Fat-Specific DsbA-L Overexpression Promotes Adiponectin Multimerization and Protects Mice From Diet-Induced Obesity and Insulin Resistance

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The antidiabetic and antiatherosclerotic effects of adiponectin make it a desirable drug target for the treatment of metabolic and cardiovascular diseases. However, the adiponectin-based drug development approach turns out to be difficult due to extremely high serum levels of this adipokine. On the other hand, a significant correlation between adiponectin multimerization and its insulin-sensitizing effects has been demonstrated, suggesting a promising alternative therapeutic strategy. Here we show that transgenic mice overexpressing disulfide bond A oxidoreductase-like protein in fat (fDsbA-L) exhibited increased levels of total and the high-molecular-weight form of adiponectin compared with wild-type (WT) littermates. The fDsbA-L mice also displayed resistance to diet-induced obesity, insulin resistance, and hepatic steatosis compared with WT control mice. The protective effects of DsbA-L overexpression on diet-induced insulin resistance, but not increased body weight and fat cell size, were significantly decreased in adiponectin-deficient fDsbA-L mice (fDsbA-L/$Ad^{-/-}$). In addition, the fDsbA-L/$Ad^{-/-}$ mice displayed greater activity and energy expenditure compared with adiponectin knockout mice under a high-fat diet. Taken together, our results demonstrate that DsbA-L protects mice from diet-induced obesity and insulin resistance through adiponectin-dependent and independent mechanisms. In addition, upregulation of DsbA-L could be an effective therapeutic approach for the treatment of obesity and its associated metabolic disorders. ***Diabetes*** **61:2776–2786, 2012**

Adiponectin is a 30-kDa adipokine with anti-inflammatory, anti–insulin resistance, antioxidant, and antiatherosclerotic properties (1–3). Adiponectin circulating in plasma exists in three major forms: trimer, hexamer, and high-molecular-weight (HMW) multimer (4–7). Serum adiponectin levels are significantly reduced in obese human subjects (8) and patients with insulin resistance (9), type 2 diabetes, and coronary artery disease (10). On the other hand, high plasma adiponectin levels are associated with increased insulin sensitivity (11), lowered incidence rate of type 2 diabetes independent of obesity (12), decreased risk of coronary artery disease (13), and extended longevity (14,15).

Adiponectin is the only known adipokine whose levels are downregulated in obesity (8). Pharmacological studies have demonstrated that acutely enhancing the globular form of adiponectin in mice significantly increased fatty acid oxidation and reduced body weight (16,17). Transgenic overexpression of full-length adiponectin or the globular form of adiponectin has been shown to increase energy expenditure, insulin sensitivity, and fatty acid oxidation (16–20). Taken together, these results suggest that increasing serum adiponectin levels might be an attractive therapeutic approach for the treatment of obesity-induced metabolic diseases. However, the serum levels of adiponectin are extremely high, ranging between 1 and 20 μg/mL (21). Such a high concentration, which is at least three orders of magnitude higher than the levels of other adipokines, such as leptin and interleukin-6 (IL-6), results in technical difficulties in the development of adiponectin-based antidiabetic and antiatherogenic strategies.

An important finding in the adiponectin research field is that complex distribution, rather than the total levels of adiponectin, is associated with improved insulin sensitivity in response to thiazolidinedione stimulation in mice and humans (22). Consistent with this finding, the HMW form of adiponectin has been demonstrated as having major biological functions in regulating glucose homeostasis (23–25). In contrast, impairment of adiponectin multimerization affects both secretion and function of this adipokine and is associated with diabetes and hypoadiponectinemia (4,6). These findings suggest that increasing the ratio of the HMW form rather than the total levels of adiponectin might provide an effective alternative therapeutic strategy.

We have recently identified the disulfide bond A oxidoreductase-like protein (DsbA-L) as a key regulator of adiponectin multimerization in 3T3-L1 cells (26). In addition, we have found that overexpression of DsbA-L, the levels of which are significantly reduced in obese mice and human subjects (26), protected adiponectin from endoplasmic reticulum (ER) stress–induced downregulation in 3T3-L1 cells (27). However, whether overexpression of DsbA-L promotes adiponectin multimerization and improves insulin sensitivity in vivo remains unknown.

In the current study, we show that adipose tissue–specific overexpression of DsbA-L increases adiponectin multimerization and stability in mice. The fat-specific DsbA-L transgenic mice (fDsbA-L) exhibited enhanced activity and energy expenditure and increased resistance to diet-induced

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obesity and insulin resistance. Our study also indicates that in addition to regulating adiponectin multimerization and function, DsbA-L has an additional beneficial effect on energy homeostasis. Taken together, our study suggests that increasing the expression levels of molecules such as DsbA-L could be an effective therapeutic approach for the treatment of obesity-induced insulin resistance and associated metabolic diseases.

RESEARCH DESIGN AND METHODS

Material. Polyclonal antibodies to adiponectin and DsbA-L were generated as described previously (26). Antibodies against β-actin, AMP-activated protein kinase (AMPK), phospho-AMPK (P-AMPK), Akt (protein kinase B), phospho-Akt (P-Akt), IL-6, F4/80, and tumor necrosis factor-α (TNF-α) were from Cell Signaling Technology (Danvers, MA). The anti–β-tubulin 2.1 antibody was from Sigma-Aldrich.

Generation of fat tissue–specific DsbA-L overexpression mice (fDsbA-L) and adiponectin-deficient fDsbA-L mice (fDsbA-L/$Ad^{-/-}$). The mouse DsbA-L cDNA fused with a fragment encoding the myc tag was subcloned into a plasmid downstream of the 5.4-kb murine adipocyte fatty acid–binding protein 4 (FABP4/aP2) promoter. The DsbA-L transgene was excised from the plasmid microinjected into the pronuclei of fertilized C57BL/6J mouse eggs by the Transgenic Mice Core of UTHSCSA. Transgenic founders were identified by Southern blot analysis of the *Eco*R1-digested genomic DNA with a DsbA-L cDNA probe spanning between exons 7 and 8 (Supplementary Fig. 1*A*, left), and by PCR amplification of tail genomic DNA with a 0.3-kb aP2/DsbA-L cDNA fragment amplified by aP2-specific (sense, 5′-ATCATTGCCAGGGAGAAC-3′) and DsbA-L–specific (antisense, 5′-TGCTTCAGGAGAGGAATC-3′) primers that recognize both aP2 and DsbA-L (Supplementary Fig. 1*A*, right). Quantification of transgene copies was performed by Southern blot analysis. Two independent lines of DsbA-L transgenic mice were generated. To generate adiponectin-deficient fDsbA-L mice (fDsbA-L/$Ad^{-/-}$), the fDsbA-L mice were first bred with adiponectin-null ($Ad^{-/-}$) mice (28) to obtain fDsbA-L/$Ad^{+/-}$ mice. The later were then bred with $Ad^{+/-}$ mice to generate fDsbA-L/$Ad^{-/-}$, fDsbA-L, $Ad^{-/-}$, and wild-type (WT) control littermates.

Food intake, body weight, and body composition. Mouse food intake and body weight were measured on a weekly basis. The total weekly food intake of a mouse was calculated by measuring the food added subtracted by the food left in the cage divided by the number of mice in the cage. Mouse daily food intake was calculated by total weekly food intake divided by 7. To check body composition, mice were anesthetized by intraperitoneal injection with avertin (120 mg/kg animal body weight). Bone mineral density, fat mass, lean mass, and percentage of fat were determined using dual-energy X-ray absorptiometry (DEXA) (GE Medical Systems, Madison, WI).

Western blot and determination of the adiponectin multimerization. The expression and phosphorylation levels of various proteins in mouse tissue homogenates and cell lysates were detected by Western blot with specific antibodies. Adiponectin multimerization was determined by gel filtration using an AKTA purifier system (GE Healthcare Bio-Sciences Corp., Piscataway, NJ) as described in our previous study (26). Quantification of the relative change in protein levels (expressed as percentage of control protein levels, arbitrarily set as 1.0) was performed by analyzing Western blots using the Scion Image Alpha 4.0.3.2 program (Scion Corp.) and was normalized for the amount of protein loaded in each experiment.

Hematoxylin and eosin and Oil Red O staining. For hematoxylin and eosin (H&E) staining, adipose tissue was fixed with a buffer containing 10% formalin for 24 h and embedded in paraffin. Tissue sections (10-mm thick) were stained with H&E. For Oil Red O staining, liver tissues were flash-frozen by liquid nitrogen, overhanging in a tragacanth-based gel (7% tragacanth in water). Tissues were sectioned by cryostat, and the slides were stained with Oil Red O and H&E according to standard protocols.

Adipocyte morphometric evaluation. Five mice per experimental group were analyzed, with four representative images per section being obtained for a total of six tissue sections per mouse. Adipocyte morphometry was visualized by H&E staining, and images were taken using a Zeiss Plan Apochromat ×20 objective. Adipocyte area and number were determined using the NIH ImageJ software.

Glucose and insulin tolerance test. Mice at the age of 5 weeks were fed with a high-fat diet (HFD) (45% kcal from fat, D12451; Research Diets Inc., New Brunswick, NJ) for 4 months. For glucose tolerance test (GTT), mice were fasted overnight, followed by an intraperitoneal injection of 2 g/kg glucose. For insulin tolerance test (ITT), mice were fasted for 5 h, followed by an intraperitoneal injection of 0.075 units/kg insulin. Blood glucose levels were measured before and after glucose or insulin injection, respectively, using an automatic glucometer (Rightest GM300; Bionime Corp.). The incremental area under the curve (AUC) over the basal value of GTT during the time period of 120 min was calculated using the trapezoidal rule and used as an index of glucose tolerance. The incremental area below the basal value of ITT during the time period of 90 min was calculated using the trapezoidal rule and used as an index of insulin tolerance.

Hyperinsulinemic-euglycemic clamp. The left carotid artery and right jugular vein of the HFD-fed mice were cannulated by surgery under anesthetized conditions, followed by at least a 5-day recovery period. Insulin sensitivity in the mice was assessed directly with a hyperinsulinemic-euglycemic clamp as described previously (29).

Energy expenditure. Before each experiment, the mice were placed in the Multiple Animal Respirometry System (MARS; Sable Systems International, Las Vegas, NV) for at least 12 h to acclimate to a new environment. Oxygen consumption (V_{O_2}), carbon dioxide production (V_{CO_2}), and the activity of each animal in live-in cages were measured for 48 h, including two complete light cycles (two for 14 h) and two complete dark cycles (two for 10 h). The respiratory quotient, which provides information on the metabolizable substrates being used, was calculated by the ratio V_{CO_2}/V_{O_2}. Activity monitoring was performed simultaneously with metabolic measurements via the MAD-1 Motion/Activity Detector.

Statistics. Statistical analysis of the data was performed using ANOVA or Student *t* test. Statistical significance was set at P values of <0.05 and <0.01.

RESULTS

Generation of fat-specific DsbA-L transgenic mice. We have recently identified DsbA-L as a critical regulator of adiponectin multimerization in 3T3-L1 adipocytes (26). To determine the functional role of DsbA-L in adiponectin multimerization in vivo, we generated adipose-specific DsbA-L transgenic mice (fDsbA-L) using the murine FABP4/aP2 promoter (Fig. 1*A*). Western blot analysis revealed that the myc-tagged DsbA-L is specifically expressed in fat tissues (Fig. 1*B*), including white adipose tissue (WAT), brown adipose tissue (BAT), and macrophage, of the fDsbA-L transgenic mice with an approximately twofold higher expression level compared with that of the endogenous protein (Fig. 1*C–E*). A similar expression level of DsbA-L was found in an independent transgenic mouse line (data not shown).

Overexpression of DsbA-L enhances adiponectin multimerization in vivo. To determine whether overexpression of DsbA-L promotes adiponectin multimerization and stability in vivo, we measured total adiponectin levels and the ratio of each multimer to total adiponectin levels in both adipose tissue and serum. Consistent with our previous finding that DsbA-L promotes adiponectin assembly and stability in 3T3-L1 adipoctyes (26), the protein levels of adiponectin and the ratio of the HMW form of this adipokine are increased in adipose tissue (Fig. 2*A–D*) and serum (Fig. 2*E–H*) of the fDsbA-L mice compared with the control mice. Overexpression of DsbA-L greatly protected mice from HFD-induced downregulation of adiponectin levels (Fig. 2*A* and *B*) and its secretion (Fig. 2*E* and *F*). Gel filtration studies revealed a significant increase in the HMW form of adiponectin in WAT (Fig. 2*C* and *D*) and serum (Fig. 2*G* and *H*) of the fDsbA-L mice compared with WT littermates under HFD-feeding conditions. Similar results were also observed in another DsbA-L overexpression transgenic mouse line (data not shown). These results provide the first evidence that DsbA-L promotes adiponectin multimerization in vivo.

Overexpression of fDsbA-L in mice increased resistance to diet-induced obesity. To determine whether overexpression of DsbA-L has an effect on energy homeostasis, we compared body weight and food intake between the fDsbA-L transgenic mice and WT littermates. On normal chow, the fDsbA-L mice showed little difference in food intake and body weight compared with WT littermates (data not shown). There was also no significant difference in food intake between fDsbA-L and WT littermates fed

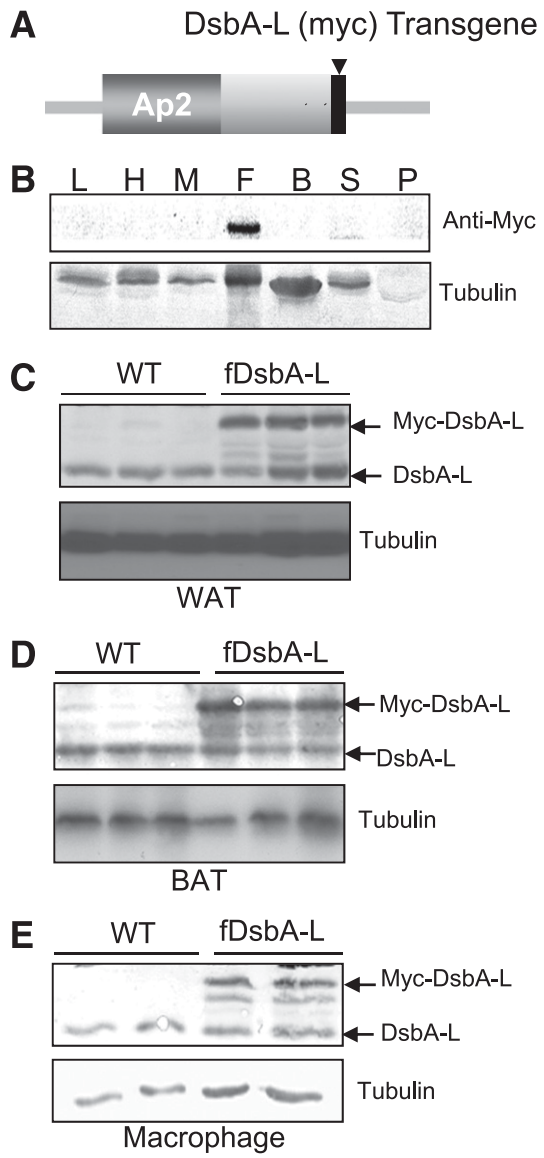

FIG. 1. Expression of the myc-tagged DsbA-L transgene in mouse tissues. *A*: The fDsbA-L transgene construct. An aP2 promoter was used to drive the expression of myc-tagged mouse *DsbA-L* gene. *B*: Western blot analysis of tissue homogenates of the fDsbA-L transgenic mice using an anti-myc antibody. B, brain; F, fat; H, heart; L, liver; M, muscle; P, pancreas; S, spleen. The expression of the myc-tagged and endogenous DsbA-L in WAT (*C*), BAT (*D*), and macrophages (*E*) isolated from WT and fDsbA-L transgenic mice was analyzed by Western blot using an anti–DsbA-L antibody. Tubulin was used as a loading control.

with an HFD (Supplementary Fig. 1*B*). However, the body weight, epididymal fat pad, and fat cell size of the fDsbA-L mice were notably reduced compared with WT littermates (Fig. 3*A*–*C*). Consistent with these findings, DEXA analysis revealed that the fDsbA-L mice had a significantly lower fat content compared with the WT littermates (Fig. 3*D*). There was no significant difference in the activity between the fDsbA-L and WT mice during the light cycle, but the total activity of the fDsbA-L mice was significantly higher than that of the WT littermates during the dark cycle (Fig. 3*E*). Consistent with these findings, the overall metabolic rate of the fDsbA-L mice, expressed as a function of lean body mass, was significantly greater than that of the WT control mice in the dark cycle (Fig. 3*F*). The respiratory quotient (V_{CO_2}/V_{O_2}) was similar between fDsbA-L and WT control mice (data not shown), suggesting that there is no difference in substrate utilization between these mice. Taken together, these results suggest that increased metabolic rate may provide a mechanism by which overexpression of DsbA-L protects mice from diet-induced obesity.

The fDsbA-L mice are resistant to HFD-induced insulin resistance. The fDsbA-L mice displayed increased glucose and insulin tolerance when compared with WT littermates under normal chow diet (Supplementary Fig. 1*C* and *D*), but the difference between fDsbA-L and WT mice did not reach statistical significance. Under HFD, the fDsbA-L mice showed a significantly enhanced glucose and insulin tolerance compared with WT littermates (Fig. 4*A*–*D*). Hyperglycemic clamp experiments revealed that insulin had a greater suppressive effect on hepatic glucose production in fDsbA-L mice compared with WT mice under the HFD condition (Fig. 4*E*). The fDsbA-L mice also exhibited a higher insulin-mediated glucose infusion rate (Fig. 4*F* and *G*) and lower levels of fasting triglycerides compared with control mice (Supplementary Fig. 2*A*). In agreement with previous findings that the HMW form of adiponectin has a major insulin-sensitizing effect in the liver (2,25,28,30), AMPK phosphorylation and insulin-stimulated Akt phosphorylation were significantly enhanced in the liver (Fig. 4*H* and *I*) and WAT (Supplementary Fig. 2*B* and *C*), but not in skeletal muscle (Supplementary Fig. 2*D* and *E*), of the fDsbA-L mice compared with WT littermates.

Fat-specific overexpression of DsbA-L protects mice from HFD-induced inflammation and hepatic steatosis. Since liver is the major organ for adiponectin action in vivo (2,25,30), we asked whether fat-specific overexpression of DsbA-L protects mice from HFD-induced inflammation and liver dysfunction. In agreement with the findings of others (31,32), HFD feeding resulted in a large increase in macrophage infiltration into adipose tissue (Fig. 5*A* and *B*). The HFD-induced fatty liver was significantly protected in the fDsbA-L mice (Fig. 5*C*). Overexpression of DsbA-L also greatly reduced HFD-induced macrosteatosis and accumulation of lipid droplets in the liver (Fig. 5*D*). Consistent with these findings, the expression levels of TNF-α and IL-6 in WAT were significantly reduced in the fDsbA-L mice compared with WT littermates fed with an HFD (Fig. 5*E*).

Targeted deletion of the adiponectin gene diminishes the beneficial effects of DsbA-L on insulin resistance and hepatic steatosis in mice. To determine whether the beneficial effect of fat-specific overexpression of DsbA-L is mediated by adiponectin multimerization and action, we generated fat-specific DsbA-L transgenic mice in which the adiponectin gene targeted is disrupted (fDsbA-L/$Ad^{-/-}$) (Fig. 6*A*). There was little difference in food intake (data not shown), body weight (Fig. 6B and Supplementary Fig. 3*A*), and fat mass (Fig. 6*C*) between HFD-fed fDsbA-L/$Ad^{-/-}$ and fDsbA-L mice. In addition, the promoting effect of DsbA-L on activity and energy expenditure was not significantly affected in the fDsbA-L/$Ad^{-/-}$ mice compared with fDsbA-L mice (Supplementary Fig. 3*B* and *C*). However, the protective effects of DsbA-L on diet-induced liver steatosis (Fig. 6*D*) and insulin resistance (Fig. 6*E* and *F* and Supplementary Fig. 3*D* and *E*) were markedly reduced in fDsbA-L/$Ad^{-/-}$ mice compared with fDsbA-L mice, suggesting that the protective effect of DsbA-L overexpression on diet-induced insulin resistance and liver steatosis is mainly mediated by adiponectin action. These results also suggest that HFD feeding could have an effect on insulin

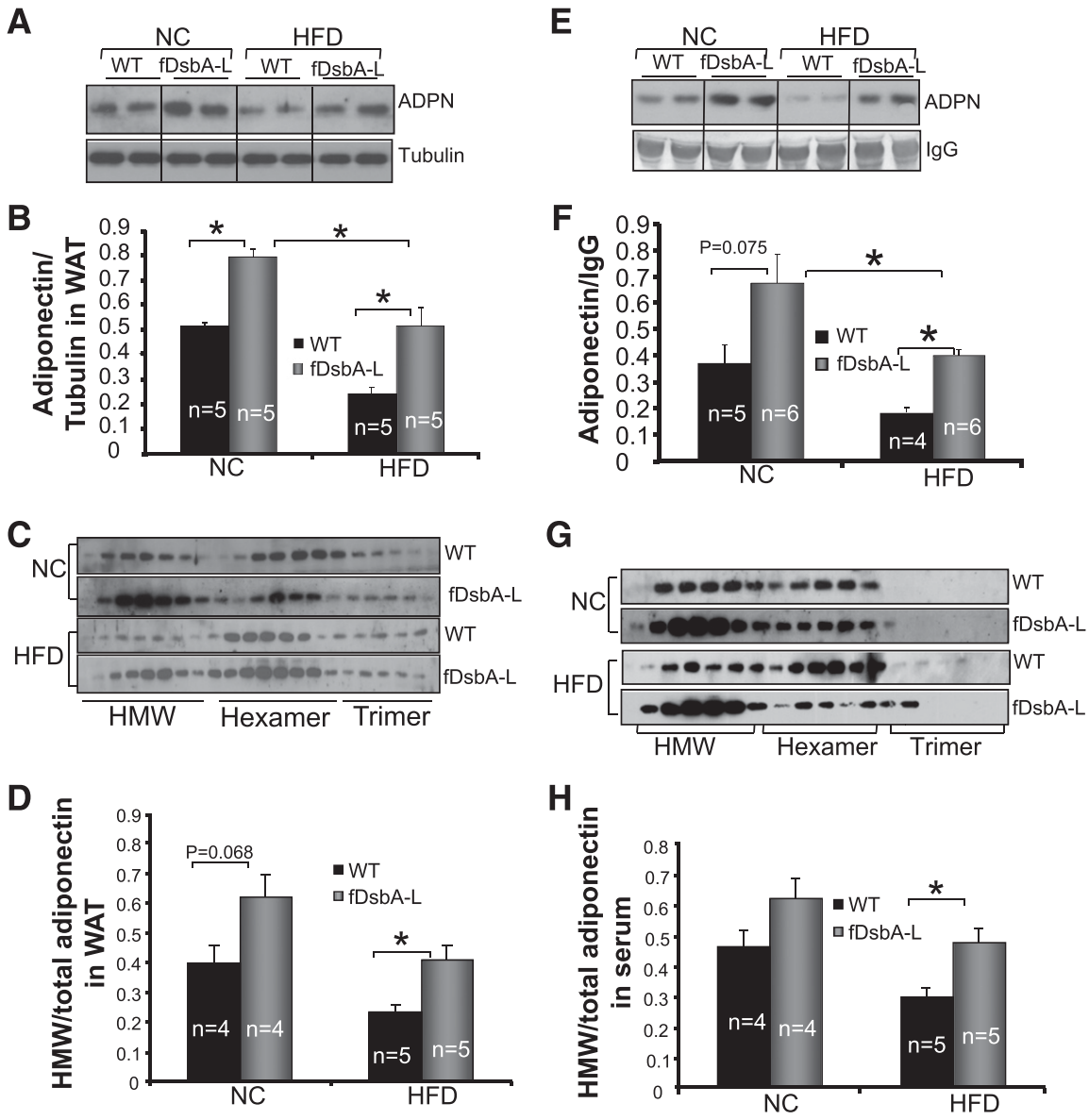

FIG. 2. Overexpression of DsbA-L enhanced adiponectin multimerization in vivo. *A*: The protein levels of adiponectin (ADPN) in WAT of fDsbA-L transgenic and WT control mice. Male fDsbA-L transgenic and WT littermates (5 weeks of age) were fed with normal chow diet (NC) or 45% HFD for 16 weeks. Mice were killed and WAT was isolated. The expression levels of adiponectin in tissue homogenates were determined by Western blot with an anti-adiponectin antibody. β-Tubulin was used as a loading control. *B*: The relative expression levels of adiponectin in *A* were quantified. Data represent mean ± SEM. **P* < 0.05. *C*: The relative expression levels of adiponectin multimers in WAT were determined by gel-filtration chromatography, followed by Western blot. *D*: The ratio of HMW to total adiponectin in WAT as shown in *C* were quantified. Data represent mean ± SEM. **P* < 0.05. *E*: The serum adiponectin levels of fDsbA-L mice and WT littermates fed with NC or HFD were determined by Western blot with an anti-adiponectin antibody. IgG was used as a loading control. *F*: The relative expression levels of adiponectin in *E* were quantified. Data represent mean ± SEM. **P* < 0.05. *G*: The relative expression levels of adiponectin multimers in serum of fDsbA-L transgenic and WT mice were determined by gel-filtration chromatography, followed by Western blot using an anti-adiponectin antibody. *H*: The ratio of HMW to total adiponectin in serum as shown in *G* were quantified. Data represent mean ± SEM. **P* < 0.05.

resistance and liver dysfunction independent of its causative role in obesity.

The protective effect of fat-specific overexpression of DsbA-L on diet-induced obesity could be partially mediated by an adiponectin-independent mechanism. The above results suggest that enhanced adiponectin levels and multimerization play a major role in the insulin-sensitizing effect of DsbA-L in vivo. However, fat-specific overexpression of DsbA-L appears to have some additional beneficial effects in addition to promoting adiponectin multimerization and function. To further test this possibility, we compared the physiological and metabolic properties of the fDsbA-L/$Ad^{-/-}$ and $Ad^{-/-}$ littermates. There is no significant difference in food intake, body weight, and insulin sensitivity between fDsbA-L/$Ad^{-/-}$ mice and $Ad^{-/-}$ littermates under normal chow conditions (data not shown). However, the fDsbA-L/$Ad^{-/-}$ mice displayed significantly less body weight and smaller fat cell size compared with $Ad^{-/-}$ mice under HFD (Fig. 7*A* and *B*). In addition, the fDsbA-L/$Ad^{-/-}$ mice were more active and displayed higher energy expenditure throughout the light and dark cycle compared with the $Ad^{-/-}$ mice under HFD (Fig. 7*C* and *D*), suggesting that overexpression of DsbA-L had additional beneficial effects on diet-induced obesity through an

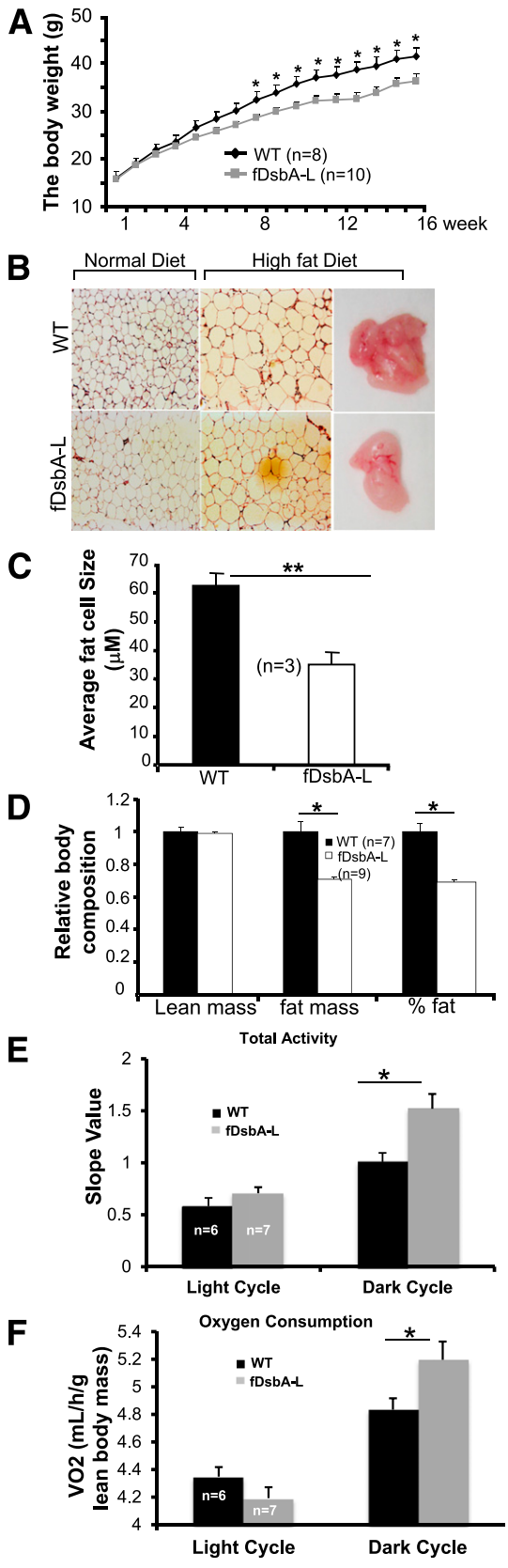

FIG. 3. fDsbA-L mice are resistant to HFD-induced obesity. *A*: Body weight gain of fDsbA-L mice and WT littermates. Male, 5-week-old, fDsbA-L mice and WT littermates were fed with 45% HFD for 16 weeks. Mouse body weight was measured weekly. *B*: Representative H&E images of epididymal fat pad and cell size from HFD-fed fDsbA-L and WT control mice. *C*: The average fat cell size of fDsbA-L mice and WT littermates was analyzed from the H&E-stained sections (200 cells were counted per slide) using the NIH ImageJ program. *D*: Tissue composition of the HFD-fed fDsbA-L mice (n = 9) and WT littermates (n = 7) was analyzed by DEXA. The total activity (*E*) and oxygen consumption (*F*) of the HFD-fed fDsbA-L mice and WT littermates were measured during a 48-h period, including two complete light cycles and two complete dark cycles. The average of oxygen consumption was normalized to lean body mass. The data represent mean ± SEM. *P < 0.05 and **P < 0.01. (A high-quality digital representation of this figure is available in the online issue.)

adiponectin-independent mechanism. However, diet-induced insulin resistance was similar in the fDsbA-L/$Ad^{-/-}$ and $Ad^{-/-}$ mice (Fig. 7*E–H*). Additionally, there was no significant difference in the glucose infusion rate and suppression of hepatic glucose production between fDsbA-L/$Ad^{-/-}$ mice and the $Ad^{-/-}$ mice fed with HFD (Supplementary Fig. 3*F* and *G*). Consistent with this, the fDsbA-L/$Ad^{-/-}$ mice and $Ad^{-/-}$ mice showed little difference in AMPK activity in both WAT and liver (data not shown). Taken together, these results suggest that adiponectin action plays a major role in mediating the protective effect of DsbA-L on diet-induced insulin resistance and liver steatosis.

DISCUSSION

Adiponectin is an anti–insulin resistant and anti-inflammatory adipokine that has great potential as a therapeutic target for various obesity-associated diseases such as type 2 diabetes, nonalcoholic steatohepatitis, and atherosclerosis (33). However, targeting adiponectin as a therapeutic intervention turns out to be difficult. Bacterially expressed full-length adiponectin, which lacks critical posttranslational modification, is essentially inactive (34), making large-scale production of this adipokine unfeasible. Efforts to increase adiponectin levels in vivo are also very challenging due to extremely high levels of endogenous adiponectin in vivo (35).

It has been shown that adiponectin oligomer distribution, rather than its absolute levels, correlates with a thiazolidiedione-mediated increase in insulin sensitivity (22). In addition, impairment in adiponectin multimerization has been shown to be associated with diabetes and hypoadiponectinemia (4,6). These important findings suggest that promoting adiponectin multimerization rather than its total cellular levels could provide an effective approach for the treatment of obesity-related diseases. Several mechanisms, including hydroxylation (36), glycosylation (37), succination (38), and disulfide bond formation (7,39), have been found to regulate adiponectin multimerization. However, the key molecules regulating these modification processes remain largely unknown.

We have recently found that DsbA-L promotes adiponectin multimerization in 3T3-L1 adipocytes (26). In addition, overexpression of DsbA-L prevents ER stress–induced and autophagy-dependent downregulation of adiponectin in 3T3-L1 adipocytes (27). Although these results suggest that DsbA-L plays a key role in regulating adiponectin multimerization and stability, the in vivo function of this protein remains unknown. In the current study, we show that DsbA-L promotes adiponectin multimerization in vivo (Fig. 2). In addition, we have demonstrated that fat-specific overexpression of DsbA-L protects mice from HFD-induced adiponectin downregulation, insulin resistance, and hepatic steatosis (Figs. 2–5). These results not only demonstrate a critical role of DsbA-L in promoting adiponectin multimerization in vivo but also provide direct evidence to support the notion that enhancing adiponectin multimerization

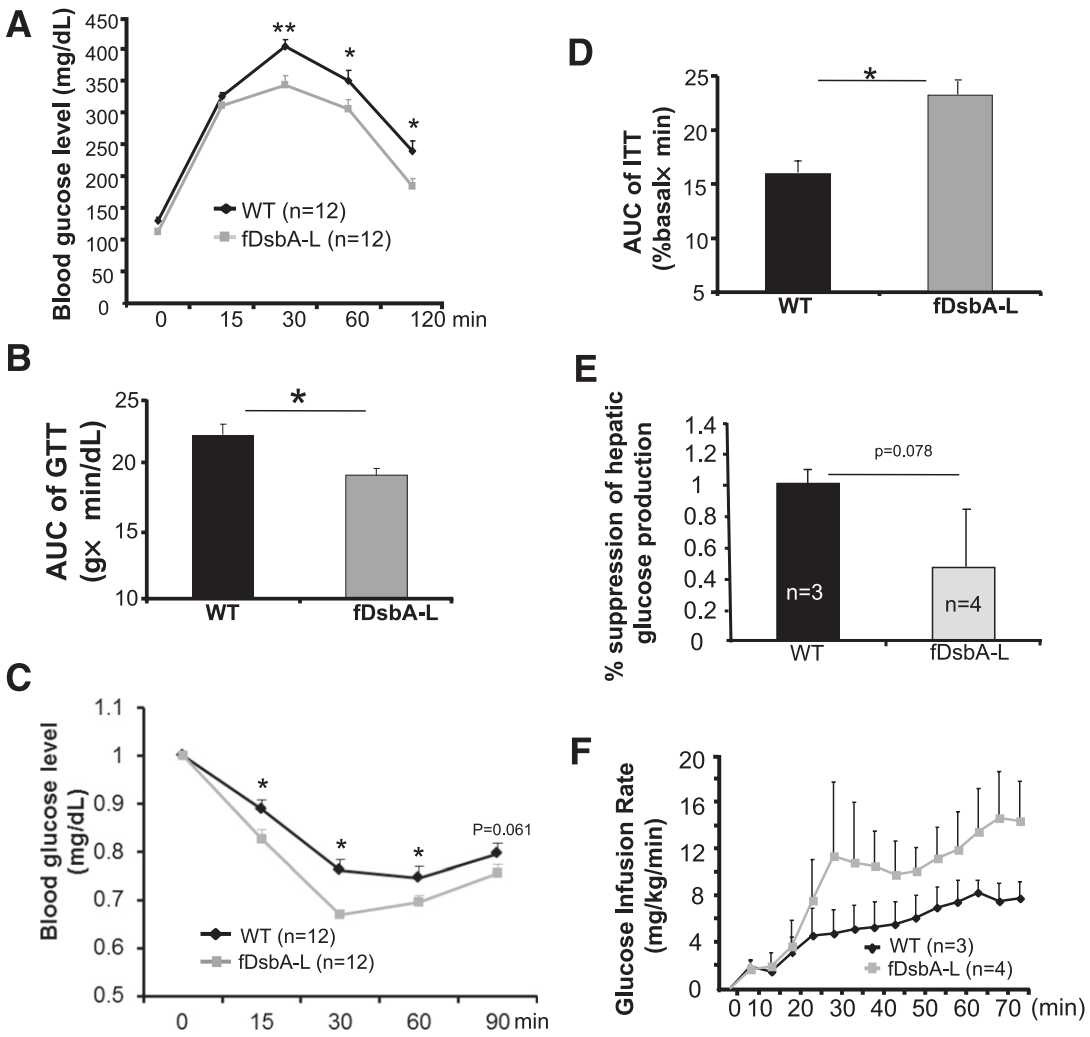

FIG. 4. Fat-specific overexpression of DsbA-L protects mice from HFD-induced insulin resistance. GTT (*A*) and ITT (*C*) were performed on male fDsbA-L and WT littermates fed with a 45% HFD for 16 weeks. *B* and *D*: The AUC for the data shown in *A* and *C* was calculated using the trapezoidal rule. The data represent mean ± SEM. **P* < 0.05. The suppressive effect of insulin on hepatic glucose production (*E*) and the glucose infusion rates (*F*) in the HFD-fed fDsbA-L mice and WT control mice were determined by hyperinsulinemic-euglycemic clamp experiments. *G*: AUC of glucose infusion rate in *F* was presented. The data represent mean ± SEM. **P* < 0.05. *H*: Phosphorylation (Thr^{172}) and the protein levels of AMPK in liver of the indicated mice were determined by Western blot. *I*: The insulin-stimulated phosphorylation of Akt (Thr^{308}) and its protein level in liver of the indicated mice were determined by Western blot. HFD-fed fDsbA-L mice and WT littermates were fasted for 16 h and injected with 5 units/kg insulin or an equal volume of saline for 5 min. Mice were killed and liver, muscle, WAT, and BAT were isolated. Data were quantified using the NIH ImageJ program. **P* < 0.05; ***P* < 0.01. Ins, insulin.

could be an effective strategy for the treatment of obesity-associated metabolic diseases.

We previously found that incubation with DsbA-L alone was insufficient to promote adiponectin multimerization in vitro (26), suggesting that additional factors may be necessary for adiponectin multimerization in intact cells. Interestingly, adiponectin has been shown to be covalently bound to the ER chaperone ERp44 (39). Ero1-Lα (endoplasmic reticulum oxidoreductin 1–like protein α), another ER chaperone, promotes adiponectin release from ERp44 (7,39). It is possible that DsbA-L may promote the release of adiponectin from ERp44 by interacting with Erol-Lα in cells, and thus facilitating adiponectin multimerization and secretion. Further studies will be needed to test this hypothesis.

The claim that adiponectin plays a role in regulating food intake has been controversial. Disruption of adiponectin gene expression did not significantly affect food intake and body weight (28,40,41). In addition, viral-mediated adiponectin expression ameliorated adiponectin deficiency–induced insulin resistance in liver or muscle but did not significantly affect the body weight of the mice (17,40). However, there is some data suggesting that the trimer form of adiponectin may function as a starvation hormone by regulating AMPK in the central nervous system to promote food intake (42). Although the origin of the low-molecular forms of adiponectin in the brain remains to be determined, the HMW form of this adipokine appears to be absent in the brain, probably due to the factor that formation of the HMW multimer prevents the crossing of the adipokine over the blood-brain barrier (42). Since overexpression of DsbA-L promotes the formation of the HMW form of adiponectin, it is conceivable that overexpression of DsbA-L has no major effect on food intake.

Our results suggest that enhanced energy expenditure may contribute to the antiobesity effect of DsbA-L. In agreement with this view, the activity of fDsbA-L mice was significantly increased compared with WT littermates. Additionally, the fDsbA-L mice displayed increased O_2

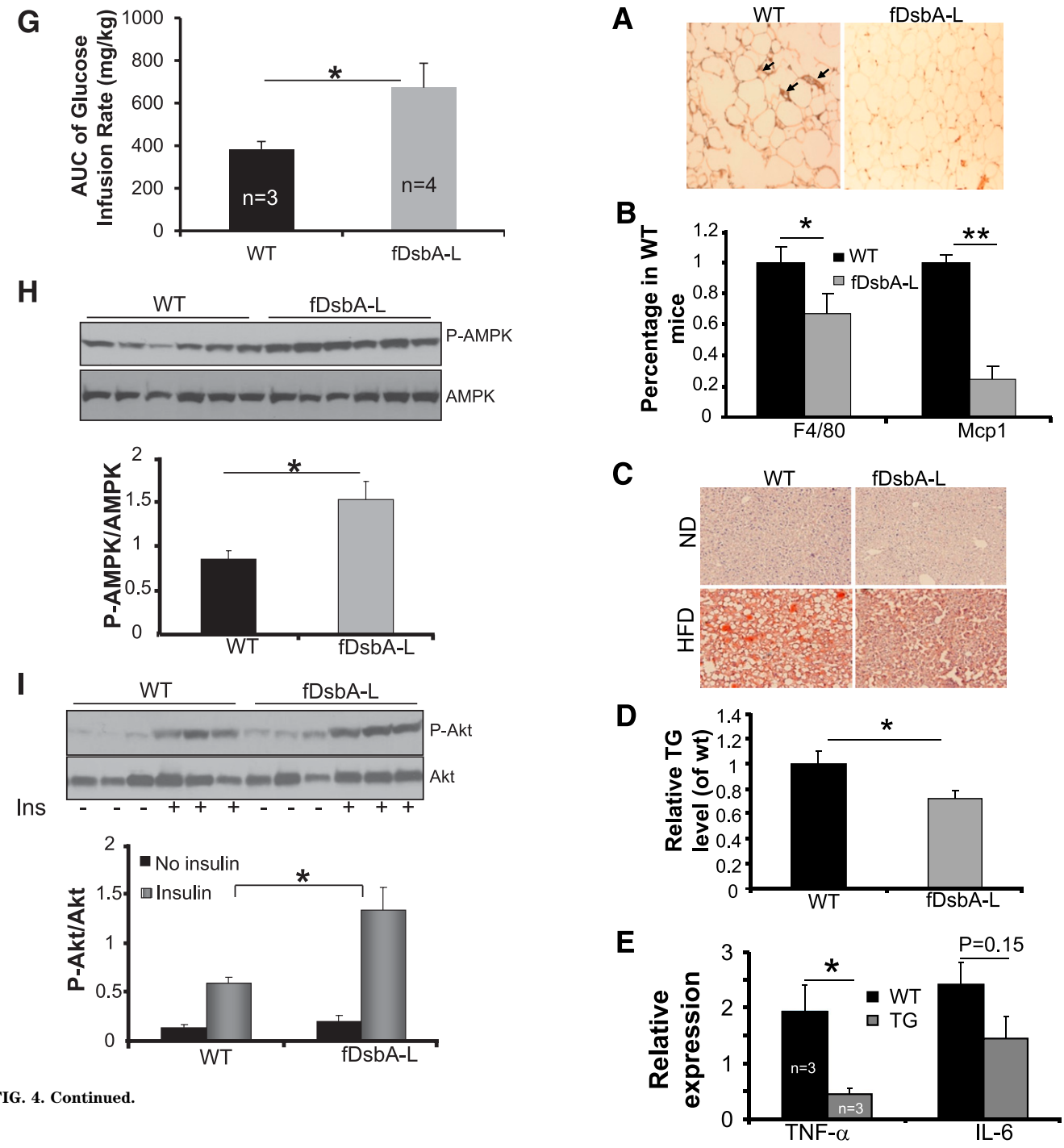

FIG. 4. Continued.

consumption compared with WT littermates (Fig. 3*F*). How fat-specific overexpression of DsbA-L leads to increased activity remains unknown, but acute peripheral administration of adiponectin has been shown to reduce body weight gain and visceral adiposity in obese mice, concurrently with enhanced rectal temperature and uncoupling protein 1 expression in BAT (43). In addition, adiponectin levels have been shown to be significantly correlated with thyroid hormones (44,45), which may contribute to increased activity. However, disruption of adiponectin expression only slightly impaired the effects of DsbA-L on body weight, fat mass, and energy expenditure on HFD (Fig. 6*B* and *C* and Supplementary Fig. 4*B* and *C*), suggesting that DsbA-L may have additional beneficial effects on energy homeostasis in addition to regulating adiponectin multimerization and

FIG. 5. Overexpression of DsbA-L in fat tissue reduced obesity-induced inflammation and hepatic steatosis. *A*: Macrophage infiltration into adipose tissue of HFD-fed fDsbA-L mice and control littermates as demonstrated by immunohistochemistry analysis with an anti-F4/80 antibody. *Arrows*: To point out infiltrated microphage. *B*: The mRNA levels of F4/80 and Mcp1 in gonadal WAT of the indicated mice were determined by quantitative real-time PCR. Glyceraldehyde-3-phosphate dehydrogenase was used as a control. n = 5–6 per group. *C*: Liver tissues were isolated from HFD-fed fDsbA-L and WT control mice, sectioned, and analyzed by Oil Red O staining. ND, normal diet. *D*: The triglyceride (TG) content in the liver of the fDsbA-L and WT control mice was determined using the triglyceride assay kit from Cayman Chemical Company and normalized to liver weight (wt). *E*: The expression levels of IL-6 and TNF-α in WAT of HFD-fed fDsbA-L and WT control mice were determined by Western blot and quantified by Scion Image Alpha 4.0.3.2 program. The expression levels were normalized to β-tubulin in each sample. n = 3. MCP1, monocyte chemoattractant protein-1. *P < 0.05; **P < 0.01. (A high-quality digital representation of this figure is available in the online issue.)

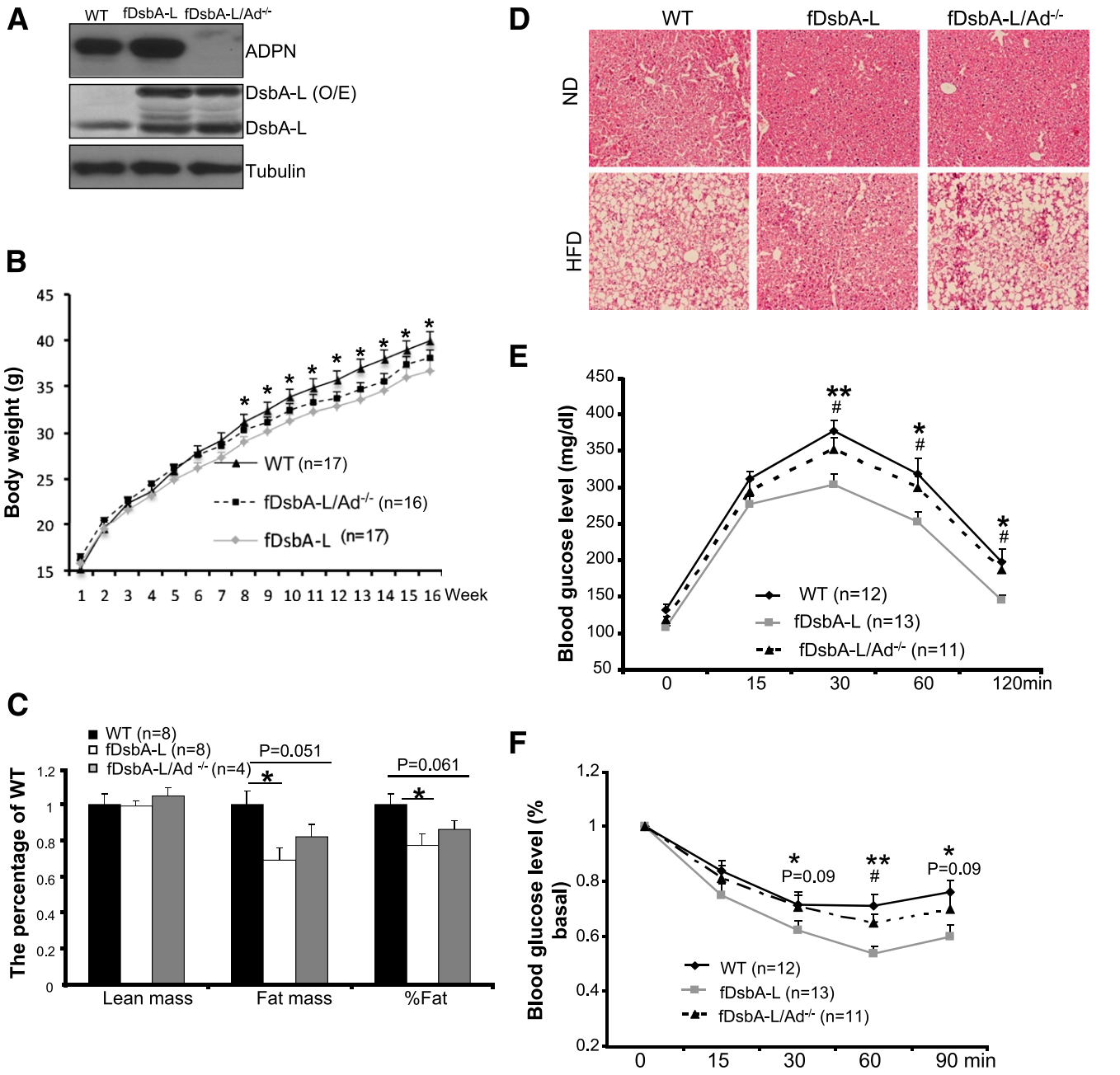

FIG. 6. Disruption of adiponectin expression diminishes the beneficial role of DsbA-L in mice. *A*: The expression levels of adiponectin and DsbA-L in fDsbA-L, fDsbA-L/*Ad*$^{-/-}$, and WT control mice were determined by Western blot with specific antibodies. Tubulin was used as a loading control. ADPN, adiponectin; O/E, overexpression. *B*: Five-week-old WT, fDsbA-L, and fDsbA-L/*Ad*$^{-/-}$ mice were fed with a 45% HFD for 16 weeks. The body weight gains of the indicated mice were measured weekly. *C*: Tissue composition of the HFD-fed DsbA-L (n = 8), fDsbA-L/*Ad*$^{-/-}$ (n = 4), and WT control mice (n = 8) was analyzed by DEXA. *D*: Representative H&E histological sections of liver from the indicated mice are shown. ND, normal diet. GTT (*E*) and ITT (*F*) were performed on the indicated mice. For GTT and ITT, 2 g/kg glucose and 0.075 units/kg insulin were used, respectively. Data represent means ± SEM. The statistical analysis was performed by ANOVA. *$P < 0.05$ and **$P < 0.01$ (fDsbA-L vs. WT); #$P < 0.05$ (fDsbA-L vs. fDsbA-L/*Ad*$^{-/-}$). (A high-quality digital representation of this figure is available in the online issue.)

function. Consistent with this, HFD-fed fDsbA-L/*Ad*$^{-/-}$ mice were leaner and more active compared with adiponectin-null mice, further suggesting an adiponectin-independent mechanism by which DsbA-L regulates energy homeostasis in vivo. It is possible that, in addition to adiponectin, DsbA-L may regulate the biosynthesis and secretion of other adipokines that exert an antiobesity effect. Alternatively, the overexpressed DsbA-L may enhance resistance to obesity through an autonomous action in adipocytes. Further investigations will be needed to test these possibilities.

Our results demonstrate that DsbA-L is an important regulator of adiponectin multimerization in vivo. Consistent with this, it has recently been found that whole-body knockout of DsbA-L (glutathione S-transferase [GST]-κ1) slightly reduced serum adiponectin levels and caused glomerular nephropathy (46). However, very recently, the same group reported that knockout of DsbA-L/GST-κ1 had no effect on total adiponectin levels, adiponectin multimerization, insulin tolerance, and glucose tolerance compared with WT control mice under HFD (47). One possible

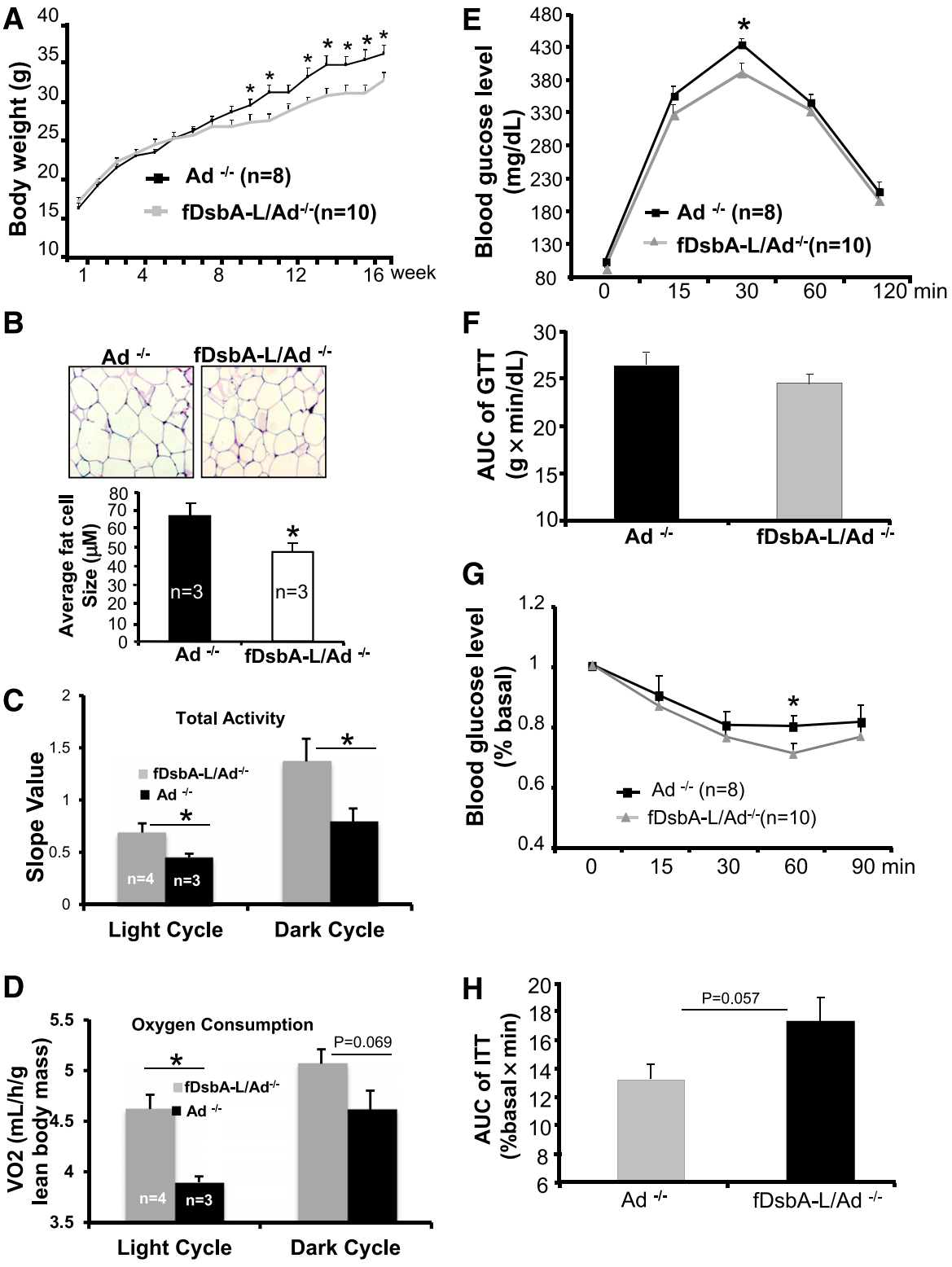

FIG. 7. DsbA-L could protect mice from HFD-induced obesity via an adiponectin-independent mechanism. Male fDsbA-L/$Ad^{-/-}$ and $Ad^{-/-}$ mice (7 weeks of age) were fed with a 45% HFD for 16 weeks. *A*: Body weight gains of the fDsbA-L/$Ad^{-/-}$ and $Ad^{-/-}$ mice. *B*: Representative H&E images showing fat cell size of epididymal fat from fDsbA-L/$Ad^{-/-}$ and $Ad^{-/-}$ male mice. The effects of DsbA-L overexpression on activity (*C*) and energy expenditure (*D*) were determined in a 48-h period including two dark and two light cycles. Oxygen consumption was normalized to lean body mass. *E*: The effects of DsbA-L overexpression on glucose tolerance in fDsbA-L/$Ad^{-/-}$ and $Ad^{-/-}$ mice. For GTT, 2 g/kg glucose was used. *F*: The AUC for the data shown in *E* was calculated using the trapezoidal rule. *G*: The effects of DsbA-L overexpression on insulin tolerance in fDsbA-L/$Ad^{-/-}$ and $Ad^{-/-}$ mice. For ITT, 0.075 units/kg insulin was used. The GTT and ITT data represent mean ± SEM. *$P < 0.05$ (ANOVA). *H*: The AUC for the data shown in *G* were calculated using the trapezoidal rule. The data represent mean ± SEM. *$P < 0.05$. (A high-quality color representation of this figure is available in the online issue.)

explanation for these controversies is that knockout of DsbA-L in vivo led to a compensatory increase in the expression of molecules that promote adiponectin expression and multimerization in adipose tissues. However, the finding that HFD feeding increased the levels of HMW adiponectin in both WT and DsbA-L knockout mice, which is contradictory to the findings of many others in the field (8,37,48–50), raises some concerns about the experimental conditions under which the experiments were performed. Thus, it remains to be determined whether knockout of DsbA-L under more physiologically relevant conditions affects adiponectin multimerization and function in vivo.

In summary, we have provided strong evidence for an in vivo role of DsbA-L in promoting adiponectin multimerization and function. Our study also demonstrates that enhanced adiponectin multimerization is sufficient to suppress obesity-induced insulin resistance and liver damage, suggesting that upregulation of DsbA-L could be an effective therapeutic approach for the treatment of obesity-induced insulin resistance and liver steatosis.

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M.L. and F.L. designed the experiment; researched data; wrote, reviewed, and edited the manuscript; and contributed to discussion. R.X. researched data and contributed to discussion. S.A.W., N.Z., L.B.S., K.A., L.Z., H.C., and G.X. researched data. C.A.W., S.N.A., N.M., R.A.D., and R.A. reviewed and edited the manuscript. P.E.S. and L.Q.D. reviewed and edited the manuscript and contributed to discussion. F.L. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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