Supporting Information

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SI Text

Materials. 3T3-L1 CAR cells stably expressing a truncated receptor for coxasckievirus and adenovirus (CAR) was a gift of Dr. Jianhua Shao (University of Kentucky). The cDNA encoding mouse DsbA-L was cloned by PCR from a mouse cDNA library and subcloned into the mammalian expression vector pCDNA 3.1 myc/His A inframe with the myc-tag (Invitrogen). The S16A and S19A mutants of DsbA-L were generated by site-directed mutagenesis. The cDNAs encoding HA-tagged and FLAG-tagged full length and the globular domain of adiponectin were described previously (7, 12). Antisera to DsbA-L and adiponectin were raised in rabbits using full-length DsbA-L or GST-adiponectin (amino acids 14–43) fusion protein as an antigen, respectively. The mouse monoclonal antibodies used in this study include antibodies to adiponectin (Chemicon), FLAG-epitope and tubulin (Sigma), PDI (ABR Affinity Bioreagents), and myc-tag (produced in house with the myc 1–9E10.2 cell clone from ATCC). The rabbit polyclonal antibodies used in this study include antibodies to leptin, and calnexin (Sigma), resistin (Chemicon), and P44/42 ERK (Cell Signaling). The cell lysis buffer contained 50 mM Hepes pH 7.6, 150 mM NaCl, 1% Triton-X, 10 mM NaF, 20 mM sodium pyrophosphate, 20 mM β-glycerophosphate, 1 mM sodium orthovanadate, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM phenylmethylsulonyl fluoride. All chemicals were from Sigma.

DsbA-L Expression in Mice. The white adipose tissues from normal diet or HFD-fed mice were gifts from Dr. Wenhong Cao (The Hamner Institutes for Health Sciences, Research Triangle Park, NC). 6–8 week old C57BL/6 mice (10 mice/group) were feed with normal (standard) chow diet or high fat diet (Research Diets #D12330: 58.0 kcal% fat, 16.0 kcal/% protein, and 26 kcal% carbohydrate) for 5 months. The body weight, fasting insulin level and fasting glucose level were determined (Table S4). Protein levels of adiponectin in serum and expression of adiponectin and DsbA-L in white adipose tissue were determined by Western blot.

Expression and Secretion of Adipokines in 3T3-L1 Adipocytes. Parental, scramble, and DsbA-L RNAi 3T3-L1 cells were differentiated into mature adipocytes. At day 8 of differentiation, cells were incubated with a serum-free DMEM containing 0.05% BSA for 24 h. The cells were dissolved in a buffer containing 10 mM Tris (pH = 7.4), 1% Triton-X-100 and protein inhibitor cocktails. Both cell lysates and the cell culture medium collected were centrifuged (15,000 g, 10 min) to remove cell debris, and were subjected to quantitative analysis of various adipokines. The concentrations of adiponectin and lipocalin-2 were measured using our in house ELISA (23, 44). The protein levels of PAI-1 (total), resistin and TNFα were determined using the multiplex immunoassay kits from the LINCO. To measure adiponectin in cell culture medium by Western blot, the conditioned media were collected and concentrated with iCON concentrators (Pierce). Fifty micrograms of protein from total cell lysates and 30 μg protein from the cell culture medium were loaded for the Western blot analysis. All of the results were from three independent experiments, with triplicates in each study. Data are expressed as means ± standard deviation (SD).

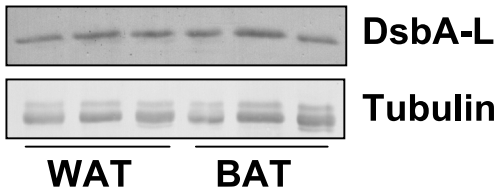

Fig. S1. Expression of DsbA-L in mouse white and brown adipose tissues. Proteins in brown (BAT) and white adipose tissue (WAT) of four-month old - C57BL/6 male mice were detected by Western blot using indicated antibodies.

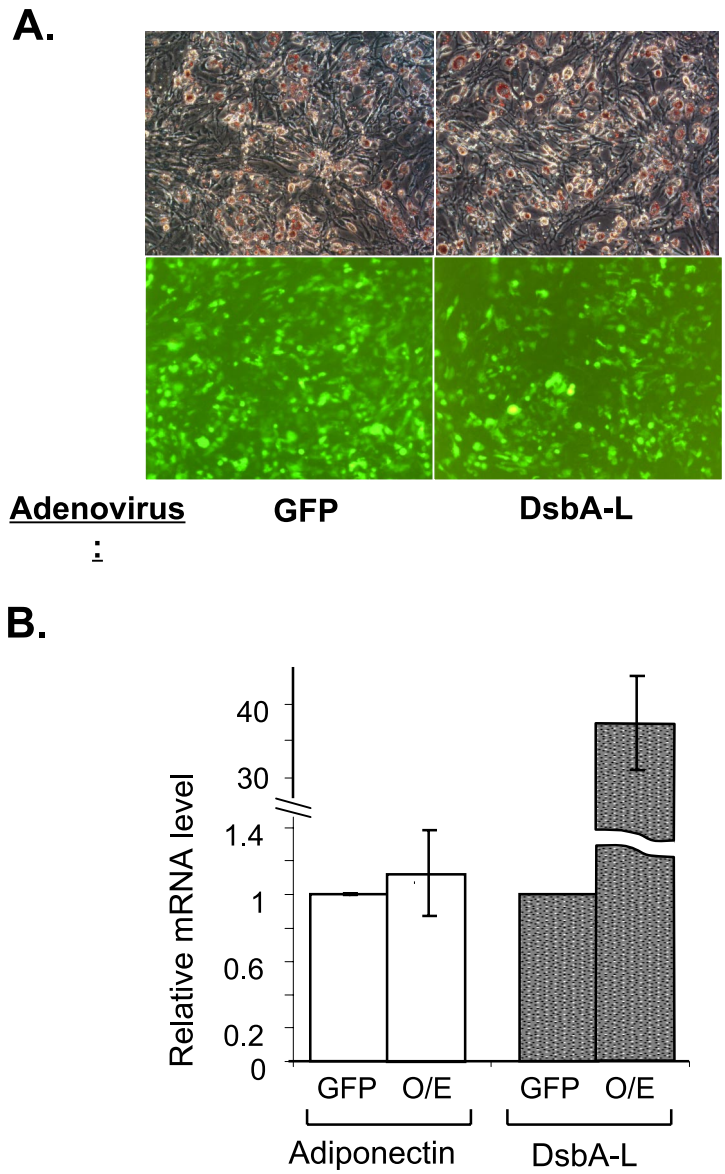

Fig. S2. The effect of DsbA-L overexpression or suppression on cell adipogenesis. (*A*) At day 9, 3T3-L1 CAR cells was assessed by Oil Red O staining (*Upper*). The efficiency of adenovirus-mediated infection was assayed 24 h post infection by GFP fluorescence microscopy (*Lower*). (*B*) Relative mRNA levels of adiponectin and DsbA-L in the GFP and DsbA-L-overexpressing 3T3-L1 CAR adipocytes were determined by real-time PCR. (*C*) Suppression of DsbA-L did not affect 3T3-L1 cell differentiation. Scramble or DsbA-L-shRNA cells were grown on cover slides and differentiated according to standard protocol. At differentiation day 0 or 10, cells were collected and adipogenesis was assessed by Oil Red O staining. (*D*) RNA was isolated from DsbA-L-suppressed or scramble control 3T3L1 adipocytes at differentiation day 10. RNA levels were semiquantified by analyzing the RT-PCR products visualized by agarose gel electrophoresis and ethidium bromide staining using the NIH IMAGE program. The RNA concentrations were normalized with GADPH levels. Data were expressed as means ± SEM from 3 independent experiments.

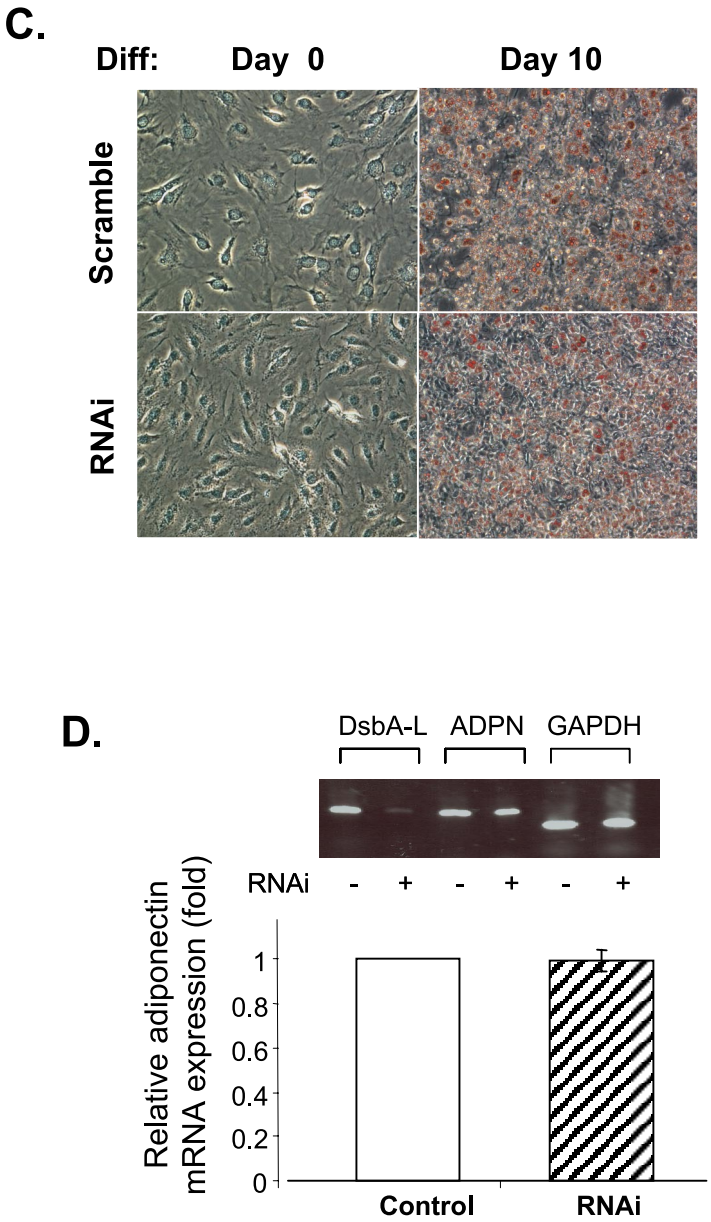

Fig. S3. Suppression of DsbA-L inhibits rosiglitazone-inducing adiponectin secretion. Differentiated 3T3-L1 scramble cells and DsbA-L-suppressed cells were treated with the vehicle control, TNFα or rosiglitazone (RGZ) for 24 h. Cells were lysed and cell lysates were resolved by non-reducing PAGE. The expression levels of DsbA-L, tubulin, and adiponectin oligomers in cell lysates (*A*) or cell culture medium (*B*) were determined by Western blot using specific antibodies as indicated.

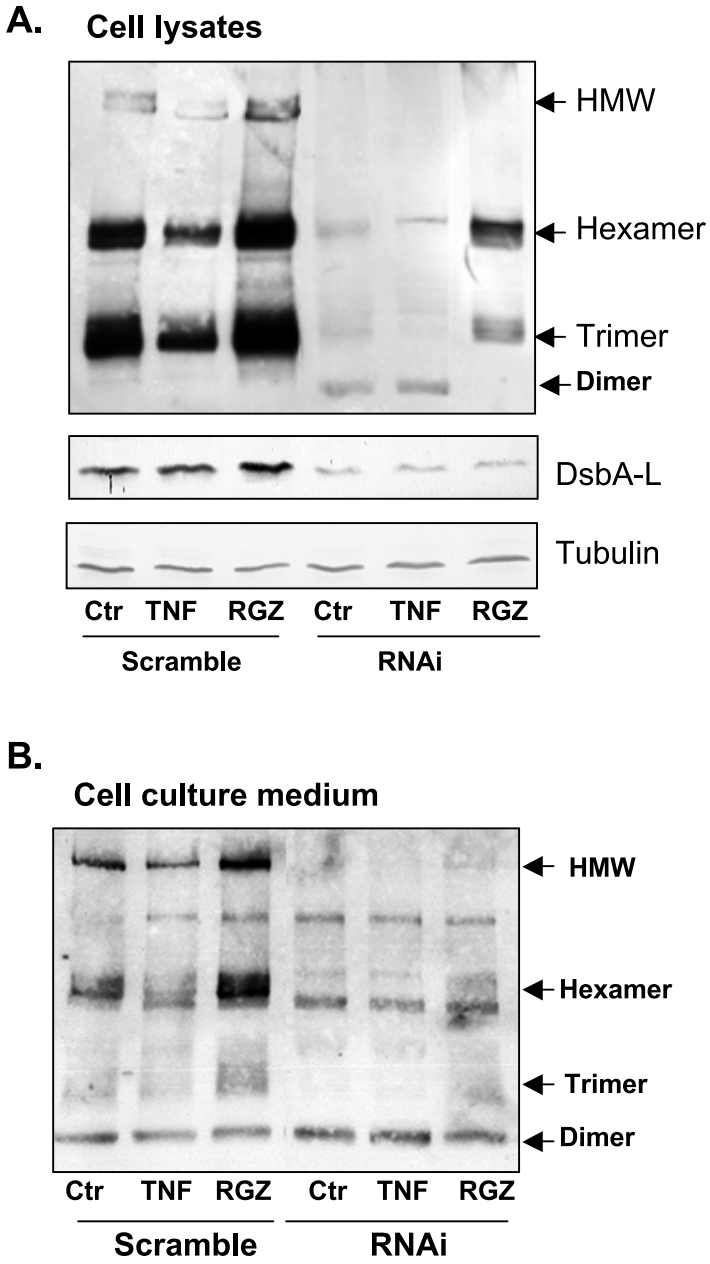

Fig. S4. DsbA-L is a key regulator of adiponectin stability and multimerization. (*A*) Alignment of sequence surrounding the CXXC motif of DsbA with those in TcpG, HCCA, PDILT, and DsbA-L. (*B*) Alignment of the sequence surrounding the SXXS motif in DsbA-L from different species. (*C*) FLAG-tagged C39S mutant of adiponectin was coexpressed with or without myc-tagged wild-type or mutants of DsbA-L in CHO/IR cells. Adiponectin and DsbA-L protein expression was determined by Western blot with specific antibodies. (*D*) Lysates from CHO/IR cells transiently expressing C39S mutant of adiponectin and wild-type or mutants of DsbA-L were resolved by nonreducing PAGE. Adiponectin multimerization was detected by Western blot with antibody to flag. Data are representative of at least three independent experiments with similar results.

Table S1. Effects of RNAi-mediated down-regulation of DsbAL on production of various adipokines in 3T3-L1 adipocytes

		Scrambled	RNAi
Adiponectin	CL, ng/mg protein	560.4 ± 45.8	294.8 ± 24.9**
	CM, ng/mL	197.5 ± 16.9	8.4 ± 0.8***
Resistin	CL, ng/mg protein	18.1 ± 1.9	16.7 ± 2.0
	CM, ng/mL	5.7 ± 1.1	6.0 ± 0.9
PAI-1	CL, ng/mg protein	66.8 ± 9.3	69.0 ± 7.6
	CM, ng/mL	31.3 ± 2.4	32.2 ± 3.4
Lipocalin-2	CL, ng/mg protein	151.2 ± 14.6	163.0 ± 14.1
	CM, ng/mL	12.4 ± 1.5	11.8 ± 1.4
TNFα	CL, ng/mg protein	8.6 ± 1.5	9.2 ± 1.9
	CM, ng/mL	2.2 ± 0.4	2.5 ± 0.5

CL, cell lysates; CM, conditioned culture medium.
**$P < 0.01$; *** $P < 0.001$ vs. scrambled RNAi.

Table S2. Characteristics of the study subjects recruited for quantification of adipose tissue DsbA-L mRNA expression

	Lean ($n = 23$)	Overweight/obese ($n = 12$)	P value
Age, years	43.1 ± 6.1	45.6 ± 6.3	0.207
BMI, kg/m^2	21.5 ± 1.8	27.1 ± 3.9	<0.001
Serum adiponectin, μg/mL	8.4 ± 3.3	5.9 ± 2.4	<0.01
Fasting glucose, mmol/L	5.7 ± 2.1	6.0 ± 2.4	0.223
Fasting insulin, μU/mL	5.4 ± 2.3	10.2 ± 7.1	<0.01

Table S3. Characteristics of the study subjects selected for analysis of DsbA-L protein levels

	Lean ($n = 4$)	Overweight/obese ($n = 4$)	P value
Age, years	36.4 ± 3.8	35.3 ± 4.1	0.234
BMI, kg/m^2	19 ± 2	29.5 ± 2.5	<0.01
Serum adiponectin, μg/mL	7.0 ± 2.6	5.6 ± 2.1	<0.05
Fasting glucose, mmol/L	5.7 ± 1.9	6.2 ± 2.2	0.137

Table S4. Characteristics of ND and HFD mice

	ND ($n = 5$)	HFD ($n = 15$)	*P* value
Body weight, g	36.9 ± 1.5	56.0 ± 3.5	<0.001
Fasting glucose level, mmol/L	7.1 ± 0.2	7.0 ± 0.3	0.655
Fasting insulin level, mU/L	14.2 ± 3.4	120.5 ± 41.8	0.023
HOMA-IR	4.5 ± 1.1	37.3 ± 3.3	0.027
QUICKI	0.315 ± 0.01	0.248 ± 0.01	<0.001