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Dynamic changes in DICER levels in adipose tissue control metabolic adaptations to exercise

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DICER is a key enzyme in microRNA (miRNA) biogenesis. Here we show that aerobic exercise training up-regulates DICER in adipose tissue of mice and humans. This can be mimicked by infusion of serum from exercised mice into sedentary mice and depends on AMPK-mediated signaling in both muscle and adipocytes. Adipocyte DICER is required for whole-body metabolic adaptations to aerobic exercise training, in part, by allowing controlled substrate utilization in adipose tissue, which, in turn, supports skeletal muscle function. Exercise training increases overall miRNA expression in adipose tissue, and up-regulation of miR-203-3p limits glycolysis in adipose under conditions of metabolic stress. We propose that exercise training-induced DICER-miR-203-3p up-regulation in adipocytes is a key adaptive response that coordinates signals from working muscle to promote whole-body metabolic adaptations.

microRNA | adipose tissue | exercise | metabolic flexibility | cross-talk

Aerobic exercise training (AET) affects cellular metabolism in an integrative manner, conditioning the organism to changes in energy homeostasis (1, 2). Among these adaptations, AET increases oxidative capacity (3, 4), improves glucose utilization (5), increases insulin sensitivity (6–8), and accelerates lipid turnover at the whole-body level (9–11). These changes are associated with improved metabolic flexibility (defined as optimal responsiveness to couple fuel utilization to fuel availability) and increased physical performance, which ultimately protects against metabolic dysfunction (12).

Adipose tissue is a key site for the regulation of integrative metabolism. Adipocytes serve as the main source of substrates for ATP synthesis during conditions of continuous negative energy balance (13). This process is mediated, at least in part, by 5′-AMP-activated protein kinase (AMPK), a heterotrimeric energy-sensing protein complex that is activated in response to increases in the AMP/ATP and ADP/ATP ratios (14, 15). Conditions that consume ATP, such as dietary restriction or exercise, often activate AMPK (16–18). In turn, AMPK activation improves oxidative metabolism and increases fuel utilization (19–21). For example, in adipocytes, exercise-induced elevation of catecholamines signals through the β-adrenergic receptor pathway to induce glycolysis (22) and lipolysis (23, 24). The former is required to maintain ATP levels in adipocytes, while the latter produces glycerol and free fatty acids to fuel the exercising body. Approximately 30 to 40% of all fatty acids released during lipolysis are reesterified by adipocytes into triacylglycerol (25). This lipid recycling consumes ATP and activates AMPK (26), which subsequently enhances oxidative metabolism and inhibits lipolysis to prevent excessive triacylglycerol hydrolysis (27). Hence, adipose tissue serves as an important energy supplier during exercise and in the recovery period after exercise (11). This requires a robust regulatory network to balance substrate utilization, availability, and storage.

Significance

Aerobic exercise elicits an integrated metabolic response that involves multiple tissues and confers beneficial effects to metabolic health. Here we found that this integrative response involves energy-sensing pathways in muscle and fat and circulating factors that lead to the upregulation of the type III endoribonuclease DICER in adipose tissue and the consequent increase of microRNAs. Upon upregulation, DICER and the microRNA-203-3p inhibit glucose utilization by fat cells and favor oxidative metabolism. In turn, this supports the exercised muscle with adequate substrate availability. When this pathway is disrupted, whole-body metabolism is affected, and exercise performance is impaired. Thus, adipose tissue DICER integrates signals from the exercising muscle to allow a proper metabolic response to exercise training.

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MicroRNAs (miRNAs) are good candidate molecules to orchestrate metabolic adaptations in adipose tissue. These small noncoding RNAs control complex gene networks by fine-tuning the translation of multiple target messenger RNAs (mRNAs). The miRNA expression is decreased in adipose tissue of mice upon aging (28) and obesity (29), and this is caused by a down-regulation of the type III endoribonuclease DICER, which is the rate-limiting enzyme for the biogenesis of most miRNAs in adipocytes (29). Accordingly, adipose-specific *Dicer* knockout mice (AdicerKO) develop aggravated obesity- and age-associated insulin resistance compared to wild-type (WT) littermates, and a significant fraction of these mice die prematurely (29–31). In contrast, dietary restriction up-regulates DICER protein, and thus miRNA expression, in adipose tissue in mice (28), and this plays a role in improving insulin sensitivity and oxidative metabolism (31). While AET affects miRNA expression in skeletal muscle in both mice and humans, this effect does not appear to be due to changes in DICER levels in skeletal muscle (32, 33).

Since AMPK activation is important for the metabolic effects of dietary restriction and aerobic exercise (34, 35), we hypothesized that exercise training may affect DICER levels and miRNA biogenesis in adipose tissue in a manner similar to dietary restriction, and that this may serve as part of a regulatory loop that affects skeletal muscle function and exercise performance. Here we found that exercise training increases DICER abundance in adipose tissue of mice and humans. In mice, this up-regulation depends on AMPK signaling in skeletal muscle and adipocytes, and it leads to an overall increase in miRNA expression in adipose tissue. These exercise training-induced changes in adipose tissue miRNA levels are essential to confer an increase in physical performance, as well as whole-body and adipose tissue metabolic fitness, and these adaptations are lost in AdicerKO mice. Among the exercise-induced miRNAs, we show that miR-203-3p plays a cell-autonomous role to limit glycolysis in adipocytes. Thus, DICER serves as an important factor in adipose tissue to integrate signals from exercising skeletal muscle and, in turn, promotes metabolic flexibility to the organism.

Results

Exercise Training Up-Regulates Components of the miRNA Processing Pathway in Adipose Tissue of Mice and Humans. To determine whether key components of the miRNA processing pathway may be involved in the beneficial effects of aerobic exercise on metabolism, we assessed DICER and Argonaute-2 (AGO2; an endoribonuclease required for miRNA action downstream of DICER) expression in adipose tissue and skeletal muscle of mice subjected to short-term AET (1 h of treadmill running per day with increasing speed and inclination, for 3 d). Four hours after the last exercise bout, this short-term AET induced a twofold increase in DICER mRNA and protein in subcutaneous (s.c.) white adipose tissue (sWAT), but not in muscle or other adipose depots (Fig. 1 *A* and *B*), whereas *Ago2* was not affected by exercise (*SI Appendix*, Fig. S1*A*). Eight weeks of AET resulted in an even more robust and generalized change in the expression/abundance of components of the miRNA-processing pathway in adipose tissues, with up-regulation of DICER in epididymal white fat (eWAT, 1.93-fold for mRNA and 3.33-fold for protein), s.c. WAT (sWAT, 2.83-fold for mRNA and 1.81-fold for protein) and brown adipose tissue (BAT, 1.34-fold for mRNA and 5.53-fold for protein) (Fig. 1 *C* and *D*). With chronic training, AGO2 was also up-regulated in eWAT (3.81-fold for mRNA and 1.73-fold for protein) and BAT (2.03-fold for protein), but not in sWAT (*SI Appendix*, Fig. S1 *B* and *C*). Again, neither *Dicer* nor *Ago2* were altered in gastrocnemius muscle (Gas) (Fig. 1 *A* and *C* and *SI Appendix*, Fig. S1*B*). As expected, these exercise training-induced responses were accompanied by reduced body weight, decreased visceral adiposity, and increased running capacity, with no change in glucose tolerance (*SI Appendix*, Fig. S1 *D–G*).

Likewise, DICER abundance was increased by 5.2-fold in s.c. adipose tissue of humans after 6 wk of high-intensity interval training (Fig. 1*E* and *SI Appendix*, Fig. S1*H*). Exercise training increased DICER levels in both younger (36 ± 11 y of age) and older (63 ± 6 y of age) adults, although there was a large individual variation. Some individuals, particularly in the younger cohort, had 10- to 25-fold increases in DICER, while others, especially in the older cohort, did not respond at all. DICER levels appeared higher in older vs. younger individuals, although the data may not be comparable since the samples were from different cohorts. Thus, exercise training induces DICER abundance in adipose tissue of both mice and humans.

AMPK Signaling Up-Regulates DICER Levels in Adipocytes. Exercise is known to activate AMPK in skeletal muscle (16, 17, 36, 37), and we found this is also true in adipose tissue of acutely exercised mice (*SI Appendix*, Fig. S2*A*). Since AMPK has been implicated in DICER regulation (38, 39), we tested whether AMPK regulates DICER levels in adipose tissue, by feeding WT mice the AMPK activator metformin for 30 d. We found that abundance of DICER in adipose tissue was up-regulated in comparison to vehicle-treated controls (*SI Appendix*, Fig. S2*B*). Likewise, treatment of 3T3-F442A adipocytes in vitro with metformin or the AMP mimetic AICAR increased *Dicer* mRNA expression by 2.7- and 3.4-fold, respectively (*SI Appendix*, Fig. S2*C*). Treatment of 3T3-L1 adipocytes with the β-adrenergic agonist isoproterenol also resulted in a rapid, transient increase in phosphorylation of AMPK on Thr172 and its target acetyl-CoA carboxylase on Ser79, as well as a later increase in DICER abundance (Fig. 2*A* and *SI Appendix*, Fig. S2*D*). In contrast, basal and isoproterenol-induced phosphorylation of ACC-Ser79 was lost in adipocytes with knockdown of AMPKα1 (Fig. 2*A* and *SI Appendix*, Fig. S2 *D* and *E*). Knockdown of AMPKα1 also markedly reduced DICER and AGO2 abundance and blocked isoproterenol-induced DICER up-regulation (Fig. 2*A*). Knockdown of AMPK α1 did not affect levels of the DICER partner protein TRBP (*SI Appendix*, Fig. S2*D*). Thus, AMPK regulates DICER mRNA expression and protein abundance in adipose tissue in a cell autonomous manner.

Exercise-Induced Increase in Adipose Tissue DICER Is Dependent on AMPK Activation in Adipocytes. To determine whether AMPK is required for the effect of AET on DICER levels in adipose tissue in vivo, we generated adipose-specific AMPKα1/2 knockout mice (fAMPK-KO) (*SI Appendix*, Fig. S2*F*), and subjected these mice to short-term exercise training. As above, DICER protein abundance was increased by 2.2-fold in WT mice after exercise, while this effect was abrogated in fAMPK-KO mice (Fig. 2*C*). Interestingly, this was associated with a 1.6-fold increase in *Dicer* mRNA in sWAT of both control and fAMPK-KO mice (Fig. 2*B*), indicating that AMPK regulates DICER abundance through a posttranscriptional mechanism. Consistent with the data in Fig. 1*A* and *B*, DICER protein abundance was not altered in BAT or eWAT in response to short-term aerobic exercise, and the loss of AMPK did not affect DICER levels in these tissues (*SI Appendix*, Fig. S2*G*). Together, these results demonstrate that AMPK is important for exercise-induced DICER up-regulation in s.c. adipocytes in vivo and that this occurs posttranscriptionally.

Exercise-Induced Increase in Adipose Tissue DICER Is Dependent on AMPK Activation in Muscle. AMPK activation in skeletal and cardiac muscle is an important driver of metabolic changes that occur during exercise, which includes the release of a variety of molecules from muscle into the circulation (e.g., myokines or exerkines) (33, 40). To determine the role of AMPK signaling in muscle for regulating DICER abundance in adipose tissue, mice overexpressing a muscle-specific kinase-dead AMPKα2 isoform

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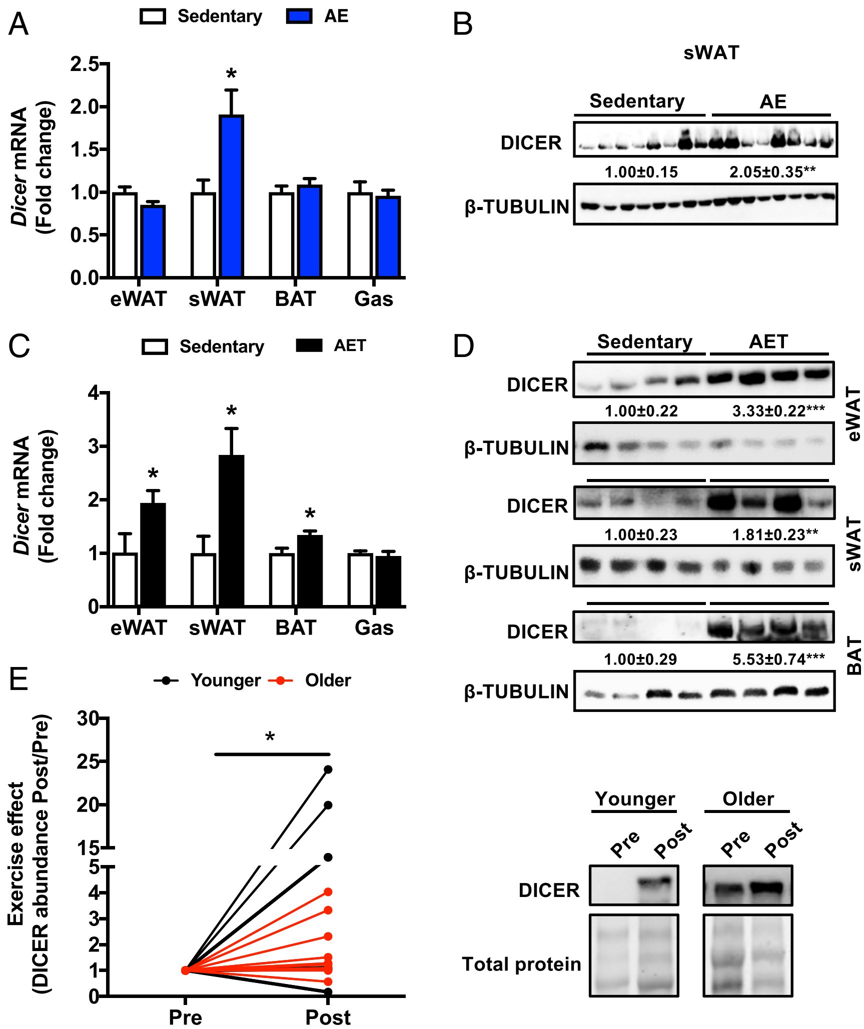

Fig. 1. Exercise up-regulates DICER in adipose tissues. (*A* and *B*) Twelve-week-old mice were subjected to the short-term aerobic exercise (AE) protocol and killed 4 h after the last bout. DICER mRNA expression (*A*) and protein content (*B*) were measured ($n = 8$ per group). *$P < 0.05$, **$P < 0.01$ (Unpaired Student *t* test). (*C* and *D*) Twelve-week old mice were subjected to 8 wk of AET and killed at the end of the protocol after overnight fasting. DICER mRNA expression (*C*) and protein content (*D*) were measured ($n = 7$ per group). In (*C*), *$P < 0.05$ (unpaired Student *t* test). In (*D*), **$P < 0.01$ and ***$P < 0.001$ (unpaired Student *t* test). (*E*) DICER protein abundance in human s.c. adipose tissue of seven 36 ± 11-y-old males (younger; black line) and nine 63 ± 6-y-old males (older; red line) pre- and post-6 wk of high-intensity interval training on a bicycle ergometer three times a week. Each high-intensity training session consisted of five 1-min intervals interspersed with 1.5-min rest. *$P < 0.05$ vs. pre (Paired Student *t* test). Data are expressed as means ± SEM.

(AMPK-KD) in skeletal and cardiac muscles, which has been shown to reduce AMPK activation by 70 to 99% (41), were subjected to AET using a combination of running wheel and treadmill exercise for a period of 6.5 wk. Despite attenuated muscle AMPK signaling and impaired metabolic response to exercise (42), running distance was similar between AMPK-KD mice and WT mice throughout the study (43). Consistent with our earlier observations, AET increased DICER 1.9-fold in sWAT and 1.5-fold in eWAT in WT mice (Fig. 2*D*), but this response was absent in AMPK-KD mice (Fig. 2*D*). There were no changes in AMPK levels in adipose tissue of AMPK-KD versus WT mice or after exercise training (*SI Appendix*, Fig. S2 *H* and *I*). Thus, the up-regulation of DICER in adipose tissue in response to AET requires AMPK activity in cardiac and skeletal muscle. The notion that this increase in adipose tissue DICER was secondary to a circulating factor released by muscle is supported by the observation that differentiated adipocytes treated in vitro for 4 h with serum from exercised mice exhibited a 1.7-fold increase in DICER protein (Fig. 2*E*). Moreover, DICER protein was increased 2.5-fold in sWAT of sedentary WT mice following infusion with serum from exercised mice for 3 d (Fig. 2*F*). Collectively, these data indicate that AET up-regulates DICER in adipose tissue through a mechanism involving release of circulating molecules controlled by the muscle in an AMPK-dependent manner.

Lack of Dicer in Adipocytes Limits Performance and Alters Metabolic Gene Expression in Skeletal Muscle in Response to AET in Mice. To investigate whether *Dicer* expression in adipocytes is required for the metabolic adaptation to exercise, mice with AdicerKO and WT littermates were subjected to AET while challenged with 60% high-fat diet (HFD) (*SI Appendix*, Fig. S3*A*). As previously described (30), AdicerKO mice exhibited signs of lipodystrophy with smaller eWAT fat pads, no changes in sWAT, and a hypertrophic BAT (*SI Appendix*, Fig. S3*B*). AdicerKO mice also had higher levels of intramuscular triglycerides (*SI Appendix*, Fig. S3*C*). Despite these changes in body composition, daily food intake was similar among the groups (*SI Appendix*, Fig. S3*D*), and there were no changes in resting VO_2 consumption or VCO_2 production (*SI Appendix*, Fig. S3 *E* and *F*) after 8 wk of AET.

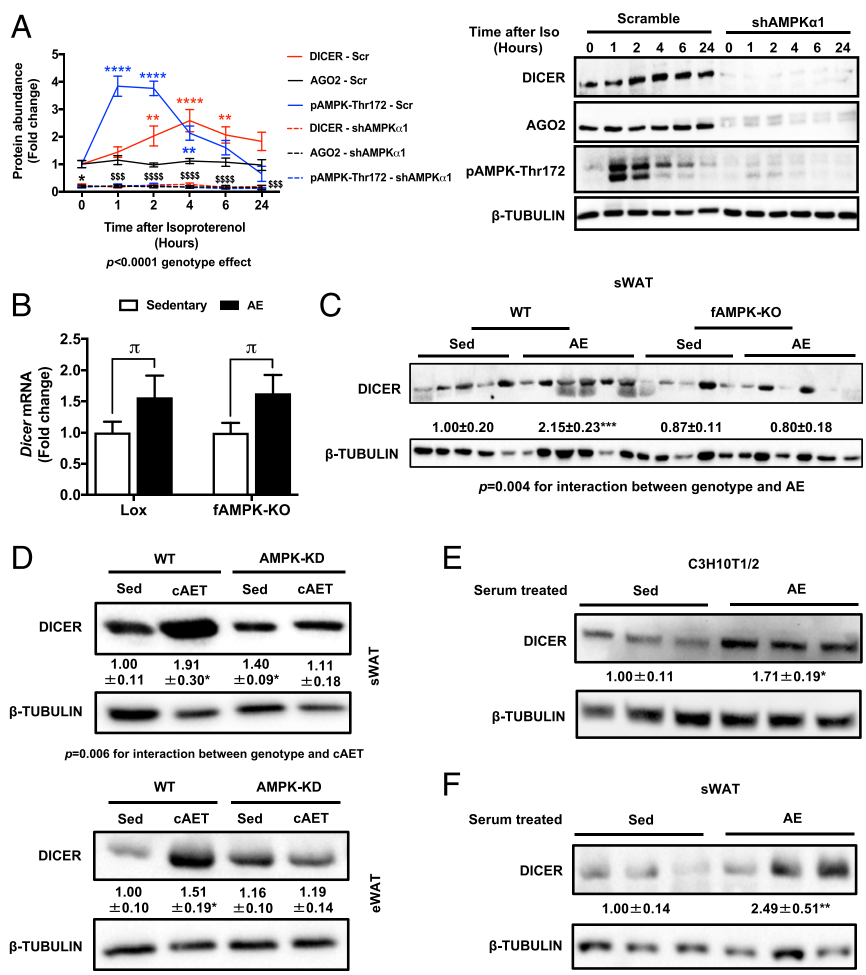

Fig. 2. Exercise requires AMPK activation in adipocytes and muscle cells to induce DICER abundance in adipose tissue. (*A*) (*Left*) Protein abundance of DICER, AGO2, and phosphorylated AMPK-Thr172 in differentiated AMPKα1 knockdown (shAMPKα1) or scramble control (Scr control) 3T3-L1 adipocytes upon isoproterenol (Iso) stimulation (10 μM) over a 24-h time course. (*Right*) Representative immunoblots of triplicates. This experiment was repeated twice. $*P < 0.05$, $**P < 0.01$ and $****P < 0.0001$ vs. respective time point 0. $^{\$\$\$}P < 0.001$, and $^{\$\$\$\$}P < 0.0001$ vs. respective Scr $P < 0.0001$ for genotype effect (two-way-ANOVA with Tukey's multiple comparison test). (*B*) RT-qPCR for *Dicer* in sWAT of fAMPK-KO and WT controls subjected to the short-term AE protocol and killed 4 h after the last exercise bout ($n = 7$ per group). $^{\pi}P < 0.05$ for exercise effect (Two-way-ANOVA with Tukey's multiple comparison test). (*C*) Immunoblotting for DICER in sWAT of fAMPK-KO and WT mice subjected to the short-term AE protocol and killed 4 h after the last exercise bout ($n = 7$ per group). $***P < 0.001$ vs. WT Sed and $P = 0.004$ for interaction between genotype and short-term aerobic exercise (two-way-ANOVA with Tukey's multiple comparison test). (*D*) Female mice overexpressing a kinase dead AMPKα2 subunit specifically in skeletal muscle (AMPK-KD) or WT were trained using the combined AET (cAET) protocol or served as sedentary controls. DICER protein content was analyzed in sWAT and eWAT ($n = 13$ to 15 per group). $*P < 0.05$ vs. WT Sed (unpaired Student *t* test) and $P = 0.006$ for interaction between genotype and cAET effects in sWAT (two-way-ANOVA with Tukey's multiple comparison test). (*E*) DICER protein abundance in C3H10T1/2 adipocytes treated with 7.5% conditioned serum from sedentary (Sed) or exercised (AE) mice for 4 h. This experiment was repeated three times. $*P < 0.05$ (unpaired Student *t* test). (*F*) DICER abundance in sWAT from sedentary C57BL/6NTac mice infused 3 d with conditioned serum (300 μL to 350 μL) from Sed or AE mice ($n = 6$ per group). $**P < 0.01$ (unpaired Student *t* test). Data are expressed as means ± SEM.

Importantly, exercise attenuated HFD-induced body weight gain and intraabdominal fat gain in WT mice, but these health-promoting effects were not observed in AdicerKO mice (Fig. 3*A* and *SI Appendix*, Fig. S3 *A* and *B*). Likewise, AET improved exercise performance in WT, but not in AdicerKO, mice (Fig. 3*B*).

Consistent with an impaired metabolic adaptation to exercise training, the expression of the mitochondrial fatty acid transporter carnitine palmitoyltransferase 1A (*Cpt1a*) gene was up-regulated in skeletal muscle of WT mice after exercise but was down-regulated in AdicerKO mice. Interestingly, *Cpt1a* levels in untrained AdicerKO mice were higher compared with WT littermates (Fig. 3*C*). A similar pattern was found for expression of *Slc2a4* (solute carrier family 2, member 4—*Glut4*) (Fig. 3*C*). In addition, expression of *Ppard*, which encodes a transcription factor known to promote running capacity by repressing glycolytic gene expression in muscle (44), was increased by AET in WT mice but not AdicerKO mice (Fig. 3*D*). Finally, the expression of the slow-twitch fiber marker troponin T1 (*Tnnt1*) was increased in AdicerKO after exercise training, with no changes in fast-twitch fiber markers, L-lactate concentration, ATP levels or mitochondrial DNA (*SI Appendix*, Fig. S4). Of note, *Dicer* levels were not changed in muscle of AdicerKO versus WT mice (30, 45). Together, these results indicate that DICER deficiency in adipose tissue leads to altered metabolic gene expression in skeletal muscle in response to exercise training which, in turn, contributes to reduced exercise performance in AdicerKO mice.

Dicer Deficiency in Adipocytes Impairs the Acute Metabolic Response to Exercise. Middle-aged AdicerKO mice (>6 mo of age) develop lipodystrophy and metabolic dysfunction (30, 31) that could, at least in part, contribute to the impaired metabolic response to exercise observed in these mice. To investigate whether the lack

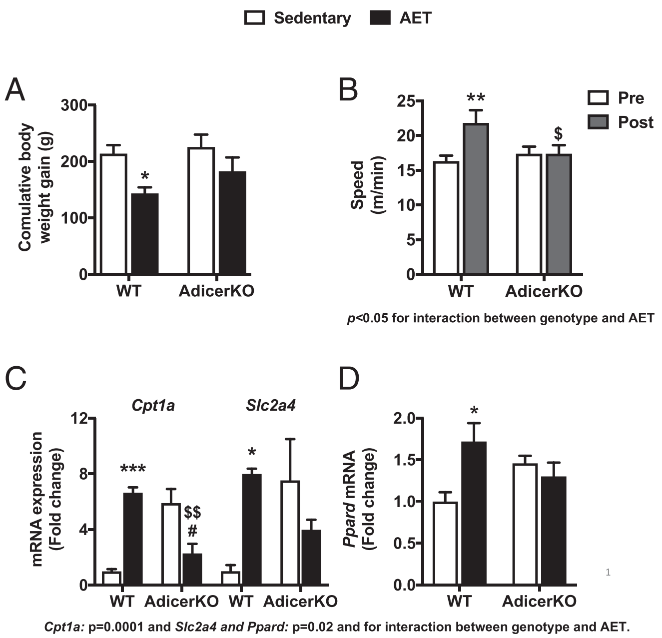

Fig. 3. AdicerKO mice are less responsive to AET. Mice were subjected to 13 wk of HFD combined with the AET protocol or sedentary condition. (*A*) Cumulative body weight gain. $*P < 0.05$ vs. WT Sedentary. (*B*) Maximum speed reached in the maximum effort test (Pre, before AET; Post, after AET). $**P < 0.01$ vs. WT Pre, $^{\$}P < 0.05$ vs. WT Post and $P < 0.05$ for interaction between genotype and AET effect. (*C* and *D*) RT-qPCR of *mCpt1, Slc2a4*, and *Ppard* in Gas. WT mice ($n = 6$ per group) and AdicerKO ($n = 4$ per group). $*P < 0.05$, $***P < 0.001$ vs. WT Sedentary. $^{\$\$}P < 0.01$ vs. WT AET. $^{\#}P < 0.05$ vs. AdicerKO sedentary. Two-way-ANOVA with Tukey's multiple comparison test. Data are expressed as means ± SEM.

of *Dicer* in adipocytes affects the acute response to exercise prior to development of these metabolic derangements, 2-mo-old control and AdicerKO mice were subjected to a single bout of strenuous exercise, and metabolic function of Gas muscle and sWAT—a depot not affected in size or morphology by exercise or *Dicer* deletion (*SI Appendix*, Fig. S3*B* and ref. 31)—were assessed. Branched-chain amino acid (i.e., valine) oxidation was increased in muscle of WT mice by 11.5-fold in response to exercise, whereas, in AdicerKO mice, this increase was much smaller (2.2-fold) and not significant (Fig. 4 *A* and *B*). In contrast, in sWAT, valine oxidation was increased by exercise regardless of the genotype (*SI Appendix*, Fig. S5*A*). In both tissues, there was no change in palmitate oxidation (Fig. 4*C* and *SI Appendix*, Fig. S5*A*), and glucose oxidation in sWAT was significantly lower in AdicerKO mice than in WT mice after exercise (Fig. 4*D*). These results demonstrate that *Dicer* deficiency in adipocytes alters the acute metabolic response to exercise in both skeletal muscle and adipose tissue even in the absence of lipodystrophy.

Lack of Dicer in Adipocytes Promotes Adipose Tissue Glucose Metabolism. In contrast to the decrease in glucose oxidation observed in adipose tissue following acute exercise, AdicerKO mice exhibited increased glucose uptake in eWAT and sWAT when compared to WT mice after 8 wk of AET (Fig. 5*A*); this occurred with no changes in skeletal muscle glucose uptake (*SI Appendix*, Fig. S5*B*). There were also higher L-lactate levels in the sWAT of AdicerKO mice after training compared to WT mice (Fig. 5*B*) indicating increased anaerobic glycolysis, but these changes were not sufficient to modify the circulating levels of L-lactate (*SI Appendix*, Fig. S5*C*). ATP content in sWAT was unaltered (Fig. 5*C*).

In agreement with a metabolic shift toward anaerobic glycolysis, five of the eight analyzed genes involved in key steps of glycolysis were up-regulated in sWAT of AdicerKO mice as compared with controls (Fig. 5*D*). These included *Slc2a1* (glucose transporter 1 insulin-independent—GLUT1), *Gpi1* (glucose-6-phosphate isomerase1), *Pkm2* (pyruvate kinase isozyme M2), *Pgam* (phosphoglycerate mutase), and *Ldha* (lactate dehydrogenase A). Only *Pkm1* (pyruvate kinase isozyme M1) was down-regulated after AET, and this occurred regardless of the genotype. In contrast, glucose-6-phosphate dehydrogenase mRNA levels were lower in trained AdicerKO mice in comparison to trained WT mice, suggesting that flux through the pentose phosphate pathway is inhibited in exercised AdicerKO mice (*SI Appendix*, Fig. S5*D*). Similarly, genes involved in mitochondrial biogenesis and function (e.g., *Ppargc1a, Nrf1*, and *Mfn1*) were down-regulated in sWAT of AdicerKO mice, particularly in the trained group (*SI Appendix*, Fig. S5*E*). Despite these alterations, citrate synthase activity was increased in AdicerKO mice (*SI Appendix*, Fig. S5*F*), whereas mitochondrial DNA was unaltered (*SI Appendix*, Fig. S5*G*), indicating that the perturbations in mitochondrial function were unrelated to changes in mitochondrial mass. Together, these results demonstrate that lack of DICER in adipocytes shifts cellular metabolism toward anaerobic glycolysis, particularly after exercise training.

miR-203-3p Limits Glycolysis and Lactate Release in Adipocytes. To identify miRNAs that may coordinate these metabolic changes, we performed small RNA sequencing in sWAT of WT and AMPK-KD mice without and with AET. We chose this model since DICER is up-regulated by exercise training in sWAT of WT, but not AMPK-KD, mice, enabling us to detect the most DICER-sensitive miRNAs. Pair-wise comparisons revealed 50 differentially expressed miRNAs ($P < 0.05$) when comparing exercised mice to sedentary controls, of which 46 were up-regulated by exercise in WT mice but not in

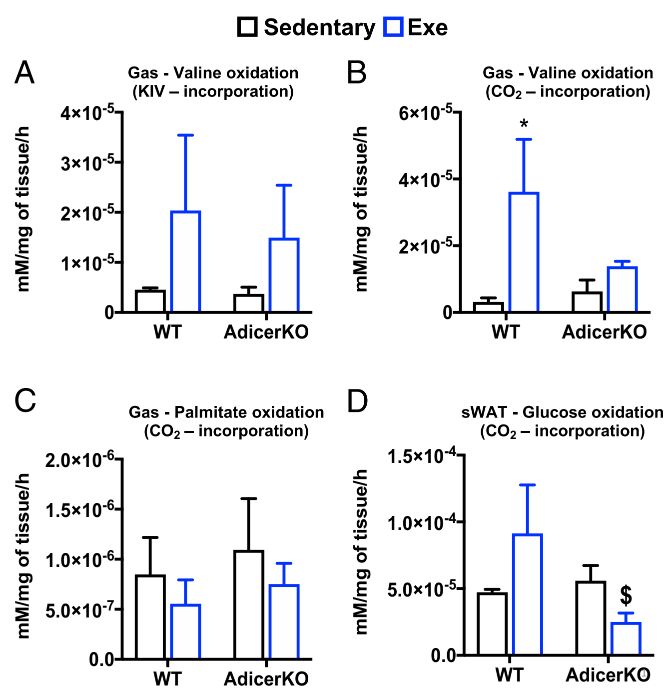

Fig. 4. Skeletal muscle and sWAT metabolism is altered in exercised AdicerKO mice. Ex vivo (*A* and *B*) valine or (*C*) palmitate oxidation in gastrocnemius skeletal muscle (Gas) and (*D*) glucose oxidation in sWAT of 12-wk old mice subjected to one maximum effort test and killed at the end of the session (Exe). Valine incorporation into alpha-ketoisovalerate (KIV), valine, palmitate, or glucose incorporation into CO_2 was measured ($n = 4$ per group). $*P < 0.05$ vs. WT Sedentary and $^{\$}P < 0.05$ vs. WT Exe. Two-way-ANOVA with Tukey's multiple comparison test. Data are expressed as means ± SEM.

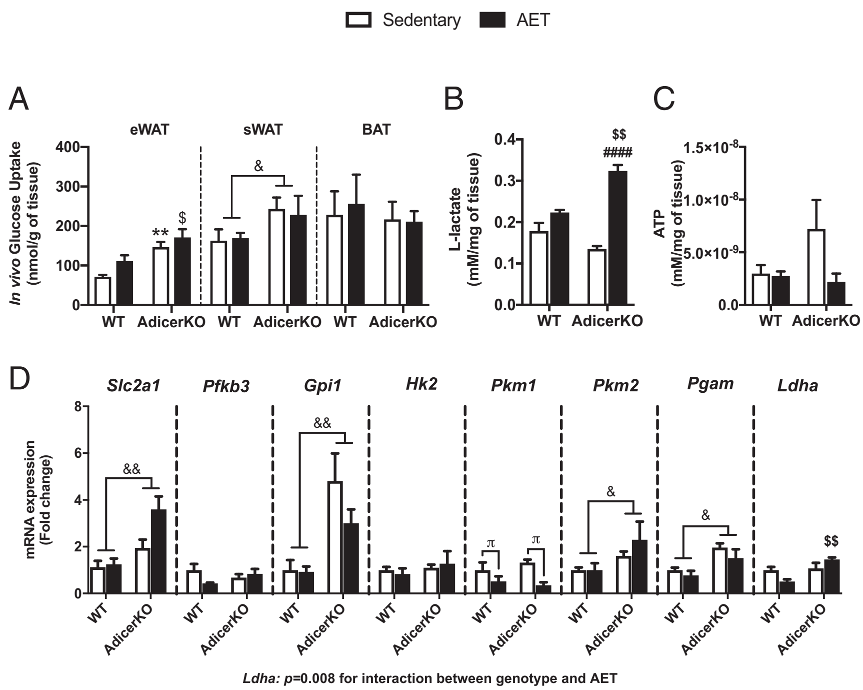

Fig. 5. Increased glycolysis in adipose tissue of trained AdicerKO mice. Male mice were subjected to HFD and 8 wk of AET or kept sedentary. WT mice ($n = 6$ per group) and AdicerKO ($n = 4$ per group). (*A*) Adipose tissue in vivo glucose uptake. (*B*) L-lactate concentration in sWAT. (*C*) ATP concentration in sWAT. (*D*) RT-qPCR of glycolytic genes in sWAT. *Ldha*: $P = 0.008$ for interaction between genotype and AET. $^{**}P < 0.01$ vs. WT sedentary, $^{\#\#\#\#}P < 0.0001$ vs. AdicerKO sedentary. $^{\$}P < 0.05$ and $^{\$\$}P < 0.01$ vs. WT AET. $^{\&}P < 0.05$ and $^{\&\&}P < 0.01$ for genotype effect. $^{\pi}P < 0.05$ for exercise effect. Two-way-ANOVA with Tukey's multiple comparison test. Data are expressed as means ± SEM.

AMPK-KD mice (Fig. 6 *A* and *B*). One of these miRNAs was miR-203-3p, and we have previously observed that this miRNA is tightly correlated with *Dicer* expression in adipose tissue in the context of AET (*SI Appendix*, Fig. S6*A*), caloric restriction, aging (28), and obesity (29).

To further explore the role of miR-203-3p, we transfected 3T3-L1 preadipocytes with miR-203-3p mimic or a scramble control, differentiated these cells into adipocytes, treated them with isoproterenol for 6 h to activate AMPK, and measured the expression of glycolytic genes and the level of L-lactate in the media. Isoproterenol increased L-lactate production in scramble control cells (Fig. 6*C*). This increase in L-lactate production after isoproterenol treatment was reduced in adipocytes overexpressing miR-203-3p (Fig. 6*D*). This was also associated with up-regulation of *Slc2a1*, *Pfkb3*, and *Ldha*, and down-regulation of *Gpi* and *Pgam* mRNA (*SI Appendix*, Fig. S6*B*). We found a 75% (miR-203-3p + Vehicle) and 64% (miR-203-3p + Isoproterenol) decrease in PGAM protein content in cells overexpressing miR-203-3p (Fig. 6*D*), suggesting that miR-203-3p up-regulation limits lactate production by, at least in part, decreasing the protein content of an enzyme present in the distal part of glycolysis. Taken together, these data indicate that induction of DICER and miR-203-3p in adipose tissue in response to exercise training is a key adaptive response to ensure appropriate control of energy homeostasis of adipocytes.

Discussion

Exercise training has beneficial effects on glucose and energy homeostasis. Recent studies have shown that these positive effects include both direct effects on muscle (46) and indirect effects via changes in adipose (40, 47) and other tissues (48). In the present study, we show that part of these indirect effects involves an effect of exercise training to up-regulate the miRNA processing enzyme DICER in adipose tissue of both humans and mice, and this, in turn, leads to increased miRNA biogenesis, including up-regulation of miR-203-3p. This phenomenon is dependent on AMPK activation in both muscle and adipose tissue and involves circulating factors. Mice lacking the ability to up-regulate miRNAs in adipose tissue (i.e., AdicerKO) have reduced work performance in response to exercise training and exhibit metabolic alterations in skeletal muscle and adipose tissue. These results provide mechanistic insights and indicate that adipose tissue's ability to produce miRNAs plays a role in the metabolic adaptation to AET.

Among the adipose tissue miRNAs affected by exercise training, miR-203-3p appears to be highly sensitive to changes in the level of DICER. Thus, in conditions where DICER levels are elevated, such as after exercise training (this study) and dietary restriction (28), miR-203-3p is up-regulated. In contrast, miR-203-3p is down-regulated in adipose tissue with aging (28) and obesity (29, 49), which are conditions where DICER is also down-regulated. Moreover, miR-203-3p and DICER have been implicated in brown fat differentiation and thermogenesis (30, 49–51), suggesting that miR-203-3p is a key miRNA downstream of DICER in adipocytes. The miR-203-3p also plays a role in cancers where DICER levels are also low (52–54), and, in these cells, miR-203 has been shown to suppress cell proliferation and migration (55–57).

Our results provide evidence that high expression of miR-203-3p also inhibits isoproterenol-induced glycolysis in adipocytes. When miR-203-3p is overexpressed, lactate production and expression of the glycolytic genes *Gpi* and *Pgam* are reduced in adipocytes. The impact of miR-203-3p on PGAM protein levels is particularly robust. PGAM converts 3-phosphoglycerate to 2-phosphoglycerate and thus serves as a bottleneck in glycolysis and the pentose phosphate pathway when inhibited (58). Whether PGAM is a direct target of miR-203-3p remains to be determined, but computational target prediction using online

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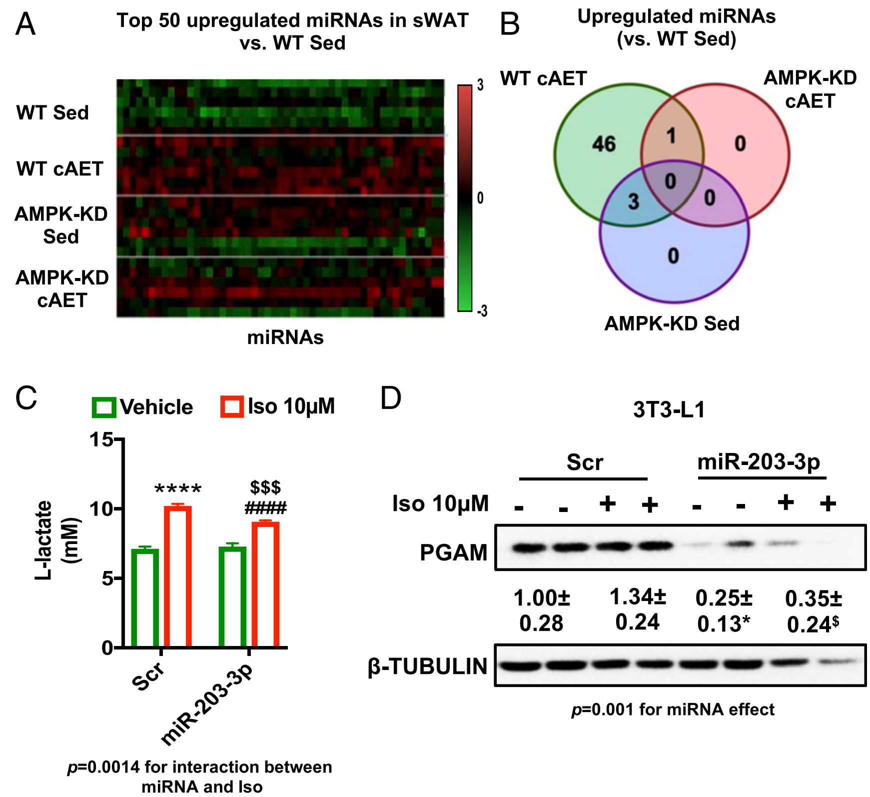

Fig. 6. The miR-203-3p limits isoproterenol-induced glycolysis in adipocytes. (*A*) Heat map and hierarchical clustering of miRNA expression in female WT and AMPK-KD mice, Sed or subjected to cAET. $n = 6$ per group. (*B*) Venn diagram representing the 50 miRNAs found to be significantly changed in sWAT using pairwise comparisons ($P < 0.05$ Student t test, $n = 6$ per group) of the miRNA sequencing data. All miRNAs are up-regulated in WT mice in response to cAET. (*C*) The 3T3-L1 adipocytes overexpressing miR-203-3p or its scramble control (Scr) were treated with vehicle (water) or 10 μM Iso for 6 h. L-lactate concentration in the culture media. $P = 0.0014$ for interaction between miRNA and Iso; $n = 3$ replicates per condition. These experiments were repeated twice. (*D*) Immunoblotting of PGAM. $P = 0.001$ for miRNA effect. $^{*}P < 0.05$, $^{****}P < 0.0001$ Scr vehicle. $^{\$}P < 0.05$ and $^{\$\$\$}P < 0.001$ vs. Scr Iso. $^{\#\#\#\#}P < 0.0001$ vs. miR-203-3p vehicle. Two-way-ANOVA with Tukey's multiple comparison test. Data are expressed as means ± SEM.

available tools (i.e., miRBASE, Target Scan, and DIANA) does not show any direct interaction. However, HIF-1α, a transcription factor known to promote glycolysis, is a target of miR-203-3p (59), and this may explain why several glycolytic genes are suppressed in response to miR-203-3p overexpression.

At the cellular level, metabolic stressors like exercise or beta-adrenergic stimulation triggered by isoproterenol treatment activate different catabolic pathways to rapidly load the mitochondria with substrates for ATP production. The kinetics of activation and deactivation of these pathways depend on negative feedback loops (60). We propose that DICER up-regulation in adipocytes serves as a negative feedback signal in glycolysis, while also eliciting the activation of oxidative pathways, thus contributing to the glucose-sparing effect of the Randle cycle (61, 62). Consistent with this notion, loss of *Dicer* in adipocytes increases anaerobic glycolysis and abrogates oxidative metabolism.

AMPK is an energy sensor which responds to metabolic stress and acts to orchestrate the shift between glycolysis and lipid oxidation, while promoting mitochondrial oxidative capacity (63). Here we show that AMPK is also a positive regulator of DICER in adipocytes and is required for exercise- and isoproterenol-induced up-regulation of DICER. This regulation appears, at least in part, to be cell autonomous, as AMPK knockdown in cultured adipocytes dramatically decreases DICER expression. These results are in line with a recent report showing that AMPK disruption compromises miRNA biogenesis and promotes lipid accumulation in hepatocytes (64).

How AMPK controls DICER levels in adipocytes is a matter for future studies. However, one may speculate that the RNA-binding protein AUF1 is involved, given that, in HeLa cells, AMPK phosphorylates AUF1 in response to metformin treatment, thus shuttling AUF1 to the nucleus and stabilizing *DICER1* mRNA levels, thereby increasing DICER1 abundance (39). Interestingly, when triggered, this pathway reduces senescence of human cells. In mouse adipose tissue, DICER is also reduced with aging, obesity, and senescence (28, 29), while metformin treatment, exercise (this study), and dietary restriction (28, 31) reverse this pattern.

Our results also show that exercise-mediated AMPK activation in skeletal muscle controls DICER expression in adipose tissue. Indeed, serum of exercised mice is sufficient to up-regulate DICER in adipocytes, indicating that some circulating molecule, dependent on muscular activity, induces DICER expression in adipose tissue. Examples of cross-talk between adipose tissue and skeletal muscle during exercise have been reported (40). Muscle-derived molecules such as irisin, meteorin-like 1, β-aminoisobutyric acid, and kynurenic acid are increased with exercise and have been implicated in adipose tissue oxidative metabolism and thermogenesis (65–69). On the other hand, exercise results in changes in adipose tissue that are beneficial to whole-body glucose homeostasis (47, 70), including improved mitochondrial function, decreased inflammation, altered adipokine secretion, and modified glucose and lipid metabolism (71, 72). Interestingly, adipose tissue DICER controls the same processes (30, 31). Moreover, adipose tissue-secreted miRNAs are implicated in the regulation of liver and skeletal

muscle function (73, 74). Although miR-203-3p has been found in circulating exosomes, and brown adipose tissue appears to be an important contributor to the circulating pool of exosomal miR-203-3p (73), we do not see changes in serum miR-203-3p in WT or AdicerKO mice in response to the acute exercise training protocol, suggesting that circulating miR-203-3p itself does not play a role in intertissue communication after exercise. Whether other exercise-induced adipose tissue miRNAs mediate such communication is a subject for future studies.

Here we propose a mechanism where changes in miRNA processing in adipocytes affect substrate utilization by the adipose tissue and, in turn, influence skeletal muscle function and its response to exercise, as well as whole-body metabolism. Fatty acids derived from adipose tissue normally contribute ∼50% of the total energy supply during moderate aerobic exercise in humans (75, 76). This mechanism is driven by metabolic changes that modulate nutrient metabolism in adipose and skeletal muscle (5, 44, 77). For example, controlled glucose metabolism in muscle helps delay hypoglycemia after strenuous exercise and increases exercise performance in mice (44) and humans (5, 77). Moreover, rats with an increased capacity of skeletal muscle to shift from glucose to lipid or amino acid oxidation during exercise can run 3 times longer (3). In contrast, exercise performance is impaired in mice with abrogated adipose tissue lipolysis (78–80). Likewise, when skeletal muscle is forced to use lipids as the exclusive energy source, incomplete organic acid intermediates can accumulate in the mitochondria, decreasing the energy influx to the trichloroacetic acid cycle (TCA) (81), which will impair exercise performance (82). When DICER levels are low in adipocytes, increased adipose tissue anaerobic glycolysis is expected to increase glucose uptake from the blood and decrease lipid utilization. The latter would then remain in circulation to be used as substrate for oxidation or accumulation in other tissues. Consistent with this notion, free fatty acids are increased in the blood stream of AdicerKO mice (30). Moreover, AdicerKO mice exhibit ectopic accumulation of triglycerides in the skeletal muscle (*SI Appendix*, Fig. S3C). Thus, skeletal muscle of mice lacking DICER in adipocytes exhibits changes consistent with an impaired metabolic response to exercise training, including reduced capacity for branched-chain amino acid oxidation. Altogether, these alterations in skeletal muscle and adipose tissue create a scenario of metabolic inflexibility to limit performance and exercise adaptation.

Collectively, our results demonstrate a form of cross-talk between adipose tissue and muscle during aerobic exercise which involves adipose tissue miRNAs. Under metabolic stress, AMPK is activated in both of these tissues, and, in adipose tissue, this leads to up-regulation of DICER. When DICER is up-regulated, miRNAs are generated to counterbalance adipose tissue energy demands and metabolism. For example, higher levels of miR-203-3p in adipocytes inhibit glycolysis induced by energy-demanding conditions, such as β-adrenergic stimulus. This will, in part, confer adequate substrate availability for skeletal muscle during and after exercise, thus allowing the metabolic flexibility required for adaptation to exercise training.

Methods

Mouse Models. Male mice were used in all cases unless stated otherwise. Mice were maintained at a 12-h light–dark cycle with ad libitum access to tap water and chow or 60% HFD (D12492; Research Diets). Protocols for animal use in Brazil were approved by the IACUC of the Universidade Federal de São Paulo (CEP-0218/11, CEP-0237/12, and CEUA4603261015) and Universidade Estadual de Campinas (4759-1/2017 and 4749-1/2017) and were in accordance with NIH guidelines. For animal use in Denmark, experiments were performed in accordance with the European Directive 2010/63/EU of the European Parliament and of the Council for the protection of animals used for scientific purposes. Ethical approval was given by the Danish Animal Experiments Inspectorate (#2012-15-2934-00026). All mice were housed in our animal facilities for at least 1 wk prior to the beginning of the experiments. Details are in *SI Appendix*.

High-Intensity Interval Training (Human Exercise Protocol). Human participants were exercised as previously described (83). In short, two groups of men, a younger group (average age 36 ± 11 y, n = 7) and an older group (63 ± 6 y, n = 9) underwent 6 wk of high-intensity interval training on a bicycle ergometer three times a week. Each high-intensity training session consisted of five 1-min intervals interspersed with 1.5-min rest. The two groups of participants were exercised in two independent studies (83, 84). The study was approved by the Ethical Committee of Copenhagen (journal no. H-3-2012-024) and complied with the Danish Data Protection Agency and the guidelines of the Helsinki Declaration.

AET Protocol. Training was performed at 60% of the maximum speed obtained in the maximum effort test, for 1 h·d^{-1}, 5 d per week for 8 wk. Mice were killed at the end of the protocol after overnight fasting, and tissues were harvested, snap-frozen in liquid nitrogen, and stored at −80 °C until further use. Details are in *SI Appendix*.

Short-Term Exercise Training Protocol. On day 1, mice ran for 2 min at 8 m/min and 3 min at 10 m/min as warm-up. Then they ran for 40 min at 14 m/min and finished at 8 m/min for the last 5 min as cooldown (0% inclination). On day 2, the treadmill was raised to a 5% inclination. Mice ran for 5 min at 10 m/min, then 40 min at 14 m/min, and 5 min at 8 m/min. On day 3, the treadmill was raised to a 10% inclination. Mice ran for 5 min at 10 m/min, then 40 min at 16 m/min, and 5 min at 8 m/min. All mice were killed 4 h after the last bout of exercise, and tissues were snap-frozen and stored at −80 °C. Details are in *SI Appendix*.

Combined AET. Mice were exercised for 6.5 wk in a combination of free access to running wheel and 1 h·d^{-1}, 5 d per week of 16 m/min treadmill running (Columbus Instruments), as described earlier (43). Details are in *SI Appendix*.

Single Bout of Exercise Protocol. Eight-week-old mice were subjected to a single bout of exercise on a treadmill for 1 h at 16 m/min with 5% incline. Mice were killed right after exercise (0 h) or 4 h, 10 h, or 24 h after treadmill running. Details are in *SI Appendix*.

In Vivo Serum Supplementation. Serum (300 μL to 350 μL) was infused at an infusion rate at 30 μL/min for three consecutive days. On day 1, serum from mice subjected to one bout of exercise or sedentary controls was infused, on day 2, serum from mice subjected to two bouts of exercise or sedentary controls was infused, and, on day 3, serum from mice subjected to three bouts of exercise or sedentary controls was infused. Mice were killed 4 h after the last infusion, and tissues were harvested, snap-frozen in liquid nitrogen, and stored at −80 °C until further use. Details are in *SI Appendix*.

In Vitro Serum Supplementation. C3H10T1/2 cells differentiated into adipocytes were treated for 4 h with 7.5% mouse serum in complete media. Details are in *SI Appendix*.

Metabolic Phenotyping. Oxygen consumption and carbon dioxide production were measured in fed animals through a computer-controlled, open-circuit calorimeter system (LE405 gas analyzer; Panlab-Harvard Apparatus). Details are in *SI Appendix*.

Glucose Tolerance. Mice were injected intraperitoneally with glucose (1 g/kg body weight; Sigma-Aldrich) after overnight fasting. Blood samples were collected at the indicated time points through a small cut at the tail tip, and glucose levels were measured using a glucometer (Accu-Chek, Roche).

Substrate Utilization and Citrate Synthase Activity. Citrate synthase activity and valine, glucose, and palmitic acid conversion into CO_2 were performed as previously described (31).

In Vivo Glucose Uptake. Eight-week-old mice were given a 60% HFD 4 wk prior to the beginning of the training protocol and were maintained on the same diet until sacrifice. When 12 wk old, mice were subjected to the AET protocols as described previously. After overnight fasting, mice were intraperitoneally injected with [^{3}H]-2-deoxy-D-glucose in phosphate-buffered saline solution (0.15 μCi per gram of body weight), and, 30 min later, animals were killed by decapitation, blood samples were collected, and tissues were weighted and snap-frozen. Tissues were digested in 5 M NaOH solution for 1 h at 70 °C, and a sample was used to determine [^{3}H]-2-deoxy-D-glucose levels using the Tri-Carb 4910TR liquid scintillation counter. Glucose uptake

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was calculated using blood glucose and [^{3}H]-2-deoxy-D-glucose levels as a reference and normalized by grams of tissue.

Lactate and ATP Analysis. To measure the level of circulating lactate, 24 h after the last training section, we collected 10 μL of blood in a tube containing 150 μL of 4% trichloroacetic acid. Details on lactate and ATP quantification in blood and cells are in *SI Appendix*.

Triglycerides Analysis. For tissue triglyceride measurement, we used the Abcam triglyceride quantification kit (ab65336) according to the manufacturer's protocol.

Cell Culture.

Cell maintenance. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM—4.5g/L glucose) growth medium supplemented with 10% fetal bovine serum (FBS) and 1% of penicillin/streptomycin (P/S) at 37 °C and 5% CO_2. Stable AMPKα1 knockdown cell generated by lentivirus.

Adipocyte differentiation. Confluent preadipocytes were given DMEM containing 10% FBS, isobutylmethylxanthine (500 μM), dexamethasone (1 μM), insulin (500 nM), and rosiglitazone (1 μM). Two days after induction, cells were switched to the maintenance medium containing 10% FBS, insulin (500 nM), and rosiglitazone (1 μM) for 4 d. C3H10T1/2 adipocyte differentiation was induced according to ref. 85.

AMPK activation and inhibition. To activate AMPK, cells were treated with 1 mM of AICAR (5-Aminoimidazole-4-carboxamide 1-β-D-ribofuranoside, Acadesine, N1-(β-D-Ribofuranosyl)-5-aminoimidazole-4-carboxamide; Sigma A9978) diluted in DMSO, or 0.5 mM metformin (Sigma, D150959) or 10 μM isoproterenol (Sigma, I6504) diluted in autoclaved MilliQ water. For AMPK inhibition, cells were treated with 50 μM of dorsomorphin/compound C (Abcam, ab120843).

miRNA-203-3p overexpression. The 3T3-L1 preadipocytes were transfected at 100% confluence with 50 nM of mouse miR-203-3p mimic or the scramble control (abmGood; Applied Biological Materials). Details are in *SI Appendix*.

miRNA Sequencing. NEBNext Multiplex Small RNA library Prep Set for Illumina kit (#E7300) was used for small RNA library preparation after the manufacturer's instruction. Raw data are deposited at Gene Expression Omnibus (GEO) (accession number GSE115408). Details are in *SI Appendix*.

Western Blot Analyses. Western blot analyses were performed as described (86). The following primary antibodies were used: DICER (Ab13502 for detection of mouse DICER and Ab14601 for human DICER) and TRBP (ab157812) from Abcam; AGO2 (2997), total AMPKα1/2 (2532), pAMPK (2532), and β-tubulin (2146) from Cell Signaling; total ACC (streptavidin/HRP, P039701-2) from Agilent; pACC-Ser79 (07-303) from Millipore; and PGAM1/4 (D-5) (sc-365677) from Santa Cruz. Mini-Protean TGX Stain-Free Gels (#456–8086, Bio-Rad) were used for quantification of total protein in human adipose tissue samples.

Gene Expression. Gene expression analysis was performed by qPCR as described (30). Details are in *SI Appendix*.

Statistics. Results are expressed as the mean ± SEM (SEM) unless indicated otherwise. Normal distribution was tested using a Shapiro-Wilk test. We used Student's *t* test to compare two independent groups, one-way ANOVA was used to compare more than two groups, and two-way ANOVA followed by Tukey's multiple comparison test was used when data had more than one categorical independent variable (such as genotype, time and/or exercise). Statistical analyses were run in Graph Prism and R. Statistical significance was considered when $P < 0.05$.

Data Availability. Raw sequencing data are deposited at GEO (accession no. GSE115408). All study data are included in the text and *SI Appendix*.

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