Regulation of Lipolytic Response and Energy Balance by Melanocortin 2 Receptor Accessory Protein (MRAP) in Adipocytes

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Melanocortin 2 receptor accessory protein (MRAP) is highly expressed in adrenal gland and adipose tissue. In adrenal cells, MRAP is essential for adrenocorticotropic hormone (ACTH)–induced activation of the cAMP/protein kinase A (PKA) pathway by melanocortin 2 receptor (MC2R), leading to glucocorticoid production and secretion. Although ACTH was known to stimulate PKA-dependent lipolysis, the functional involvement of MRAP in adipocyte metabolism remains incompletely defined. Herein, we found that knockdown or overexpression of MRAP in 3T3-L1 adipocytes reduced or increased ACTH-induced lipolysis, respectively. Moreover, an unbiased proteomics screen and coimmunoprecipitation analysis identified Gαs as a novel interacting partner of MRAP. An MRAP mutant disabled in Gαs association failed to augment the activation of PKA and lipolytic response to ACTH. Furthermore, compared with wild-type mice, transgenic mice (aP2-MRAP) overexpressing MRAP fat specifically exhibited increased lipolytic response to ACTH. When fed a high-fat diet (HFD), the transgenic mice displayed a significant decrease in the gain of adiposity and body weight as well as an improvement in glucose and insulin tolerance. These phenotypes were accompanied by increased adipose expression of genes for mitochondrial fatty acid oxidation and thermogenesis, and overall energy expenditure. Collectively, our data strongly suggest that MRAP plays a critical role in the regulation of ACTH-induced adipose lipolysis and whole-body energy balance.

Obesity, which is characterized by the excessive accumulation of triglycerides (TGs) in adipose tissue, has been implicated to predispose many metabolic disorders, such as hyperlipidemia, insulin resistance, and type 2 diabetes. Lipolysis is a process of TG hydrolysis via which free fatty acids (FFAs) are released from adipocytes for intracellular fatty acid (FA) oxidation or for supplying energy substrates to other tissues (1). Impaired lipolysis in obesity is known to cause dysregulated adipocyte metabolism and FA efflux, which contribute to ectopic TG storage, lipotoxicity, and insulin resistance in other peripheral tissues (2–4). Accordingly, the precise mechanisms underlying the lipolytic dysfunction need to be fully elucidated in order to overcome obesity and its complications.

In response to fasting, adipocyte lipolysis is mainly stimulated by β-adrenergic hormone norepinephrine (NE) and catalyzed sequentially by adipose triglyceride lipase (ATGL), hormone-sensitive lipase (HSL), and monoacylglycerol lipase (MGL) (1,5). During chronic cold exposure, lipolytic activation by NE leads to the upregulation of thermogenic gene program in brown adipose tissue (BAT) and the appearance of brown-like (beige) adipocytes in subcutaneous white adipose tissue (WAT) (6). A promising therapeutic concept for treating obesity and its associated disorders is through expanding the beige adipocyte population and thereby enhancing whole-body energy expenditure (7).

Emerging evidence indicates that apart from NE, adrenocorticotropic hormone (ACTH) also plays an important role in adipocyte lipolysis (8–13). ACTH activity is mediated exclusively by a G protein–coupled receptor (GPCR) known as the melanocortin 2 receptor (MC2R), the activation of which results in elevated intracellular cAMP levels and increased protein kinase A (PKA) activity (14,15). The MC2R is the smallest member of the melanocortin receptor

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family, which consists of five GPCRs (MC1R–MC5R) with diverse physiological roles (16). Early studies have shown that MC2R activation by ACTH requires the MC2R accessory protein (MRAP) (17–20). In the adrenal gland, MC2R and MRAP together promote the synthesis and secretion of glucocorticoids (GCs). In fact, inactivating MRAP mutations account for ~20% of the familial GC deficiency type 2 cases in humans (17).

Interestingly, MRAP was originally identified to be a fat-specific protein and highly enriched in differentiated adipocytes (21). A recent study showed that MRAP is a direct target gene of PPARγ (22), a master transcription factor for adipogenesis. However, whether and how MRAP is involved in the ACTH-MC2R signaling to lipolytic activation has not been determined. In the current study, we explored the effects of MRAP in adipocytes by using both loss- and gain-of-function approaches. Moreover, using an unbiased proteomics approach, we identified Gαs, the stimulatory subunit of the heterotrimeric G protein complex, as a specific binding protein of MRAP. Experiments using transgenic mice overexpressing MRAP fat specifically further demonstrated an in vivo role for MRAP in regulating adiposity and insulin resistance associated with diet-induced obesity (DIO).

RESEARCH DESIGN AND METHODS

Antibodies and Reagents

Anti–β-actin antibody (no. A1978) and anti-FLAG M2 Affinity gel (no. A2220) were obtained from Sigma-Aldrich. Anti–phospho-HSL antibody (no. 4126), anti-HSL antibody (no. 4107), anti-aP2 (FABP4) antibody (no. 2120), and anti–phospho-PKA substrate antibody were purchased from Cell Signaling Technology. Affinity-purified rabbit polyclonal antibody for MRAP was generated against a peptide corresponding to the COOH-terminal CLRRASLQTTEEPGRRAGTDQWLT sequence in mouse MRAP by FabGennix International Inc. Horseradish peroxidase–linked secondary antibodies were purchased from Jackson ImmunoResearch Laboratories. The protease inhibitor mini tablets (catalog no. 11 836 170 001) were obtained from Roche Diagnostics. The glycerol assay kit (catalog no. LIP-1-NC-L1) was purchased from Zen-Bio. The RNeasy Plus Mini Kit (catalog no. 74134) was purchased from Qiagen. ACTH and isoproterenol (ISO) were obtained from Sigma-Aldrich. Reagents for tissue culture were obtained from Invitrogen.

RNA Extraction and Real-time PCR

Total RNA was isolated using the RNeasy Plus Mini Kit according to the manufacturer's instruction. cDNA synthesis and subsequent real-time PCR (RT-PCR) analysis were performed as described previously (23). The sequences of PCR primers are available upon request. Data were analyzed using the comparative cycle threshold ($^{\Delta\Delta}$Ct) method. The mRNA levels of genes normalized to β-actin were presented as relative to the control. PCR product specificity was verified by postamplification melting curve analysis and by running products on an agarose gel.

PCR Cloning of cDNA and Site-Directed Mutagenesis

The complete open reading frame of mouse MRAP was cloned into pRK vector without any tags, or pKF vector with a COOH-terminal FLAG epitope tag as before (24). Deletion mutations were generated by using the QuikChange site-directed mutagenesis kit (Agilent) according to the manufacturer's guidelines.

Cell Culture and Protein Overexpression

3T3-L1 preadipocytes were maintained and differentiated as described previously. HepG2 cells were cultured in high-glucose DMEM supplemented with 10% newborn calf serum, 100 units/mL penicillin G sodium, and 100 μg/mL streptomycin sulfate. For overexpression of MRAP in 3T3-L1 adipocytes, recombinant adenoviruses encoding mouse MRAP-FLAG (Ad-MRAP-FLAG) and MRAPΔ14–17-FLAG (Ad-MRAPΔ14–17-FLAG) under the control of a cytomegalovirus promoter were custom generated by Vector Biolabs. Adenoviral infection of differentiated 3T3-L1 adipocytes was conducted using our established protocol as described previously (25). For overexpression of MRAP or G protein subunits in HepG2 cells, cells cultured in high-glucose DMEM supplemented with 10% FBS were transfected with DNA plasmids using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Small Interfering RNA–Mediated Gene Knockdown

For small interfering RNA (siRNA)–mediated knockdown experiments, differentiated 3T3-L1 adipocytes were electroporated, as described previously (24), and then plated in six-well plates. After 3 days of incubation, cells were processed for designated assays. The following double-stranded stealth siRNA oligonucleotides (Invitrogen) for MRAP were used: set 1, sense 5′-UGGCUACCUUCGUGGUGCUCCUCUU-3′ and antisense 5′-AAGAGGAGCACCACGAAGGUACCA-3′; set 2, sense 5′-UAGACCUCAUUCCUGUGGACGAGAA-3′ and antisense 5′-UUCUCGUCCACAGGAAUGAGGUCUA-3′. Control oligonucleotides with comparable GC content were also from Invitrogen.

Immunoblotting Analysis

Preparation of tissue and cell lysates and subsequent immunoblotting analysis were performed as described previously (23). After SDS-PAGE and transfer to nitrocellulose membranes, individual proteins were blotted with primary antibodies at appropriate dilutions. For competition assay, the membranes were incubated with anti-MRAP antibody in the absence or presence of MRAP peptide (CLRRASLQTTEEPQRRAGTDQWLT). After an overnight incubation, peroxide-conjugated secondary antibodies were incubated with the membrane at a dilution of 1:5,000. The signals were then visualized by chemiluminescence (Supersignal ECL; Pierce).

Immunoprecipitation

Liver tissues or cells were lysed in cell lysis buffer containing 50 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 1% Triton X-100, 1 mmol/L dithiothreitol, and protease tablet

inhibitors (one tablet per 10 mL buffer). Anti-Flag M2-conjugated gels were incubated with the lysates for 4 h at 4°C. The beads were then washed four times with lysis buffer, and the bound proteins were eluted in SDS buffer and analyzed by immunoblotting or mass spectrometry.

Immunofluorescence Staining

Cells were fixed with 3% paraformaldehyde in PBS for 20 min, quenched with 100 mmol/L glycine in PBS for 20 min, and then blocked with 1% BSA in PBS for 1 h. The cells were then exposed to anti-FLAG antibody for 2 h at room temperature. After three washes with PBS, the cells were treated for 1 h with the Alexa Fluor anti-mouse 568 secondary antibody (Invitrogen). Samples were mounted on glass slides with Vectashield mounting medium with DAPI and examined under a Zeiss LSM 510 inverted confocal microscope.

Human Adipose Tissue Collection

Human subcutaneous abdominal adipose tissue (2–6 g) was obtained from patients undergoing elective abdominal/gynecological laparoscopic surgeries at the Mayo Clinic Arizona Hospital. Patients were females, average age 40 years old, with an average BMI 40 kg/m^2, otherwise healthy with no active or recent use of glucocorticosteroid medications, and nonsmokers. No known history of cardiometabolic disease was reported by any of the participants. The study was approved by the Institutional Review Board of the Mayo Clinic and registered at clinicaltrials.gov (NCT02378077).

Mass Spectrometry

The immunoprecipitated samples were resolved by 10–20% SDS-PAGE gels and visualized by Coomassie blue staining. Then, the gel portions were excised, destained, dehydrated, dried, and subjected to trypsin digestion. The resulting peptides were subjected to liquid chromatography–mass spectrometry analysis.

Animal Experiments

To generate transgenic mice with tissue-specific overexpression of MRAP in adipose tissue, murine MRAP cDNA full sequence (National Center for Biotechnology Information reference sequence: NM_029844) was subcloned into a pBluescript II SK(+) vector containing a 5.4-kB adipocyte FA binding protein (aP2) promoter (Addgene) with *Sma*I and *Not*I restriction enzyme sites on the 5′ and 3′ ends, respectively. The completed aP2-G0S2-poly(A) construct was confirmed by sequencing. Through the Mayo Clinc Transgenic and Knockout Mouse Core, the transgene fragment was released by *Sal*I digestion, purified, and microinjected into fertilized eggs of C57BL/6J mice. Tail DNA genotyping revealed that seven independent transgenic founder lines were obtained, of which five lines underwent successful germ line transmission. aP2-MRAP transgenic mice and wild-type (WT) C57BL/6J littermates were maintained in the animal facility at Mayo Clinic Arizona. All mice were given free access to water and were fed a standard chow diet (test diet no. 5001, 10% calories as fat).

For high-fat diet (HFD) treatment, mice were fed a 60% HFD (Research Diets, D12492, 60% calories as fat) beginning at 7 weeks of age. Peritoneal macrophages were collected by peritoneal lavage from mice that had received, 4 days previously, an intraperitoneal injection of 2 mL of 3% Brewer thioglycollate medium (BD Biosciences). All animal protocols were approved by the Mayo Clinic Institutional Animal Care and Use Committee.

Metabolic Cage Analysis

Female mice were individually placed in a PhenoMaster metabolic cage unit (TSE Systems) for a multiday study. Standard 12-h light (7:00 A.M. to 7:00 P.M.) and dark (7:00 P.M. to 7:00 A.M.) cycles were maintained throughout the experiment. Mice were allowed an environmental acclimation period of 24 h prior to starting the experiment at 7:00 A.M. Mice were allowed to eat and drink ad libitum.

Glucose and Insulin Tolerance Tests

For the glucose and insulin tolerance tests, fasted mice were injected intraperitoneally with glucose (2 g/kg) or insulin (0.85 units/kg). Blood glucose levels were monitored at indicated times from the tail vein using a glucometer (Freestyle; Abbott Diabetes Care).

Plasma Metabolite Analysis

Plasma TG (Thermo Fisher Scientific), cholesterol (Wako), and FFAs were measured according to the manufacturer's protocols. Plasma insulin was measured using ELISA reagent kits purchased from Millipore.

Adipose Tissue Histology

When mice were killed, inguinal adipose tissue or BAT were rapidly fixed in 10% neutral buffered formalin at 4°C overnight and embedded in paraffin. Paraffin sections were cut, mounted on glass slides, and stained with hematoxylin-eosin. Sections were photographed at ×20 magnifications.

Assays for Lipolysis

In vitro lipolysis was measured as the amounts of FFAs and glycerol released into the medium. 3T3L-1 adipocytes in six-well plates were washed twice with PBS and starved with phenol red–free DMEM containing 2% BSA for 3 h. Then cells were incubated in 2 mL of the same medium in the presence or absence of ISO or ACTH for another 3 h. For ex vivo lipolysis, inguinal fat pads isolated from mice or human subcutaneous fat pads were incubated at 37°C in 1.0 mL phenol red–free DMEM containing 2% FA-free BSA with or without ACTH or ISO. The amounts of FFAs (Wako) and glycerol (Zenbio) in the medium were determined by using assay kits according to the manufacturer's instructions. The lipolytic output was normalized with either protein concentrations in the lysates or the total mass of fat tissue used. For in vivo lipolysis, female mice were injected with 1 mg/kg ISO, 10 mg/kg ACTH, or saline as a vehicle control. Plasma was collected at 1 h postinjection for the determination of the plasma FFA levels (Wako).

Statistical Analysis

Values are expressed as mean ± SD unless otherwise indicated. Statistical significance was evaluated by Student t test. Differences were considered significant at $P < 0.05$.

RESULTS

Expression Pattern of MRAP Protein

First, we examined the protein expression of MRAP in various mouse tissues by immunoblotting with a newly generated antibody. As shown in Fig. 1*A*, a major cluster of bands slightly heavier than 25 kDa were detected mainly in adrenal gland as well as in gonadal WAT. These bands were only abolished by an MRAP peptide competing for the antibody but not by a nonspecific peptide (Supplementary Fig. 1), validating their specific identity as MRAP protein. This is consistent with the previous observations that MRAP protein, despite a predicted size at14 kDa, is often glycosylated and as a result migrates as a higher molecular weight smear on SDS-PAGE (18,26–28). Consistently, treatment of WAT extracts with PNGase F reduced a majority of the 25-kDa bands to below 20 kDa (Fig. 1*B*), confirming N-glycosylation as a key modification in MRAP. In addition, immunoblotting analysis in combination with the peptide competition confirmed a differentiation-dependent expression of MRAP in 3T3-L1 adipocytes (Fig. 1*C*). The MRAP protein was only detected in aP2-expressing mature adipocytes but not in preadipocytes. aP2 is a specific marker

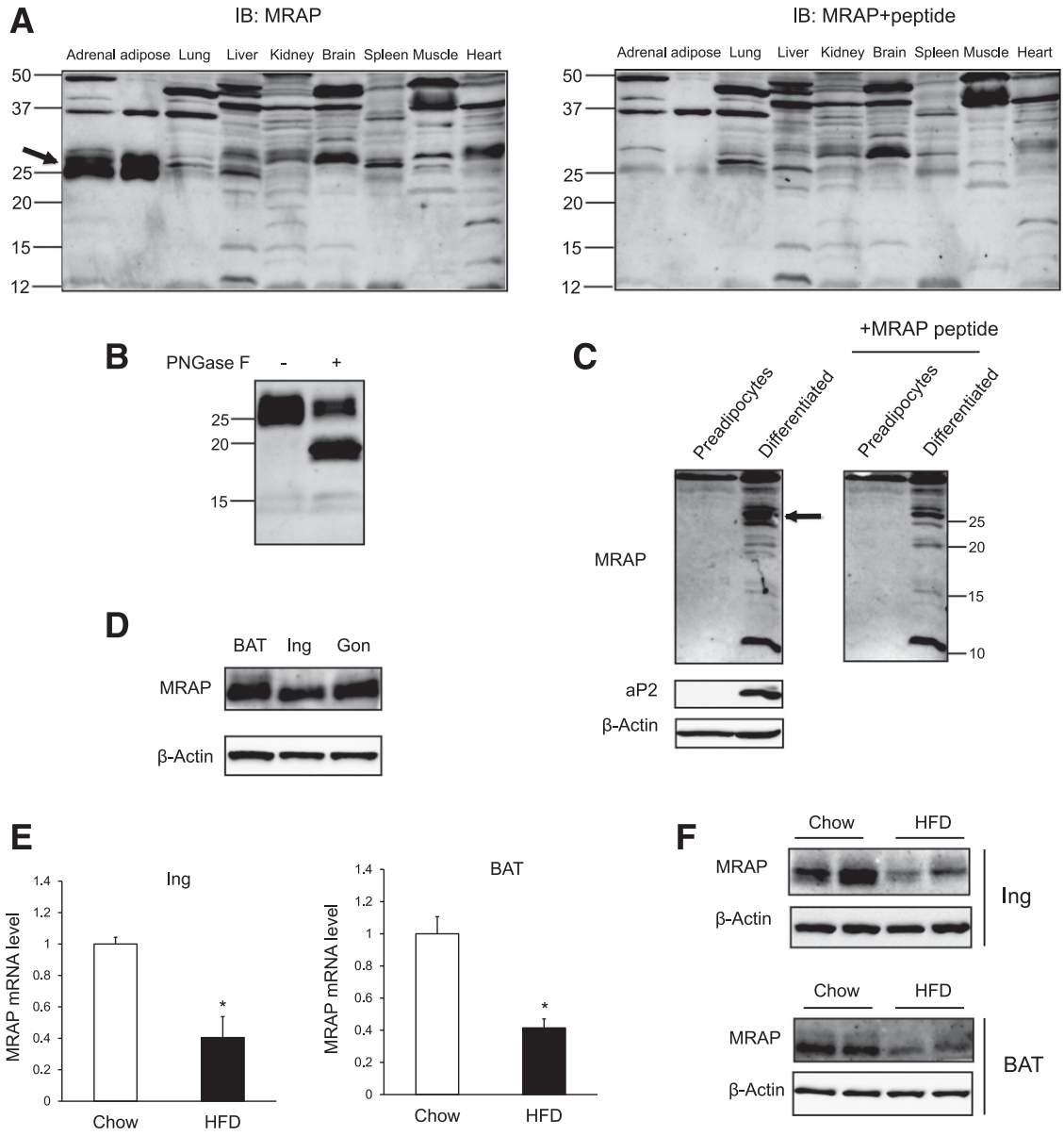

Figure 1—MRAP is highly expressed in adipose tissue and reduced by HFD feeding. *A:* Tissue distribution of MRAP protein in mice was detected by immunoblotting (IB) using an anti-MRAP antibody in the presence or absence of MRAP peptide. Adipose lysate was extracted from the gonadal adipose tissue. Arrow indicates MRAP protein. *B:* Lysate from inguinal adipose tissue was treated with PNGase and then immunoblotted with MRAP antibody. *C:* Expression of MRAP protein was analyzed by immunoblotting in lysates from undifferentiated 3T3-L1 preadipocytes or differentiated 3T3-L1 adipocytes. Arrow indicates MRAP protein. *D:* Immunoblotting analysis of MRAP expression in adipose tissues. Gon, gonadal adipose tissue; Ing, inguinal adipose tissue. *E* and *F:* RT-PCR or immunoblotting analysis of MRAP expression in adipose tissues from mice fed chow diet or HFD for 12 weeks. Data were shown as mean ± SE (n = 3 per group). *$P < 0.05$ vs. chow.

protein that emerges in the later stage of adipogenic differentiation. Moreover, MRAP protein was found to be expressed in different adipose tissues, including interscapular BAT and inguinal and gonadal WAT (Fig. 1*D*). Interestingly, both MRAP mRNA and protein levels were markedly decreased in both BAT and inguinal WAT of HFD-fed mice compared with those of chow-fed mice (Fig. 1*E* and *F*). Together, these results demonstrate that MRAP is abundantly expressed in mature adipocytes, and its expression in vivo is subject to downregulation in DIO.

MRAP Regulates ACTH-Stimulated Lipolysis

MRAP is necessary for activation of the cAMP-PKA pathway by MC2R upon ACTH stimulation. Since PKA activation is a major driver of lipolytic response in adipose tissue, we next tested whether MRAP would be involved in the ACTH-stimulated lipolysis. To this end, we used two distinct siRNA oligonucleotides (oligos) to knock down the expression of MRAP in differentiated 3T3-L1 adipocytes. Figure 2*A* showed that compared with a GC-matched control oligo, both MRAP siRNAs caused a >85% reduction of MRAP mRNA levels. The effectiveness of knockdown was also confirmed by immunoblotting analysis of MRAP protein (Fig. 2*B*). Importantly, knockdown of MRAP significantly decreased phosphorylation of PKA substrates and HSL stimulated by ACTH as well as attenuated the rate of ACTH-induced release of FFAs and glycerol, although no differences in PKA activity or lipolysis were observed under basal conditions (Fig. 2*C*–*E*). In comparison, ISO-stimulated lipolysis was unaffected by the absence of MRAP (Fig. 2*F* and *G*). Therefore, MRAP is only indispensable for the full-scale activation of lipolysis by ACTH in adipocytes. Furthermore, a previous study by Xue et al. (11) demonstrated that ACTH stimulated lipolysis in isolated human subcutaneous adipocytes. In agreement, we found that ACTH was able to significantly stimulate release of FFAs and glycerol in isolated human subcutaneous fat explants, although to a lesser extent when compared with the β-adrenergic agonist ISO (Fig. 2*H* and *I*). These results indicate that the MRAP-MC2R system is functional in the regulation of lipolysis in human adipose tissue.

MRAP Binding to Gαs Is Critical for ACTH Stimulation of PKA and Lipolysis

To gain further insight into the mechanistic role of MRAP, we searched for novel binding proteins of MRAP using an unbiased proteomic approach. Specifically, we expressed MRAP protein COOH-terminally tagged with a FLAG epitope (MRAP-FLAG) in HepG2 cells using an empty vector as control (Fig. 3*A*). After anti-FLAG immunoprecipitation, mass spectrometry analysis identified one potential MRAP binding partner as Gαs, the stimulatory subunit of the heterotrimeric G protein complex that transduces extracellular signals from GPCRs to intracellular activation of the cAMP/PKA pathway. The specific interaction between Gαs and MRAP was demonstrated by anti-FLAG coimmunoprecipitation when MRAP-FLAG and Gαs were coexpressed in cells (Fig. 3*B*). In comparison, there also was a modest interaction of MRAP with Gαi, but no interaction was detected between MRAP and Gαq (Fig. 3*C* and *D*).

Mouse MRAP is a 127–amino acid protein that contains a central hydrophobic transmembrane domain (residues 37–59). Whereas deletion of either the transmembrane domain (MRAPΔ37–59) or the COOH-terminal region (MRAPΔ63–127) severely impacted protein expression, an MRAP mutant lacking the NH_2-terminal portion (MRAPΔ2–35) was stable and could be expressed at a level comparable to that of the WT protein (Fig. 3*E*). Interestingly, this mutant was markedly impaired in its ability to interact with Gαs as revealed by the coimmunoprecipitation assay. Analysis of three smaller deletions of the NH_2-terminal domain showed that residues 14–17 in MRAP were critically needed for mediating the specific interaction with Gαs (Fig. 3*F*).

To elucidate the functional relevance of the MRAP-Gαs interaction, we overexpressed either WT MRAP or MRAPΔ14–17 in 3T3-L1 adipocytes via adenoviral infection. Compared with the cells infected with Ad-null, cells infected with Ad-MRAP-FLAG and Ad-MRAPΔ14–17-FLAG expressed encoded proteins respectively at similar levels (Fig. 4*A*). As shown in Fig. 4*B*, expression of the WT MRAP significantly enhanced PKA substrate and HSL phosphorylation under the treatment by ACTH. However, no change in phosphorylation of PKA substrates or HSL was observed with MRAPΔ14–17, the mutant deficient in binding to Gαs. Whereas neither form of MRAP conferred any impact on basal lipolysis, expression of the WT MRAP but not MRAPΔ14–17 significantly increased the rates of FFA and glycerol release upon ACTH stimulation (Fig. 4*C* and *D*). As revealed by immunofluorescent staining (Fig. 4*E*), MRAPΔ14–17 was still capable of localizing to the plasma membrane in HepG2 cells and differentiated 3T3-L1 adipocytes (Fig. 4*E*). Taking together, these data suggest that the interaction between MRAP and Gαs is a necessary step for ACTH/MC2R to mediate PKA activation and lipolytic stimulation in adipocytes.

Overexpression of MRAP Enhances ACTH-Stimulated Lipolysis In Vivo

To examine the in vivo function of MRAP in adipose tissue, we generated a transgenic mouse model that overexpresses MRAP under the control of a fat-specific aP2 promoter. Immunoblotting analysis of isolated WAT showed an around fourfold increase in MRAP protein expression in aP2-MRAP mice over the endogenous levels in WT animals (Fig. 5*A*), indicating a successful overexpression of MRAP in adipose tissue. In comparison, there was no detectable expression of MRAP in macrophages isolated from either WT or transgenic mice (Fig. 5*A*). Whereas HFD expectedly induced a significant decrease in endogenous MRAP levels in the WAT of WT animals, the aP2-MRAP mice on HFD were able to largely maintain their adipose MRAP expression (Fig. 5*B*). Transgenic expression of MRAP did not appear to impact basal and fasting-induced lipolysis as aP2-MRAP mice exhibited no changes in fed or fasted plasma FFA levels (Fig. 5*C*). Whereas administration of ACTH and ISO

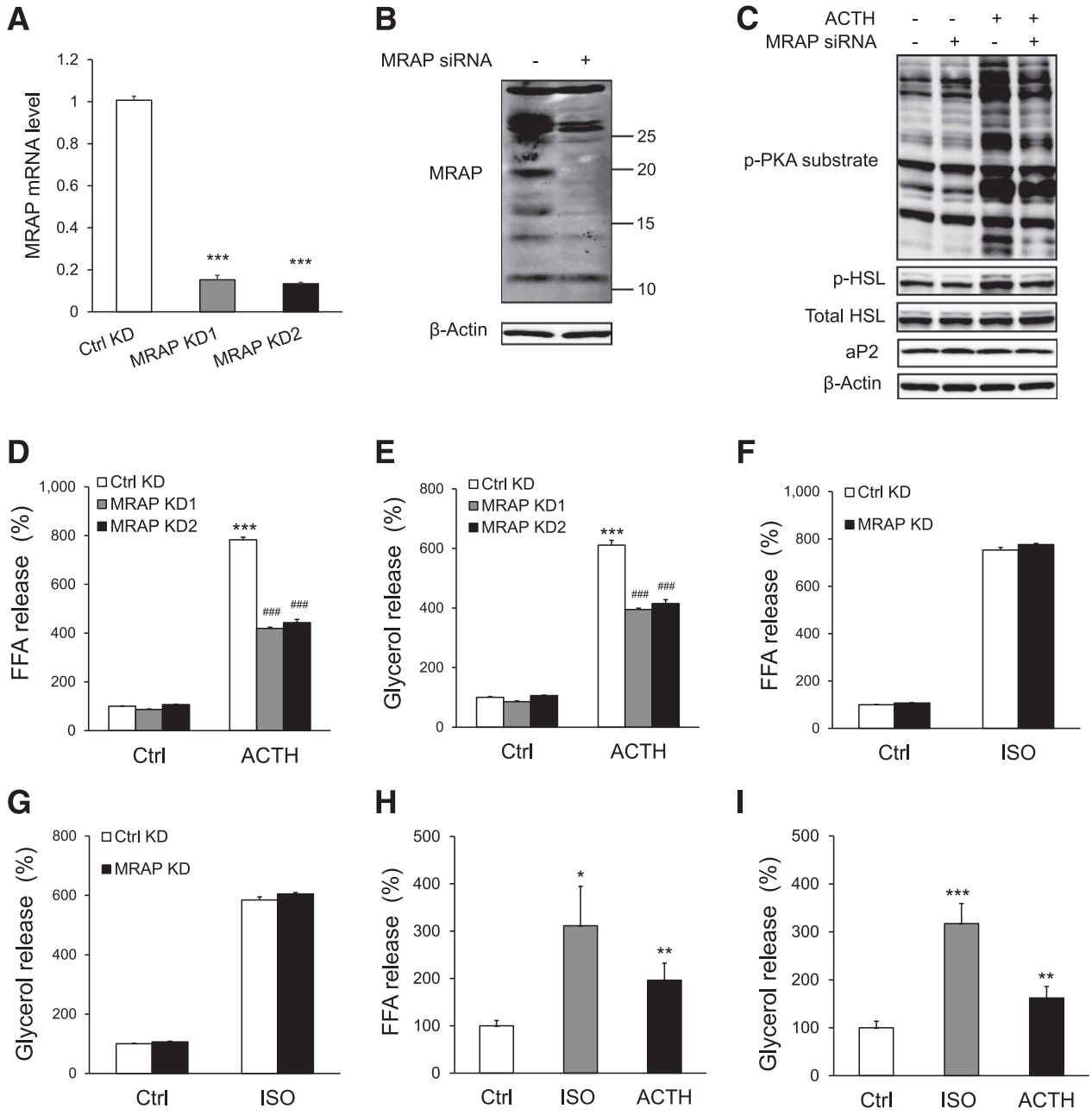

Figure 2—MRAP mediates ACTH-induced lipolysis in adipocytes. *A–G:* Knockdown (KD) of MRAP in differentiated 3T3-L1 adipocytes was achieved by transfecting cells with two distinct MRAP-specific siRNA oligonucleotides. After 3 days of siRNA treatment, cells were collected for RT-PCR analysis (*A*) or immunoblotting (MRAP KD1) (*B*) or were treated with 20 nmol/L ACTH or 2 μmol/L ISO for 3 h followed by immunoblotting (*C*) and lipolysis assay (*D–G*). siRNA for MRAP KD1 was used in *C*, *F*, and *G*. RT-PCR data were normalized to β-actin. FFA and glycerol releases were normalized with intracellular protein contents. $^{***}P < 0.001$ vs. control (Ctrl) group; $^{###}P < 0.001$ vs. Ctrl KD group with ACTH treatment. *H* and *I:* Fat explants from human subcutaneous adipose tissue were treated with 2 μmol/L ISO or 0.2 μmol/L ACTH for 3 h. FFA and glycerol releases were measured and normalized with the total mass of explants. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ vs. Ctrl group. Data represent three independent experiments.

both increased plasma FFA levels in the WT mice, aP2-MRAP only displayed enhanced lipolytic response to ACTH but not to ISO (Fig. 5*D*). The effect of MRAP overexpression was further confirmed by analysis of the lipolytic output from isolated fat explants. Compared with those from the WT mice, WAT explants from the aP2-MRAP mice showed a 35% and 60% increase in the release rates of glycerol and FA, respectively, in response to ACTH treatment (Fig. 5*E* and *F*). By contrast, no significant changes were observed with ISO-induced lipolysis in the fat explants of WT and aP2-MRAP mice (Fig. 5*G* and *H*). Collectively, these findings suggest that adipose overexpression of MRAP is capable of enhancing lipolysis mediated by ACTH/MC2R signaling but not in response to β-adrenergic stimulation. It is unlikely

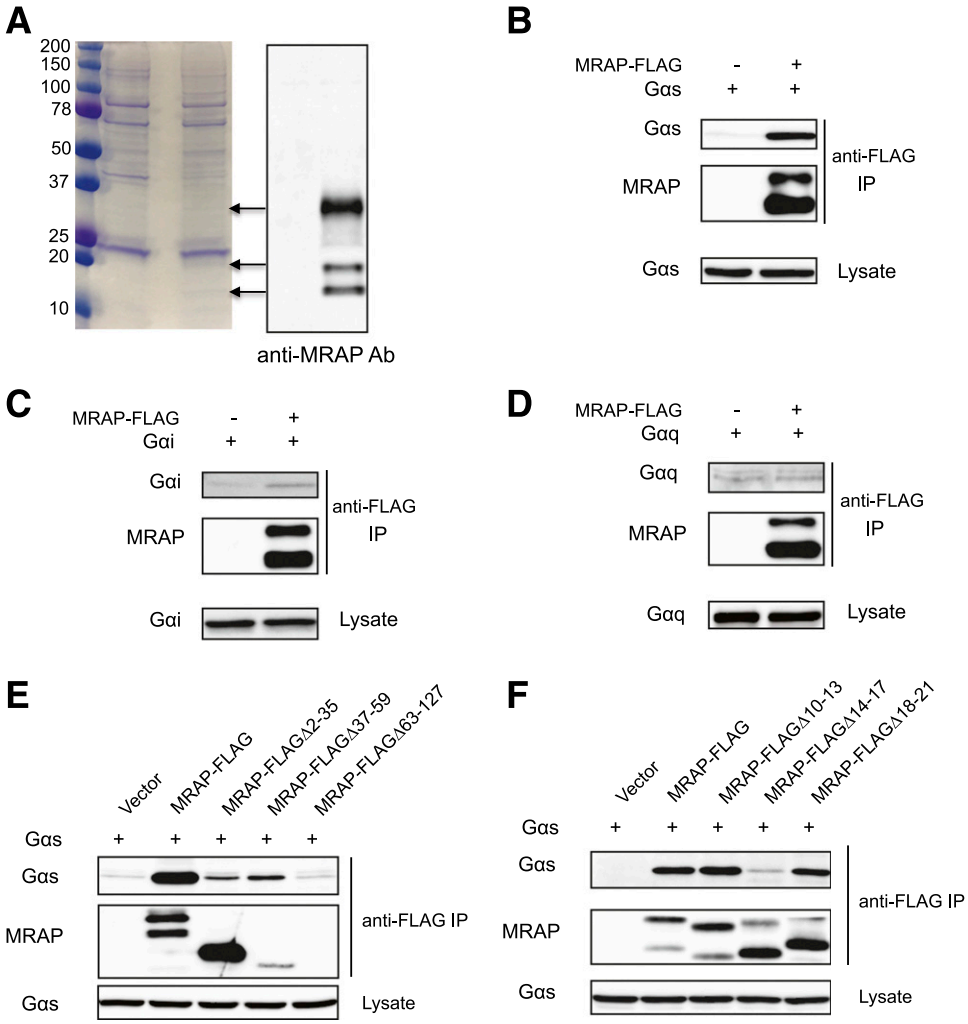

Figure 3—MRAP specifically interacts with Gαs. *A:* Eight-week-old C57BL/6J male mice were injected with 1×10^9 pfu of Ad-null or Ad-MRAP-FLAG via the retro-orbital sinus. Three days later, the liver was collected and lysed. Then the lysate was separated on the 10–20% SDS-PAGE gel and was stained with Coomassie blue or immunoblotted with anti-MRAP antibody (Ab). The arrows indicate MRAP-FLAG bands. *B–D:* The interaction between MRAP and G proteins. HepG2 cells were cotransfected with MRAP-FLAG vector along with vector alone, Gαs, HA-Gαi, or HA-Gαq vector. Immunoprecipitation (IP) was performed by anti-FLAG gels. MRAP and Gαs proteins were detected by anti-FLAG and anti-Gαs antibody, respectively. Gαi and Gαq proteins were detected by anti-HA antibody. *E* and *F:* Mapping the domain of MRAP responsible for its interaction with Gαs. HepG2 cells were cotransfected with Gαs vector along with vector alone, MRAP-FLAG, or mutant vectors followed by immunoprecipitation. HIG2 and Gαs proteins were detected by anti-FLAG and anti-Gαs antibody, respectively.

that the observed lipolytic stimulation is caused by changes in the ACTH receptor, since the MC2R expression was found to be decreased but not increased in the WAT of transgenic mice (Supplementary Fig. 2).

MRAP Overexpression in Adipose Tissue Increases Overall Energy Expenditure and Thermogenesis

aP2-MRAP mice and WT littermates were fed either a standard chow diet or an HFD. On the chow diet, there were no differences in body weight, fat pad weight, or organ weight between WT and aP2-MRAP mice at 18 weeks of age (data not shown). However, on an HFD, aP2-MRAP mice were leaner and gained weight at a slower rate than their WT littermates (Fig. 6*A* and *B*). Figure 6*C* further showed that inguinal and gonadal WAT and BAT weighed significantly less in aP2-MRAP mice compared with respective depots in WT mice, whereas there was no difference in the weight of liver between these two groups (Fig. 6*C*). Histological evaluation of inguinal WAT isolated from aP2-MRAP mice showed a noteworthy decrease in adipocyte size (Fig. 6*D*). Moreover, cells in the BAT of transgenic mice contained less fat and maintained multilocular lipid droplets when compared with those in WT mice (Fig. 6*D*). Furthermore, quantitative PCR analysis revealed that the inguinal WAT and BAT in aP2-MRAP mice both exhibited a significant increase in the expression of genes for FA oxidation (Cpt1a, Pdk4, Vlcad, PPARα, and PGC1α), mitochondria content (Cox8b, Cox7a1, and Hsp60), and beige adipocyte markers (Cidea and Ucp1) (Fig. 6*E* and *F*).

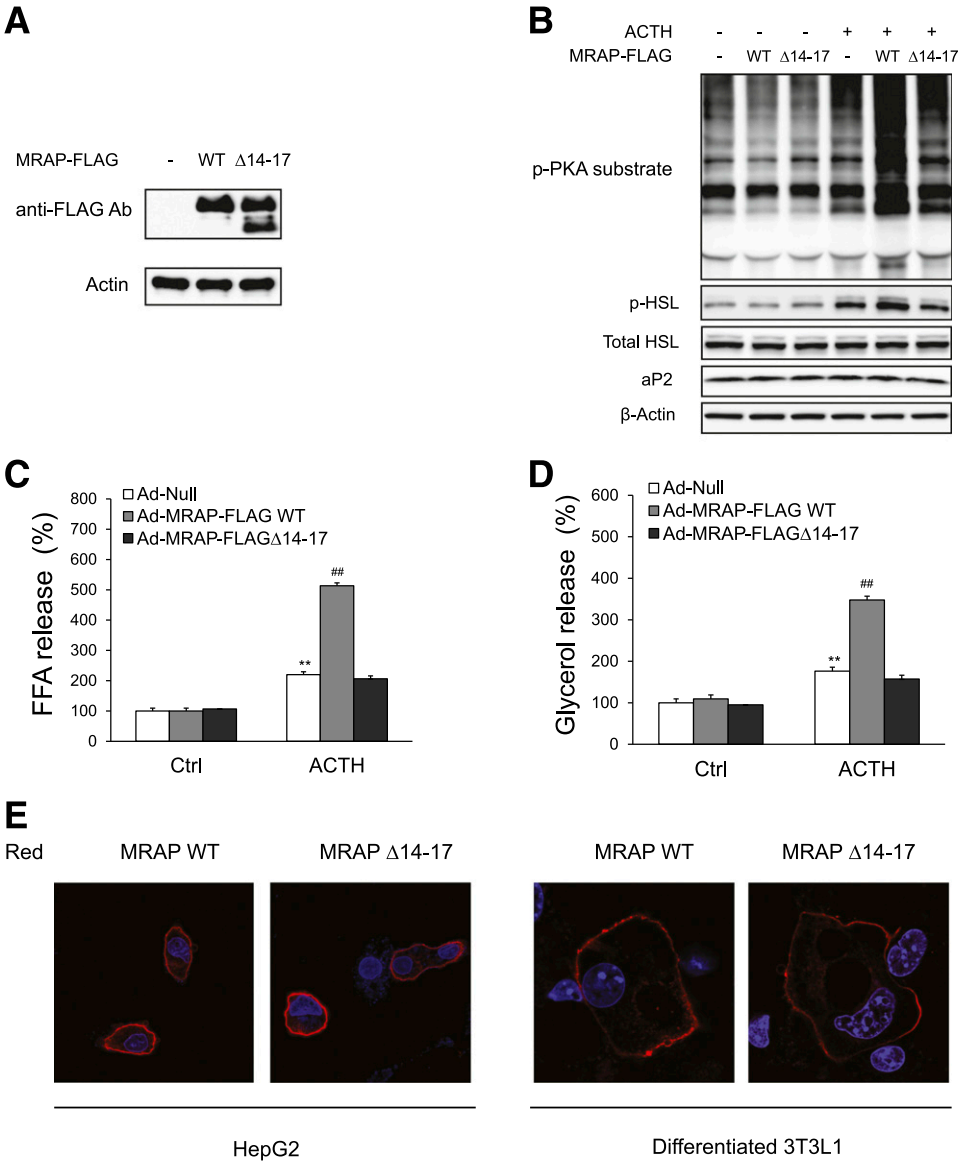

Figure 4—Amino acids 14–17 of MRAP are required for ACTH-induced PKA activation and lipolysis. Differentiated 3T3L-1 adipocytes in six-well plates were infected with 1×10^8 pfu/well of Ad-null, Ad-MRAP-FLAG, or Ad-MRAPΔ14–17-FLAG for 3 days. After 3 h of treatment with PBS or 1 nmol/L ACTH, media were collected for the measurements of FFAs and glycerol, and cells were lysed for immunoblotting. *A:* Immunoblotting of MRAP under ACTH-stimulated conditions using anti-FLAG antibody (Ab). *B:* Immunoblotting using anti–phospho-PKA substrate antibody, anti–phospho-HSL antibody, anti–total HSL antibody, and anti-aP2 antibody, respectively. *C:* FFA levels secreted in the media. *D:* Glycerol levels secreted in the media. $^{**}P < 0.01$ vs. Ad-null control (Ctrl); $\#\#P < 0.01$ vs. Ad-null ACTH. Data represent three independent experiments. *E*: MRAP tagged with FLAG was introduced into differentiated 3T3L1 cells by adenovirus infection or into HepG2 cells by plasmid transfection, and then immunofluorescence was performed by anti-FLAG antibody. Red indicates MRAP or its mutant and blue indicates nucleus stained by DAPI.

Next, we subjected WT and aP2-MRAP mice to a multi-day metabolic cage study to examine the effect of adipose MRAP on global energy homeostasis. Compared with WT mice, aP2-MRAP mice consumed more oxygen (Fig. 7*A*) and produced more carbon dioxide (Supplementary Fig. 3*A*) and heat (Fig. 7*B*) especially during the dark periods, indicating increased energy expenditure when mice were active. Meanwhile, adipose overexpression of MRAP did not appear to affect movement (Supplementary Fig. 3*B*) or food intake (Supplementary Fig. 3*C*). Taken together, these results suggest that when fed with an HFD, the upregulation of whole-body energy expenditure and heat generation in aP2-MRAP mice may account for the delay in their body weight and fat mass gain.

Adipose Overexpression of MRAP Improves Insulin Sensitivity and Hyperlipidemia in HFD-Fed Mice

Given that increased adiposity is often associated with hyperlipidemia and abnormality of glucose metabolism, we further evaluated the impact of MRAP transgene on insulin sensitivity and hyperlipidemia in HFD-fed mice. Although it did not change plasma FFA levels (Supplementary Fig. 4*A*), adipose overexpression of MRAP significantly decreased

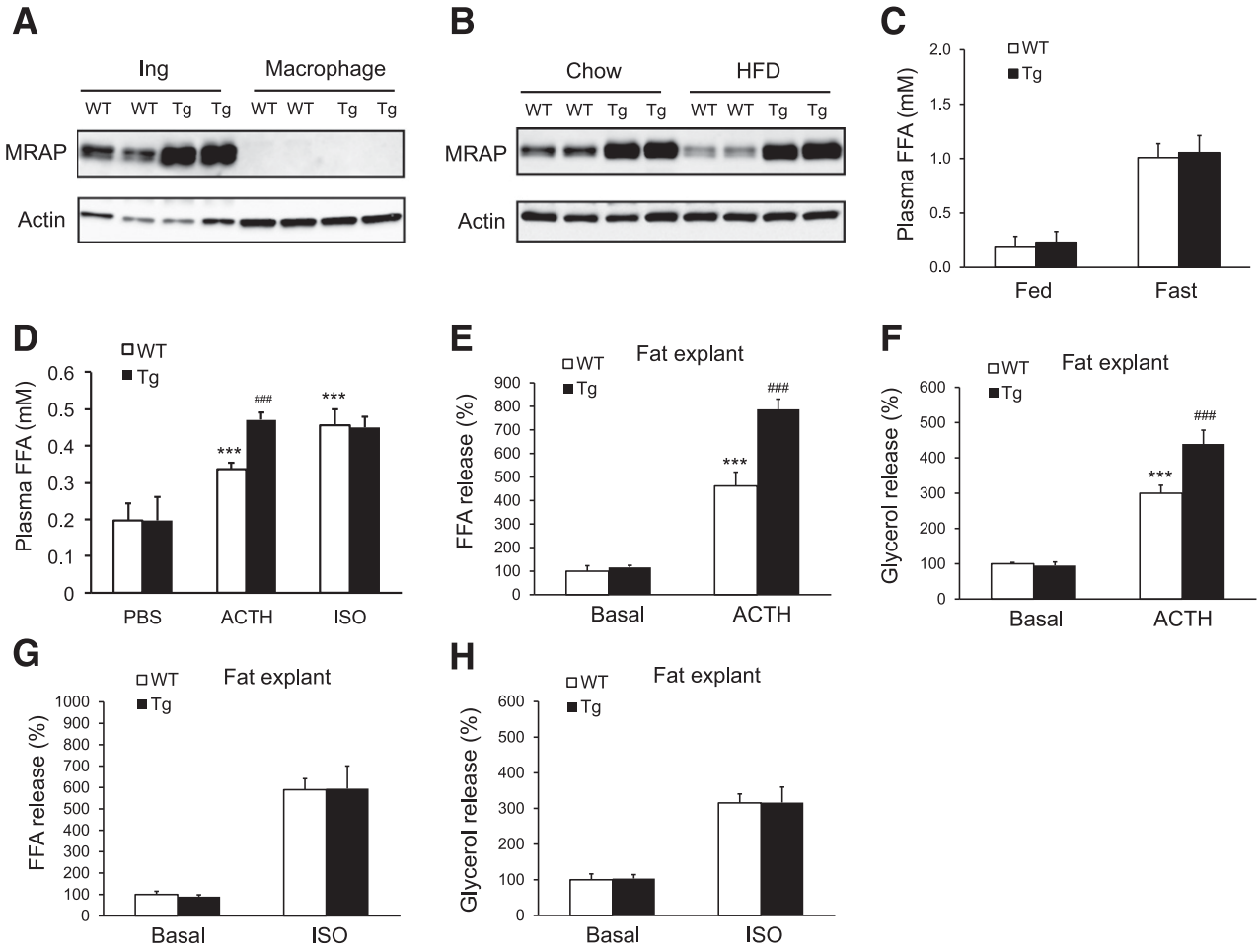

Figure 5—MRAP promotes lipolysis induced by ACTH in vivo. *A:* Immunoblotting analysis of MRAP expression in inguinal adipose tissues (Ing) and peritoneal macrophages collected from WT or aP2-MRAP (transgenic [Tg]) female mice. *B*: Immunoblotting analysis of MRAP expression in inguinal adipose tissue from mice fed chow diet or HFD for 12 weeks. *C:* Plasma FFA levels at fed conditions or after a 16-h fasting in 12-week-old female mice (n = 10 per group). *D:* The evaluation of lipolysis in vivo. Thirteen-week-old female mice were injected with saline, 10 mg/kg ACTH, or 1 mg/kg ISO. One hour later, plasma FFA levels were measured. n = 6 per group. *E–H:* Measurement of lipolysis in fat explants. Inguinal fat explants from 14-week-old female mice were incubated in the presence or absence of 2 nmol/L ACTH or 2 μmol/L ISO for 3 h. Then, FFA and glycerol releases were measured and normalized with the total mass of fat explants (n = 5 per group). All data were shown as mean ± SE. ***$P < 0.001$ vs. PBS group or basal condition; ###$P < 0.01$ vs. WT mice treated with ACTH.

plasma TG and cholesterol levels in the transgenic mice (Supplementary Fig. 4*B* and *C*). In addition, there was a significant decrease in the fasted plasma levels of both insulin and glucose in aP2-MRAP mice compared with WT mice (Fig. 7*C* and *D*). Improved glucose and insulin tolerance further confirmed improved insulin sensitivity in the transgenic animals (Fig. 7*E* and *F*). Thus, overexpression of MRAP in adipose tissue can significantly protect against HFD-induced hyperlipidemia and insulin resistance.

DISCUSSION

Dysregulation of adipocyte lipolysis, resulting in elevated circulating FFAs, is associated with obesity and its comorbidities, including insulin resistance and type 2 diabetes (2–4). The current study explored the function of MRAP in adipose lipolysis and relevant underlying mechanisms. Using cultured adipocytes with MRAP overexpression and knockdown, we have shown that MRAP is indispensable for generating the lipolytic response to ACTH. Moreover, we have identified GαS as a new binding partner of MRAP and have provided data to suggest that in adipocytes, the MRAP-GαS interaction is required for relaying signals from ACTH to the activation of PKA and downstream induction of lipolysis. Furthermore, by using the aP2-MRAP transgenic mice, we have obtained in vivo evidence in support of a positive role of adipose MRAP in maintaining the overall energy homeostasis and metabolic health.

By using a custom-generated antibody, we detected an abundant expression of MRAP protein in adipose tissue and adrenal gland in mice. The predicted size of mouse MRAP protein is ~14 kDa. However, the endogenous protein displayed a multiple banding pattern, with the majority of bands clustering at ~25 kDa. The identity of these bands was further confirmed by the peptide competition assay as

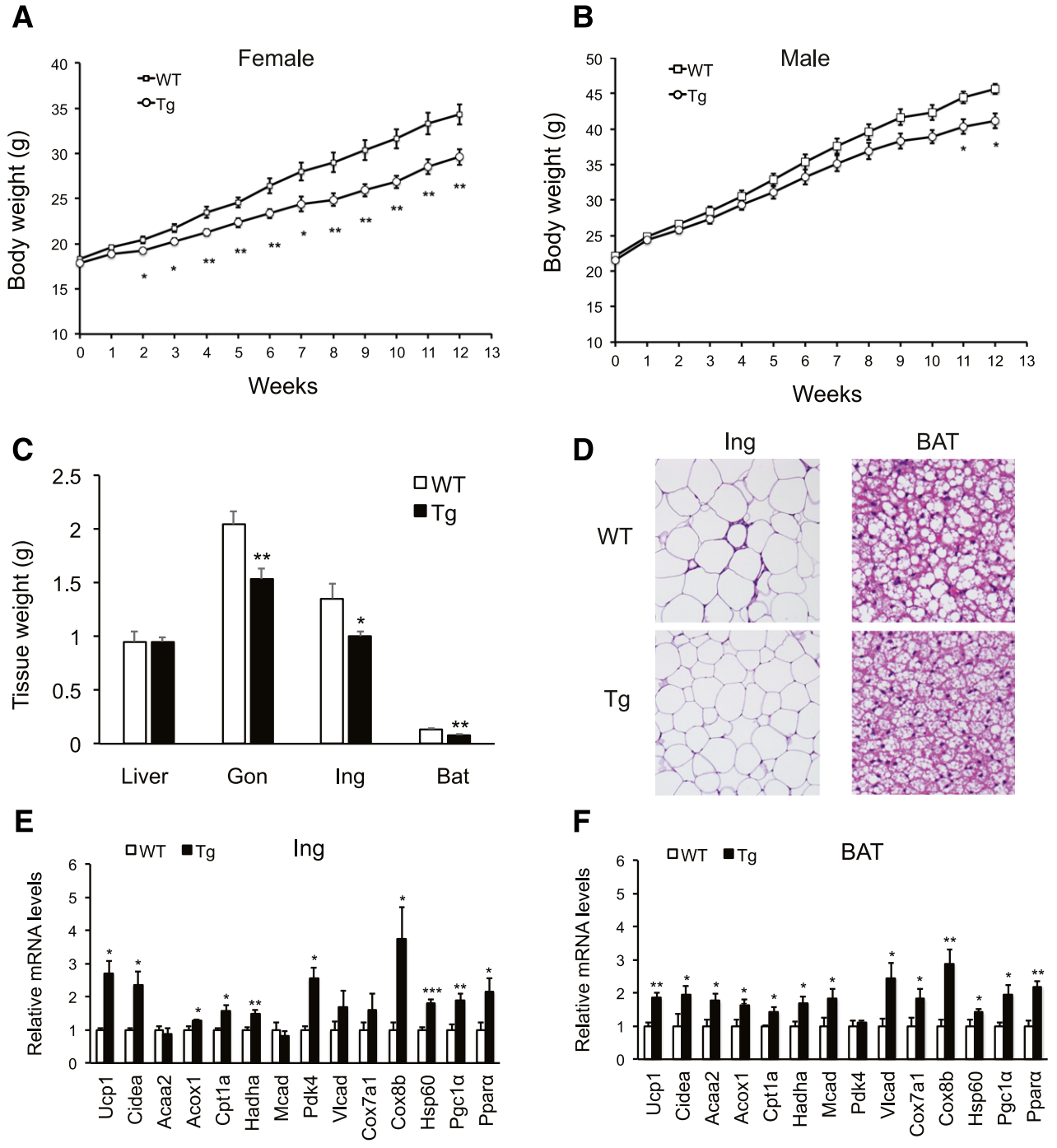

Figure 6—aP2-MRAP mice have a lean phenotype. Time course of body weight over 12 weeks of HFD feeding in female (*A*) or male (*B*) mice (n = 10–14 per group). *C:* Organ and fat pad weights in female mice after 12 weeks of HFD feeding (n = 10–14 per group). Gon, gonadal adipose tissue. *D:* Representative hematoxylin-eosin staining images of inguinal adipose tissue (Ing) or BAT from WT and transgenic (Tg) mice. RT-PCR was used to determine the mRNA levels of genes in inguinal adipose tissue (*E*) or BAT (*F*) after 12 weeks of HFD feeding. Data normalized to β-actin were expressed relative to the control (n = 3–4 per group). All data were shown as mean ± SE. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ vs. WT group.

well as their disappearance upon MRAP knockdown using specific siRNA. Previously, exogenously expressed MRAP showed either a single or multiple bands ranging from 16 to 36 kDa (18,26–28). The discrepancies between results from these different studies are likely to be related to the expression levels of exogenous versus endogenous proteins as well as the extent of posttranslational modifications. In this regard, glycosylation is known to cause varying shifts in MRAP migration on SDS-PAGE (18,26–28).

MRAP is an MC2R accessory protein that facilitates the folding, maturation, and localization of the receptor to the plasma membrane (29,30). Its predominant expression in

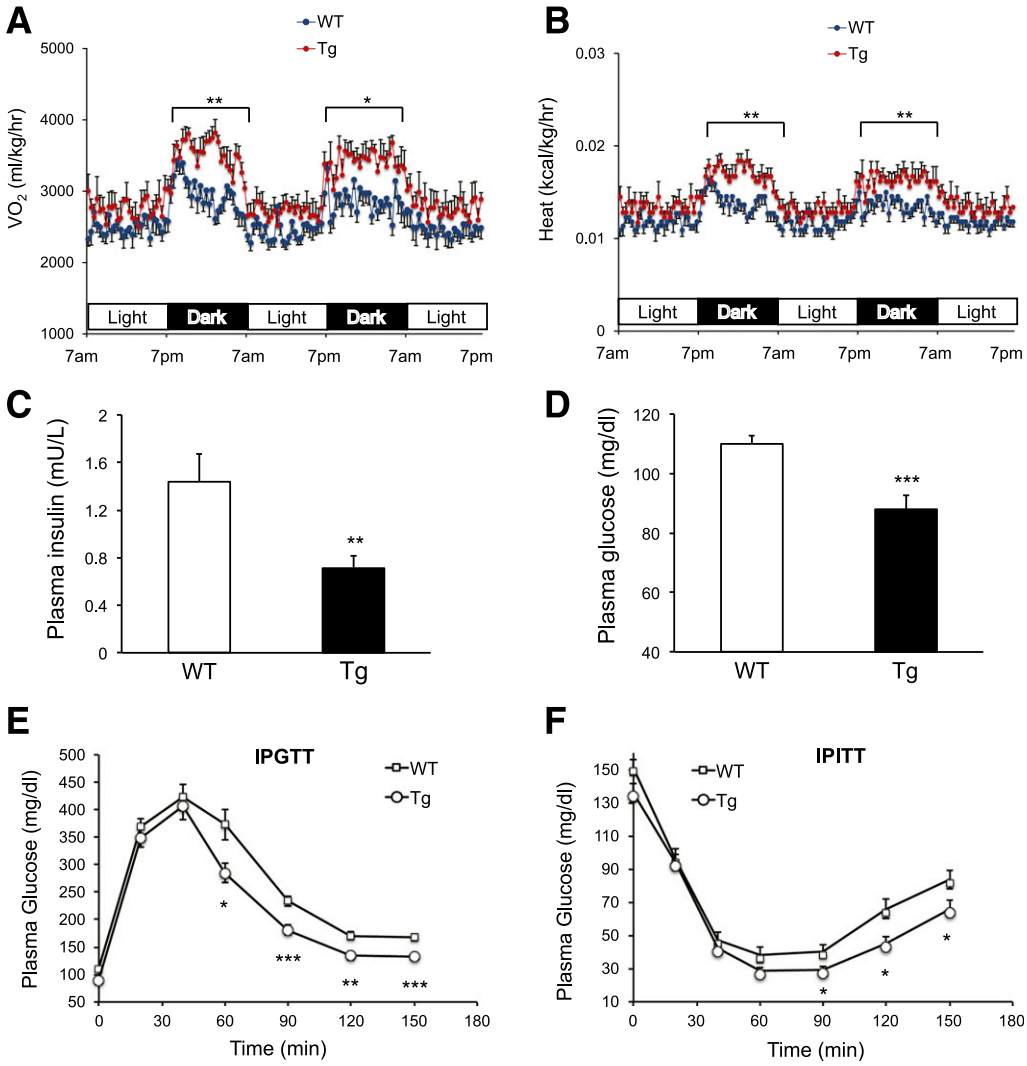

Figure 7—Adipose overexpression of MRAP improves whole-body energy expenditure and insulin sensitivity in HFD-fed mice. *A* and *B:* Female mice fed HFD for 12 weeks were subjected to a multiday metabolic cage study. Oxygen consumption (*A*) and heat production (*B*) were measured. Six hour–fasted plasma insulin (*C*) and glucose levels (*D*) in female mice after 11 weeks of HFD feeding. *E:* Intraperitoneal glucose tolerance test (IPGTT) in 16 h–fasted female mice after 10 weeks of HFD feeding. *F:* Intraperitoneal insulin tolerance test (IPITT) in 6 h–fasted female mice after 11 weeks of HFD feeding. All data were shown as mean ± SE (n = 10–12 per group). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ vs. WT group.

adipose tissue along with the adrenal gland implies a unique function of MRAP in adipocytes. Indeed, our data demonstrate that in differentiated 3T3-L1 cells, MRAP knockdown decreased while its overexpression increased ACTH-induced lipolysis. In addition, our mass spectrometry and coimmunoprecipitation analyses identified Gαs as a new specific partner of MRAP. Subsequent mutagenesis experiments revealed that an NH_2-terminal four–amino acid sequence (^{14}YEYY^{17}) of MRAP is crucial for its association with Gαs as well as for mediating downstream signal to the activation of PKA and lipolysis. In a classic GPCR signaling model, the receptor is coupled to Gαs upon ligand binding and functions as a guanine nucleotide exchange factor (GEF) to activate Gαs. However, as the shortest melanocortin receptor and the smallest known GPCR, MC2R may not possess the capability to interact directly with Gαs. Early studies demonstrate that an active MC2R is a dimer in a heterohexameric complex with an antiparallel MRAP dimer (18,28,31). Liang et al. (32) have suggested that binding of ACTH to MC2R is a two-step process that involves two critical motifs in this ligand (HFRW and KKRRP). The KKRRP segment of ACTH engages the receptor first and causes conformational changes that enable HFRW binding. A separate study later showed that MRAP is required for MC2R to recognize and bind ACTH (20). Our findings add to this model by suggesting that MRAP also recruits Gαs to the same functional complex. This resembles the way in which receptor activity–modifying proteins (RAMPs) bridge the interaction between calcitonin receptor–like receptor (CLR) and Gαs (33,34). The fact that the MRAP mutant

lacking the ^{14}YEYY^{17} motif is disabled in activating PKA and lipolysis indicates the recruitment of Gαs being a critical step in transducing the signal downstream of the ACTH/MC2R/MRAP unit.

The hypothalamic-pituitary-adrenal axis is one of the main stress response pathways, and it has been studied extensively in relation to energy metabolism. GCs play a key role in adipocyte gene expression and metabolism that promotes central fat deposition. Previous studies performed in rodents showed that GCs mediate an inhibitory effect on BAT development and activity via the GC receptor (10,35–37). Dexamethasone was also found to induce inhibition of UCP1 expression and metabolic rate in cultured human brown adipocytes (38). However, a recent human study showed that BAT is activated acutely in vivo after administration of GCs (39), indicating the involvement of complex mechanisms underlying the context-specific and temporal roles of GCs. In comparison, ACTH, the stimulator of adrenal generation of GCs, has been shown consistently to directly activate adipocyte lipolysis (8–13), which results in thermogenic activation of BAT and beiging conversion of WAT. Based on our result that there is a decreased expression of MRAP in both BAT and WAT of mice on HFD, we postulate that DIO may cause ACTH resistance in these fat depots. The results derived from our study of the aP2-MRAP transgenic mice, where MRAP is constitutively expressed in adipose tissue, are in support of this hypothesis.

In line with the lipolysis data determined as plasma FFA levels, the fat explants from aP2-MRAP mice showed a higher lipolytic rate in response to ACTH but not to ISO treatment. Given that both fasting and ISO treatment–induced lipolysis are mediated by β-adrenergic receptors, it is reasonable that adipose overexpression of MRAP enhances only ACTH-induced lipolysis. Consistent with increased lipolytic response to ACTH, BAT and inguinal WAT of the aP2-MRAP mice were able to maintain the expression of genes for FA oxidation (Cpt1a, Pdk4, Vlcad, PPARα, and PGC1α), mitochondria content (Cox8b, Cox7a1, and Hsp60), and beige or brown adipocyte markers (Cidea and Ucp1). As a result, the transgenic mice on an HFD were able to maintain energy expenditure and heat production at high levels, leading to significant resistance to the development of HFD-induced obesity, hyperlipidemia, and glucose and insulin intolerance.

In summary, we have demonstrated that MRAP is an important regulator of lipolysis and is a major contributor to the development of obesity, hyperlipidemia, and insulin resistance in response to HFD feeding. It has been extensively reported that ACTH can stimulate lipolysis in rodent adipose tissues and mouse adipocytes via MC2R-dependant cAMP/PKA activation. However, the reports on the lipolytic effects of ACTH in human adipose tissue were less than consistent. In this regard, the current study provided evidence that ACTH is capable of stimulating lipolysis in human subcutaneous fat explants, suggesting that MC2R signaling to lipolysis is conserved in human adipocytes. Although ACTH is capable of stimulating lipolysis via MC2R/MRAP, its promoting effect on the secretion of GCs in the adrenal gland has limited its therapeutic usage. Our findings suggest a possibility that modulation of MRAP expression in adipose tissue could be a putative treatment avenue to alleviate energy imbalance in obesity.

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Author Contributions. X.Z. designed and performed the research, analyzed data, and wrote the manuscript. A.M.S. and L.E.C. performed research and analyzed the data. E.A.D.F. performed research and revised the manuscript. J.L. designed and supervised the study and wrote the manuscript. J.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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