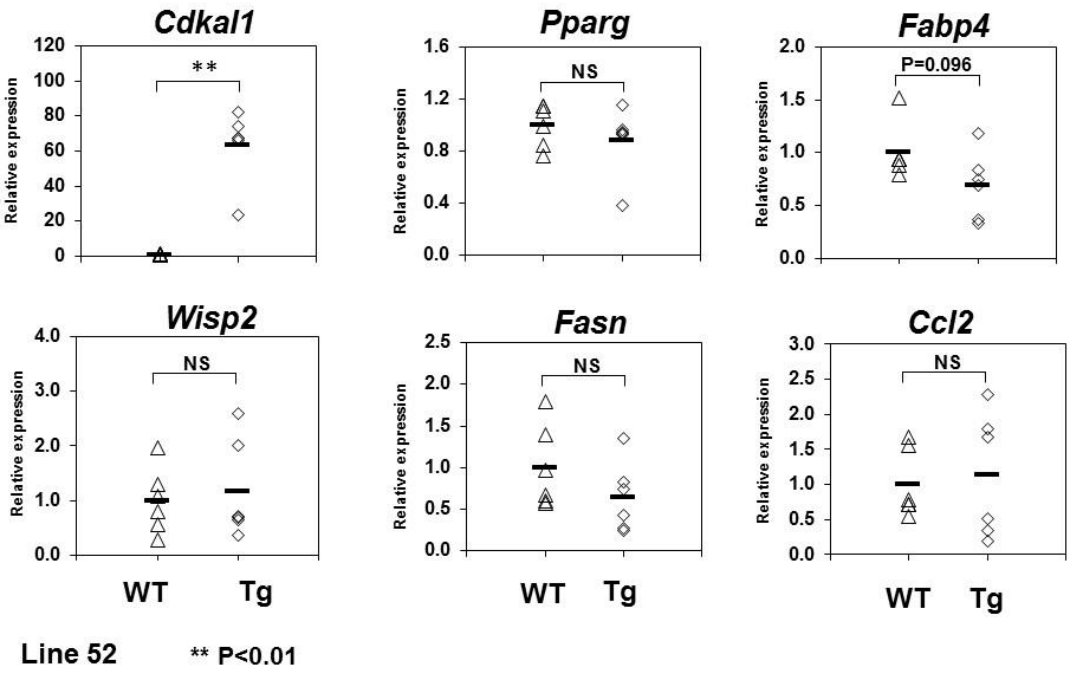


Figure 26. Comparison of mRNA expression in scWAT between WT and Tg mice on a HFD (by qPCR).

Real-time PCR analysis of gene expression of Cdkal1, *Pparg*, *Fabp4*, *Fasn* and *Wisp2* in scWAT from Cdkal1 transgenic and the wild type mice, N=6 each group, 16 weeks old (HFD for 12 weeks). Data are presented as mean SEM. *p< 0.05.

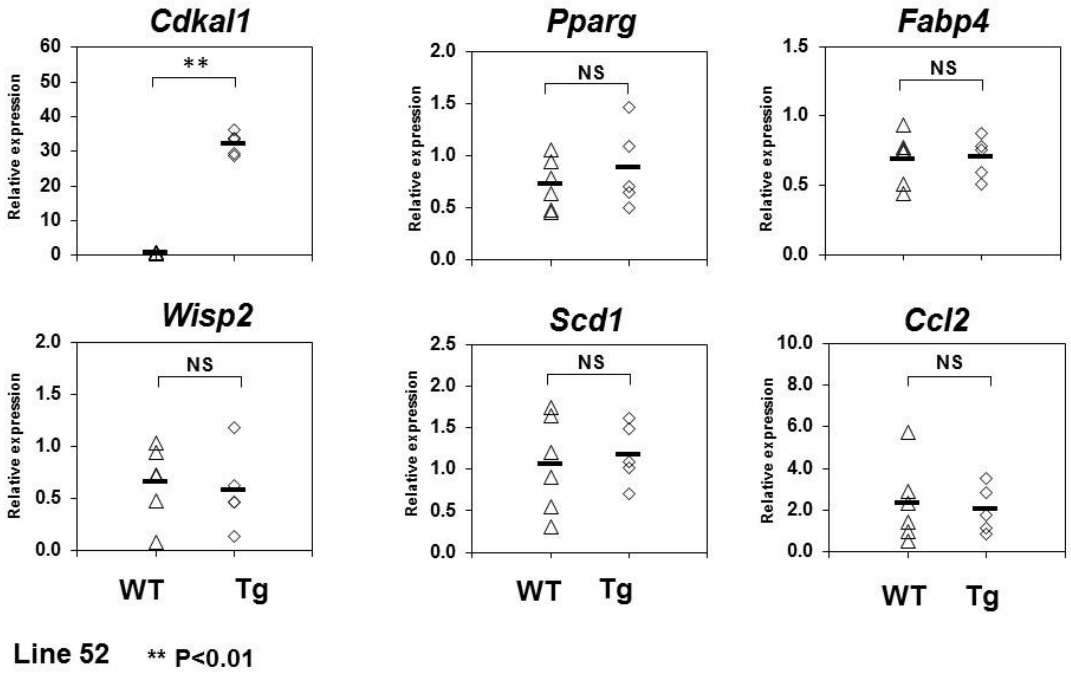


Figure 27. Comparison of mRNA expression in eWAT between WT and Tg mice on a HFD (by qPCR).

Real-time PCR analysis of gene expression of Cdkal1, *Pparg*, *Fabp4*, *Scd1* and *Wisp2* in eWAT from Cdkal1 transgenic and the wild type mice, N=6 each group, 16 weeks old (HFD for 12 weeks). Data are presented as mean SEM. *p< 0.05.

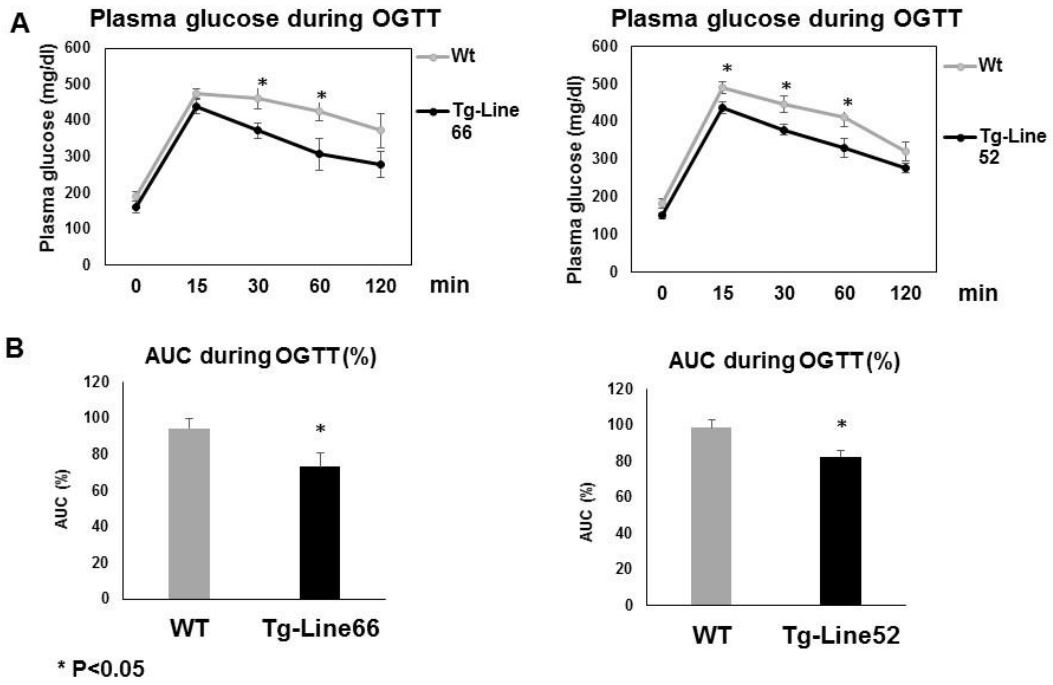


Figure 28. Plasma glucose during OGTT under a HFD of Cdkal1 transgenic and the wild type mice.

Plasma glucose of Line 66 (left top panel, N=5-8, 19 weeks old, HFD for 14 weeks) and Line 52 (right top panel, N=6 each groups, 16 weeks old, HFD for 12 weeks) in 0min, 15min, 30min, 60min, 120min after intake a concentration of 1.5g/kg glucose (A). AUC of the curves of (A) during OGTT. The wild type mice (grey bar) and Cdkal1 transgenic mice (black solid bar) (B).

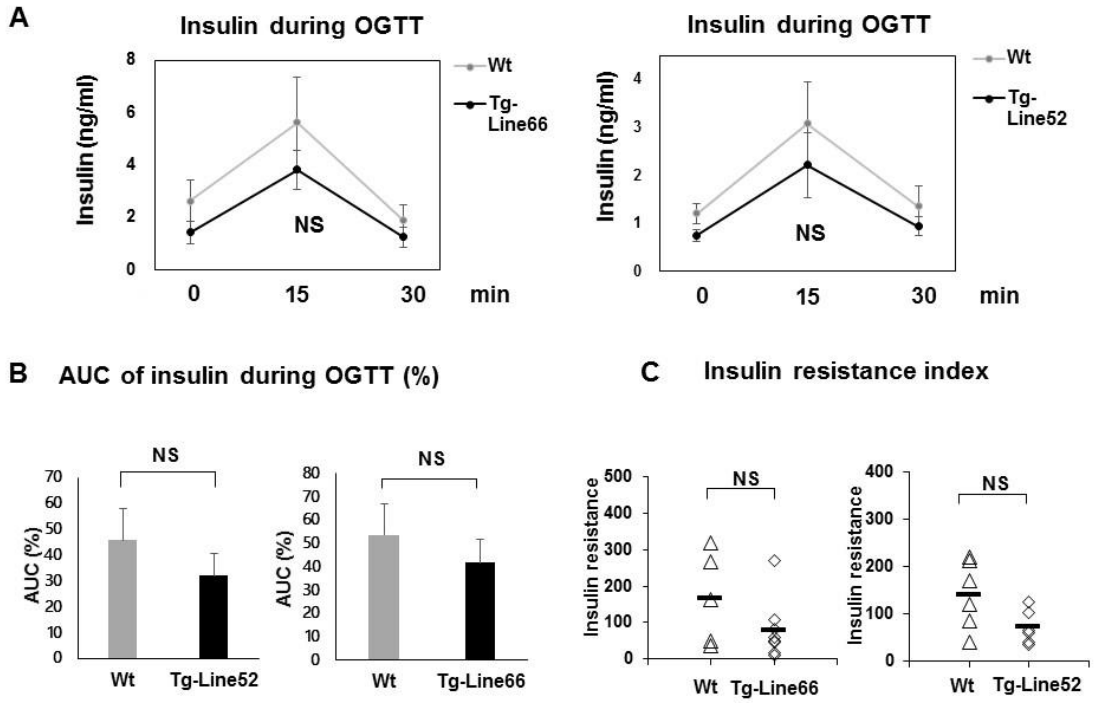
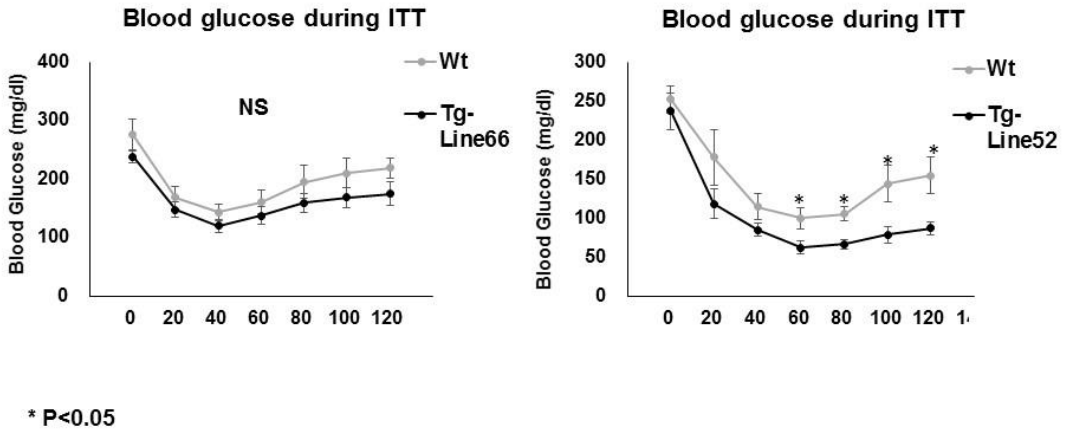


Figure 29. Insulin and insulin resistance index during OGTT of Cdkal1 transgenic and the wild type mice on a HFD.

Plasma insulin of Line 66 (left top panel, N=5-8, 19 weeks old, HFD for 14 weeks) and Line 52 (right top panel, N=6 each group, 16 weeks old, HFD for 12 weeks) in 0min, 15min, 30min after intake a concentration of 1.5g/kg glucose (A). AUC of the curves of (A) during OGTT. (C) Insulin resistance index during OGTT of (B). Insulin resistance index=Fasting glucose (mg/dl) x insulin (ng/mL).



* P<0.05

Figure 30. Plasma glucose during ITT under a HFD.

Plasma glucose of Line 66 (left side, N=5-8, 19 weeks old, HFD for 14 weeks) and Line 52 (right side, N=6 each groups, 16 weeks old, HFD for 12 weeks) in 0min, 20min, 40min, 60min, 80min, 100min, 120min after inject a concentration of 0.75U/kg insulin. The wild-type mice (grey curve) and Cdkal1 transgenic mice (black solid curve).

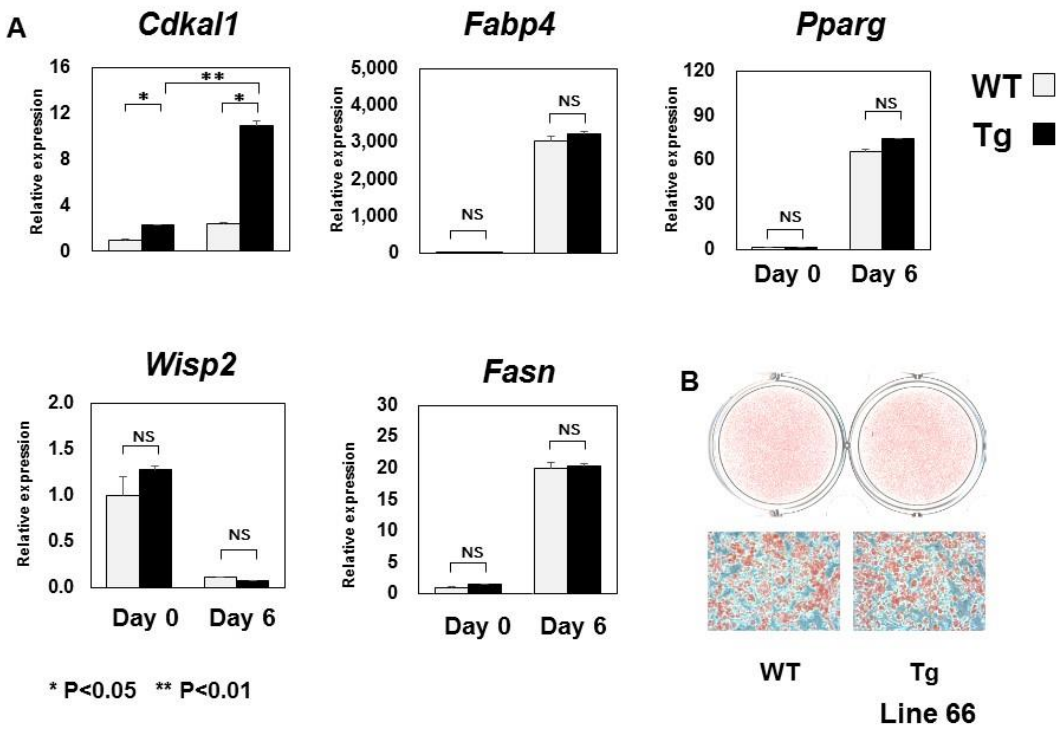


Figure 31. Gene expression and lipid accumulation of primary adipocytes isolated from the wild-type and Cdkal1 transgenic mice.

scWAT primary cells isolated from 5 mice (6-8 weeks old) of each group were mixed, digested, and plated into dishes, induced to differentiate. The expression of genes were analyzed by real-time qPCR before (day 0) and 6 days after induction (day 6). Data are presented as mean SEM. *p< 0.05, **p< 0.01, N=2 (A). Oil red O staining (bottom panel, right) of primary cells from (A), day 6 after differentiation (B).

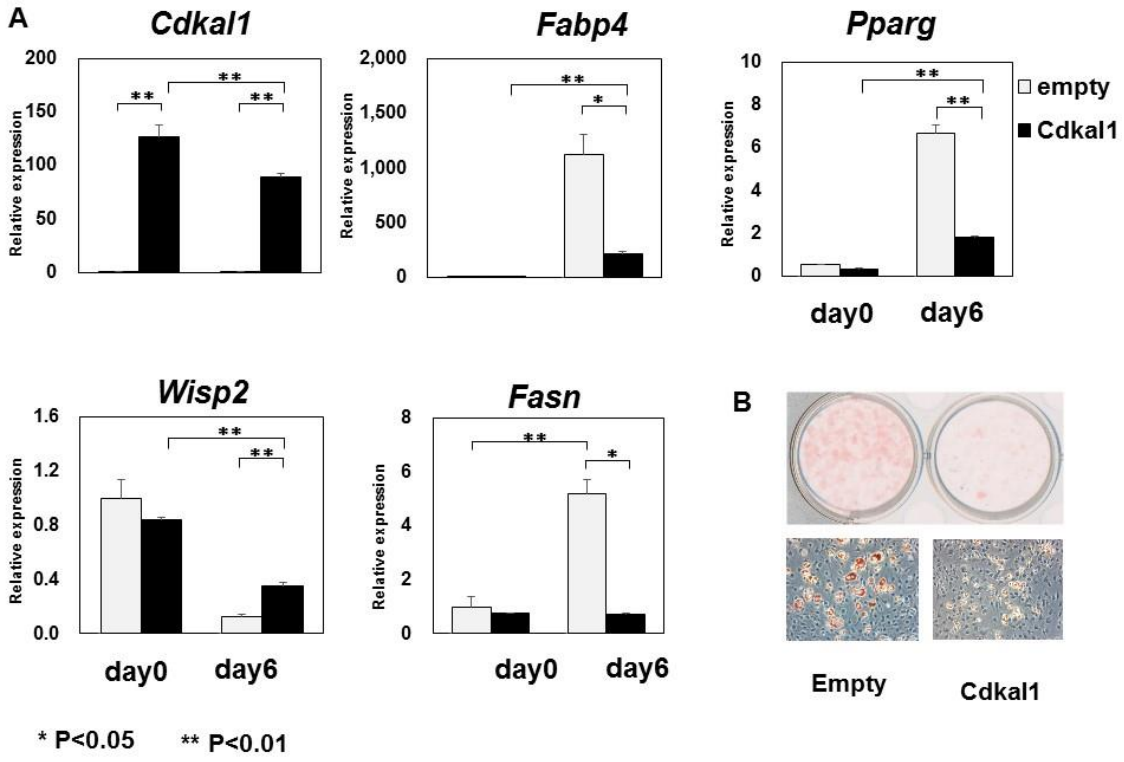


Figure 32. Retrovirus-mediated overexpression of Cdkal1 in primary adipocytes isolated from wide type mice.

Real-time PCR analysis of genes expression in scWAT primary adipocytes with control or Cdkal1-expressing retroviral vectors at before (day 0) and 6 days after induction (day 6). Data are presented as mean SEM. *p< 0.05, **p< 0.01 (A). Oil red O staining (bottom panel, right) of scWAT primary cells with control or Cdkal1-expressing retroviral vectors 6 days after induction of differentiation (N=3) (B).

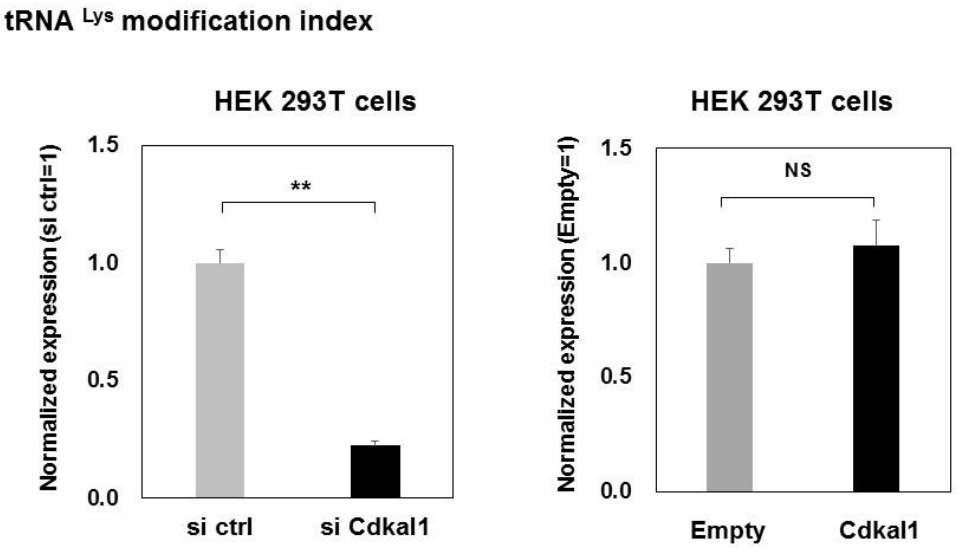


Figure 33. Comparing of tRNA^{Lys} modification index in Cdkal1 knockdown and Cdkal1-overexpressing HEK 293T cell.

Real-time PCR analysis of tRNA^{Lys} modification index in HEK 293T cells, with control or siCdkal1, and control or Cdkal1-expressing retroviral vectors. Cells were collected 48 hours after transfection. Data are presented as mean SEM. **p< 0.01, N=3. HEK293T cells used for this experiment were the same as used for the western blot of figure 5. Therefore, overexpression of Cdkal1 was confirmed.

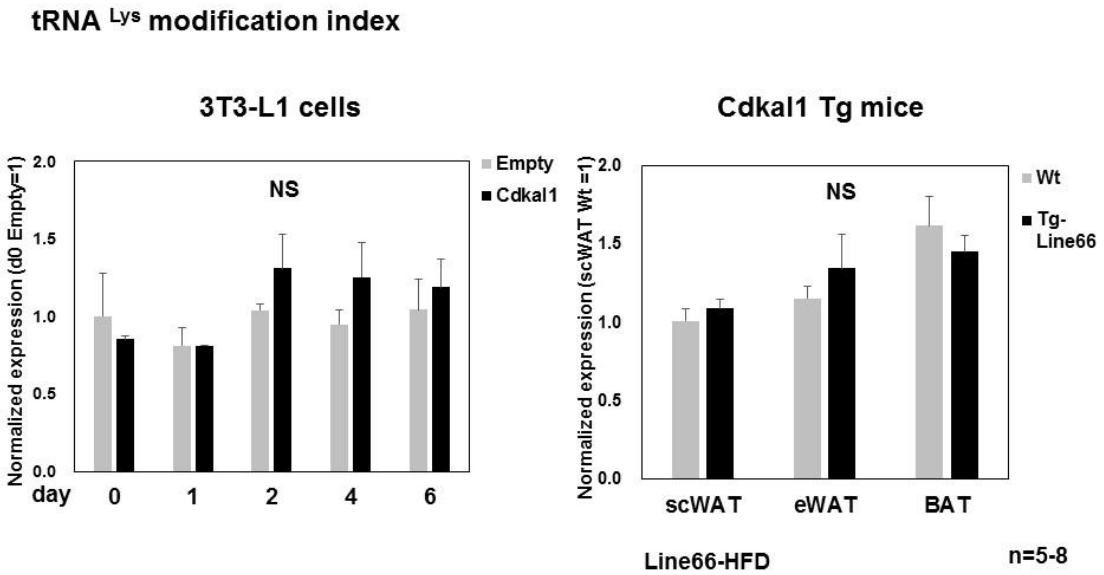


Figure 34. tRNA^{Lys} modification index of Cdkal1-overexpressing 3T3-L1 cells and Cdkal1 transgenic mice.

Real-time PCR analysis of tRNA^{Lys} modification index in 3T3-L1 cells with control or Cdkal1-expressing retroviral vectors at before (day 0) and 0.5, 1, 2, 4, 6 days after induction (left chart). Real-time PCR analysis of tRNA^{Lys} modification index of scWAT, eWAT and BAT in Cdkal1 transgenic mice and littermate on a HFD (right chart), N=5-8, 19 weeks old, HFD for 14 weeks.

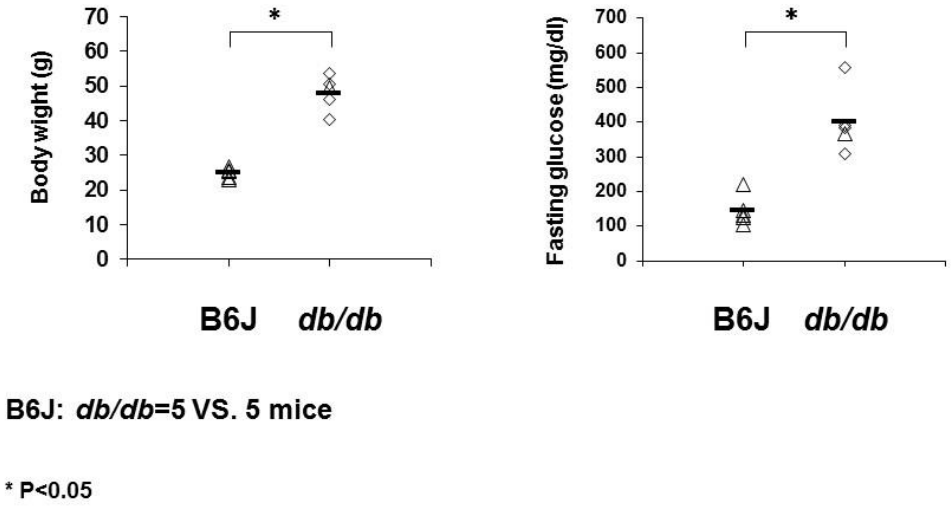


Figure 35. Body weight and blood glucose of *db/db* mice compared to control mice. Body weight (left) and 18h fasting glucose (right) of *db/db* mice (diamond plots) and control mice (triangle plots) of 15 weeks old. N=5 each groups. Data are presented as mean SEM. *p< 0.05.

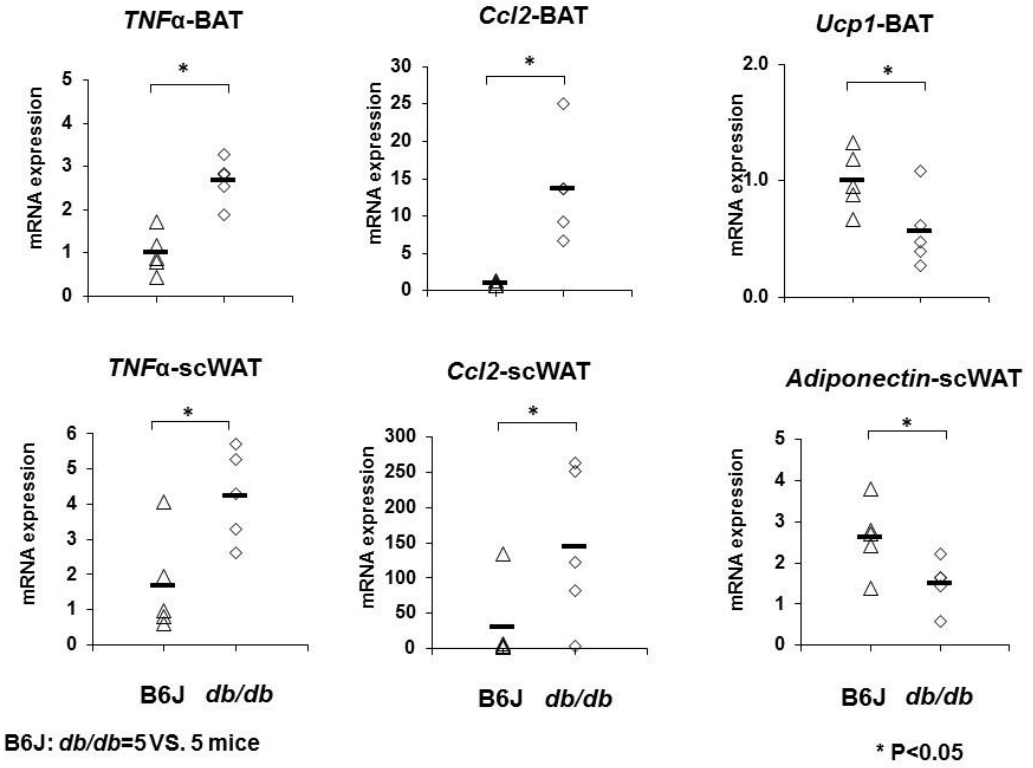


Figure 36. Gene expressions of *db/db* mice compared to control mice.

Tnfα (left), *Ccl2* (middle), *Ucp1* (right) mRNA expression level in BAT (top panel) and *Tnfα* (left), *Ccl2* (middle), *Adipoq* (right) expression in scWAT were analyzed by real-time qPCR. N=5 each group, 15 weeks old. Data are presented as mean SEM. *p< 0.05.

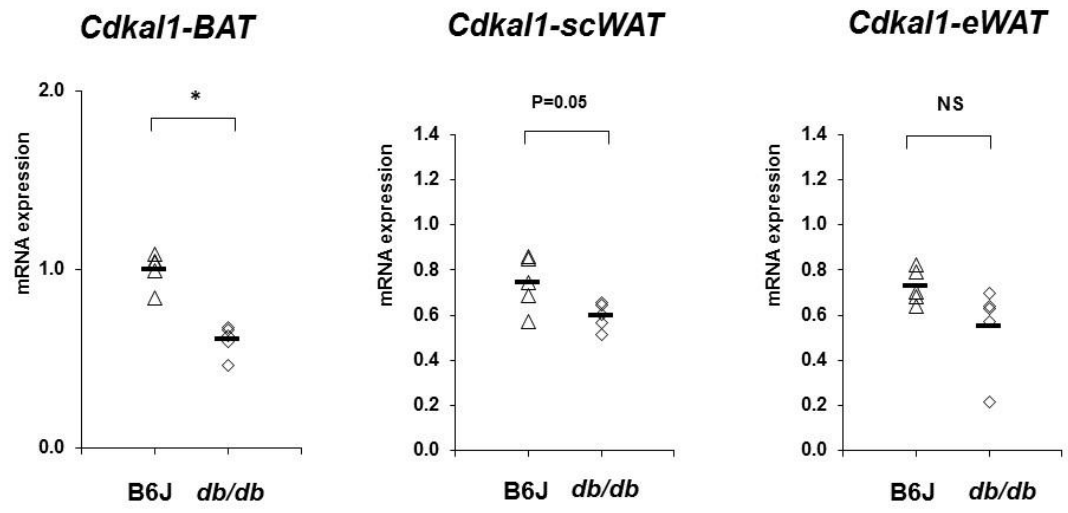


Figure 37. *Cdkal1* mRNA expression in *db/db* mice compared to control mice.
Cdkal1 mRNA expression was measured by real-time qPCR in BAT (left), scWAT (middle) and eWAT (right). N=5 each group, 15 weeks old. Data are presented as mean SEM. *p< 0.05.

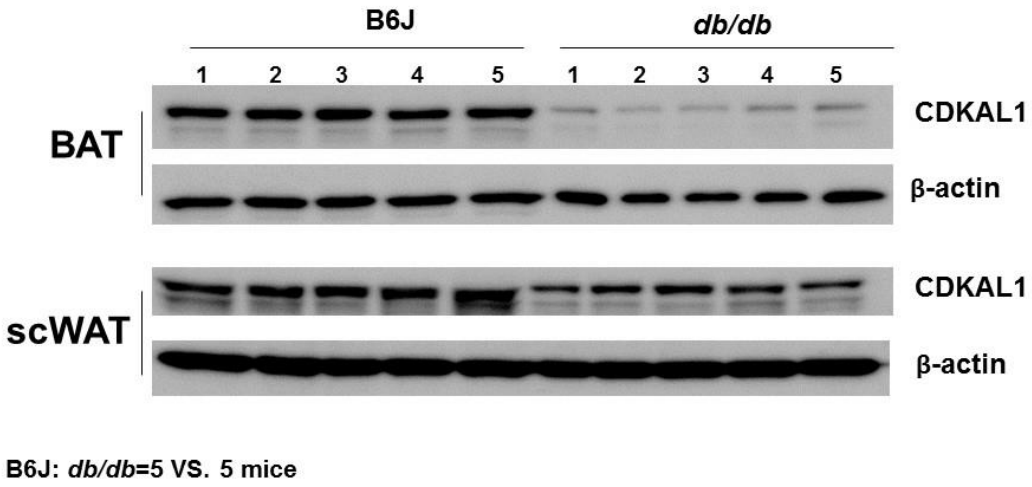


Figure 38. CDKAL1 protein level in *db/db* mice compared to control mice.
CDKAL1 protein expression was measured by western blot in BAT (upper panel) and scWAT (lower panel). N=5 each group, 15 weeks old. Exposure time 1 min.

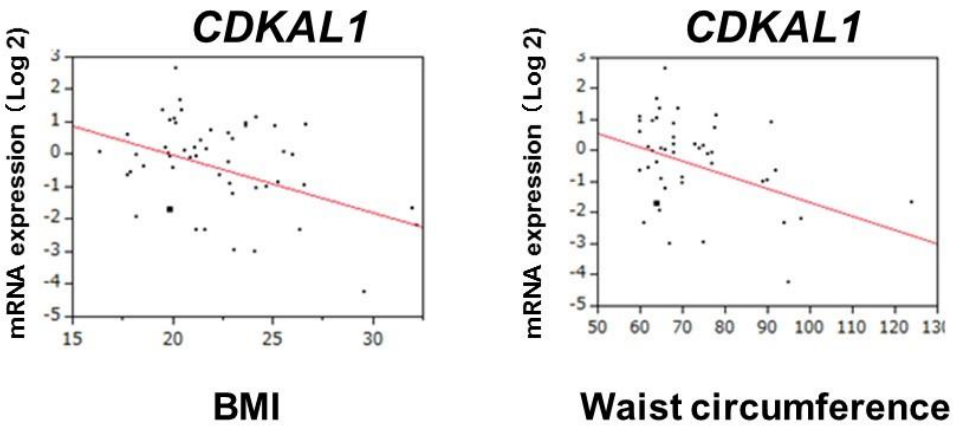


Figure 39. *CDKAL1* has an inverse correlation with BMI and waist circumference in human adipose tissue.

Unpublished data from a collaboration of Department of Diabetes and Metabolic Diseases and Department of Stomach and Esophageal Surgery at the University of Tokyo Hospital. Subcutaneous adipose tissue (~1cm^3) was collected from 102 patients undergoing a gastric surgery in the University of Tokyo Hospital (71 male and 31 female subjects, the average of age was 67.5±12.5 years old, BMI was 22.3±3.0, waist was 82.6±9.0 cm).

Table 1. qPCR Primers (from 5’ to 3’)

Cdkal1 Fwd	CATCCATCAGCAAGCCACT
Cdkal1 Rev	TGATTCCCAAGCCTATTTCTG
Wisp2 Fwd	TCCTCTGCATTCTCTCAATGG
Wisp2 Rev	GTGTCCAAGGACAGGCACA
36B4 Fwd	AGATGCAGCAGATCCGCAT
36B4 Rev	GTTCTTGCCCATCAGCACC
Fabp4 Fwd	CACCGCAGACGACAGGAAG
Fabp4 Rev	GCACCTGCACCAGGGC
Pparg Fwd	CCATTCTGGCCCACCAAC
Pparg Rev	AATGCGAGTGGTCTTCCATCA
Dio2 Fwd	CTGCGCTGTGTCTGGAAC
Dio2 Rev	GGAGCATCTTCACCCAGTTT
Ucp1 Fwd	GGCCTCTACGACTCAGTCCA
Ucp1 Rev	TAAGCCGGCTGAGATCTTGT
Ppara Fwd	GACAAGGCCTCAGGGTACCA
Ppara Rev	GCCGAATAGTTCGCCGAAA
*Fasn*_Fwd	GCTGCGGAAACTTCAGGAAAT

*Fasn*_Rev	AGAGACGTGTCACTCCTGGACTT
Ccl2 Fwd	CATCCACGTGTTGGCTCA
Ccl2 Rev	GATCATCTTGCTGGTGAATGAGT
Tnfa Fwd	ATCATCTTCTCAAAATTCGAGTGA
Tnfa Rev	TTGAGATCCATGCCGTTGG
Adiponectin Fwd	CCGGAACCCCTGGCAG
Adiponectin Rev	CTGAACGCTGAGCGATACACA