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Inhibition of PPARγ, adipogenesis and insulin sensitivity by MAGED1

Qinghua Wang1,2,*, Jing Tang1,*, Shujun Jiang1,3, Zan Huang1,4, Anying Song1, Siyuan Hou1, Xiang Gao1 and Hai-Bin Ruan5

1State Key Laboratory of Pharmaceutical Biotechnology and MOE Key Laboratory of Model Animal for Disease Study, Model Animal Research Center, Nanjing Biomedical Research Institute, Nanjing University, Nanjing, Jiangsu, China
2Laboratory Animal Center, Nantong University, Nantong, Jiangsu, China
3School of Traditional Chinese Medicine, China Pharmaceutical University, Nanjing, Jiangsu, China
4Laboratory of Gastrointestinal Microbiology, Jiangsu Key Laboratory of Gastrointestinal Nutrition and Animal Health, College of Animal Science and Technology, Nanjing Agricultural University, Nanjing, Jiangsu, China
5Department of Integrative Biology and Physiology, University of Minnesota Medical School, Minneapolis, Minnesota, USA

Correspondence should be addressed to X Gao or H-B Ruan: gaoxiang@nju.edu.cn or hruan@umn.edu

*(Q Wang and J Tang contributed equally to this work)

Abstract

Peroxisome proliferator-activated receptor-γ (PPARγ) is a master regulator of adipogenesis and a target of the thiazolidinedione (TZD) class of antidiabetic drugs; therefore, identifying novel regulators of PPARγ action in adipocytes is essential for the future development of therapeutics for diabetes. MAGE family member D1 (MAGED1), by acting as an adaptor for ubiquitin-dependent degradation pathways and a co-factor for transcription, plays an important role in neural development, cell differentiation and circadian rhythm. Here, we showed that MAGED1 expression was downregulated during adipogenesis and loss of MAGED1 promoted preadipocyte proliferation and differentiation in vitro. MAGED1 bound to PPARγ and suppressed the stability and transcriptional activity of PPARγ. Compared to WT littermates, MAGED1-deficient mice showed increased levels of PPARγ protein and its target genes, more CD29+CD34+Sca-1+ adipocyte precursors and hyperplasia of white adipose tissues (WATs). Moreover, MAGED1-deficient mice developed late-onset obesity as a result of decreased energy expenditure and physical activity. However, these mice were metabolically healthy as shown by improved glucose clearance and insulin sensitivity, normal levels of serum lipids and enhanced secretion of adipokines such as leptin and adiponectin. Taken together, our data identify MAGED1 as a novel negative regulator of PPARγ activity, adipogenesis and insulin sensitivity in mice. MAGED1 might therefore serve as a novel pharmaceutical target to treat obesity-associated insulin resistance.

Key Words
- obesity
- insulin sensitivity
- PPARγ stability
- energy expenditure
- MAGE gene family

Introduction

The obesity epidemic continues to rise as a global health challenge. Obesity is a major risk factor for high blood pressure, hyperlipidemia, diabetes, heart disease, cancers, etc. (Ng et al. 2014, Ogden et al. 2014). The white adipose tissue (WAT) serves as a critical integrator of energy balance and glucose homeostasis by storing excess energy to prevent ectopic fat accumulation in non-adipose tissues and by secreting various adipokines to modulate whole-body metabolism (Ouchi et al. 2011). Adipocyte hyperplasia, or adipogenesis, is the process of adipocyte
differentiation from pre-adipocytes, which reside in the stromal vascular fraction (SVF) of WAT (Rosen & MacDougald 2006, Gesta et al. 2007, Tran & Kahn 2010). The nuclear receptor peroxisome proliferator-activated receptor-γ (PPARγ) is a master regulator of adipogenesis and most pro- and anti-adipogenic factors seem to function at least in part by regulating PPARγ expression and/or activity (Farmer 2006, Rosen & MacDougald 2006). The thiazolidinedione (TZD) class of antidiabetic drugs agonize PPARγ to activate adipogenesis and sensitize insulin action (Spiegelman 1998). The PPARγ protein is short-lived as it can be ubiquitinated and degraded through the proteasome (Floyd & Stephens 2002, van Beekum et al. 2009, Kim et al. 2014). Activation by TZDs further accelerates PPARγ degradation; therefore, understanding how PPARγ stability is controlled will shed light on new strategies to fine-tune this metabolic master regulator to treat type 2 diabetes.

Melanoma antigen (MAGE) family member D1 (MAGED1), also known as neurotrophin receptor-interacting MAGE (NRAGE) and DLXIN-1, plays an important role in transcription, cell signaling and protein stability (Di Certo et al. 2007, Doyle et al. 2010, Wang et al. 2010, Mouri et al. 2012). Growing evidence demonstrates that MAGED1 interacts with proteins such as DLX5 (Masuda et al. 2001), p75NTR (Salehi et al. 2000), ITA and XIAP (Jordan et al. 2001), UNCSH1 (Williams et al. 2003), ROR2 (Matsuda et al. 2003) and RORα (Wang et al. 2010) to regulate neural development, cell apoptosis and proliferation and circadian rhythm. We and others have showed that MAGED1-knockout mice exhibit a shortened circadian period and bouts (Wang et al. 2010), impaired learning and memory (Yang et al. 2015) and an osteoporotic phenotype (Liu et al. 2015). However, the role of MAGED1 in energy and glucose metabolism has not been studied.

Recently, MAGE family proteins were shown to bind really interesting new gene (RING) domain proteins to form active E3 ubiquitin ligases (Doyle et al. 2010). For example, the MAGEC2-TRIM28 complex targets p53 for proteasomal degradation (Doyle et al. 2010). The MAGEL2-TRIM27 promotes K63-linked ubiquitination of WASH, thus facilitating endosomal F-actin nucleation and retromer-mediated transport (Hao et al. 2013). Likewise, MAGED1 interacts with RING proteins including PRAJA-1 (Sasaki et al. 2002), and TRIM28 to a less extent (Doyle et al. 2010). In addition, MAGED1 controls the degradation of the anti-apoptotic factor CHE-1 (Di Certo et al. 2007), and the activity and ubiquitylation of the serotonin transporter (Mouri et al. 2012). In this study, we report that MAGED1 interacts with PPARγ and decreases its stability. Loss of MAGED1 leads to enhanced preadipocyte proliferation and adipogenesis. MAGED1-deficient mice develop obesity but lack metabolic abnormalities including dyslipidemia, impaired glucose tolerance and insulin resistance.

**Materials and methods**

**Animals**

C57BL/6J mice from Jackson Laboratory and Maged1-knockout (KO) mice (Wang et al. 2010) were maintained in an AAALAC-accredited, specific pathogen-free facility in Model Animal Research Center of Nanjing University. All animal protocols were approved by Institutional Animal Care and Use Committee of Nanjing University, in accordance with published guidelines in the Principles of Laboratory Animal and the Care and Use of Laboratory Animals (National Research Council 1996). All mice were maintained under 12-h light/darkness cycles (light on at 08:00 h and off at 20:00 h) and fed with acidified water and standard chow *ad libitum*. Maged1-KO mice had been backcrossed to C57BL/6J mice for at least 15 generations.

**Serum biochemistry parameters measurements**

Mice were fasted for overnight or 6 h or refeed for 2 h as indicated. Whole blood was collected from the eye socket vein. Serum was collected after a centrifugation at 3000 g for 15 min. Lipid metabolites in plasma including total cholesterol, triglyceride (TG), high-density lipoprotein and low-density lipoprotein (VLDL) were quantified by colorimetric assays with a 7020 automatic analyzer (Hatakichi High Technology, Japan).

**Glucose and insulin tolerance tests**

For oral glucose tolerance test, mice were fasted from 17:00 to 09:00 h and injected with D(-)-glucose (Sigma, 2 g/kg body weight). For insulin tolerance test (ITT), mice were fasted from 09:00 to 15:00 h and injected with 0.75 U insulin (Novo Nordisk) per 1 kg body weight. Blood glucose concentrations were determined by GLUCOCARD II Test Meters (Arkray, Minneapolis, MN, USA).

**Metabolic characterization and body composition analyses**

The Comprehensive Lab Animal Monitoring System was used to determine food intake, physical activity, oxygen...
consumption and carbon dioxide production. All mice were maintained in a 12:12 light/darkness cycle at 22°C and acclimated for 2 days. Data were recorded for at least 72 h for analyses.

A dual-energy X-ray absorptiometry system (GE Medical System Lunar, Madison, WI, USA) was used to determine body composition. Mice were anesthetized with Avertin (Sigma Aldrich) and kept on a heat plate for data acquisition.

**Histology**

Tissues were collected and fixed in 4% formalin (in PBS) overnight, embedded in paraffin and sectioned at a thickness of 5 μm. Slides were dewaxed with xylene, rehydrated with descending grades of ethanol and then rinsed with distilled water. Tissue sections were then stained with hematoxylin and eosin. ImageJ was used for quantification of adipocyte size and area from histological slides. In brief, images were transformed into 8-bit and the image threshold was set to selectively visualize adipocyte prior analyses. All the images were acquired with the Coolscope Microscope (Nikon).

For immunohistochemistry, tissue sections were deparaffinized, antigen was retrieved by steaming for 10 min, followed by incubation with blocking solution (5% BSA, 1.5% BSA, and 1 mg/mL collagenase D (Roche)). Then, the mixture was blocked with 5% BSA for 1 h, incubated with anti-BrdU primary antibody (rat monoclonal, Abcam) overnight at 4°C and secondary antibody conjugated with cyanine 5 (eBioscience). Images were observed with a confocal laser-scanning microscope (FV1000, Olympus Micromaging).

**Isolation and differentiation of adipose SVF cells**

Adipose tissues were excised, washed with PBS and minced in digestion buffer (100 mM HEPES pH 7.4, 120 mM NaCl, 50 mM KCl, 5 mM glucose, 1 mM CaCl₂, 1.5% BSA, and 1 mg/mL collagenase D (Roche)). Then, the mixture was digested at 37°C for 30 min with shaking at 85 rpm. The slurry was filtered through 40 μm cell strainer to remove undigested bulks and large adipocytes. After that the aqueous phase was centrifuged for 5 min at 500 g, the pellet was resuspended in preadipocytes culture medium (DMEM/F12 with Glutamine, 10 mM HEPES, and 25 μg/mL sodium ascorbate). Preadipocytes were seeded on 6 cm dishes, and medium was changed next morning. Cells were passaged at confluence and frozen for further use.

For adipocyte differentiation, preadipocytes were cultured on poly-lysine-coated dishes to confluence in preadipocyte medium. Cells were kept at confluence for another 24 h to arrest the cell cycle into the G1 phase. Cells were then induced by basic differentiation medium containing the adipogenic cocktail (850 nM Insulin, 0.5 μM dexamethasone, 250 μM isobutylmethylxanthine (IBMX) and 1 μM rosiglitazone) for 48 h. Cells were then kept in basic preadipocyte culture medium with 850 nM insulin till analyses.

To make Oil-Red O stock, 500 mg of Oil-Red O was dissolved in 100 mL of isopropanol and kept stirring overnight, avoiding of light. The solution was filtered and stored in dark cabinet for further use. Adipocytes were fixed with 4% formalin for 1 h, washed with 1× PBS three times and stained with the Oil-Red O solution for 1 h. Cells washed with deionized water for four times were imaged. Finally, 1 mL isopropanol was added to dried wells to dissolve Oil-Red O for the colorimetric detection.

**Flow cytometry**

WATs were excised, washed in PBS solution twice and then minced in the digestion buffer (100 mM HEPES pH 7.4, 120 mM NaCl, 50 mM KCl, 5 mM glucose, 1 mM CaCl₂, 1.5% BSA, and 1 mg/mL collagenase D (Roche)). Tissues were transferred to 15 mL tubes and incubated at 37°C with vibrations at 87 rpm. Cell pellets were obtained by centrifugation at 500 g for 5 min and resuspended in 1 mL PBS. After filtering through 100 μm strainers, cells were fixed in 70% ethanol for 20 min and incubated with 0.1% TritonX-100 in 5% BSA solution for 1 h. Cells were stained with antibodies against Sca-1-PE, CD29-PE-Cy5 and CD34-APC (BD Biosciences) on ice for 1 h. Samples were analyzed with a FACSCalibur flow cytometer and the CellQuest software (BD Biosciences).

For MTT assay, passage two preadipocytes were seeded at a density of 8 × 10^3 per well in 96-well plates. A total of 20 μL of 5 mg/mL MTT were added to each well after 48 h of culture. Cells were incubated at 37°C for 4 h. Culture medium was removed carefully, 150 μL MTT solvent was added and plates were shaken at 60 rpm for 15 min avoiding of light. The absorbance was read at 590 nm with a reference filter of 620 nm.

**Mouse embryonic fibroblasts preparation and culture**

Embryos at day 12.5 were dissected, washed in PBS and transferred into drops of digestion solution (0.25% trypsin, 0.04% EDTA). Tissues were minced and incubated in 3 mL of digestion solution at 37°C for 15 min. Three milliliters of DMEM plus 10% FBS were added and then incubated at 37°C with occasional shaking for another 15 min. Cells were pelleted by centrifugation at 300 g for 5 min and resuspended in DMEM containing 10% FBS for the culture.
SV40T antigen integrated lentivirus was used to infect the P2 mouse embryonic fibroblasts (MEFs). Puromycin-selected cells were considered as immortalized MEFs.

**Cell culture, transient transfection and luciferase assay**

HEK293, NIH/3T3 and 3T3-L1 cells were cultured in DMEM medium supplemented with 10% FBS at 37°C, 5% CO2. 3T3-L1 cells were differentiated similarly as primary SVFs. Briefly, overconfluent 3T3-L1 cells were induced with 1 μg/mL Insulin, 1 μM dexamethasone, 0.5 mM IBMX and 1 μM troglitazone for 2 days, and then maintained with 1 μg/mL insulin and 1 μM troglitazone for another 6 days. Cells were harvested at indicated days for RNA and protein extraction.

Cells were transiently transfected with equal molar amounts of luciferase-fusion PPARγ1 and PPARγ2 constructs or pGL3-basic backbone in antibiotics-free growth media using the Lipofectamine 2000 reagent according to the manufacturer’s protocol (Invitrogen). pRL-tK (Promega) containing the Renilla luciferase gene was co-transfected in order to normalize transfection efficiencies. To examine the ability of MAGED1 in regulation the transcription of Pparg, pCDNA3.1-MAGED1 vector, fragments those containing the proximal promoters of Pparg1 (−1500bp) and Pparg2 (−677, −2275bp) incorporated into the luciferase-fusion reporter constructs, and pRL-tK were transfected. Cells were then lysed at 48h after transfection, and the luciferase activities were determined using the dual-luciferase reporter assay system (Promega).

**Quantitative RT-PCR**

Total RNA from adipocyte tissues was extracted with RNAiso plus (9108, TaKaRa). Primary cDNA was synthesized by the PrimeScript RT Kit together with the gDNA Eraser kit (RR047A, TaKaRa). Quantitative RT-PCR was performed using the SYBR Premix Ex Taq (RR420A, TaKaRa) in an ABI 7700 sequence detector (Applied Biosystems). The relative abundance of target gene transcripts was normalized to the 18S sequence detector (Applied Biosystems). The relative mRNA levels of both Pparg1 and Pparg2 constructs were determined using the 2−ΔΔCt method (Protein assay dye reagent concentrate, Cat. No. 500-0006, Bio-Rad).

Statistical analyses

Data were presented as the means ± s.e.m. Statistical differences were determined by unpaired Student’s t-test (for two groups) or one-way ANOVA with Tukey’s multiple comparisons test (for three or more groups).

**Results**

**MAGED1 negatively regulates adipogenesis**

To determine the potential role of MAGED1 in adipogenesis, we first examined its expression during the differentiation of 3T3-L1 preadipocytes. We found that levels of both Maged1 mRNA and MAGED1 protein gradually decreased after the adipogenic induction (Fig. 1A and B). In the gonadal WATs (gWAT) in mice, the expression of Maged1 mRNA reduced when adipogenesis progressed (Fig. 1C). This expression trend was confirmed in primary MEFs, immortalized MEFs, and adipocyte-derived MEFs (Fig. 1D–I). The expression of Maged1 mRNA was also examined in the gonadal WATs of mice treated with 4-hydroxytamoxifen (4-OHT), a MAGED1 inhibitor. As expected, Maged1 mRNA expression was significantly increased in 4-OHT-treated mice compared to the control group (Fig. 1J). Together, these results suggest that MAGED1 negatively regulates adipogenesis.
was induced by high-fat diet (HFD) feeding (Fig. 1C). These data indicate that MAGED1 expression is negatively associated with adipogenesis. To directly determine the effect of MAGED1 on adipogenesis, we differentiated WT and MAGED1-KO MEFs into the adipogenic lineage with IBMX, dexamethasone and insulin (collectively termed as MDI) or MDI plus triiodothyronine (T3). MAGED1-deficient MEFs showed increased efficiency in adipogenesis when compared to WT MEFs (Fig. 1D). Consistent with this result, the adipogenic response of SVF cells from gWAT was more robust in KO mice than in WT mice (Fig. 1E). We found that the expression of marker genes, such as total Pparg, Pparg2 and Adipoq, was significantly upregulated in induced KO adipocytes, particularly at late time points (Fig. 1F, G and H). The nuclear receptor PPARγ is a master regulator of adipogenesis (Farmer 2006, Rosen & MacDougald 2006), and levels of PPARγ protein were evidently increased in induced KO adipocytes, when compared to WT controls (Fig. 1I). Collectively, these data demonstrate that MAGED1 is a negative regulator of adipogenesis.

MAGED1 inhibits the stability and activity of PPARγ

In its MAGE domain, MAGED1 contains two LXXLL motifs that are required for interactions with nuclear receptors (Supplementary Fig. 1A, see section on supplementary data given at the end of this article). Indeed, immunoprecipitation showed that MAGED1 bound to both PPARγ1 and PPARγ2 (Fig. 2A). We then assayed protein stability using cycloheximide (CHX), which is an inhibitor of protein biosynthesis (Ruan et al. 2012). MAGED1 overexpression reduced the stability of both PPARγ1 and PPARγ2 (Fig. 2B and C). In addition, using a PPAR response element (PPRE)-driven luciferase reporter, we found that MAGED1 prevented the induction of luciferase activity by PPARγ1 in 3T3-L1 cells (Fig. 2D) and NIH/3T3 cells (Supplementary Fig. 1B). PPARγ binds to its own promoter and activates its own transcription (Lefterova et al. 2008, Nielsen et al. 2008). We also observed decreased Pparg1 and Pparg2 promoter activities when MAGED1 was overexpressed in 3T3-L1 cells (Fig. 2E) and NIH/3T3 cells (Supplementary Fig. 1C, D, E and F). On the other hand, MAGED1 knockdown in NIH/3T3 cells increased Pparg2 promoter activities (Supplementary Fig. 1G). These data suggest that MAGED1 may inhibit adipogenesis by suppressing the stability and activity of PPARγ.

Increased adiposity in MAGED1-KO mice

We then sought to determine whether MAGED1 regulated adipogenesis in vivo. In young male mice when there was
no difference in body weight (Fig. 3A), no difference in WAT weight was observed at 2 weeks (Fig. 3B). At 3 weeks of age, there was a trending increase in weight of gonadal and posterior subcutaneous WAT in KO mice (Fig. 3C). At 4 weeks of age, compared to WT mice, KO mice had significantly heavier gonadal, posterior subcutaneous and interscapular WAT and showed a tendency to increase in inguinal and perilrenal fat weight (Fig. 3D). No change in brown adipose tissue (BAT) weight was observed (Fig. 3B, C and D), indicating that the effect of MAGED1 on adipogenesis is either specific to WAT only or not evident in BAT at this developmental stage. We then further analyzed gWAT and found no changes in adipocyte number or size at 2 weeks (Fig. 3E, F and G). We could observe adipocyte hyperplasia starting at 3 weeks of age (Fig. 3E) and adipocyte hypertrophy not until 4 weeks of age (Fig. 3F, G and H). These data demonstrate that loss of MAGED1 in mice results in increased adiposity in early life.

Consistent with the inhibitory effect of MAGED1 on PPARγ in vitro, we found that PPARγ protein levels were substantially upregulated in gWAT in KO mice (Fig. 4A). mRNA levels of Pparg and its target genes involved in adipogenesis, lipogenesis and glucose metabolism were all increased in KO mice (Fig. 4B). These results demonstrate that loss of MAGED1 increases PPARγ levels and promotes adipogenesis in vivo.

Cell proliferation is essential for adipocyte differentiation (Fajas 2003). The MTT assay showed that SVF cells from KO mice had higher proliferation rates than WT cells in vitro (Fig. 4C). Regulators of cell cycle progression, including Cyclin D1 and A2, increased their expression in KO SVF cells (Fig. 4D). To directly assess adipocyte proliferation in vivo, we performed bromodeoxyuridine (BrdU) labeling in mice from postnatal day 7 to 14. Immunostaining in gWAT showed that significantly more BrdU-tracked adipocytes were present in KO mice than WT mice (Fig. 4E and F). We then performed flow cytometry of isolated SVF cells from gWAT and found KO mice had more CD29⁺CD34⁺Sca-1⁺ adipocyte precursors (Fig. 4G) (Rodeheffer et al. 2008, Berry & Rodeheffer 2013), suggesting an increased adipogenic potential in MAGED1-deficient mice.
Figure 3
Increased adiposity in young MAGED1-KO mice. (A) Body weight of 2–4 weeks old WT and KO mice (n=7–13). (B, C and D) Weight of different fat depots in 2-week-old (B, n=7–13), 3-week-old (C, n=6–11) and 4-week-old (D, n=5–16) mice. (E and F) Adipocyte numbers (E) and cell sizes (F) in gWAT (n=20). (G) Representative H&E images of gWAT. (H) Frequency distribution of adipocyte sizes in gWAT (n=20). Data presented as mean ± s.e.m. *P<0.05, **P<0.01 and ***P<0.001 by two-tailed t-test.

Figure 4
Enhancement of adipogenesis in MAGED1 KO mice. (A) Expression of PPARγ in gWAT shown by Western blotting. (B) Quantitative RT-PCR of PPARγ-target genes in gWAT in 4-week-old mice (n=5). (C) MTT assay of cultured preadipocytes (n=11–14). (D) Expression of Cyclin D1 and A2 in culture SVF cells. (E) Immunostaining of BrdU in gWAT from mice injected with BrdU from postnatal day 7 to 13. (F) Quantification of the percentage of BrdU+ adipocytes (n=7). (G) Frequency of CD29+CD34+Sca-1+ adipocyte precursors in gonadal SVF cells (n=3). Data presented as mean ± s.e.m. *P<0.05, **P<0.01 and ***P<0.001 by two-tailed t-test.
MAGED1-KO mice develop late-onset obesity

We then sought to determine the long-term energy homeostasis in MAGED1-KO mice. We found that KO males gained more weight than WT mice starting from around week 19 (Fig. 5A). Body composition analyses showed that there was no difference in lean mass and only fat weight and percentage were increased in KO mice (Fig. 5B). Consistent with findings in young mice (Fig. 3), all WAT depots were hypertrophic (Fig. 5C). Similarly, KO females also developed late-onset obesity (Fig. 5D), suggesting no sex difference in the effect of MAGED1 on body weight control. We then profiled serum levels of adipokines and lipids. Similar to increased mRNA levels of \( \text{Lep} \) and \( \text{Adipoq} \) in KO adipose tissue (Fig. 4), levels of serum LEPTIN (Fig. 5E and F) and ADIPONECTIN (Fig. 5G and H) in KO mice were higher than WT mice, even before obesity was apparent. However, we did not observe any changes in serum levels of triglyceride, cholesterol or free fatty acids at all ages we examined (Fig. 5I, J and K), indicating that increased fat was mostly stored in adipocytes and did not induce lipotoxicity in KO mice.

To determine what caused obesity in KO mice, we performed metabolic cage studies. At 6 weeks of age when no difference in body weight was observed, KO male mice consumed less food at some time points, but total food intake was comparable between WT and KO mice (Fig. 6A). A tendency of reduced RER in KO mice was observed (Fig. 6B). Total energy expenditure indicated by calculated heat production was reduced in KO mice during early hours into the dark phase (Fig. 6C). Reduced physical activity was also observed in KO mice, particularly during the dark phase (Fig. 6D). We then repeated the metabolic cage study in 4-month-old male mice and body weight-matched mice were selected to avoid confounding factors in calculating energy metabolism (Tschop et al. 2012). We found that 4-month-old KO mice consumed less food during the dark phase (Fig. 6E). RER was consistently lower in KO mice during the dark phase and the early light phase (Fig. 6F), as a result of reduced food intake and/or

Figure 5
Late-onset obesity in MAGED1-deficient mice. (A) Growth curve of male WT and KO mice \((n=9)\). (B) Body composition of 7 months old male WT and KO mice \((n=9)\). (C) Adipose weight of 1-year-old male WT and KO mice \((n=4–5)\). (D) Growth curve of female WT and KO mice \((n=10–11)\). (E and F) Serum levels of leptin in fed (E) and fasted (F) mice \((n=3–6)\). (G and H) Serum levels of adiponectin in fed (G, \(n=6–9\)) and fasted (H, \(n=6–7\)) mice. (I, J and K) Serum levels of triglyceride (I), cholesterol (J) and free fatty acid (K) \((n=5–10)\). Data presented as mean ± S.E.M. or scatter dot blot with lines at mean and s.e.m. * \(p<0.05\), ** \(p<0.01\) and *** \(p<0.001\) by two-tailed t-test.
preferred fat utilization. Heat production (Fig. 6G) and physical activity (Fig. 6H) were consistently lower in KO mice during the dark phase. However, we did not observe any changes in the morphology (Supplementary Fig. 2A) or UCP1 expression (Supplementary Fig. 2B) in BAT in KO mice. Taken together, these data indicate that MAGED1 KO mice develop energy imbalance and late-onset obesity.

**Improved glucose metabolism and insulin sensitivity in MAGED1 KO mice**

Activation of PPARγ in adipose tissues improves glucose tolerance and insulin sensitivity in diabetic patients and animal models (Spiegelman 1998). We then assessed systemic glucose metabolism in MAGED1-KO mice. Glucose levels in KO mice were significantly lower than WT mice during glucose tolerance tests at ages of 3 months (Fig. 7A) and 6 months (Fig. 7B). ITTs demonstrated that KO mice had improved insulin sensitivity compared to WT mice before and after their body adiposity was increased (Fig. 7C and D). The reduction in fasting glucose concentrations in KO mice was apparent at 3 and 6 months but lost when mice were more than 1 year old (Fig. 7E and F). However, these old KO mice still had lower fasting insulin levels (Fig. 7G) and reduced scores of the homeostatic model assessment of insulin resistance (HOMA-IR, Fig. 7H). Similar levels of fasting serum glucagon suggested that the improved glucose metabolism was not a result of functional changes in pancreatic α cells (Fig. 7I). Hepatic glucose production was not affected in KO mice, shown by the pyruvate tolerance test (Supplementary Fig. 3A) and gluconeogenic gene expression (Supplementary Fig. 3B). Glucose-stimulated insulin secretion in vivo (Supplementary Fig. 3C) and in isolated pancreatic islets (Supplementary Fig. 3D) was also normal, indicating intact β cell function in KO mice. These data demonstrate that obesity in MAGED1-KO mice was not associated with an impairment in glucose metabolism, possibly because enhanced adipogenesis and lipid-storing ability eliminated the deleterious effects of lipids on tissues like liver and pancreas.

Finally, we assessed insulin signaling directly. Treating MEFs with increasing concentrations of insulin for 5 min (Fig. 8A) or 100 nM insulin for various time points (Fig. 8B) showed that AKT phosphorylation and total IRS1 levels were upregulated while phosphorylated JNK was decreased in KO cells, indicating that loss of MAGED1 sensitizes insulin signaling. Consistently,
phosphorylation of AKT at S473 was increased in gWAT of KO mice, compared to WT controls (Fig. 8C). Levels of JNK phosphorylation, however, were comparable between genotypes (Fig. 8C). Obesity generally is associated with a state of chronic, low-grade inflammation (Xu et al. 2003). However, the expression of inflammatory genes including Il6 and Tnfa was not changed in gWAT in KO mice (Fig. 8D). Similarly, serum levels of TNFα were not elevated in KO mice at all ages examined (Fig. 8E). Taken together, our data demonstrate that loss of MAGED1 in mice enhances insulin sensitivity and improves glucose metabolism.

### Discussion

MAGE family consists of more than 50 genes, most of which are localized in clusters in the X chromosome (Chomez et al. 2001, Lee & Potts 2017). They can be broadly categorized into the cancer-testis antigen type I that includes MAGE-A, -B and -C, and the ubiquitous type II that includes MAGE-D, -E, -F, -G, -H, -L and NECDIN. While type I MAGEs control germ cell development and function as oncogenes to drive tumorigenesis, type II MAGEs are important for neurodevelopment (Lee & Potts 2017). MAGEL2 and NECDIN are two of the five genes inactivated in Prader–Willi syndrome (PWS), a genetic condition that causes hyperphagia and severe obesity in affected children. Mice lacking the Magel2 gene phenocopy human PWS, being overweight with increased adiposity (Bischof et al. 2007, Mercer et al. 2013). Disruption of the Necdin gene in mice also results in hypothalamic and behavioral defects reminiscent of human PWS (Muscatelli et al. 2000). NECDIN is highly expressed in preadipocytes and loss of NECDIN promotes adipogenesis in vitro and adipose hyperplasia in vivo (Bush & Wevrick 2012, Fujiwara et al. 2012). Here, we showed that MAGED1 is another MAGE family member that negatively regulates...
adipogenesis and MAGED1 deficiency leads to adipose hyperplasia and obesity in mice. It will be interesting to determine in the future whether other type II MAGEs also modulate adipogenesis and systemic metabolism and whether a unified mechanism can be adopted.

Besides promoting adipogenesis, MAGED1-KO mice also show reduced food intake and physical activity when compared to WT littermates, suggesting possible functional defects in the hypothalamus. In supporting its neuronal function, MAGED1 was previously shown to regulate neuronal differentiation and survival (Salehi et al. 2000), modulate circadian rhythm (Wang et al. 2010) and be involved in depression (Mouri et al. 2012), learning and memory formation (Yang et al. 2015), and social interactions and sexual behavior (Dombret et al. 2012). Of note, Dombret et al. also observed that MAGED1-KO mice develop very-late-onset obesity (when 1 year old) that is associated with hyperphagia and reduced motor activity (Dombret et al. 2012). However, obesity could be seen in our MAGED1-KO mice much earlier in both sexes (Fig. 5) and increased adipogenesis could be observed as early as 1 month old. In addition, food intake is reduced in our MAGED1-KO mice (Fig. 6). These discrepancies between the two studies could be a result of different knockout strategies used. Dombret et al. deleted exons 4–12 of the Maged1 gene and a truncated MAGED1 fragment translated from the remaining exons 2–3 could be detected. We replaced exons 3 through 8 with the PGK-Neo cassette, and we could not detect any Maged1 mRNA or residual MAGED1 protein (data not shown). Nevertheless, future investigations using tissue-specific Maged1 deletions would help delineate the potential role of MAGED1 in hypothalamic control of metabolism.

Elegant biochemical and biophysical studies by the Potts group discovered that MAGE proteins bind to and activate the E3 RING ubiquitin ligases (Doyle et al. 2010, Hao et al. 2013, Lee & Potts 2017). They found that MAGED1 forms a strong complex with PRAJA-1 and a weak complex with TRIM28 (Doyle et al. 2010). A previous study using yeast two-hybrid screening also identified PRAJA-1 as a MAGED1-interacting protein (Sasaki et al. 2002). The E3 ligase activity of PRAJA-1 causes a decrease in MAGED1 protein level, thus controlling the transcription function of the homeodomain protein DLX5. Here, we demonstrated that MAGED1 interacts with both PPARγ1 and PPARγ2. Overexpression of MAGED1 reduces the stability and transcriptional activity of PPARγ1 and PPARγ2. Various E3 ligases have been reported to control PPARγ protein levels, including NEDD4 (Li et al. 2016) and RING proteins including ARIH2, c-CBL, LRSAM-1, RNF4, SIAH2,
MKRN1 and TRIM23 (Kilroy et al. 2012, Kim et al. 2014, Watanabe et al. 2015). Future experiments are warranted to determine whether and which RING proteins mediate the interaction and ubiquitination of PPARγ proteins by MAGED1.

In MEF cells where PPARγ is not expressed, we found that MAGED1 KO increased IRS1 expression and insulin signaling activity (Fig. 8). IRS1, a critical mediator of the insulin pathway, has been extensively shown to be regulated by ubiquitination-dependent degradation (Xu et al. 2008, Song et al. 2013, Yang et al. 2016). We suspect that MAGED1 may function as an adaptor protein for E3 ligases to target IRS1 for degradation. Therefore, in MAGED1-KO mice, increased IRS1 expression and insulin signaling can also function upstream to activate PPARγ and promote adipogenesis.

Taken together, MAGED1 negatively regulates PPARγ stability and activity, adipogenesis and insulin sensitivity. Harnessing MAGED1 may represent a novel approach for the treatment of obesity, diabetes and related metabolic diseases.

**Supplementary data**
This is linked to the online version of the paper at https://doi.org/10.1530/JOE-18-0349.

**Declaration of interest**
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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**Author contribution statement**
Q W and J T performed most of the experiments and data analyses with the help from S J, Z H, A S and S H. Q W wrote the manuscript, together with J T. X G supervised the project and contributed to manuscript editing. H B R conceived the project, performed experiments, analyzed data and wrote the manuscript.

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