



Supplementary Information for

**Feedback regulation of Arid5a and Ppar-gamma2 maintains adipose tissue homeostasis**

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## **Extended information of Materials and Methods**

### **Cell lines, plasmids and stable cell line preparation**

The 3T3L-1 cells lines were purchased from the ATCC, USA. The cells were cultured in DMEM containing 1% glucose (Nacalai Tesque, Kyoto, Japan), 10% FCS, 100 I.U./ml penicillin and 100 µg/ml streptomycin (Nacalai Tesque). Immortalized MEF cells were prepared from WT and Arid5a<sup>-/-</sup> mice as previously described (1). Stable Arid5a<sup>-/-</sup> 3T3-L1 cells were prepared using the CRISPR Cas9 plasmid following the manufacturer's instructions (sc-431969 and sc-431969-HDR, Santa Cruz, USA). To generate stable cell lines overexpressing Arid5a, HA-tagged Arid5a plasmid vector was prepared on a PCX4 blasticidin plasmid backbone. This vector using lentivirus with the CMV promoter, was transfected to prepare stable cell lines. Flag-tagged Ppar-γ2 and Arid5a expression vectors were prepared with pcDNA3.1 using the infusion kit expression kit (Thermo Fisher Scientific, Waltham, USA) according to the manufacturer's instructions.

### **Histology**

Visceral adipose tissue and sections of liver from mice were collected, fixed with 4% formaldehyde, paraffin-embedded, sectioned and stained with eosin and hematoxylin. Bright field images were captured with a Keyence BZ-X710 microscope.

### **Quantitative real-time PCR**

RNA from cells and tissues was isolated using the RNeasy Mini kit or lipid RNA extraction kit (Qiagen, Hilden, Germany). cDNA was prepared using the reverse transcription kit (ReverTra Ace® qPCR RT Master Mix with gDNA Remover (Toyobo, Japan) was used according to the manufacturer's instructions. Real-time PCR was carried out using power up SYBR Green master mix (Life Technologies, Carlsbad, USA) according to manufacturer's instructions. The comparative delta delta Ct method normalized to beta-actin was used to determine the relative expression value. The sequences of the PCR primers for all genes are shown in supplementary Table 1.

### **Adipogenesis, red oil O staining and lipid quantification**

We used 3T3-L1 and immortalized MEF cells for the adipogenesis experiments *in vitro*. The adipogenesis protocol was adopted from Kassotis et al with some modifications(2). In brief, cells were cultured under normal conditions in DMEM with 10% fetal bovine serum and penicillin and streptomycin. After the cells reached approximately 80-90% confluence, the medium was replaced with adipocyte differentiation medium consisting of DMEM, 10% fetal bovine serum and 1  $\mu$ M dexamethasone (Nacalai Tesque), 1  $\mu$ M insulin (Sigma), 1 mM isobutylxanthine (Nacalai Tesque) and 5  $\mu$ M rosiglitazone (Wako chemicals, Tokyo, Japan). After three days in differentiation medium, the 3T3-L1 cells were switched to maintenance medium (DMEM, 10% FBS, 1  $\mu$ M insulin, 1  $\mu$ M dexamethasone for 7 to 10 days). The maintenance medium was changed every 3 days. After 7-10 days, the cells were washed with PBS, fixed with 4% PFA, and stained with red oil O stain as previously described (3). The cells were further counterstained with

hematoxylin, washed several times with warm water and analyzed under a microscope. Phase contrast images were captured. Lipid amount in cells during adipogenesis was quantified according to Kraus et al (4). Briefly, the red oil O stained cells were incubated with isopropanol for 10 minutes in room temperature with gentle agitation and the absorbance was measured at 510 nm.

### **High fat studies**

For the high fat study experiment, mice were fed either High Fat diet 32 (crude fat content 32%, fat origin calorie rate 60% kcal) or CLEA Rodent Diet Quick Fat (crude fat content: 14.3%) from CLEA Japan Inc. The food was replaced with fresh food every three days. The weight of the mice was measured every week or every month.

### **Insulin tolerance test and glucose tolerance test**

The insulin tolerance test (ITT) and glucose tolerance test (GTT) were performed as described previously. Briefly, ITT mice were fasted for 6 hours followed by injection of insulin IP (Sigma) at a dose of 1.25 U/kg body weight. For GTT, mice were fasted 6 hours, and glucose (1 mg/kg of body weight) was injected intraperitoneally. Blood was drawn from the tail at 0, 30, 60, 90, 120 and 180 min after insulin or glucose injection, and the level of glucose was measured using a glucometer (OneTouch® Ultra® 2 meter).

### **Total steroid measurement and fatty acid uptake assay**

Total cortisol in the serum of mice was measured using the Cortisol Parameter Assay Kit (RnD, Minneapolis, USA) according to the manufacturer's instructions. Adipokines in

serum or cell lysates were measured with a Proteome Profiler Mouse Adipokine Array Kit (RnD system) according to the manufacturer instructions.

#### **Arid5a localization (Immunofluorescence)**

Arid5a-overexpressing 3T3-L1 cells were cultured in histology culture plates. At day 0 and day 3 of adipogenesis, the cells were fixed with a 1:1 acetone: methanol mixture, followed by incubation with HA antibody (Cell Signaling Technology, Danvers, USA) diluted (1:1000) in PBST overnight. The cells were washed several times with PBST followed by staining with anti-rabbit Alexa Fluor 488-conjugated IgG for one hour. After one hour, the cells were washed, stained with 3  $\mu$ M DAPI and analyzed with a Keyence BZ-X710 fluorescence microscope.

#### **FACS analysis**

Surface expression of CD36 and mitochondrial analysis of 3T3-L1 and MEF cells during adipogenesis were analyzed by FACS analysis. For CD36 analysis, the cells were stained with anti-CD36 antibody (Biolegend, San Diego, USA) and analyzed by FACS using a standard protocol. For mitochondrial analysis, Mito Tracker Green and Red (CMXRos) (Thermo Fisher Scientific, Waltham, USA) were added to the cell culture one hour before analysis, followed by standard FACS analysis (5). All analyses were performed using a FACS Aria II or FACS Canto (BD).

## **Western blot**

Cells or tissues were lysed in RIPA buffer cocktail (Santa Cruz Biotechnologies, Dallas, USA) with additional protease inhibitor, PMSF and NaOrth (Santa Cruz Biotechnologies). The extracted proteins were run on a 5-20% SDS PAGE gel (Nacalai Tesque). The proteins were transferred to PVD nitro-cellulose membrane using the semi dry transfer system. Membranes were blocked with 5% milk for 1-2 hrs, and after three washes with TBST buffer, the membrane was incubated with primary antibodies overnight. This process was followed by the addition of secondary antibodies and development using chemo-luminescence reagents. The luminescence of the developed membrane was read using a luminescent image analyzer (ImageQuant LAS 4000) and edited with Image J software. The following antibodies were used for western blotting: anti-Arid5a (Cat. no ab81149; Abcam, Cambridge, UK), anti- $\beta$ -actin (Cat no 4970; Cell Signaling Technology, Danvers, USA), anti-Ppar- $\gamma$  (Cat. no 2443, Cell Signaling Technology) anti-HA antibody (Cat no C29F4, Cell Signaling Technology).

## **Luciferase activity**

For promoter assays, the 1000-kB promoter of the mouse Ppar- $\gamma$ 2 gene or segments of the promoter were amplified using the polymerase chain reaction (PCR) from mouse genomic DNA and then ligated into the basic pGL3 vector (Promega, Madison, USA) using the infusion kit. The prepared Ppar- $\gamma$ 2 promoter vector was confirmed by sequencing. The 3T3-L1 cells were transfected with Ppar- $\gamma$ 2 promoter vector or pGL3-luciferase plasmid (control). Renilla was used as an internal control. After 1-3 days of transfection, luciferase activity was measured using the Dual luciferase reporter assay

system. The firefly luciferase activity for each construct was normalized with respect to *Renilla* activity and expressed as arbitrary units relative to the pGL3-basic plasmid.

### **Chromatin immunoprecipitation (CHIP) assay**

At day 3 of adipogenesis, HA tagged Arid5a overexpressing 3T3-L1 cells were processed for DNA chip analysis according to the protocol provided by Carey et al (6). Briefly, cells were fixed with formaldehyde followed by quenching with 3 M glycine. The cells were then lysed with buffer, and nuclei were isolated. Chromatin was separated by sonication, followed by overnight incubation with rabbit anti-HA antibody (Cat no C29F4, Cell Signaling Technology) or control IgG antibody (Cat no C2729, Cell Signaling Technology). Samples were washed and eluted, and cross-links were reversed with a 4-h incubation at 65°C. DNA was precipitated and analyzed by qRT-PCR.

### **RNA sequencing**

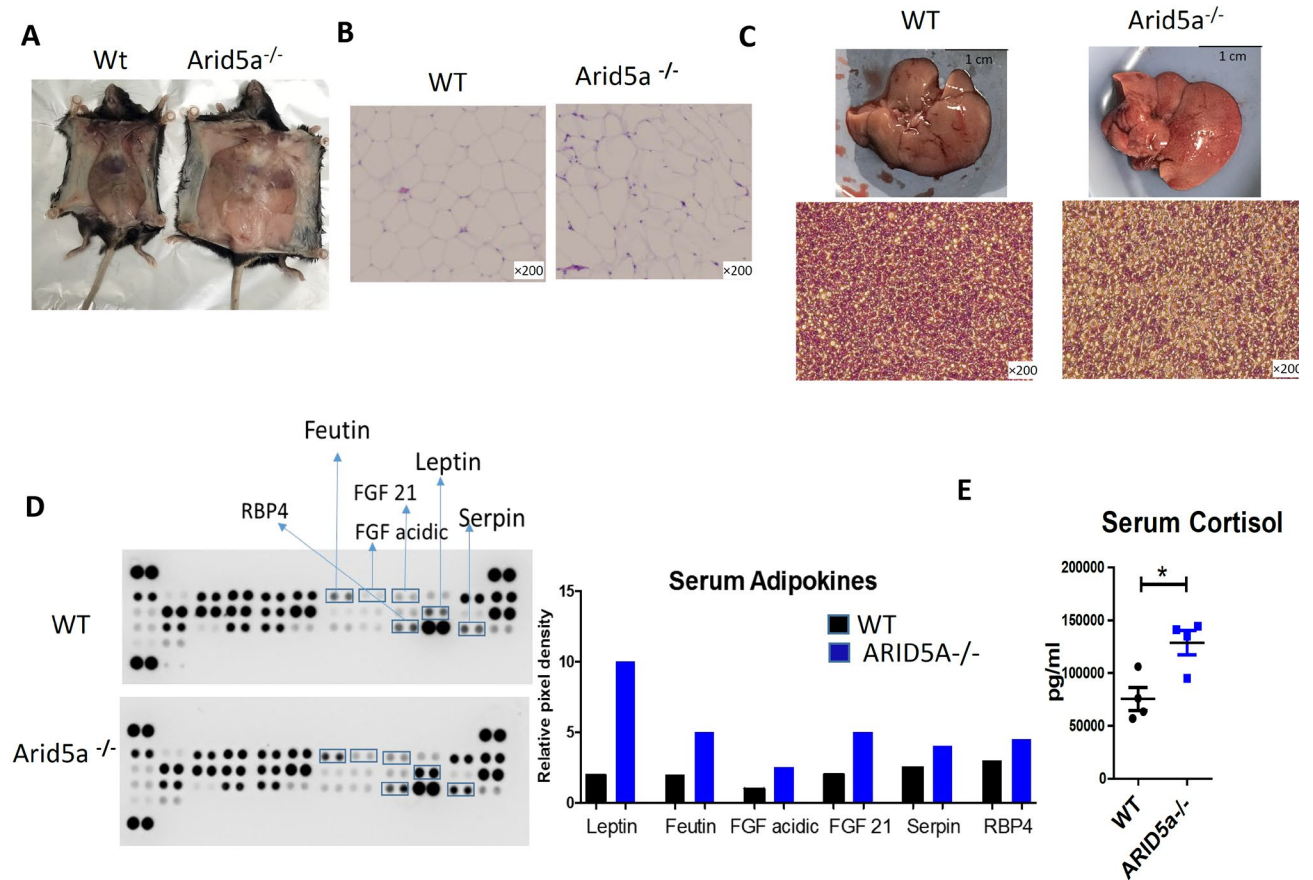
Epididymis adipose tissue from Wt and Arid5a<sup>-/-</sup> mice were collected, cut into small pieces and digested in serum free DMEM culture medium containing 1mg/ml collagenase (Worthington Biochemical Corporation Lakewood, USA) and DNase1 10 mg/ml (Sigma). After digestion, SVF was separated by centrifugation at 160 g for 5 mins (SVF are present in the cell pellet and adipocyte are present in the floating fraction). RNA was isolated from SVF by using Qiagen kit and proceeded for RNA sequencing. Library preparation was performed using a TruSeq stranded mRNA sample prep kit (Illumina, San Diego, CA) according to the manufacturer's instructions. Sequencing was performed on an Illumina HiSeq 2500 platform in a 75-base single-end mode. Illumina Casava1.8.2

software was used for base calling. The sequenced reads were mapped to the mouse reference genome sequences (mm10) using TopHat version 2.0.13 in combination with Bowtie2 version 2.2.3 and SAMtools version 0.1.19. The fragments per kilobase of exon per million mapped fragments (FPKM) were calculated using Cuffnorm version 2.2.1.



## Supplementary Figures

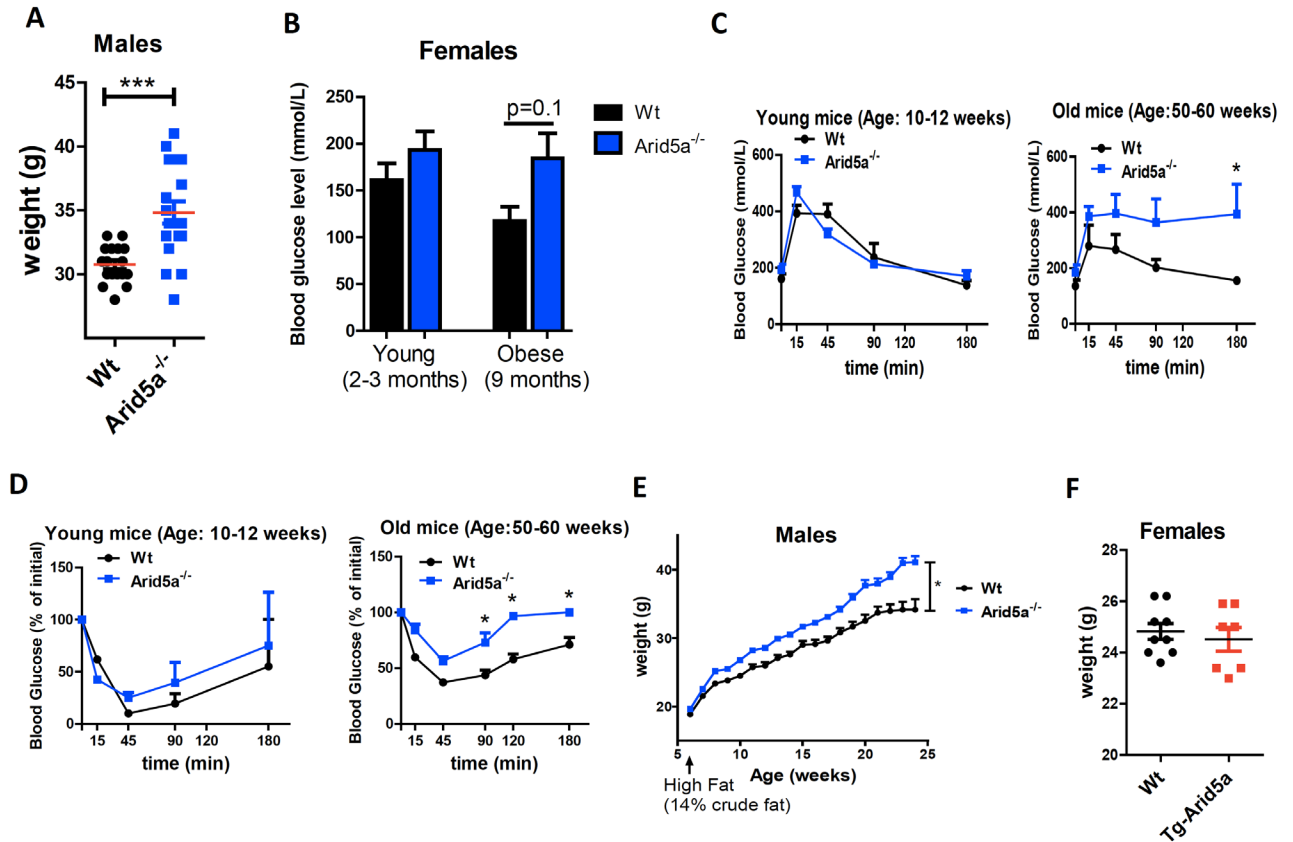
### Fig. S1



**Fig. S1.** (A) Adipose tissue distribution in female Wt and Arid5a<sup>-/-</sup> mice (age 18-20 months). (B) Microscopic images of visceral adipose taken from female Wt and Arid5a<sup>-/-</sup> at the age of 12 months. (C) Photo of whole liver (up) and microscopic image of liver section (down) from female Wt and Arid5a<sup>-/-</sup> mice fed with normal chow at 18 months of age. (D) Adipokine array analysis of serum of Wt and Arid5a<sup>-/-</sup> mice at the age of 12 months (see methods). Left figure is the X-Ray exposed membrane containing several dots that represents specific adipokine. The pixel intensity of black dot in the membrane corresponds to the concentration of specific adipokine. Right bar diagram is comparison of relative pixel density of selected adipokines between Wt and Arid5a<sup>-/-</sup> serum. Pixel density is quantified by densitometry analysis from Image J software and expressed on the scale of 0-10 (0-not detected- 10 -maximum). (E) Total cortisol level in serum

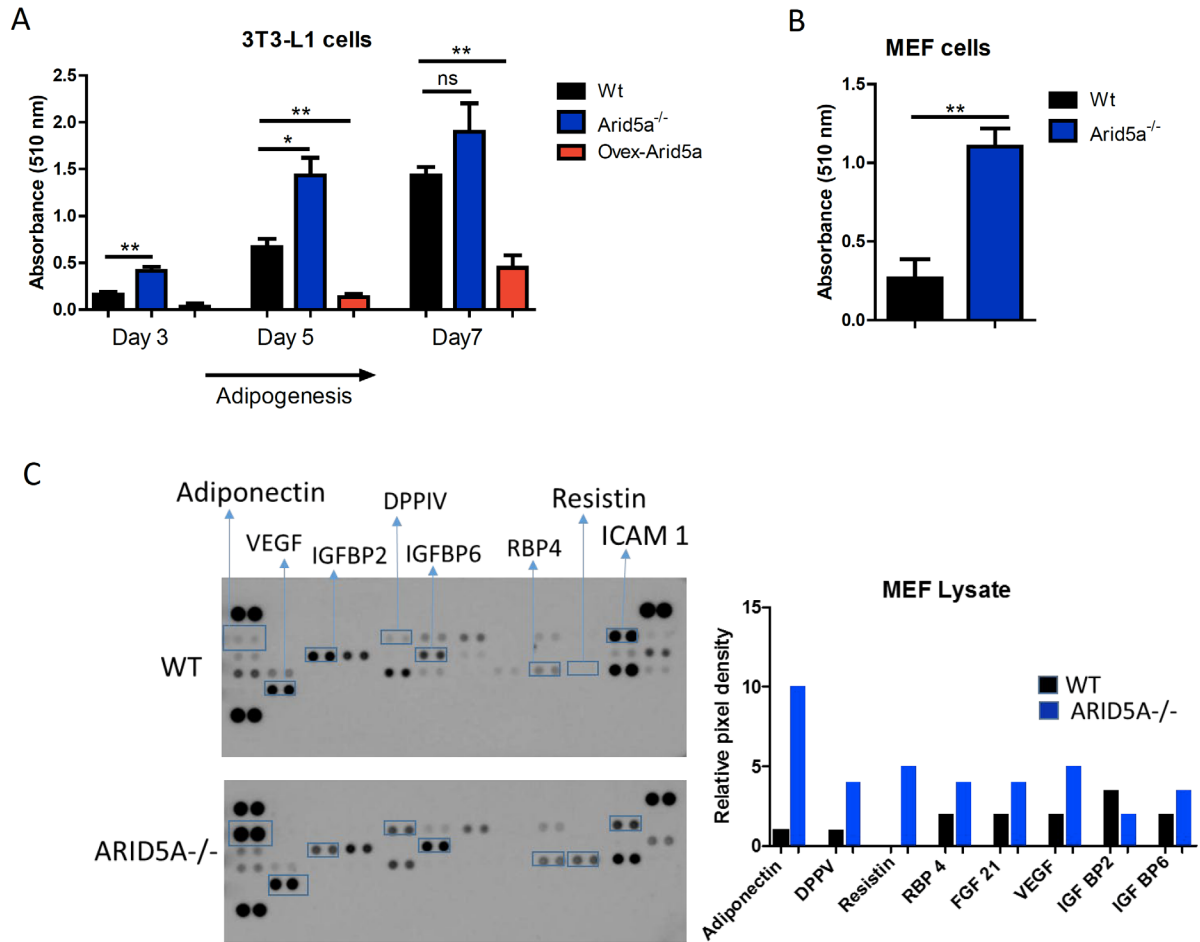
collected from WT and Arid5a<sup>-/-</sup> mice at 12 months of age. Error bars shows the means  $\pm$  SEM (\*,  $P < 0.05$ )

**Fig S2**



**Fig. S2:** (A) Comparison of total body weight of male mice with normal chow (4 % crude fat) at the age of 7 months. (B) Blood glucose level of Wt and Arid5a<sup>-/-</sup> mice measured at the age of 3 months and 12 months. (C) Glucose tolerance test performed in young (3 months) and old mice (12 months), n=5. (D) Insulin tolerance test performed in young mice (age: 10-12 weeks) and old mice (age: 50-60 weeks), n=5. (E) Monitoring of total body weight of male Wt and Arid5a<sup>-/-</sup> mice from age of six weeks moderate high fat diet (14% crude fat) n=8. (F) Total bodyweight comparison of female Wt and Tg-Arid5a mice fed with normal chow at the age of 3 months. Error bars shows the means  $\pm$  SEM. (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ).

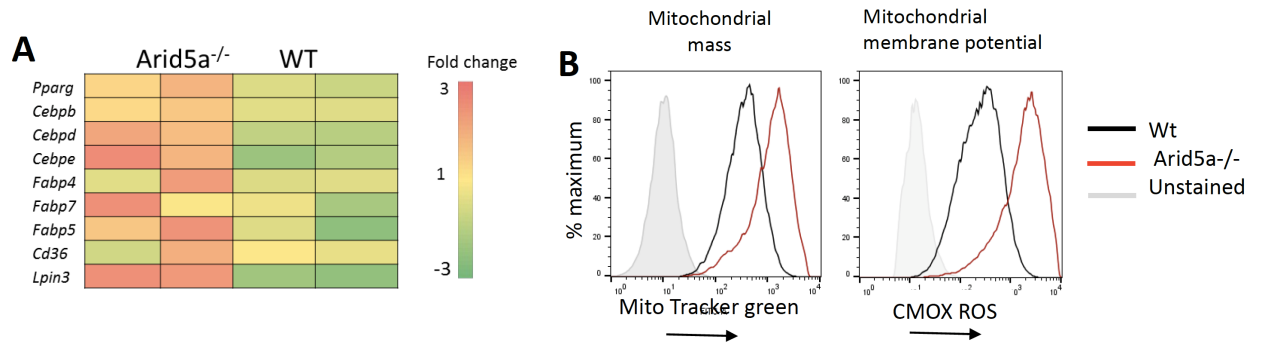
**Fig S3**



**Fig. S3: A and B)** Lipid quantification during adipogenesis process in 3T3-L1 cells (**A**) and MEF cells (**B**). Cells were subjected to adipogenesis and at day 3, 5 and 7 for 3T3-L1 cells and at day 10 for MEF cells, the lipid amount was quantified by measuring the absorbance (see method).  $n=3$ , Experiment was repeated twice. Unpaired two tailed student t test is performed for statistical analysis. Error bars shows the means  $\pm$  SEM (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ . **C)** Adipokine array analysis in lysate of Wt and Arid5a<sup>-/-</sup> MEF cells collected at day 10 of adipogenesis. Left figure is the X-Ray exposed membrane containing several dots that represents specific adipokine. The pixel intensity of black dot in the membrane corresponds to the concentration of specific adipokine. Right bar diagram is comparison of relative pixel density of selected adipokines between lysate of Wt and Arid5a<sup>-/-</sup> MEF cells. Pixel density is quantified by

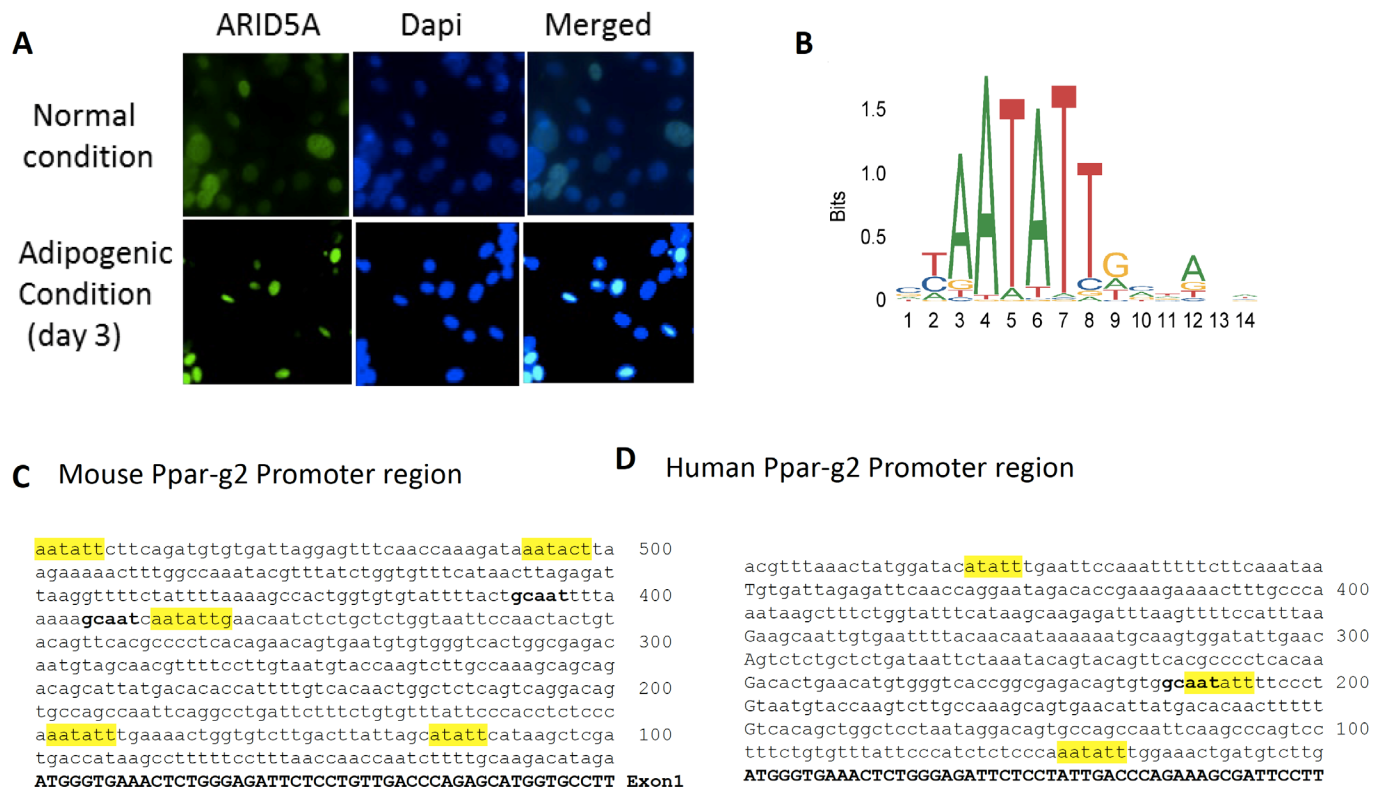
densitometry analysis from Image J software and expressed on the scale of 0-10 (0 (not detected- 10 maximum).

**Fig S4**



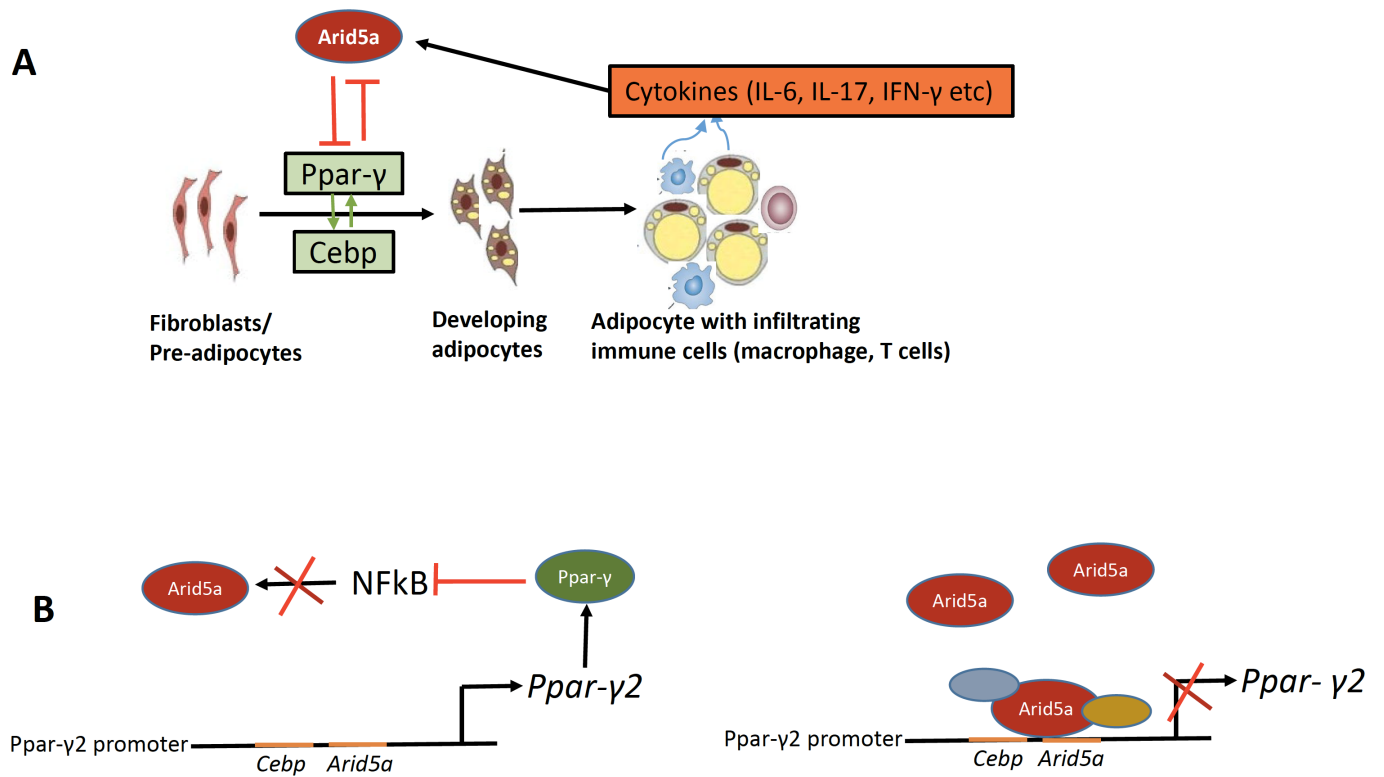
**Fig. S4. (A)** Heat map of selected genes related to lipid metabolism from RNA Seq analysis of stromal vascular fraction (SVF) from Wt mice and Arid5a<sup>-/-</sup> mice collected at 6 month of age. **(B)** FACS histogram showing mitochondrial mass (Mitotracker green) and mitochondrial membrane potential (CMXRos) in Wt and Arid5a<sup>-/-</sup> MEF cells during day 3 of adipogenesis.

**Fig S5**



**Figure S5. (A)** Subcellular localization of Arid5a in 3T3-L1 cells during day 0 and day 3 of adipogenesis. Cells were fixed, permeabilised and stained by antibodies against Arid5a (green) and nucleus by DAPI (blue). **(B)** Arid5a binding site generated through jasper database ([www.jaspar.genereg.net](http://www.jaspar.genereg.net)). **(C and D)** Promoter sequence (-500 bp from 1<sup>st</sup> exon) of mouse **(C)** and human **(D)** Ppar- $\gamma$ 2 gene showing the possible Arid5a binding site (highlighted yellow) and CEBP (bold letters).

**Fig. S6**



**Fig. S6: Hypothetical schematic representation of adipogenic homeostasis control by Ppar- $\gamma$  and Arid5a: (A)** Adipogenesis homeostasis maintained by Ppar- $\gamma$ , Cebp, Arid5a and cytokines. **(B)** Transcriptional regulation of Ppar- $\gamma$  by Arid5a.



**Table S1. Primer sequences used for RTPCR analysis**

<b>Gene</b>	<b>Forward/Reverse</b>	<b>Sequence</b>
Ppar-gamma	F	5' GCTCTGTGGACCTCTCCGTG 3'
Ppar-gamma	R	5'CATGAACTCCATAGTGGAAGCC 3'
Cd36	F	5' CTATTGGCCAAGCTATTGCGAC 3'
Cd36	R	5' GGATTCTGGAGGGGTGATG 3'
Fabp4	F	5' GATGCCTTTGTGGGAACCTG 3'
Fabp4	R	5' CACATTCCACCACCAGCTTGTC 3'
Glut1	F	5' GACCAAAAGCAACGGAGAAGAG 3'
Glut1	R	5' GTCGGAGGGCAAATCCAAGG 3'

## References

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5. Dingley S, Chapman KA, & Falk MJ (2012) Fluorescence-activated cell sorting analysis of mitochondrial content, membrane potential, and matrix oxidant burden in human lymphoblastoid cell lines. *Methods Mol Biol* 837:231-239.
6. Carey MF, Peterson CL, & Smale ST (2009) Chromatin immunoprecipitation (ChIP). *Cold Spring Harb Protoc* 2009(9):pdb prot5279.