

DISEASES AND DISORDERS

Maternal exercise via exerkinin apelin enhances brown adipogenesis and prevents metabolic dysfunction in offspring mice

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The obesity rate is rapidly increasing, which has been attributed to lack of exercise and excessive energy intake. Here, we found a previously unidentified explanation, due to lack of maternal exercise. In this study, healthy maternal mice were assigned either to a sedentary lifestyle or to exercise daily, and fetal brown adipose tissue (BAT) development and offspring metabolic health were analyzed. Compared to the sedentary group, maternal exercise enhanced DNA demethylation of *Prdm16* promoter and BAT development and prevented obesity of offspring when challenged with a high-energy diet. Apelin, an exerkinin, was elevated in both maternal and fetal circulations due to exercise, and maternal administration of apelin mimicked the beneficial effects of exercise on fetal BAT development and offspring metabolic health. Together, maternal exercise enhances thermogenesis and the metabolic health of offspring mice, suggesting that the sedentary lifestyle during pregnancy contributes to the obesity epidemic in modern societies.

INTRODUCTION

In the United States and worldwide, there is an epidemic of individuals who are overweight and obese (1) due to an excess of energy intake over expenditure. Brown adipose tissue (BAT) is a major organ burning fatty acids and glucose for nonshivering thermogenesis, which extensively consumes energy (2, 3). Brown-like adipocytes have been identified in white adipose tissue (WAT), which are termed as beige or brite adipose tissue. Similar to BAT, these adipocytes burn energy for thermogenesis; beige adipocytes exist widely in humans (4, 5). Enhancing the thermogenic function of BAT and beige adipocytes dissipates excessive energy, holding the promise to prevent obesity and associated metabolic diseases (6, 7). Initiation of brown adipogenesis and maintenance of the brown adipocyte phenotype require activity of the key transcription factor, PR domain containing 16 protein (PRDM16) (8). Its promoter is enriched with CpG sites, characterizing a key developmental gene (9), and active DNA demethylation is needed to trigger its expression and brown adipogenesis (10). Thus, facilitating DNA demethylation and expression of *Prdm16* gene is a key strategy to attenuate obesity-induced metabolic dysfunction.

Maternal obesity and poor maternal diet, especially high-fat diet (HFD) and/or high-calorie diet, are detrimental to both maternal health and fetal development, predisposing offspring to obesity and type 2 diabetes (11). In our previous study, we found that maternal obesity impairs fetal BAT development with persistent effects on its thermogenic function in later life, predisposing offspring to obesity and metabolic dysfunction (10). However, the surge in childhood obesity cannot be solely explained by obesity during pregnancy, because obesity rates in children are increasing among mothers with various body mass indices (12). Thus, additional mechanisms exist,

which are responsible for the increase in obesity rates among children in recent decades. In obese dams, exercise has been found to be protective against obesity and metabolic dysfunction in offspring (13). Because exercise in pregnant women is becoming less common regardless of maternal body mass indices, we hypothesized that just the lack of exercise during pregnancy in lean mothers can predispose offspring to obesity and metabolic dysfunction. Furthermore, exercise stimulates the secretion of exercise-induced hormone, exerkinins, including apelin, which has been increasingly implicated in the regulation of placental and fetal development (14). We further hypothesized that the elevation of apelin due to maternal exercise mediates its beneficial effects on fetal BAT development and offspring metabolic health.

By comparing lean maternal mice with and without exercise during pregnancy, we found that maternal exercise stimulates brown and beige adipose tissue development during fetal development and enhances their thermogenic function in the offspring, suggesting that the sedentary lifestyle during pregnancy contributes to the obesity epidemic in modern societies. Maternal administration of apelin mirrors the beneficial effects of exercise training on offspring metabolic health, suggesting a previously unidentified therapeutic approach for preventing the programming effect of maternal sedentary life on offspring obesity.

RESULTS

Exercise during pregnancy activates brown and beige adipose tissues

To test the effects of exercise during pregnancy on fetal development, pregnant females were exercised daily. Maternal exercise reduced the body weight gain during pregnancy, and the difference in body weight was maintained during lactation (fig. S1, A and B), despite no difference in feed intake (fig. S1, C and D). The relative interscapular BAT (iBAT) weight, defined as the iBAT wet weight-to-body weight ratio, was 37.8% higher in exercised dam, while inguinal WAT (ingWAT) was 37.1% lower compared to maternal

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control (CON) mice (fig. S1F). Exercise reduced maternal circulatory glucose levels and insulin resistance (fig. S1, G and I), suggesting that exercise has preventive effects for gestational prediabetes mellitus (13). On the other hand, no significant changes in the blood insulin and pancreatic β -cell function were observed (fig. S1, H and K). We observed that offspring from exercised mothers showed higher uncoupling protein 1 (UCP1) expression in both iBAT and ingWAT at embryonic day 18.5 (E18.5) and weaning (fig. S1, J and L).

Maternal daily exercise induces iBAT activity and browning of offspring ingWAT

At weaning, the body weight and ingWAT mass of female offspring (M-Ex) born to exercised mothers were lower, but the iBAT mass was higher compared to the offspring from sedentary CON mice (M-Ctrl). Consistently, iBAT weight was also increased in male M-Ex offspring, while ingWAT and body weight were similar

(Fig. 1, A and B). In addition, in iBAT and ingWAT of both female and male M-Ex offspring, mRNA expression of brown adipocyte markers including *Ucp1*, *Ppargc1a*, and *Prdm16* (Fig. 1C) was higher than that in M-Ctrl mice. Consistently, both female and male offspring born to exercised mothers had higher UCP1, PRDM16, and peroxisome proliferator-activated receptor γ coactivator-1 α (PGC-1 α) protein levels in iBAT and ingWAT than those from M-Ctrl offspring at weaning (Fig. 1, E and F, and fig. S2, A and B). There was no difference in litter sizes between groups (fig. S2C).

Because BAT development initiates before E15.5 (15), we further analyzed the effects of maternal exercise on fetal BAT development. In fetal iBAT from E18.5 embryos, UCP1, PRDM16, and PGC-1 α protein levels were higher in M-Ex than in M-Ctrl for both female and male fetuses (Fig. 1D). These differences were maintained in iBAT and ingWAT at weaning (Fig. 1, E and F). In agreement, small lipid droplet sizes were observed in the ingWAT of female and male

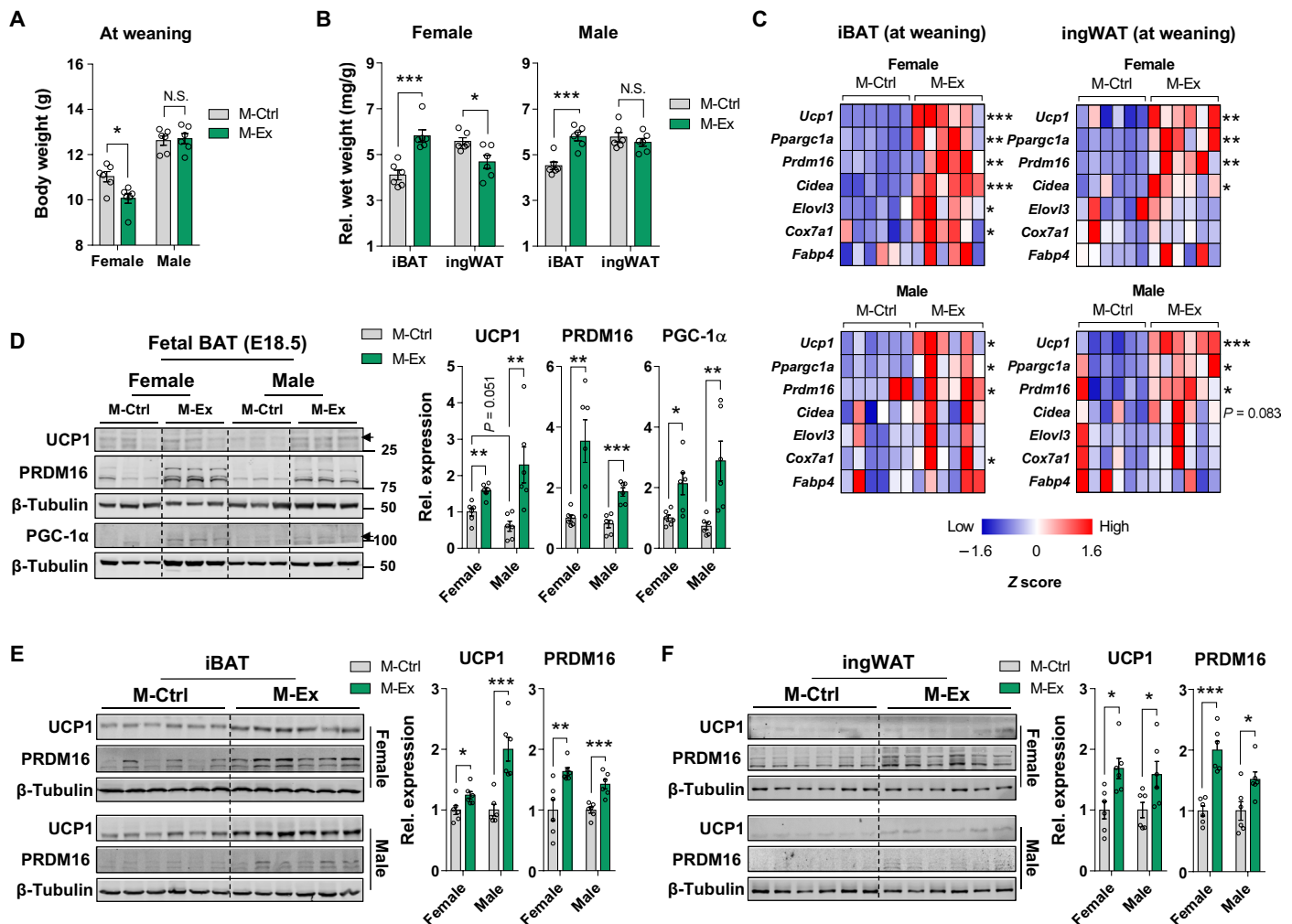


Fig. 1. Maternal exercise intervention markedly induces the expression of thermogenic markers in iBAT and ingWAT of offspring at weaning. (A and B) Body weight (A) and relative wet weight of iBAT and ingWAT (B) normalized to body weight of female and male M-Ctrl and M-Ex offspring at weaning ($n = 6$ mice per group). N.S., not significant. (C) Z scores of mRNA levels of different brown fat markers in the iBAT and ingWAT of M-Ctrl and M-Ex offspring at weaning ($n = 6$ mice per group). Expression was normalized by ΔC_t values. (D) Cropped Western blots of UCP1, PRDM16, and PGC-1 α in the iBAT (β -tubulin is the loading control) from the E18.5 fetuses of CON and EX maternal mice ($n = 6$ fetuses per group). (E and F) Cropped Western blots of UCP1 and PRDM16 (β -tubulin is the loading control) from the iBAT (E) and ingWAT (F) isolated from female and male mice of M-Ctrl and M-Ex at weaning ($n = 6$ mice per group). Data are mean \pm SEM, and each dot represents one litter. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ by two-tailed unpaired Student's t test (A to C, E, and F) or one-way analysis of variance (ANOVA) (D).

M-Ex weanling offspring, showing less lipid accumulation (fig. S2, D to H). Together, these data demonstrate that maternal exercise enhances brown and beige adipogenesis in fetuses and weanling offspring mice.

Maternal exercise enhances thermogenic function in offspring following CE

Because cold exposure (CE) stimulates thermogenesis in BAT and beige adipocytes (4), we analyzed whether the thermogenic activity of M-Ex offspring was enhanced following 2 days of CE. Following CE, the difference in iBAT mass of M-Ex and M-Ctrl offspring became even higher (Figs. 1B and 2A), which was less obvious in M-Ctrl and M-Ex offspring mice without CE (fig. S3B). Furthermore, M-Ex elicited a higher intrascapular temperature in both female and male offspring compared with M-Ctrl offspring following 2 days of CE (Fig. 2B), demonstrating enhanced thermogenesis in M-Ex offspring. Following 2 days of CE, protein levels of thermogenic markers including UCP1 and PRDM16 in iBAT and ingWAT were also higher in M-Ex offspring (Fig. 2, C and D). Immunocytochemical analysis showed stronger UCP1 staining in iBAT and ingWAT of M-Ex mice with and without CE (Fig. 2E). To determine whether the enhanced thermogenesis could improve metabolic health, we examined blood glucose and insulin levels and the presence of insulin resistance. No difference was observed between M-Ctrl and M-Ex offspring without CE. However, in the M-Ex group, CE reduced the basal glucose levels and improved insulin sensitivity (fig. S3, C to F). To assess whether the changes in altered glucose metabolism might be due to a higher glucose uptake into iBAT, we analyzed glucose transporter member 4 (GLUT4) expression in iBAT under room temperature (RT) and CE conditions and showed that the GLUT4 protein level was higher in female and male M-Ex offspring following CE (Fig. 2F). Together, our data demonstrate that M-Ex induces the thermogenic function of BAT and beige adipose tissue in both sexes of offspring.

Maternal exercise protects offspring from diet-induced obesity and insulin resistance

To further examine the protective effect of M-Ex on offspring metabolic health, we subjected offspring mice to 8 weeks of HFD feeding. Notably, female and male M-Ex offspring consumed more feed than M-Ctrl offspring (Fig. 3A). Nonetheless, female M-Ex offspring showed lower body weight gain than M-Ctrl female offspring, while there was no difference in male M-Ex offspring in body weight. However, the ingWAT weight was reduced for both female and male M-Ex offspring (Fig. 3, B and C). In addition, both sexes of M-Ex offspring demonstrated better glucose tolerance than M-Ctrl offspring (Fig. 3, E and F), consistent with lower fasting blood glucose and insulin levels in M-Ex offspring (Fig. 3, D to H). We further found a positive correlation between maternal blood glucose/insulin and their offspring, suggesting that maternal metabolic states could contribute to the improved fetal development and metabolism in their offspring (fig. S4, A to D). In agreement, hepatic lipid accumulation was observed in both female and male M-Ctrl offspring versus their corresponding M-Ex offspring (Fig. 3I).

Notably, following HFD challenge, the VO_2 and VCO_2 levels were higher in M-Ex offspring than in M-Ctrl offspring (Fig. 3, J and K, and fig. S5, A and B), whereas substrate utilization was not altered, based on unchanged respiratory exchange ratios (RERs) (fig. S5, C and D). Maternal exercise increased carbohydrate oxidation in

the female and male offspring challenged with HFD (fig. S5, E to H). These increased oxygen consumption rates (OCRs) and carbohydrate oxidation were consistent with elevated iBAT wet weight and temperature in M-Ex offspring (Fig. 4, A and B). Furthermore, up-regulation of thermogenic UCP1 and PRDM16 protein levels in iBAT and ingWAT of both female and male M-Ex offspring were paralleled with OCRs (Fig. 4, C and F). In the female and male offspring challenged with HFD, negative correlations between iBAT/ingWAT UCP1 protein levels and blood glucose/insulin resistance were found (fig. S4, E to P), demonstrating that maternal exercise protected metabolic dysfunction of offspring challenged with HFD, consistent with up-regulated brown/beige adipose thermogenic activity (4). Microscopically, lipid droplets of iBAT in female and male M-Ex offspring were smaller than those in M-Ctrl offspring, while mitochondrial density was higher in M-Ex offspring (Fig. 4, D and G), which was correlated with small sizes of lipid droplets in ingWAT of M-Ex offspring than M-Ctrl offspring (Fig. 4, E and H to J). Together, our data demonstrate that maternal exercise during pregnancy enhances fetal brown/beige adipose tissue development and protects offspring from obesity and metabolic syndromes when they consume HFD, common in Western societies.

Apelin supplementation during pregnancy promotes fetal BAT development

Protein levels of apelin were markedly up-regulated in exercised dams and their fetuses at E18.5. This difference was maintained in offspring following HFD challenge (Fig. 5A). Consistently, circulating apelin levels in exercised mothers and their fetuses were increased (Fig. 5, B and D). The elevation of apelin in fetal circulation was likely derived from fetal placenta. Exercise during pregnancy profoundly elevated apelin expression in the labyrinth zone of the placenta, the fetal side of placenta (fig. S6A). Exercise creates a hypoxic environment in the placenta (16), which stimulates vasculogenesis. These developing vascular endothelial cells are major sources of apelin secretion (17). Placental vascularization was increased because of maternal exercise (16). Furthermore, compared to other fetal organs/tissues, apelin expression was much higher in the placenta (fig. S6B), consistent with the placenta as the source of apelin in fetal circulation stimulated by maternal exercise.

To explore the possible mediatory effects of apelin, we performed apelin supplementation by daily injection of pyroglutamate apelin-13 during pregnancy (Fig. 5C). At E18.5, apelin treatment induced placental hypoxia (fig. S6C) and increased iBAT wet weight of female and male fetuses (Fig. 5E). Furthermore, apelin treatment also elevated the protein contents of brown adipocyte markers including UCP1, PRDM16, and PGC-1 α in fetal iBAT at E18.5 (Fig. 5H) and mRNA expression of *Ucp1*, *Ppargc1a*, *Prdm16*, *Cidea*, *Elovl3*, and *Cox7a1* (Fig. 5, F and G), as well as circulating apelin levels in female and male fetuses (Fig. 5D). To further analyze the mediatory roles of apelin in fetal BAT development, we conducted RNA sequencing (RNAseq) using whole transcriptome termini site (WTTs) sequencing, which showed that apelin administration during pregnancy not only enhanced brown adipogenesis, oxidative phosphorylation, mitochondrial activity, and molecular function but also inhibited lipid synthetic process and white adipocyte differentiation in the fetal BAT (fig. S7). In short, our data showed that exercise induced placental hypoxic environment, which increased vascularization and stimulated apelin expression in the placenta, elevated apelin contents in the fetal circulation, and

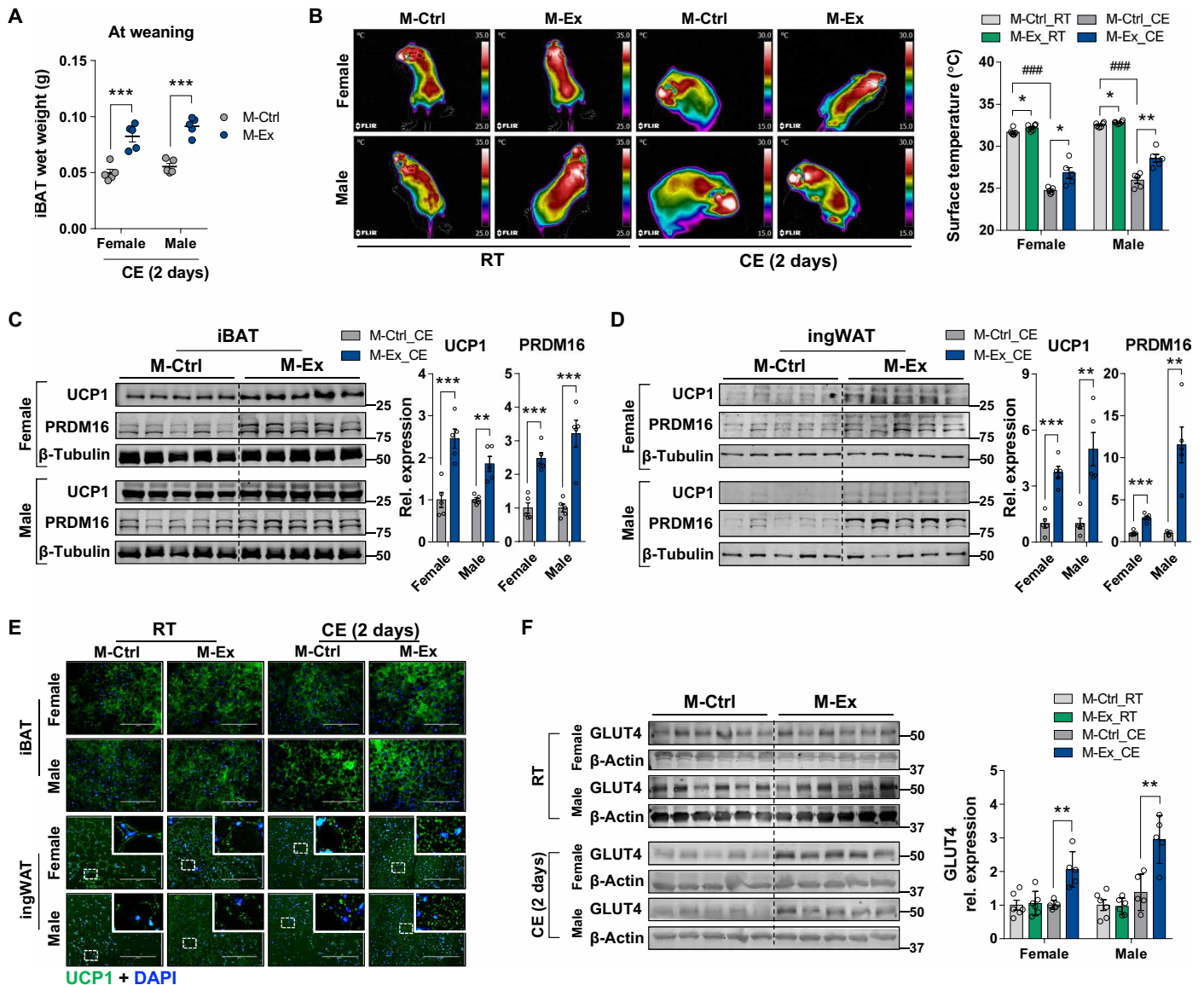


Fig. 2. Maternal exercise intervention facilitates thermogenesis in iBAT and ingWAT of offspring following CE. (A) iBAT wet weight of female and male M-Ctrl and M-Ex offspring by 2 days of CE ($n = 5$ mice per group). (B) Representative thermographic images (left) and calculated averages of intrascapular temperature (right) of female and male M-Ctrl and M-Ex offspring at room temperature (RT) and 2 days of CE ($n = 6$ litters for RT and $n = 5$ litters for CE). (C and D) Cropped Western blots of UCP1 and PRDM16 (β -tubulin is the loading control) from iBAT (C) and ingWAT (D) of female and male M-Ctrl and M-Ex offspring followed by 2 days of CE ($n = 5$ mice per group). (E) Representative images of UCP1 immunocytochemical staining of iBAT and ingWAT from M-Ctrl or M-Ex offspring at RT or CE. Scale bars, 100 μ m. DAPI, 4',6-diamidino-2-phenylindole. (F) Cropped Western blots of GLUT4 in the iBAT (β -actin was used as the loading control) isolated from M-Ctrl or M-Ex offspring at RT and after 2 days of CE ($n = 6$ litters for RT and $n = 5$ litters for CE). Data are mean \pm SEM, and each dot represents one litter. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ in M-Ctrl versus M-Ex, and $###P < 0.001$ in RT versus CE by two-tailed unpaired Student's t test (A, C, and D) or two-way ANOVA (B and F).

enhanced fetal brown adipogenesis. These data suggest a major role of apelin in mediating the beneficial effects of maternal exercise and fetal BAT development.

We further analyzed the phosphorylation of protein kinase A (PKA), a direct target of apelin receptor (APJ), and a G protein-coupled receptor. Apelin supplementation inhibited PKA phosphorylation in fetal BAT (fig. S8A), consistent with the previous report showing that APJ couples with G_i to reduce cyclic adenosine monophosphate (cAMP) synthesis (18). We further measured AMP-activated protein kinase (AMPK) phosphorylation and found that apelin markedly

activated AMPK (fig. S8, B to D), suggesting that apelin-induced thermogenesis might be due to AMPK activation (19).

Maternal exercise induces DNA hypomethylation in the *Prdm16* promoter via an apelin/ α -KG-dependent axis

Previously, we found that low α -ketoglutarate (α -KG) concentration is a limiting factor in DNA demethylation of the *Prdm16* promoter during BAT development, which regulates brown adipogenesis (10). Consistent with highly activated *Prdm16* mRNA expression (Fig. 6A), DNA methylations [5-methylcytosine (5mC)] were reduced, and DNA

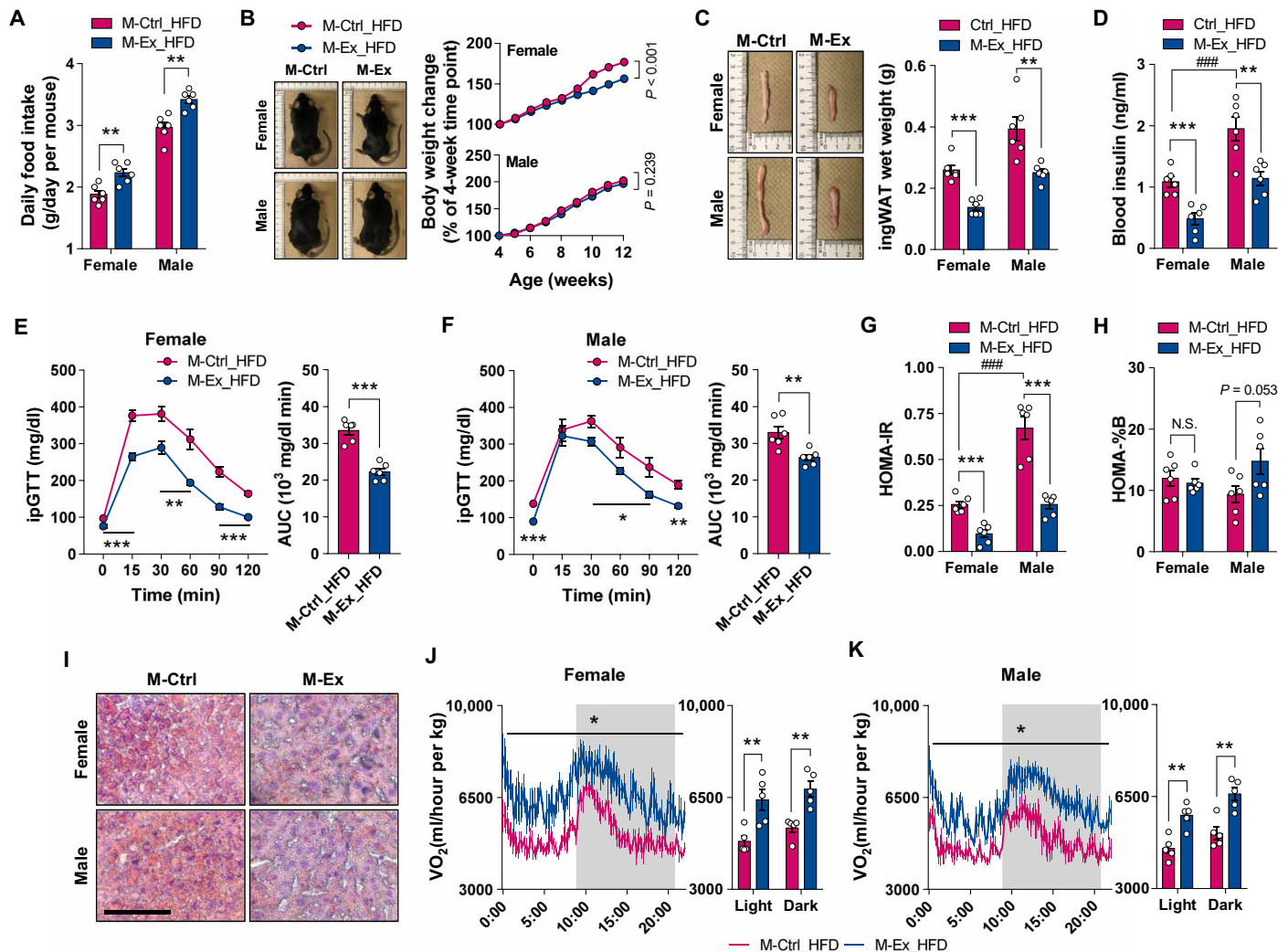


Fig. 3. Maternal exercise protects offspring from HFD-induced obesity. (A to C) Daily food intake (A), body weight gain (B), and ingWAT wet weight (C) of female and male M-Ctrl and M-Ex offspring mice that were fed an HFD for 8 weeks ($n = 6$ mice per group). (D to H) Glucose tolerance test (E and F), blood insulin (D), insulin resistance (G), and pancreatic β -cell function (H) of female and male M-Ctrl and M-Ex offspring that were fed an HFD for 8 weeks with 5-hour fasting ($n = 6$). AUC, area under the curve. (I) Hepatic Oil Red O staining of female and male M-Ctrl and M-Ex offspring. Scale bar, 100 μ m. (J and K) Time-resolved oxygen consumption and analysis of female and male M-Ctrl or M-Ex offspring that were fed an HFD for 8 weeks. Graph depicts calculated means of each litter ($n = 5$ litters). Data are mean \pm SEM, and each dot represents one litter. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ in M-Ctrl versus M-Ex, and ### $P < 0.001$ in female versus male by two-tailed unpaired Student's t test (A, C to H, J, and K) or two-way ANOVA (B).

demethylations [5-hydroxymethylcytosine (5hmC)] were elevated in the fetal BAT from exercised mothers (Fig. 6, B and C), which were maintained following HFD challenge in offspring (Fig. 6D), showing the programming effects of maternal exercise on metabolic health of offspring. Furthermore, the expression of isocitrate dehydrogenase (IDH), which catalyzes α -KG (10) production, was enhanced in M-Ex fetal BAT (Fig. 6, F and G), in agreement with elevated α -KG concentration in M-Ex fetal BAT (Fig. 6E), which was correlated with higher concentration of 5hmC, an intermediate of DNA demethylation (fig. S9, A to C).

Apelin administration reduced 5mC but increased 5hmC concentrations in fetal BAT of sedentary mice (Fig. 6H), consistent with data from M-Ex BAT. The concentrations of α -KG, IDH, and ten-eleven translocation were also elevated by apelin administration (Fig. 6, E, I,

and J). Together, our data suggest that maternal exercise enhances DNA demethylation of the *Prdm16* promoter, which promotes *Prdm16* gene expression in fetal BAT. Maternal exercise-induced apelin might mediate the beneficial effects of maternal exercise on fetal BAT development and its programming effects on offspring metabolic health (Fig. 6K).

To explore whether maternal exercise is specifically beneficial for key genes regulating brown adipogenesis, we analyzed DNA methylation of promoter regions of thermogenesis-related or unrelated genes involved in mesenchymal stem cell differentiation including *Pparg1a* (master coactivator for mitochondrial biogenesis), *zinc finger protein 423* (*Zfp423*; initiating white adipogenesis) (20), *runx-related transcription factor 2* (*Runx2*; mediating osteoblast differentiation) (21), and *paired box gene 3* (*Pax3*; early myogenic

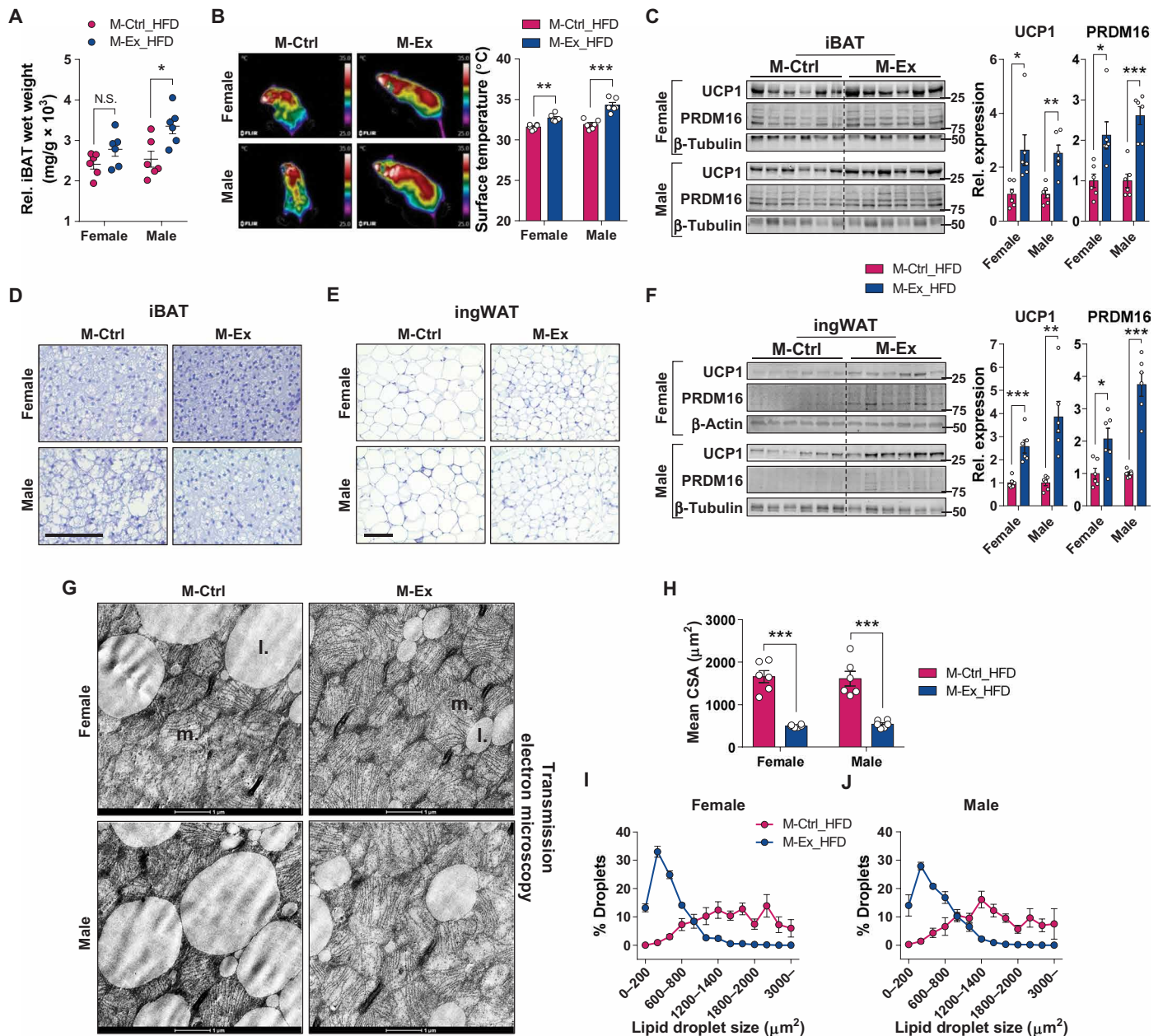


Fig. 4. Maternal exercise protects the impairment of thermogenic markers and formation in iBAT and ingWAT of the offspring challenged with HFD. (A and B) Relative iBAT wet weight (A) and representative thermographic images (left) and calculated averages of intrascapular temperature (right) (B) of female and male M-Ctrl and M-Ex offspring challenged with HFD ($n = 6$). (C and F) Cropped Western blots of UCP1 and PRDM16 protein levels in the iBAT (C) and ingWAT (β -tubulin or β -actin were used as the loading control) (F) from the female and male M-Ctrl and M-Ex offspring challenged with HFD ($n = 6$). (D and G) Hematoxylin and eosin (H&E) staining (D) and transmission electron microscopic images (G) of iBAT of female and male offspring challenged with HFD. Scale bars, 100 μm (D) and 1 μm (G); m.: mitochondria; l.: lipid droplets. (E and H to J) H&E staining (E), mean cross-sectional areas (CSAs) (H), and percent distribution of lipid droplets (I and J) in the ingWAT of female and male offspring challenged with HFD. Scale bar, 100 μm . Data are mean \pm SEM, and each dot represents one litter. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ in M-Ctrl versus M-Ex by two-tailed Student's t test (A to C, F, and H).

differentiation) (22) (fig. S9D). The DNA methylation of *Ppargc1a* promoter regions was reduced in female and male M-Ex and apelin supplementation (APN) fetuses compared to M-Ctrl and phosphate-buffered saline (PBS), respectively. On the other hand, no differences in DNA methylation of *Zfp423*, *Runx2*, and *Pax3* promoter regions were found between groups (fig. S9E).

DISCUSSION

Maternal adaptation to adverse environmental stimuli has been associated with various physiological changes in the offspring (11, 23), establishing that maternal obesity is a major factor in the development of obesity and type 2 diabetes mellitus in offspring (23). Physical exercise is a prominent therapeutic tool to combat diet-induced obesity

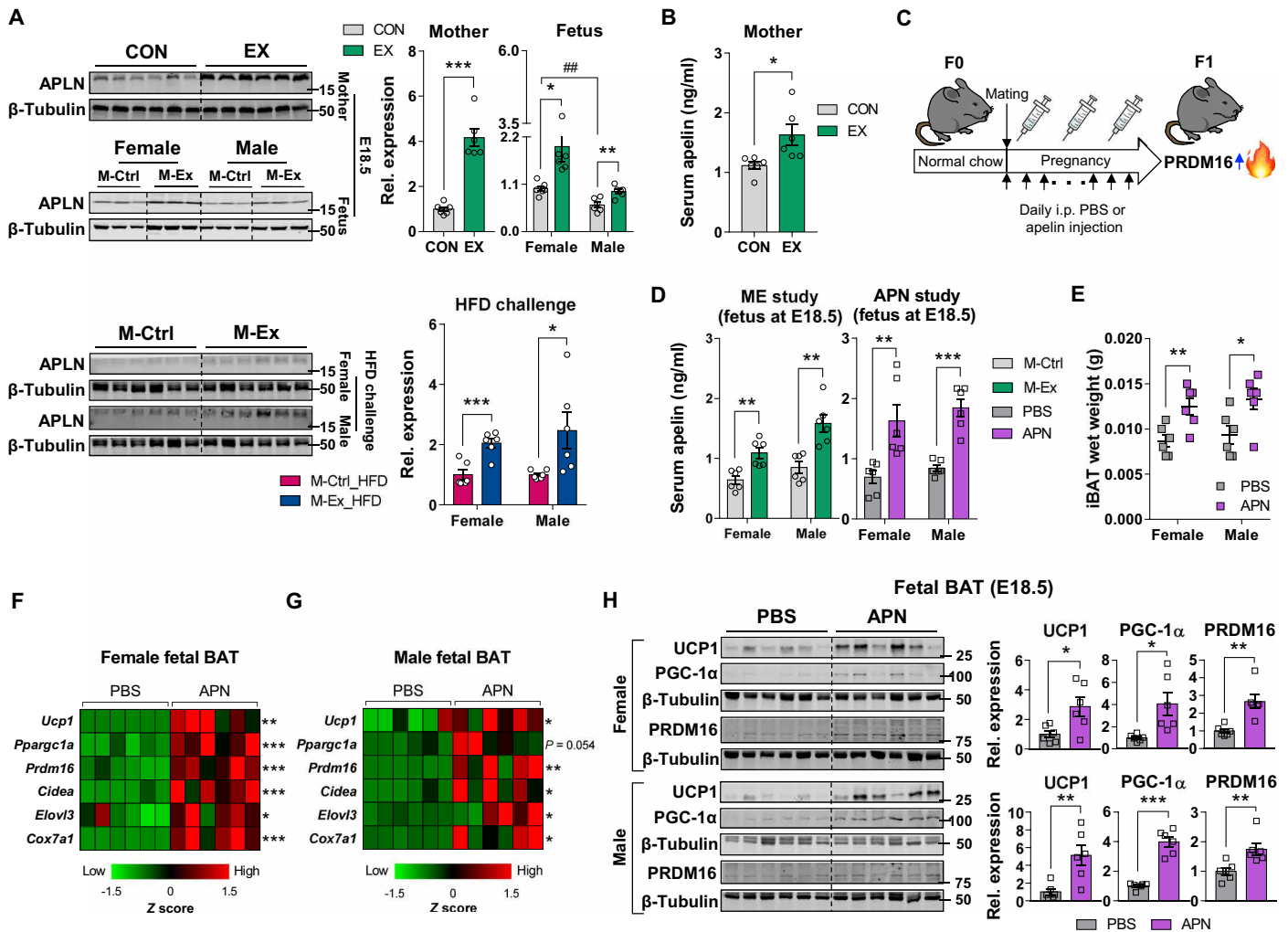


Fig. 5. Apelin supplementation induces brown adipogenesis in the fetal BAT at E18.5. (A) Cropped Western blots of apelin protein levels in the BAT (β -tubulin was used as the loading control) of fetal and offspring mice ($n = 6$). apelin (APLN) (B) Serum apelin (APLN) concentrations in maternal mice at E18.5 ($n = 6$). (C) Protocol of apelin supplementation (i.p.). (D) Serum apelin concentrations in female and male fetuses of maternal exercise (ME) and APN studies ($n = 6$). (E) BAT wet weight of female and male fetuses maternally treated with apelin ($0.5 \mu\text{mol/kg}$ per day) or phosphate-buffered saline (PBS) ($n = 6$). (F and G) Z scores of mRNA level of different brown fat markers in the female (D) and male (E) fetal BAT ($n = 6$). Expression was normalized by ΔC_t values. (H) Cropped Western blots of UCP1, PGC-1 α , PRDM16, and AMPK phosphorylation (β -tubulin was used as the loading control) in the female and male fetal BAT maternally treated with apelin or PBS ($n = 6$). Data are mean \pm SEM, and each dot represents one litter. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ in CON versus EX, M-Ctrl versus M-Ex, or PBS versus APN, and ## $P < 0.01$ in female versus male by two-tailed Student's t test (A, B, and D to H) or Z score (F and G). F0, initial filial generation; F1, first filial generation.

in adults (16, 24), which can produce clinically important alterations in BAT morphology and mitochondrial activity (25). Despite these positive reports of exercise in enhancing UCP1 expression (26), PRDM16 expression (24), and mitochondrial biogenesis (25), there were also reports showing that exercise does not affect thermogenic and mitochondrial activity of BAT (27) or even down-regulated the BAT thermogenesis (28). These inconsistent reports could be due to the difference in exercise types, intensity and duration, and animal physiological conditions (29), as well as the timing of sample collection following exercise (29). Up to now, the impacts of exercise during pregnancy on fetal development have only been examined in obese mothers (13), and its effects on fetal BAT development and long-term thermogenesis of offspring born to healthy mothers are unknown. Our data show that exercise of healthy mothers during pregnancy can im-

prove the BAT and beige adipose function in offspring, which prevents offspring from obesity and metabolic dysfunction induced by HFD.

Exercise and CE are known to stimulate brown/beige adipose tissue activities (4, 25), and their thermogenesis is due to the uncoupling of respiration from adenosine triphosphate synthesis by UCP1 (30). In agreement, we found that the fetal BAT development was facilitated by maternal exercise. Furthermore, we found that the elevation of fetal BAT function was maintained in the offspring of M-Ex mice. The basal UCP1 protein expression in iBAT and ingWAT of M-Ex offspring was elevated compared to M-Ctrl mice. In addition, CE substantially increased brown/beige adipose tissue activity in offspring born to exercised mothers (31). After challenging with HFD, offspring born to exercised mothers were protected from diet-induced obesity. Consequently, there was an improvement in glucose tolerance

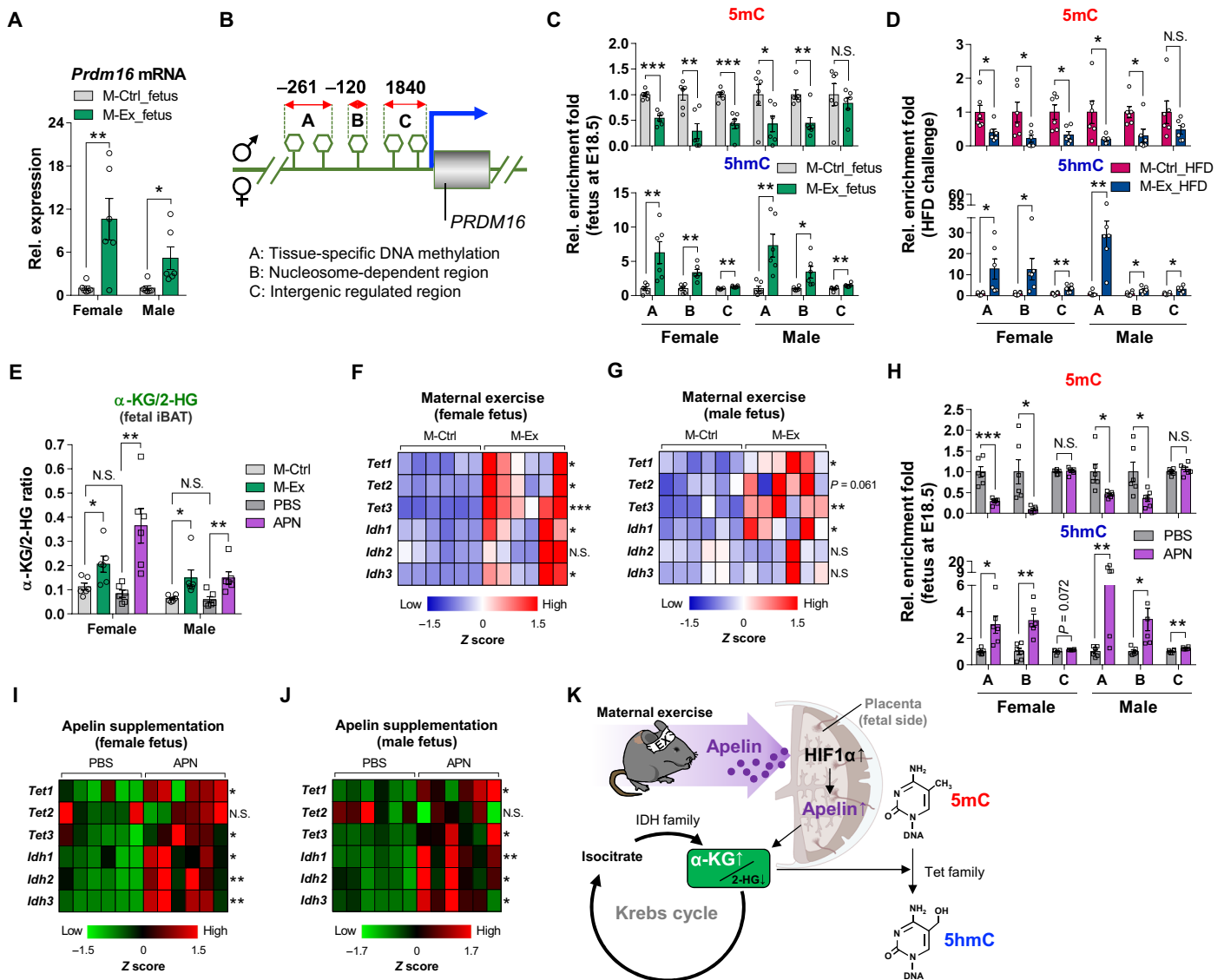


Fig. 6. Maternal exercise induces DNA demethylation of the *Prdm16* promoter through apelin/ α -KG axis. (A) mRNA expression of *Prdm16* of fetal BAT from M-Ctrl and M-Ex ($n = 6$). Expression was normalized by ΔC_t values. (B) Diagram showing three regions in the *Prdm16* promoter. (C, D, and H) 5mC and 5hmC enrichment fold of fetal BAT (ME study, C; APN study, H) and adult offspring challenged with HFD (D) ($n = 6$). (E) α -KG to 2-hydroxyglutarate (2-HG) ratio of fetal iBAT from ME or APN study ($n = 6$). (F, G, I, and J) Z scores of mRNA level of ten-eleven translocation (Tet) and IDH families in the fetal iBAT of APN study (I and J) or ME study (F and G) ($n = 6$). Expression was normalized by ΔC_t values. (K) Working model: DNA demethylation stimulated by α -KG/AMPK/maternal exercise-induced apelin. Data are mean \pm SEM, and each dot represents one litter. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ in M-Ctrl versus M-Ex or PBS versus APN by two-tailed Student's *t* test (A to J).

and insulin sensitivity and reduction of fasting glucose and insulin levels in the female and male offspring challenged with HFD. Together, these data demonstrate long-term beneficial effects of maternal exercise on metabolic health of offspring and support the preventive role of maternal exercise in glucose tolerance and metabolism of offspring born to obese maternal mice (13).

Apelin, a peptide hormone and an exercise-induced adipo-myokine (exerkine), has a key role in regulating placental trophoblast nutrient uptake and endocrine link between the mother and fetus (14), which improves fetal glucose homeostasis (32) and enhances brown adipogenesis and muscle metabolism (19, 33). Furthermore, exercise-dependent apelin secretion reverses age-related muscle loss, sarcopenia,

and promotes mitochondriogenesis and protein synthesis of muscle fibers (33). During pregnancy, exercise induces a hypoxic condition in the placenta (16, 34). Because hypoxia stimulates apelin expression and the placenta is the major site for apelin synthesis and secretion (14, 35), exercise-induced placental hypoxia might explain the elevated fetal apelin concentration. As a peptide, maternal apelin is not expected to cross the placental barrier (36). In agreement, we found that apelin was elevated following exercise in both maternal and fetal circulation and BAT, suggesting that the apelinergic system may be a therapeutic target promoting fetal brown adipogenesis, thus maintaining metabolic homeostasis. Consistently, in adults, the apelinergic system facilitates brown adipogenesis and browning of white adipocytes (19).

Astonishing, apelin treatment enhanced fetal BAT development and offspring metabolic health, which mimicked the beneficial effects of maternal exercise. Mechanistically, apelin administration not only stimulated AMPK activation and α -KG production but also enhanced DNA demethylation of the *Prdm16* promoter, and PRDM16 is a critical factor initiating and maintaining brown adipogenesis and thermogenic function (8). As a rate-limiting factor of brown adipogenesis, the elevation of α -KG in fetal BAT due to exercise promotes brown adipogenesis (10). Unfortunately, the modern way of life in Western countries makes exercise less common, including women during pregnancy (37). Our data suggest that the apelinergic system could be a therapeutic target for enhancing fetal BAT development, uncoupling the sedentary lifestyle of pregnant women with the sub-optimal metabolic health in their offspring. Of note, maternal exercise and apelin treatment likely have additional trophic effects beyond enhancing fetal BAT development, which warrants further exploration.

While the notable consistency in physiological and molecular effects is seen in fetuses between genders based on maternal exercise or apelin administration, sexual dimorphism of offspring mice occurred after challenging with HFD. During HFD challenge, the body weight of male offspring did not differ between M-Ctrl and M-Ex offspring, but M-Ex females gained less body weight compared to M-Ctrl. Overall, male offspring mice gained more weight during HFD feeding with higher inguinal fat weight and higher insulin levels than females. The likely explanation is that males tended to gain more weight and develop obesity and metabolic dysfunction under HFD challenge when compared to females (38, 39), resulting in phenotypic differences between female and male offspring after HFD challenge.

In summary, we found that maternal exercise during pregnancy markedly increases the functionality of brown/beige adipose tissues, which involves enhanced DNA demethylation of the *Prdm16* promoter and elevated α -KG concentration in fetal BAT due to maternal exercise, protecting offspring mice from obesity and metabolic dysfunction induced by HFD. The beneficial effects of maternal exercise on fetal and offspring BAT development were reproduced through maternal apelin administration. These findings suggest that physical activities during pregnancy are critical for metabolic health of offspring, the sedentary lifestyle of women during pregnancy may contribute to the obesity epidemic in modern societies, and the apelinergic system is a potential target for optimizing metabolic health of offspring born to mothers with the common sedentary lifestyle.

METHODS

Mice

Wild-type female C57BL/6J mice (the Jackson Laboratory, Bar Harbor, ME) at 8 weeks of age were randomly assigned into two groups of CON and EX (exercise) and fed ad libitum with a conventional rodent diet (CD; 10% energy from fat, D12450J, Research Diets, New Brunswick, NJ). Nonpregnant female mice were housed in a controlled room on an inverted 12-hour dark/light cycle. After 1 week of acclimation to treadmill exercise training, female mice were mated with males fed on a CD. Mating was determined by the presence of vaginal smears. At birth, the litter size was normalized to 6. Pups were weaned at postnatal day 21. Offspring from maternal CON mice were designated as M-Ctrl, and those from exercised mothers were designated as M-Ex. In the apelin supplementation study, 9-week-old C57BL/6J female mice (the Jackson Laboratory) fed a CD were further randomly assigned into two groups injected with either apelin

or PBS (PBS, $n = 6$; APN, $n = 6$). Apelin was injected daily with [pyr1]apelin-13 (AAPPTec, Louisville, KY) at 0.5 μ mol/kg per day [intraperitoneally (i.p.)] for 16 days (E1.5 to E16.5), as previously described (33). Simultaneously, age-matched pregnant C57BL/6J mice were injected with PBS and served as controls. All experimental animals were euthanized 2 days after the last apelin or PBS injection at E18.5.

Weanling offspring mice (one female and male per litter) were subjected to CE ($n = 6$ for general treatment at weaning and $n = 5$ for CE). After weaning, the remaining female and male offspring were weaned onto HFD (60% energy from fat; D12492, Research Diets) ad libitum to mimic a postweaning obesogenic environment. Maternal mice, and one female and one male per litter at weaning and after 8-week HFD challenge, were anesthetized for further analyses.

In a separate cohort of animals, at E18.5, after 5-hour fasting, maternal mice were euthanized by carbon dioxide inhalation and cervical dislocation, and female and male fetuses were collected separately (fig. S1E and Fig. 5C). Fetal sex identification was based on polymerase chain reaction (PCR) (40). All animal studies were approved by the Institute of Animal Care and Use Committees at the Washington State University.

Treadmill exercise protocol

In maternal daily exercise training, the training protocol followed the principles of progressive loading in exercise intensity and duration based on the previous report (16). Briefly, 1 week before mating, all female animals underwent adaptation on the treadmill running following the protocol of 10 min at 10 m/min, three times per week for a week. After this adaptation, animals were mated and then started to be trained from E1.5 to E16.5 for collecting fetal samples at E18.5 or from E1.5 to E20.5 for delivering pups. On the basis of the guidelines, maximal OCR (VO_2) was measured before gestation, which was applied for setting intensity of treadmill exercise training as follows: E1.5 to E7.5 (40% of VO_{2max}), E8.5 to E14.5 (65% of VO_{2max}), and E15.5 to E20.5 (50% of VO_{2max}). Each exercise stage was conducted based on the three steps: warming up (5 m/min for 10 min), main exercise (10 to 14 m/min for 40 min), and cool down (5 m/min for 10 min), being performed at the same time every morning. Sedentary CON mice were placed on the treadmill (speed set at 0 m/min) for an hour daily as previously described (16).

Cold exposure

For cold stimulation, one female and one male weanling mouse from each litter were kept at either 5° or 22°C for 2 days and fed ad libitum with water provided (4).

Indirect calorimetry

Indirect open-circuit calorimetry measurements were performed by using Comprehensive Lab Animal Monitoring System (Columbus Instruments, Columbus, OH) according to the manufacturer's protocols and guidelines. Briefly, mice were acclimated to the metabolic cages for an hour before recording. During measurement, mice were fed ad libitum with the designated diets, and water was provided (16).

Thermal imaging

Surface temperatures were measured with an E6 Thermal Imaging Infrared Camera (FLIR System, Wilsonville, OR) and analyzed with FLIR Tools Software (FLIR System) (10).

Transmission electron microscope

Tissues were processed on the basis of previous reports (6, 10).

Glucose tolerance test

Mice were fasted for 15 hours and then injected with D-glucose (2 g/kg body weight, i.p.), and blood was collected and measured from the tip of the tail at 0, 15, 30, 60, 90, and 120 min after glucose injection. Levels of blood glucose were measured with a blood glucose meter (Contour Blood Glucose Meter, Bayer, Mishawaka, IN) (10).

Biochemical analysis of serum

Following 5-hour fasting, blood for biochemical analysis was collected from heart via cardiopuncture under anesthesia and used for measuring insulin and glucose levels. Fetal blood was further collected in accordance with a previous report (16). Serum apelin and insulin concentrations were measured using an apelin-12 enzyme immunoassay (EIA) kit (Phoenix Pharmaceuticals, Belmont, CA) and a mouse high range insulin enzyme-linked immunosorbent assay kit (ALPCO, Salem, NH), and blood glucose was measured using a glucose meter (Contour Blood Glucose Meter). The homeostatic model assessment of insulin resistance (HOMA-IR) and pancreatic β -cell function (HOMA-%B) were calculated in accordance with the following formula: HOMA-IR [fasting insulin in $\mu\text{U/ml} \times (\text{fasting blood glucose in mg/dl} \times 0.055)/22.5$]; HOMA-%B $[(360 \times \text{fasting insulin in } \mu\text{U/ml})/(\text{fasting blood glucose} - 63)]$ (16).

GC-MS analysis

Frozen tissues were homogenized, and proteins were extracted using lysis buffer (20 mM tris-HCl, 4.0 mM EDTA, 20 mM NaCl, and 1% SDS). Ten milligrams of samples was used for gas chromatography-mass spectrometry (GC-MS) analysis based on a previous report (41).

RNA extraction, cDNA synthesis, and quantitative real-time PCR

Total RNA was extracted from tissues and cells by TRIzol reagent (Invitrogen, Grand Island, NY) followed by the manufacturer's guidelines. Reverse transcription was conducted to make complementary DNA (cDNA) using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) was used for measuring relative mRNA expression by real-time quantitative PCR (iQ5, Bio-Rad). The RNA expression was normalized to the 18S ribosomal RNA (10, 37). Primer sequences are listed in table S1.

Methylated DNA immunoprecipitation-PCR

DNA (20 μg) was isolated from iBAT tissues using lysis buffer (20 mM tris-HCl, 4.0 mM EDTA, 20 mM NaCl, and 1% SDS) and proteinase K solution [50 mM tris-HCl, 10 mM CaCl_2 , and proteinase K (20 mg/ml)]. The DNA was diluted with tris-EDTA (TE) buffer and sonicated. Antibodies against 5hmC (#51660), 5mC (#28692), or immunoglobulin G (IgG) (#7054) were added into denatured DNA (2 μg) diluted with 10 \times immunoprecipitation (IP) buffer (100 mM Na phosphate, 1.4 M NaCl, and 0.5% Triton X-100). The DNA-antibody complexes were pulled down with EcoMag Protein A Magnetic Particles (#MJA-102, Bioclone Inc., San Diego, CA). The captured beads were washed two times with 1 \times IP buffer and re-suspended in proteinase K solution. Immunoprecipitated DNA was used for real-time qPCR using SsoAdvanced Universal SYBR Green

Supermix (Bio-Rad). Relative folds of enrichment were normalized to that of IgG (42), and primers are listed in table S1.

RNAseq by WTTs sequencing

These experiments were conducted on the basis of a previous report (43). Briefly, 105 million raw reads were generated from Poly(A)-seq, of which 96% were kept after quality control and length filter of reads. Then, alignment was performed and mapped to the reference genome (*Mus_musculus.GRCm38*).

Western blot analysis

Proteins were isolated from tissues with lysis buffer [100 mM tris-HCl (pH 6.8), 2.0% SDS, 20% glycerol, 0.02% bromophenol blue, 5% 2-mercaptoethanol, 100 mM NaF, and 1 mM Na_3VO_4]. The concentration of homogenized protein lysates was determined by the Bradford method (Bio-Rad). The following antibodies were used for the detection of target proteins: UCP1 (#14670), phospho-PKA (#5661), total PKA (#4782), phospho-AMPK (#2535), and total AMPK (#2793) were purchased from Cell Signaling Technology (Danvers, MA). Antibodies against glucose transporter 4 (GLUT4; sc-1607), apelin (sc-293441), and HIF-1 α (sc-13515) were purchased from Santa Cruz Biotechnology (Dallas, TX). PRDM16 (PA5-20872; Thermo Fisher Scientific, Rockford, IL) and PGC-1 α (no. 66369-1-Ig; Proteintech, Rosemont, IL) were also purchased. Antibodies against β -actin and β -tubulin were obtained from the Developmental Studies Hybridoma Bank (Iowa City, IA). IRDye 680 goat anti-mouse secondary antibody (1:10,000), IRDye 800CW donkey anti-rabbit secondary antibody (1:10,000), and IRDye 800CW donkey anti-goat secondary antibody (1:10,000) were purchased from LI-COR Biosciences (Lincoln, NE). The target proteins were detected using the infrared imaging system (Odyssey, LI-COR Biosciences), and intensity of band was quantified using Image Studio Lite (LI-COR Biosciences) (16).

Histological and image analyses

Fresh tissues were fixed in 4% paraformaldehyde for 24 hours at RT and then embedded in paraffin. Sections (5 μm thick) were used for UCP1 (1:200) (#14670, Cell Signaling) immunocytochemistry (ICC) staining or hematoxylin and eosin (H&E) staining in iBAT and ingWAT as previously described (16). In addition, 5- μm -thick sections from placenta at E18.5 were used for apelin (1:50) (#11497-1-AP, Proteintech) ICC staining. Goat anti-mouse IgG2b Alexa Fluor 488 secondary antibody (#A-21141; 1:250; Thermo Fisher Scientific, Waltham, MA) and anti-rabbit IgG Alexa Fluor 488 secondary antibody (#4412; 1:250; Cell Signaling Technology) were used. The cross-sectional areas of lipid droplet were measured using ImageJ software (National Institutes of Health, Bethesda, MD). For Oil Red O staining, liver samples from the offspring were fixed in PBS containing 4% paraformaldehyde and embedded in optimal cutting temperature compound (Thermo 6502, Thermo Fisher Scientific). Sections at 10- μm thickness were stained, and images were obtained by EVOS XL Core Imaging System (Mill Creek, WA).

Statistical analyses

Data were visualized by using GraphPad Prism 7 for Windows (GraphPad Software, San Diego, CA) and analyzed using statistical analysis software (SAS Institute Inc., Cary, NC). Results were reported as mean \pm SEM. Two-tailed unpaired or paired Student's *t* test was applied for comparison. Analysis of variance (ANOVA) was used for

comparisons among multiple groups. Statistical differences were indicated as * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ (or # $P < 0.05$, ## $P < 0.01$, and ### $P < 0.001$).

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at <http://advances.sciencemag.org/cgi/content/full/6/16/eaaz0359/DC1>

[View/request a protocol for this paper from Bio-protocol.](#)

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