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RESEARCH ARTICLE

Activation of cardiac Nmnat/NAD$^+$/SIR2 pathways mediates endurance exercise resistance to lipotoxic cardiomyopathy in aging *Drosophila*

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ABSTRACT

Endurance exercise is an important way to resist and treat high-fat diet (HFD)-induced lipotoxic cardiomyopathy, but the underlying molecular mechanisms are poorly understood. Here, we used *Drosophila* to identify whether cardiac Nmnat/NAD$^+$/SIR2 pathway activation mediates endurance exercise-induced resistance to lipotoxic cardiomyopathy. The results showed that endurance exercise activated the cardiac Nmnat/NAD$^+$/SIR2/FOXO pathway and the Nmnat/NAD$^+$/SIR2/PGC-1α pathway, including up-regulating cardiac *Nmnat*, *SIR2*, *FOXO* and *PGC-1α* expression, superoxide dismutase (SOD) activity and NAD$^+$ levels, and it prevented HFD-induced or cardiac *Nmnat* knockdown-induced cardiac lipid accumulation, malondialdehyde (MDA) content and fibrillation increase, and fractional shortening decrease. Cardiac *Nmnat* overexpression also activated heart Nmnat/NAD$^+$/SIR2 pathways and resisted HFD-induced cardiac malfunction, but it could not protect against HFD-induced lifespan reduction and locomotor impairment. Exercise improved lifespan and mobility in cardiac *Nmnat* knockdown flies. Therefore, the current results confirm that cardiac Nmnat/NAD$^+$/SIR2 pathways are important antagonists of HFD-induced lipotoxic cardiomyopathy. Cardiac Nmnat/NAD$^+$/SIR2 pathway activation is an important underlying molecular mechanism by which endurance exercise and cardiac *Nmnat* overexpression give protection against lipotoxic cardiomyopathy in *Drosophila*.

KEY WORDS: Heart disease, Cardiac dysfunction, High-fat diet, Heart, FOXO

INTRODUCTION

Heart disease is the number one cause of human death in contemporary society. Many reports have shown the strong connections between obesity and cardiac dysfunction in both animals and humans (Maarman et al., 2020; Reiter et al., 2001). The epidemic of obesity and associated cardiac dysfunctions are at least partly caused by consumption of high caloric fat- and sugar-enriched foods (Pavillard et al., 2017). Because they are genetically and metabolically complex, the biology and physiology of cardiac dysfunctions induced by a high-fat diet (HFD) remain poorly understood in humans. Among invertebrate models, *Drosophila* is a good genetic tool for analyzing heart function. Importantly, as most gene families and signaling pathways are highly conserved between flies and humans, flies can be used to perform heart function-related studies well (Reiter et al., 2001). For example, recent studies show that consumption of HFD in *Drosophila* leads to excessive fat accumulation accompanied by severe heart defects, including increased frequency of arrhythmias, reduced cardiac output, increased non-contractile myocardial cells, and altered myofibrillar structure and collagen content (Birse et al., 2010; Diop et al., 2017; Guida et al., 2019; Wen et al., 2019a,b; Wen et al., 2018). Moreover, it has been identified that cardiac PGC-1, FOXO and Brummer are key antagonists of HFD-induced lipotoxic cardiomyopathy in flies (Birse et al., 2010; Diop et al., 2015). Therefore, compared with mammals, the regulatory network of lipotoxic cardiomyopathy is easier to study in *Drosophila*.

In both mammals and *Drosophila*, physical exercise is regarded as a cost-effective approach to prevent or improve some heart diseases. For example, exercise can mitigate HFD-induced cardiac fibrosis, fractional shortening reduction, triacylglycerol (TAG) accumulation and arrhythmias in both rats and flies (Boardman et al., 2017; Kesherwani et al., 2015; Wen et al., 2018). However, as both lipotoxic cardiomyopathy and cardiac exercise adaptation are associated with complex molecular mechanisms, the regulatory network of exercise-induced resistance to lipotoxic cardiomyopathy remains poorly understood. Increasing evidence suggests that the moderate exercise may be an upstream regulator of PGC-1α, FOXO, SIRT1 (the mammalian homolog of SIR2 in *Drosophila*; the silent information regulator 2, SIR2 or Sirtuin, is the most intensively discussed longevity gene in current aging research; *SIR2* gene encoding a NAD$^+$-dependent histone deacetylase initially was found to extend the lifespan of various organisms ranging from yeast to mammals) and NAD$^+$ (Radak et al., 2020). For instance, endurance exercise can protect the myocardium by reducing myocardial oxidative stress injury and apoptosis via activation of the SIRT1 signaling pathway, up-regulating the myocardial expression of SIRT1 and regulating the deacetylation of FOXO in rats (Li et al., 2017). Additionally, the reduction of PGC-1α in the heart is insufficient to cause an aging phenotype, and moderate overexpression of *PGC-1α* reduces pathological remodeling of older hearts and contributes to the beneficial effects of exercise on cardiac function in aging (Whitehead et al., 2018). Endurance exercise results in a sustained increase in NAD$^+$ levels in the gastrocnemius muscle of rats (White and Schenk, 2012). Finally, the *CG9940* gene encodes the NAD(+) synthase protein in *Drosophila*, and our previous research suggests that endurance exercise can improve heart dysfunction induced by *CG9940* knockdown (Wen et al., 2016), which may be related to the upregulation of NAD$^+$ concentration in the heart by exercise. Therefore, cardiac NAD$^+$/SIR2/PGC-1α and NAD$^+$/SIR2/FOXO may be two key pathways by which exercise combats lipotoxic cardiomyopathy induced by a

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HFD. However, no systematic studies have confirmed this speculation.

Nicotinamide mononucleotide adenylyltransferase (Nmnat) was initially identified as an NAD^+ synthase. It catalyzes the reversible conversion of NMN (nicotinamide mononucleotide) to NAD^+ in the final step of both the *de novo* biosynthesis and salvage pathways in most organisms across all three kingdoms of life. Nicotinamide adenine dinucleotide (NAD) is an essential co-cofactor that serves to mediate various biological processes, including metabolism, DNA repair and gene expression (Jadeja et al., 2020; Yaku et al., 2018). Increasing evidence shows that Nmnat is indispensable in maintaining neuronal homeostasis; for example, Nmnat is closely related to Alzheimer disease and other tauopathies (Ma et al., 2020). Nmnat is a rate-limiting enzyme present in all organisms, and it reversibly catalyzes the important step in the biosynthesis of NAD from ATP and NMN (Schweiger et al., 2001). Overexpression of *Nmnat* has been reported to increase NAD^+ levels in cells (Fang et al., 2012; Zhai et al., 2006). However, the function of the *Nmnat* gene in the heart is still unknown.

To explore whether endurance exercise or cardiac *Nmnat* overexpression can resist HFD-induced lipotoxic cardiomyopathy via activation of NAD^+/SIR2 pathways, fruit flies were used in this study. Firstly, flies were subjected to endurance exercise or fed a HFD to explore whether these two interventions can change the activity of cardiac Nmnat/NAD^+/SIR2 pathways. Next, a cardiac *Nmnat* overexpressing line was built using the UAS/hand-Gal4 system, and these flies were fed a HFD to explore whether upregulation of cardiac NAD^+/SIR2 pathways can protect the heart from lipotoxic cardiomyopathy induced by a HFD. Finally, a cardiac *Nmnat* knockdown line was constructed by RNAi to explore whether down-regulation of cardiac NAD^+/SIR2 pathways can induce lipotoxic cardiomyopathy. The cardiac *Nmnat* knockdown flies were subjected endurance exercise to explore whether this could improve lipotoxic cardiomyopathy induced by cardiac *Nmnat* knockdown. Climbing index and lifespan were also measured in cardiac *Nmnat* overexpressing and knockdown flies to find the relationship between exercise, a HFD, lipotoxic cardiomyopathy and health/lifespan.

MATERIALS AND METHODS

Fly stocks, diet and husbandry

The w^{1118} (stock number: 3605; genotype: w^{1118}) and *hand-Gal4* (stock number: 48396; genotype: w^{1118}; *P{GMR88D05-GAL4}attP2*) flies were a gift from Xiu-shan Wu (Heart Development Center of Hunan Normal University). UAS-*Nmnat*-overexpressing flies (stock number: 39699; genotype: y^1 *w*; P{UAS-Nmnat.Z}2/CyO*) were obtained from the Bloomington Stock Center. UAS-*Nmnat*-RNAi flies (stock number: v107262; genotype: *P{KK101988}VIE-260B*) were obtained from the Vienna Drosophila RNAi Center. To build different expression of the *Nmnat* gene in fly heart, male *hand-Gal4* flies were crossed to female w^{1118} flies, UAS-*Nmnat*-overexpressing flies and UAS-*Nmnat*-RNAi flies. The Gal4/upstream activating sequence (UAS) system is one of the most powerful tools for targeted gene expression. It is based on the properties of the yeast GAL4 transcription factor, which activates transcription of its target genes by binding to UAS *cis*-regulatory sites. In *Drosophila*, the two components are carried in separate lines, allowing for numerous combinatorial possibilities (Brand and Perrimon, 1993; Busson and Pret, 2007). The bipartite system is commonly used in gain-of-function analysis, and by combining this with RNA interference (RNAi) technology, it can also be applied in loss-of-function analysis (Ou and Lei, 2013). The *hand-Gal4*>w^{1118}, *hand-Gal4*>UAS-*Nmnat*-overexpressing and *hand-Gal4*>UAS-*Nmnat*-RNAi flies are referred to as *Nmnat*-Control (*Nmnat*-C), *Nmnat*-overexpressing (*Nmnat*-OE) and *Nmnat*-knockdown (*Nmnat*-KD) flies. All UAS and GAL4 insertions were backcrossed to the w^{1118} line at least 10 times to avoid excess phenotype affecting the experimental results. Female virgin fruit flies from each group were collected within 8 h after hatching; 20 flies were placed in a vial.

Normal food contained 10% yeast, 10% sucrose and 2% agar. The HFD was made by mixing 30% (w/v) coconut oil with the heated normal food (Birse et al., 2010). All HFD group flies were fed a HFD from 21 days of age and were exposed to the HFD for 2 weeks. During the experimental time course, flies were housed in a 22±1°C incubator with 50% humidity and a 12 h light/dark cycle. This environment kept the coconut oil food in a solid state as the melting point of coconut oil is about 24°C, thus ensuring that flies would not get stuck in the oily food. Fresh food was provided every other day for the duration of the experiment. Because we found aging flies were very sensitive to exercise or HFD at this time, all group flies were raised to the fourth weekend, and then the flies were given exercise training and a HFD from the first day of their fifth week of age, followed by five consecutive days of intervention and sampling on the last day of the fifth week of age.

Exercise training device and protocols

When constructing the exercise device, we took advantage of the flies' natural negative geotaxis behavior to induce upward walking. Flies in all exercise groups started exercise from when they were 21 days old, and underwent a 2 week long exercise program. Vials (2.8 cm inner diameter) containing diet and housing 25 flies each, were loaded horizontally into a steel tube that was rotated (at 0.16 rev s^{-1}) about its horizontal axis by an electric motor, with a gear regulating its shaft speed. Thus, with the accompanying rotating steel tube, each vial was rotated along its long axis, which made the flies climb ('The TreadWheel'; Lowman et al., 2018). Most flies continued to respond by climbing throughout the exercise period. The few that failed to climb were actively walking at the inner wall of the vial (Lowman et al., 2018; Wen et al., 2016; Zheng et al., 2015). Flies were exercised for 1.5 h.

Semi-intact *Drosophila* preparation and image analysis

Flies were anesthetized with FlyNap for 2–3 min. The head, ventral thorax and ventral abdominal cuticle were removed by special glass needles to expose the heart and abdomen. These semi-intact preparations removed the cranial nerve and thoracic ventral nerve cord, and blocked the output of the nervous system from affecting heart function (Fink et al., 2009). Dissections were done under oxygenated artificial hemolymph (freshly prepared artificial hemolymph solution containing 108 mmol l^{-1} Na^+, 5 mmol l^{-1} K^+, 2 mmol l^{-1} Ca^{2+}, 8 mmol l^{-1} $MgCl_2$, 1 mmol l^{-1} NaH_2PO_4, 4 mmol l^{-1} $NaHCO_3$, 10 mmol l^{-1} sucrose, 5 mmol l^{-1} trehalose and 5 mmol l^{-1} Hepes, pH 7.1. The sucrose and trehalose were added to the artificial hemolymph from refrigerated stock solutions just prior to use in order to prevent bacterial contamination (Vogler and Ocorr, 2009). These semi-intact preparations were allowed to recover, with oxygenation, for 15–20 min before filming. Image analysis of heart contractions was performed using high-speed videos of the preparations. Videos were taken a 120–130 frames s^{-1} using a Hamamatsu EM-CCD digital camera (McBain Instruments, Chatsworth, CA, USA) on a Leica DM LFS microscope (McBain Instruments) with a 10× objective lens that can be immersed in water. To get a random sampling of heart function, a single 30 s recording was made for each fly. All images were acquired and contrast

enhanced using Simple PCI imaging software (Compix, Sewickley, PA, USA). The heart physiology of the flies was assessed using a semi-automated optical heartbeat analysis program that quantifies heart rate, fractional shortening, diastolic diameter, systolic diameter and fibrillation (Fink et al., 2009). The sample size was 30 flies for each group.

ELISA

Cardiac TAG, NAD^+, malondialdehyde (MDA) and superoxide dismutase (SOD) levels were measured by ELISA (insect TAG, NAD, MDA and SOD ELISA kits, mlbio, Shanghai, China). Fly hearts were homogenized in PBS (pH 7.2–7.4), rapidly frozen with liquid nitrogen and maintained at 2–8°C after melting, then homogenized by grinders, and centrifuged for 20 min at 690–1409 ***g*** remove supernatant, as per the manufacturer's instructions. Every measurement was repeated 3 times, and for each group a total of 240 hearts was used in the ELISA. Normal levels of TAG, NAD^+, MDA and SOD were obtained to calculate changes in heart levels of each (Fig. 1).

RT-qPCR

Approximately 80 hearts from each group were homogenized in Trizol. First, 10 µg of total RNA was purified by organic solvent extraction (TRIzol, Invitrogen). The purified RNA was then treated with DNase I (RNase-free, Roche) and used to produce oligo dT-primed cDNA by reverse transcription (SuperScript II RT, Invitrogen), which was then used as a template for quantitative PCR (qPCR). The *rp49* gene was used as an internal reference for normalizing the quantity of total RNA. qPCR was performed with SYBR Green using an ABI7300 Real time PCR system (Applied Biosystems). Expression of the various genes was determined by the comparative CT method (ABI Prism 7700 Sequence Detection System User Bulletin #2, Applied Biosystems). Primer sequences were as follows: *PGC-1α* F: 5′-TGTTGCTGC-TACTGCTGCTT-3′, R:5′-GCCTCTGCATCACCTACACA-3′; *bmm* F: 5′-ACTGCACATTTCGCTTACCC-3′, R: 5′-GAGAATC-CGGGTATGAAGCA-3′ (Fig. 2); *SIRT1* F: 5′-GCCCAAGAAC-AACATAACAAGC-3′, R: 5′-CGAGATGATGCCACCTACCAC-3′; *FOXO* F: 5′-AACAACAGCAGCATCAGCAG-3′, R: 5′-CTGAACCCGAGCATTCAGAT-3′; *rp49* F: 5′-CTAAGCTGT-CGCACAAATGG-3′, R:5′-AACTTCTT GAATCCGG TGGG-3′.

Negative geotaxis assay

The climbing apparatus consisted of an 18 cm-long vial with an inner diameter of 2.8 cm, and flies were allowed to adapt to the vial for 10 min before assessing negative geotaxis. Sponges were placed in the ends of the tube to prevent escape while allowing air exchange. With a light box behind the vials, the rack was tapped down 5 times and on the fifth time, a timed digital camera snapped a picture after 8 s. The extent of climbing could be analyzed visually or using imaging software. Five pictures of each group were taken and averaged to arrive at a fixed score for each vial. The total score for all the flies in a vial was tallied, and then divided by the number of flies in the vial to generate the 'climbing index' for that trial. Each vial was subjected to five trials, and then the indexes from the five trials were averaged.

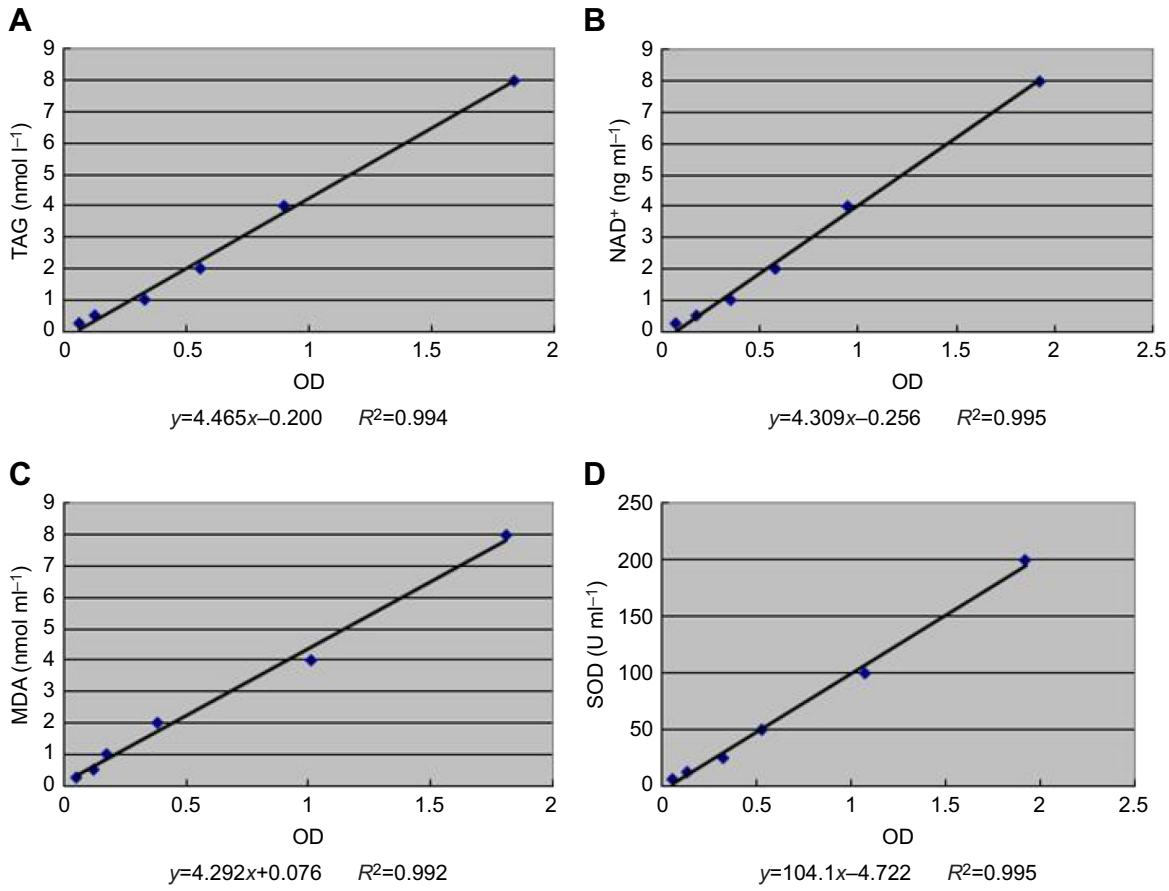

Fig. 1. The normal levels of insect cardiac TAG, NAD^+, MDA and SOD.

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Lifespan assays

The number of dead flies was recorded daily. Lifespan was estimated for each fly as the number of days the fly was alive from the day of eclosion to the day of death. Mean and median lifespan and survival curves were primarily used to characterize lifespan. Sample sizes were 200–220 flies per group.

Statistical analyses

A two-way ANOVA was used to analyze the effects of HFD and exercise on the heart in *Nmnat*-C flies. A one-way ANOVA with least significant difference (LSD) tests was used to identify differences among the *Nmnat*-C group, *Nmnat*-KD group and *Nmnat*-KD plus exercise (*Nmnat*-KD+E) group. A one-way ANOVA with LSD tests was used to identify differences among the *Nmnat*-C group, *Nmnat*-OE group and *Nmnat*-OE plus HFD (*Nmnat*-OE+HFD) group. Analyses were performed using the Statistical Package for the Social Sciences (SPSS) version 16.0 for Windows (SPSS Inc., Chicago, IL, USA), with statistical significance set at $P<0.05$. Data are represented as means±s.e.m.

RESULTS

Exercise prevents HFD-induced cardiac dysfunction and Nmnat/NAD$^+$/SIR2 pathway suppression

In both mammals and flies, there are increasing reports that a HFD can induce cardiac dysfunction, such as increased frequency of arrhythmias, reduced cardiac output and increased fibrillations. Endurance exercise has been reported to protect the heart from HFD-induced cardiac malfunction, but its molecular mechanisms of regulation are still poorly understood. Accumulating evidence suggests that endurance exercise, HFD-induced cardiac malfunction and Nmnat/NAD$^+$/SIR2 pathways are closely related. So, to explore whether endurance exercise resistance to HFD-induced lipotoxic cardiomyopathy was accompanied by activation of cardiac Nmnat/NAD$^+$/SIR2 pathways, flies were subjected to endurance exercise or fed a HFD in this study. Cardiac TAG levels and *bmm* expression were measured to monitor lipid accumulation in the heart. Cardiac *Nmnat* expression, NAD$^+$ levels, *SIR2* expression, *FOXO* expression, *PGC-1α* expression, SOD activity levels and MDA levels were measured to reflect the activity of cardiac Nmnat/NAD$^+$/SIR2 pathways. Heart rate, fractional shortening, diastolic diameter, systolic diameter and fibrillation were tested by M-mode tracing to represent heart contractility and dysfunction.

We found that the relative heart TAG content in *Nmnat*-C+E group flies was lower than that of *Nmnat*-C group flies (LSD test, $P<0.05$); relative heart TAG content in *Nmnat*-C+HFD group flies was higher than in *Nmnat*-C group flies (LSD test, $P<0.01$); relative heart TAG content in *Nmnat*-C+HFD+E group flies was lower than that of *Nmnat*-C+HFD group flies (LSD test, $P<0.01$); and there was no significant difference between *Nmnat*-C group flies and *Nmnat*-

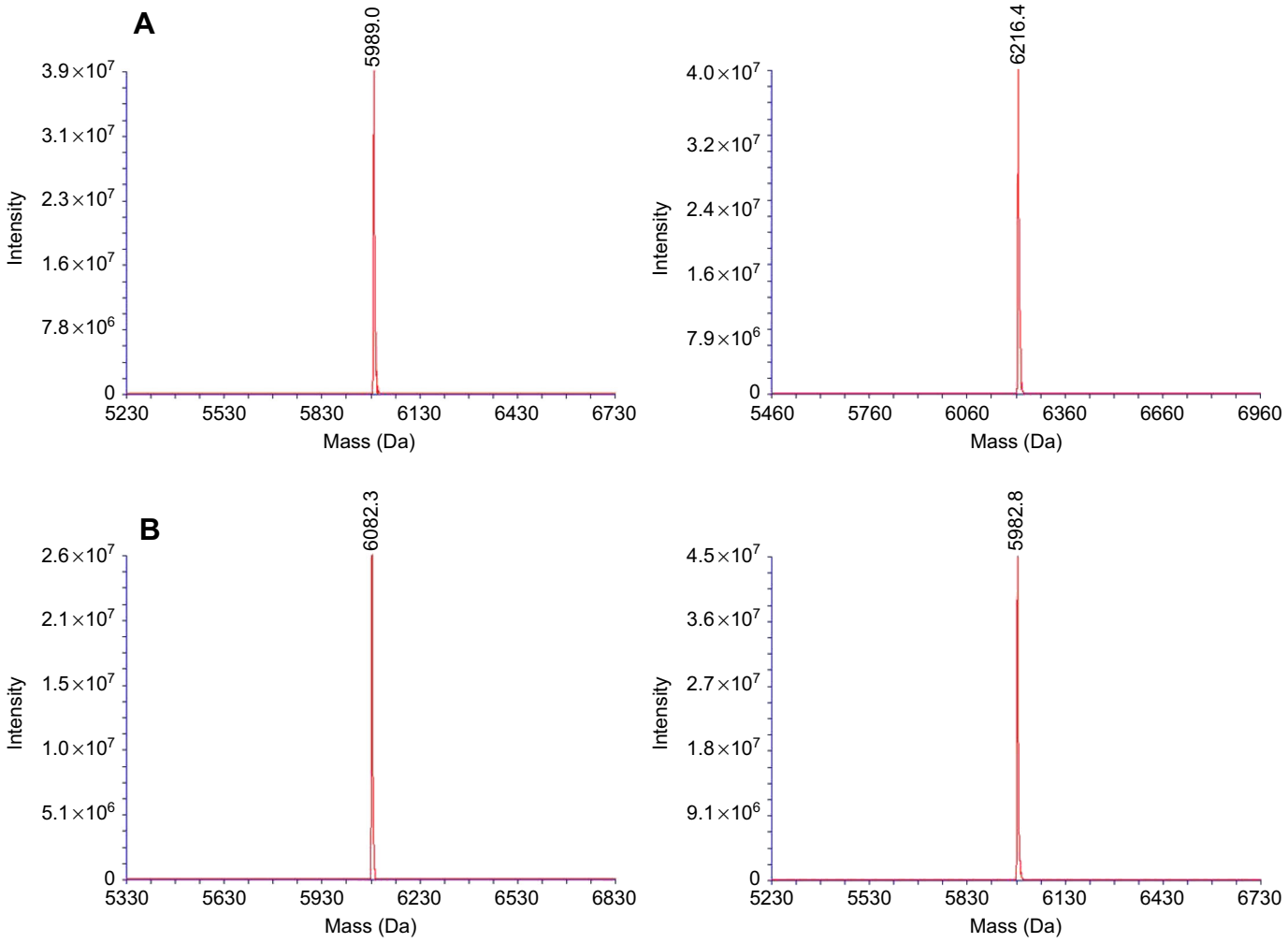

Fig. 2. Testing of PCR primers using mass spectrometry. Forward (left) and reverse (right) primers were designed for *bmm* (A) and *PGC-1α* (B). The results showed that the target gene had no high homology with other genes.

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C+HFD+E group flies in relative TAG content (LSD test, $P>0.05$) (Fig. 3A). The *Brummer* (*bmm*) gene, which encodes the *Drosophila* homolog of mammalian ATGL lipase, also mediates fat hydrolysis in this invertebrate model system (Gronke et al., 2005). A HFD can reduce *bmm* expression, and *bmm* overexpression efficiently prevents HFD-induced fat accumulation and heart dysfunction (Birse et al., 2010). The results show that relative heart *bmm* expression in *Nmnat*-C+E group flies was higher than in *Nmnat*-C group flies (LSD test, $P<0.01$); relative heart *bmm* expression in *Nmnat*-C+HFD group flies was lower than in *Nmnat*-C group flies (LSD test, $P<0.01$); relative heart *bmm* expression in *Nmnat*-C+HFD+E group flies was higher than in *Nmnat*-C+HFD group flies (LSD test, $P<0.01$); and relative heart *bmm* expression in *Nmnat*-C+HFD+E group flies was higher than in *Nmnat*-C group flies (LSD test, $P<0.05$) (Fig. 3B). These results suggest that endurance exercise resisted HFD-induced heart lipid accumulation by promoting the breakdown of TAG.

For analysis of the effect of HFD and exercise on cardiac Nmnat/NAD$^+$/SIR2 pathways, the results show that relative cardiac *Nmnat* expression, NAD$^+$ levels, *SIR2* expression, *FOXO* expression, *PGC-1α* expression and SOD activity levels in *Nmnat*-C+E group flies were higher than in *Nmnat*-C group flies (LSD test, $P<0.01$ or $P<0.05$), but cardiac MDA levels in *Nmnat*-C+E group flies were lower than in *Nmnat*-C group flies (LSD test, $P<0.05$); additionally, cardiac MDA levels in *Nmnat*-C+HFD group flies were higher than in *Nmnat*-C group flies (LSD test, $P<0.01$), but relative cardiac *Nmnat* expression, NAD$^+$ levels, *SIR2* expression, *FOXO* expression, *PGC-1α* expression and SOD activity levels in *Nmnat*-C+HFD group flies were lower than in *Nmnat*-C group flies (LSD test, $P<0.01$ or $P<0.05$). Moreover, relative cardiac MDA levels in *Nmnat*-C+HFD+E group flies were lower than in *Nmnat*-C+HFD group flies, and relative cardiac *Nmnat* expression, NAD$^+$ levels, *SIR2* expression, *FOXO* expression, *PGC-1α* expression and SOD activity levels in *Nmnat*-C+HFD+E group flies were higher than in *Nmnat*-C+HFD group flies (LSD test, $P<0.01$). Finally, there was no significant difference between *Nmnat*-C group flies and *Nmnat*-C+HFD+E group flies in cardiac *Nmnat* expression, NAD$^+$ levels, *SIR2* expression, *FOXO* expression, MDA levels and SOD activity levels, but relative heart *PGC-1α* expression in *Nmnat*-C+HFD+E group flies was higher than in *Nmnat*-C group flies (LSD test, $P<0.05$) (Fig. 4A–G). As the myofibrils and mitochondria of cardiomyocytes are critical to the contractile function of the heart, the ultrastructure of cardiomyocytes was observed by electron microscopy in 5 week old flies. The images showed that a HFD reduced the number of myofibrils and mitochondria, and destroyed the continuity between the Z-lines and the arrangement of myofibrils, and endurance exercise could effectively prevent this from happening (Fig. 4H). These results indicate that endurance exercise relieved HFD-induced lipid toxicity injury in myocardial cells by up-regulating the Nmnat/NAD$^+$/SIR2/FOXO pathway and Nmnat/NAD$^+$/SIR2/PGC-1α pathway.

For cardiac function, the results show that exercise significantly increased cardiac fractional shortening, and it notably reduced heart rate and fibrillation (two-way ANOVA, $P<0.05$ or $P<0.01$). In contrast, a HFD significantly increased heart rate and fibrillation (two-way ANOVA, $P<0.05$ or $P<0.01$), but it notably reduced fractional shortening (two-way ANOVA, $P<0.05$). Exercise and a HFD had no interaction influence on cardiac fractional shortening, heart rate and fibrillation (two-way ANOVA, $P>0.05$). Both exercise and a HFD also had no significant influence on diastolic diameter and systolic diameter, and they had no interaction influence on diastolic diameter and systolic diameter (two-way ANOVA, $P>0.05$). Further analysis and comparison of results revealed that fractional shortening in *Nmnat*-C+E group flies was higher than in *Nmnat*-C group flies (LSD test, $P<0.05$), but heart rate and fibrillation in *Nmnat*-C+E group flies were lower than in *Nmnat*-C group flies (LSD test, $P<0.05$ or $P<0.01$); in addition, heart rate and fibrillation in *Nmnat*-C+HFD group flies were higher than in *Nmnat*-C group flies (LSD test, $P<0.05$ or $P<0.01$), but fractional shortening in *Nmnat*-C+HFD group flies was lower than in *Nmnat*-C group flies (LSD test, $P<0.05$). Heart rate and fibrillation in *Nmnat*-C+HFD+E group flies were lower than in *Nmnat*-C+HFD group flies, and fractional shortening in *Nmnat*-C+HFD+E group flies was higher than in *Nmnat*-C+HFD group flies (LSD test, $P<0.01$). Finally, there was no significant difference between *Nmnat*-C group flies and *Nmnat*-C+HFD+E group flies in heart rate, fractional shortening and fibrillation (LSD test, $P>0.05$) (Fig. 5A–G). These results indicate that endurance exercise improved HFD-induced weak heart contractility and severe arrhythmia.

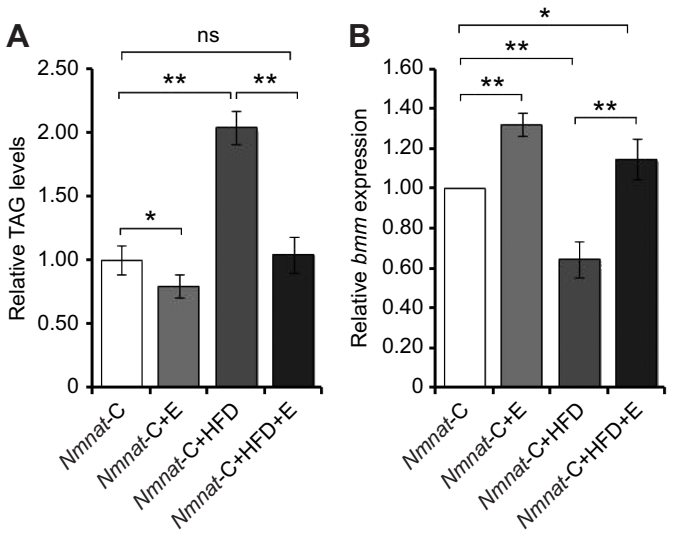

Fig. 3. Effect of a high-fat diet (HFD) and exercise on heart lipid accumulation in *Drosophila*. (A) Heart relative triacylglycerol (TAG) levels. Results are expressed as the fold-change from *Nmnat*-C flies. The sample size was 80 hearts with 3 biological replicates. (B) Heart relative *bmm* gene expression level. The sample size was 80 hearts with 3 biological replicates. A two-way ANOVA was used to identify differences among the *Nmnat*-Control (*Nmnat*-C), *Nmnat*-C plus exercise (*Nmnat*-C+E), *Nmnat*-C plus HFD (*Nmnat*-C+HFD) and *Nmnat*-C+HFD+E group flies. These experimental data were measured on the last day of the fifth week of age of flies. The genotype of *Nmnat*-C group flies is *hand-Gal4>w^{1118}*. Data are presented as means±s.e.m. *P<0.05, **P<0.01.

Exercise improves *Nmnat* RNAi-induced heart dysfunction and lipid accumulation

Functional studies in *Drosophila* and mammals have shown that loss of *Nmnat* causes neurodegeneration and a decrease in NAD$^+$ levels of cells (Fang et al., 2012; Zhai et al., 2006). Although previous results indicated that exercise-induced prevention of HFD-induced cardiac dysfunction was accompanied by up-regulation of cardiac Nmnat/NAD$^+$/SIR2 pathway activity, it still remained unclear whether cardiac Nmnat/NAD$^+$/SIR2 pathways could mediate endurance exercise resistance to HFD-induced cardiac malfunction as there was no direct evidence that cardiac Nmnat/NAD$^+$/SIR2 pathways were key players in HFD-induced lipid

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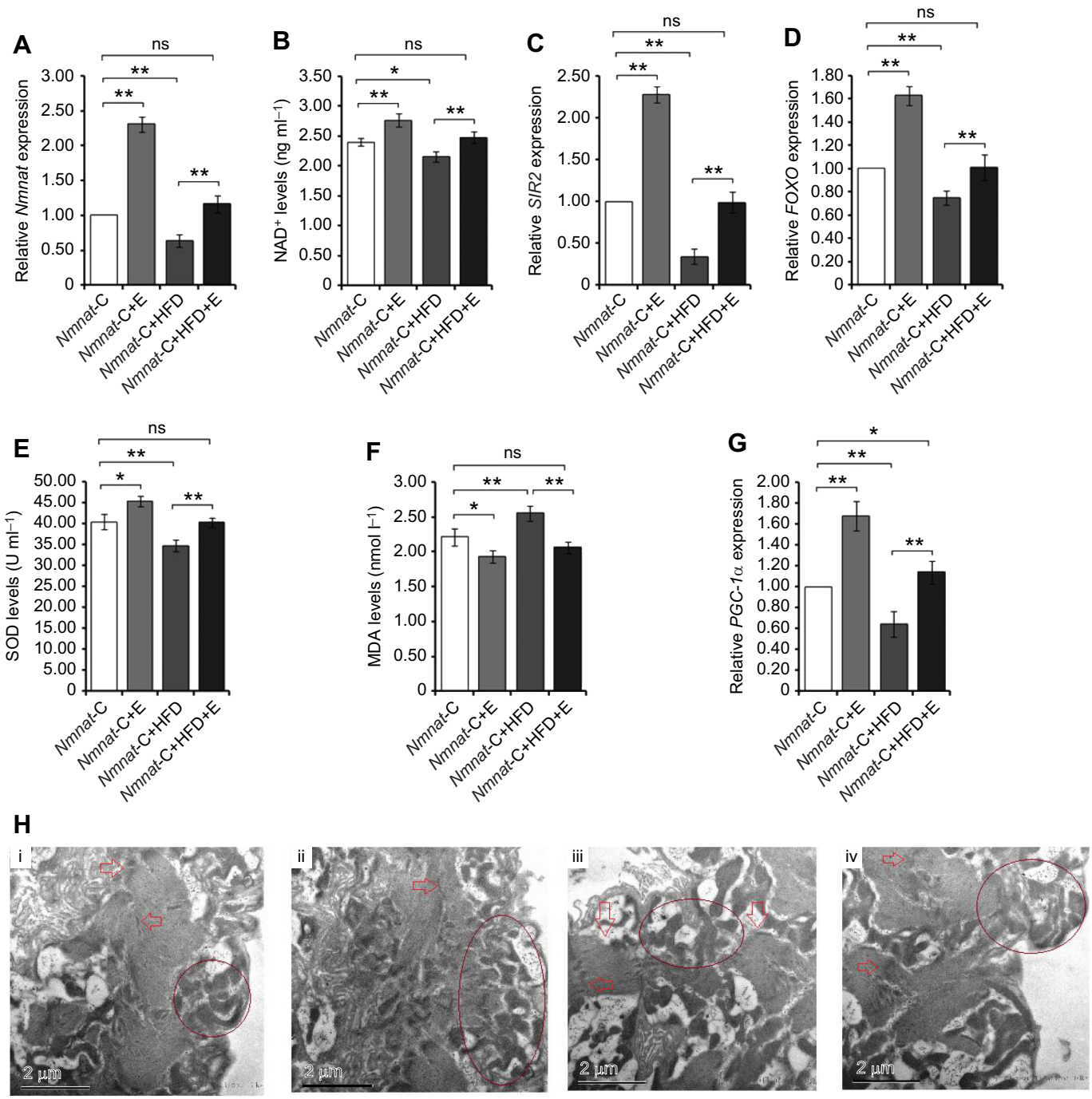

Fig. 4. Effect of a HFD and exercise on Nmnat/NAD$^+$/SIR2 pathways. (A) Heart relative *Nmnat* expression. (B) Heart NAD$^+$ levels. (C) Heart relative *SIR2* expression. (D) Heart relative *FOXO* expression. (E) Heart SOD activity levels. (F) Heart MDA levels. (G) Heart relative *PGC-1α* expression. (H) Transmission electron microscopy images: (i) *Nmnat*-C, (ii) *Nmnat*-C+E, (iii) *Nmnat*-C+HFD and (iv) *Nmnat*-C+HFD+E. Endurance exercise increased mitochondrial numbers and improved myofibril arrangement regularity in myocardial cells. The ellipsis indicates the position of the mitochondria in myocardial cells. The arrows point to the position of the Z-line in myocardial cells. A two-way ANOVA was used to identify differences among the *Nmnat*-C, *Nmnat*-C+E, *Nmnat*-C+HFD and *Nmnat*-C+HFD+E groups flies. These experimental data were measured on the last day of the fifth week of age of flies. The genotype of *Nmnat*-C group flies is *hand-Gal4>w^{1118}*. Data are presented as means±s.e.m. **P*<0.05, ***P*<0.01. The sample size of each indicator was 80 hearts, with 3 biological replicates.

toxicity in cardiomyopathy. Therefore, to explore whether cardiac Nmnat/NAD$^+$/SIR2 pathways could modulate the formation of HFD-induced lipotoxic cardiomyopathy, the cardiac *Nmnat* gene was knocked down by RNAi.

The results show that there were no significant changes in any of the cardiac measures between *Nmnat*-C group flies and *Nmnat*-C flies carrying the KD UAS (*Nmnat*-UAS-KD group flies), suggesting that the insertion of the transgenic sequence had no significant effect on the heart (LSD test, $P>0.05$) (Fig. 6A–N). Additionally, cardiac relative *Nmnat* expression in *Nmnat*-KD group flies was lower than in *Nmnat*-C group flies (LSD test, $P<0.01$), which suggests that cardiac *Nmnat* was successfully

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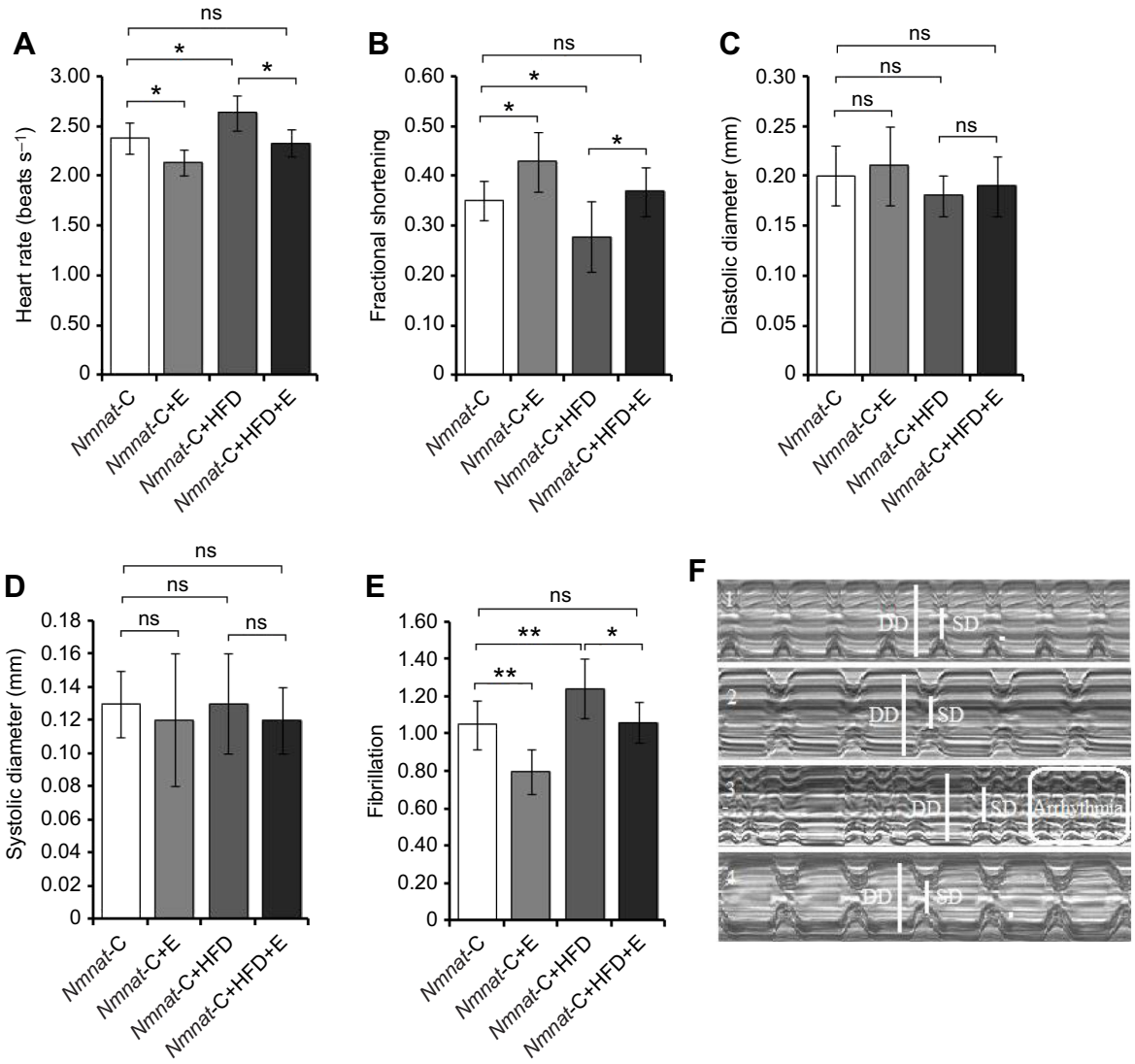

Fig. 5. Effect of a HFD and exercise on heart function. (A) Heart rate. (B) Fractional shortening. (C) Cardiac diastolic diameter. (D) Cardiac systolic diameter. (E) Fibrillation. (F) Qualitative differences in heart function parameters (3 s): fractional shortening and arrhythmia index: (1) *Nmnat*-C, (2) *Nmnat*-C+E, (3) *Nmnat*-C+HFD and (4) *Nmnat*-C+HFD+E. DD, diastolic diameter; SD, systolic diameter. A two-way ANOVA was used to identify differences among the *Nmnat*-C, *Nmnat*-C+E, *Nmnat*-C+HFD and *Nmnat*-C+HFD+E group flies. These experimental data were measured on the last day of the fifth week of age of flies. The genotype of *Nmnat*-C group flies is *hand-Gal4>w*^{1118}. Data are presented as means±s.e.m. **P*<0.05, ***P*<0.01. The sample size was 30 hearts.

knocked down by RNAi (Fig. 6A). Moreover, for cardiac Nmnat/NAD$^+$/SIR2 pathways, the results show that cardiac NAD$^+$ levels, *SIR2* expression, *FOXO* expression, SOD activity levels and *PGC-1α* expression in *Nmnat*-KD group flies were lower than in *Nmnat*-C group flies (LSD test, P<0.01) (Fig. 6B–E,G), but MDA levels in *Nmnat*-KD group flies were higher than in *Nmnat*-C group flies (LSD test, P<0.01) (Fig. 6F). These results suggest that down-regulation of cardiac *Nmnat* expression reduced the activity of cardiac Nmnat/NAD$^+$/SIR2 pathways and increased the risk of oxidative damage to myocardial cells. Moreover, cardiac TAG levels in *Nmnat*-KD group flies were higher than in *Nmnat*-C group flies (LSD test, P<0.01), and cardiac *bmm* expression in *Nmnat*-KD group flies was lower than in *Nmnat*-C group flies (Fig. 6H,I). These results suggest that down-regulation of cardiac *Nmnat* expression increased heart lipid accumulation. Finally, heart rate, diastolic diameter, systolic diameter and fibrillation in *Nmnat*-KD group flies were higher than in *Nmnat*-C group flies (LSD test, P<0.05 or P<0.01), and the fractional shortening in *Nmnat*-KD group flies was lower than in *Nmnat*-C group flies (LSD test, P<0.05) (Fig. 6J–O). These results indicate that down-regulation of cardiac *Nmnat* expression weakened the heart contractility and exacerbated cardiac arrhythmia. Therefore, we assert that cardiac *Nmnat* knockdown induces lipotoxic cardiomyopathy by down-regulating cardiac Nmnat/NAD$^+$/SIR2 pathways.

Next, to further explore the relationship between endurance exercise and cardiac Nmnat/NAD$^+$/SIR2 pathways, the cardiac *Nmnat* knockdown flies were subjected to endurance exercise. The results show that relative *Nmnat* expression, cardiac NAD$^+$ levels, *SIR2* expression, *FOXO* expression, SOD activity levels and *PGC-1α* expression in *Nmnat*-KD group flies were higher than in *Nmnat*-KD+E group flies (LSD test, P<0.01) (Fig. 6A–E,G), but MDA levels in *Nmnat*-KD+E group flies were lower than in *Nmnat*-KD group flies (LSD test, P<0.01) (Fig. 6F). These results indicate that endurance exercise improved cardiac Nmnat/NAD$^+$/SIR2 pathway activity and decreased the risk of oxidative damage to myocardial cells. In addition, cardiac TAG levels in *Nmnat*-KD+E

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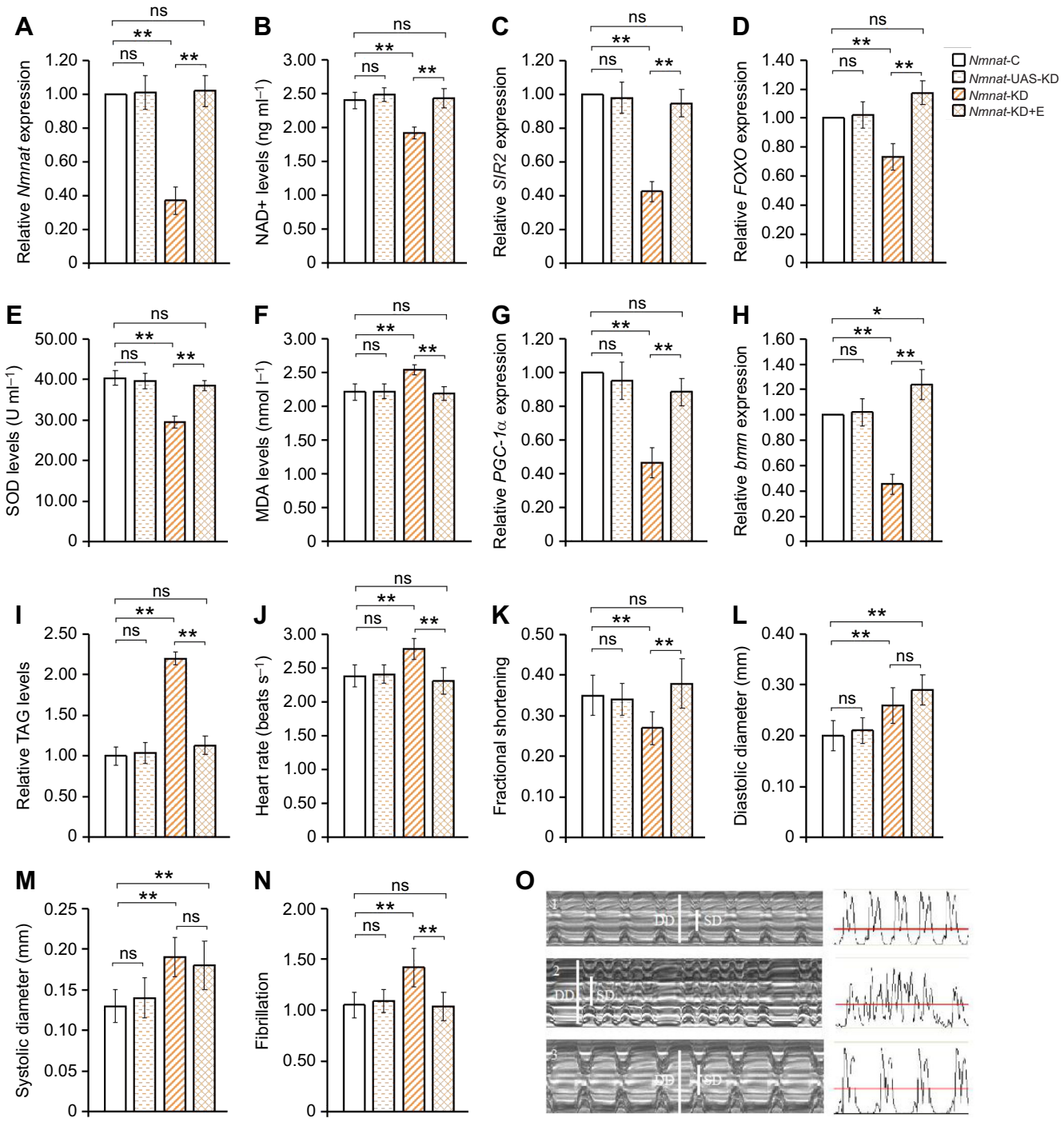

Fig. 6. Effect of cardiac *Nmnat* RNAi and endurance exercise on heart function. (A) Heart relative *Nmnat* expression. (B) Heart NAD^+ levels. (C) Heart relative *SIR2* expression. (D) Heart relative *FOXO* expression. (E) Heart SOD activity levels. (F) Heart MDA levels. (G) Heart relative *PGC-1α* expression. (H) Heart relative *bmm* expression. (I) Heart relative TAG levels. (J) Heart rate. (K) Fractional shortening. (L) Cardiac diastolic diameter. (M) Cardiac systolic diameter. (N) Fibrillation. (O) Qualitative differences in heart function parameters (3 s): fractional shortening and arrhythmia index: (1) *Nmnat*-C, (2) *Nmnat*-KD and (3) *Nmnat*-KD+E. The genotype of *Nmnat*-C group flies is *hand-Gal4>w*^{1118}. The genotype of *Nmnat*-UAS-KD group flies (which control for insertion of the UAS) is *P{KK101988}VIE-260B*. The genotype of *Nmnat*-KD group flies is *hand-Gal4>P{KK101988}VIE-260B*. These experimental data were measured on the last day of the fifth week of age of flies. Data are presented as means±s.e.m. *P<0.05, **P<0.01.

group flies were lower than in *Nmnat*-KD group flies (LSD test, P<0.01), and cardiac *bmm* expression in *Nmnat*-KD+E group flies was higher than in *Nmnat*-KD group flies (Fig. 6H,I). These results indicate that endurance exercise accelerated TAG catabolism and prevented heart lipid accumulation. Finally, the results show that heart rate and fibrillation in *Nmnat*-KD+E group flies were lower than in *Nmnat*-KD group flies (LSD test, P<0.05), and the fractional shortening in *Nmnat*-KD+E group flies was higher than in *Nmnat*-KD group flies (LSD test, P<0.05) (Fig. 6J,K,N,O). These results indicate that exercise improved heart contractility defects and cardiac arrhythmia induced by cardiac *Nmnat* knockdown. So, we can state that endurance exercise improves lipotoxic

cardiomyopathy induced by cardiac *Nmnat* knockdown by activating cardiac Nmnat/NAD^+/SIR2 pathways.

To understand the degree to which endurance exercise improves lipotoxic cardiomyopathy induced by cardiac *Nmnat* knockdown, we compared *Nmnat*-C group flies with *Nmnat*-KD+E group flies. The results show that there was no significant difference in cardiac relative *Nmnat* expression, NAD^+ levels, *SIR2* expression, *FOXO* expression, SOD activity levels, *PGC-1α* expression, TAG levels, heart rate, fractional shortening and fibrillation between *Nmnat*-C flies and *Nmnat*-KD+E flies (LSD test, $P>0.05$) (Fig. 6A–E,G,I–K,O). These results indicate that endurance exercise could almost reverse lipotoxic cardiomyopathy induced by cardiac *Nmnat* knockdown and return the heart to the state of normal cardiac *Nmnat* expression.

Cardiac *Nmnat* overexpression resists HFD-induced lipotoxic cardiomyopathy

It has been reported that overexpression of *Nmnat* could delay age-related neurodegeneration, and it also suppresses dendrite maintenance defects associated with loss of the tumor suppressor kinase Warts (Wen et al., 2011). Additionally, *Nmnat* overexpression can improve health/lifespan and locomotor activity in aging *Drosophil*a (Liu et al., 2018). Although our previous results showed that loss of *Nmnat* in the heart could cause lipotoxic cardiomyopathy, it remained unknown whether cardiac *Nmnat* gain of function could improve heart function and increase the heart's resistance to lipotoxic cardiomyopathy. So, to further confirm that cardiac *Nmnat* and cardiac Nmnat/NAD^+/SIR2 pathways are key regulators of lipotoxic cardiomyopathy, cardiac *Nmnat* overexpression was generated using UAS/hand-Gal4 in flies, and then these flies were fed a HFD.

The results show that there were no significant changes in any of the cardiac measures between *Nmnat*-C group flies and *Nmnat*-C flies carrying the OE UAS (*Nmnat*-UAS-OE group flies), suggesting that the insertion of the transgenic sequence had no significant effect on the heart (LSD test, $P>0.05$) (Fig. 7A–N). Cardiac relative *Nmnat* expression in *Nmnat*-OE group flies was higher than in *Nmnat*-C group flies (LSD test, $P<0.01$), which suggests that cardiac *Nmnat* overexpression was successfully constructed by UAS/hand-Gla4 in flies (Fig. 7A). In addition, for cardiac Nmnat/NAD^+/SIR2 pathways, cardiac NAD^+ levels, *SIR2* expression, *FOXO* expression, SOD activity levels and *PGC-1α* expression in *Nmnat*-OE group flies were lower than in *Nmnat*-C group flies (LSD test, $P<0.05$ or $P<0.01$) (Fig. 7B–E,G), but MDA levels in *Nmnat*-OE group flies were higher than in *Nmnat*-C group flies (LSD test, $P<0.05$) (Fig. 7F). These results indicate that overexpression of cardiac *Nmnat* increased the activity of cardiac Nmnat/NAD^+/SIR2 pathways and reduced the risk of oxidative damage to myocardial cells. Moreover, cardiac TAG levels in *Nmnat*-OE group flies were lower than in *Nmnat*-C group flies (LSD test, $P<0.01$), and cardiac *bmm* expression in *Nmnat*-OE group flies was higher than in *Nmnat*-C group flies (Fig. 7H,I). These results suggest that cardiac *Nmnat* overexpression reduced heart lipid accumulation. Finally, heart rate and fibrillation in *Nmnat*-OE group flies were lower than in *Nmnat*-C group flies (LSD test, $P<0.05$), and fractional shortening, diastolic diameter and systolic diameter in *Nmnat*-OE group flies were higher than in *Nmnat*-C group flies (LSD test, $P<0.05$ or $P<0.01$) (Fig. 7J–O). These results indicate that cardiac *Nmnat* overexpression enhanced heart contractility and reduced cardiac arrhythmia. Therefore, we assert that cardiac *Nmnat* overexpression improves heart function by increasing cardiac Nmnat/NAD^+/SIR2 pathways activity.

Although previous results showed that cardiac *Nmnat* overexpression increased cardiac Nmnat/NAD^+/ SIR2 pathway activity, decreased heart fat accumulation, strengthened heart contractility and reduced fibrillation, it was unclear whether this improvement of heart function induced by cardiac *Nmnat* overexpression could resist HFD-induced lipotoxic cardiomyopathy. To further explore whether cardiac *Nmnat* overexpression could counter cardiac malfunction induced by a HFD, cardiac *Nmnat* overexpressing flies were fed a HFD. The results show that there was no significant difference in cardiac NAD^+ levels, *SIR2* expression, *FOXO* expression, SOD activity levels, *PGC-1α* expression, TAG levels, *bmm* expression, heart rate, fractional shortening, diastolic diameter, systolic diameter and fibrillation between *Nmnat*-OE flies and *Nmnat*-OE+HFD flies (LSD test, $P>0.05$) (Fig. 7B–K,O), but relative *Nmnat* expression in *Nmnat*-OE+HFD group flies was lower than in *Nmnat*-OE group flies (LSD test, $P<0.05$) (Fig. 7A). Moreover, cardiac relative *Nmnat* expression, cardiac NAD^+ levels, *SIR2* expression, *FOXO* expression, SOD activity levels and *PGC-1α* expression in *Nmnat*-OE+HFD group flies were higher than in *Nmnat*-C group flies (LSD test, $P<0.05$ or $P<0.01$) (Fig. 7A–E,G), but MDA levels in *Nmnat*-OE+HFD group flies were lower than in *Nmnat*-C group flies (LSD test, $P<0.05$) (Fig. 5F). Cardiac TAG levels in *Nmnat*-OE+HFD group flies were lower than in *Nmnat*-C group flies (LSD test, $P<0.01$), and cardiac *bmm* expression in *Nmnat*-OE+HFD group flies was higher than in *Nmnat*-C group flies (Fig. 7H,I). Heart rate and fibrillation in *Nmnat*-HFD group flies were lower than in *Nmnat*-C group flies (LSD test, $P<0.05$), and fractional shortening, diastolic diameter and systolic diameter in *Nmnat*-HFD+OE group flies were higher than in *Nmnat*-C group flies (LSD test, $P<0.05$ or $P<0.01$) (Fig. 7J–O). These results indicate that cardiac *Nmnat* overexpression resists lipotoxic cardiomyopathy induced by a HFD by activating cardiac Nmnat/NAD^+/SIR2 pathways.

Impact of the cardiac *Nmnat* gene on climbing ability and longevity

It has been reported that whole-body *Nmnat* overexpression can improve health/lifespan by enhancing stress resistance and locomotor activity in aging *Drosophila* (Liu et al., 2018). Our previous study showed that a HFD causes a decrease in lifespan and mobility, but endurance exercise could protect both from the effects of a HFD (Wen et al., 2018). However, the effect of cardiac *Nmnat* expression on the climbing ability and longevity of *Drosophila* is unclear, the effect of endurance exercise on the climbing ability and longevity in cardiac *Nmnat* knockdown flies is also unknown, and the impact of a HFD on the climbing ability and longevity in cardiac *Nmnat* overexpressing flies is unclear.

The results show that either cardiac *Nmnat* overexpression or *Nmnat* knockdown did not significantly affect the climbing index in young or adult flies (LSD test, $P>0.05$) (Fig. 8A,B). However, the relative climbing index in 7 week old *Nmnat*-KD group flies was lower than in 7 week old *Nmnat*-C group flies (LSD test, $P<0.05$), and the relative climbing index in 5 or 7 week old *Nmnat*-KD+E group flies was higher than in 5 or 7 week old *Nmnat*-KD group flies and *Nmnat*-C group flies (LSD test, $P<0.05$ or $P<0.01$) (Fig. 8C). Additionally, the relative climbing index in 5 week old *Nmnat*-OE group flies was higher than in 5 week old *Nmnat*-C group flies (LSD test, $P<0.05$), and the relative climbing index in 5 or 7 week old *Nmnat*-OE+HFD group flies was lower than in 5 or 7 week old *Nmnat*-OE group flies and *Nmnat*-C group flies (LSD test, $P<0.01$) (Fig. 8D). The lifespan of cardiac *Nmnat*-KD group flies was shorter than for cardiac *Nmnat*-C group flies (a log-rank test, $P<0.05$), and the lifespan of cardiac *Nmnat*-KD+E group flies was longer than for

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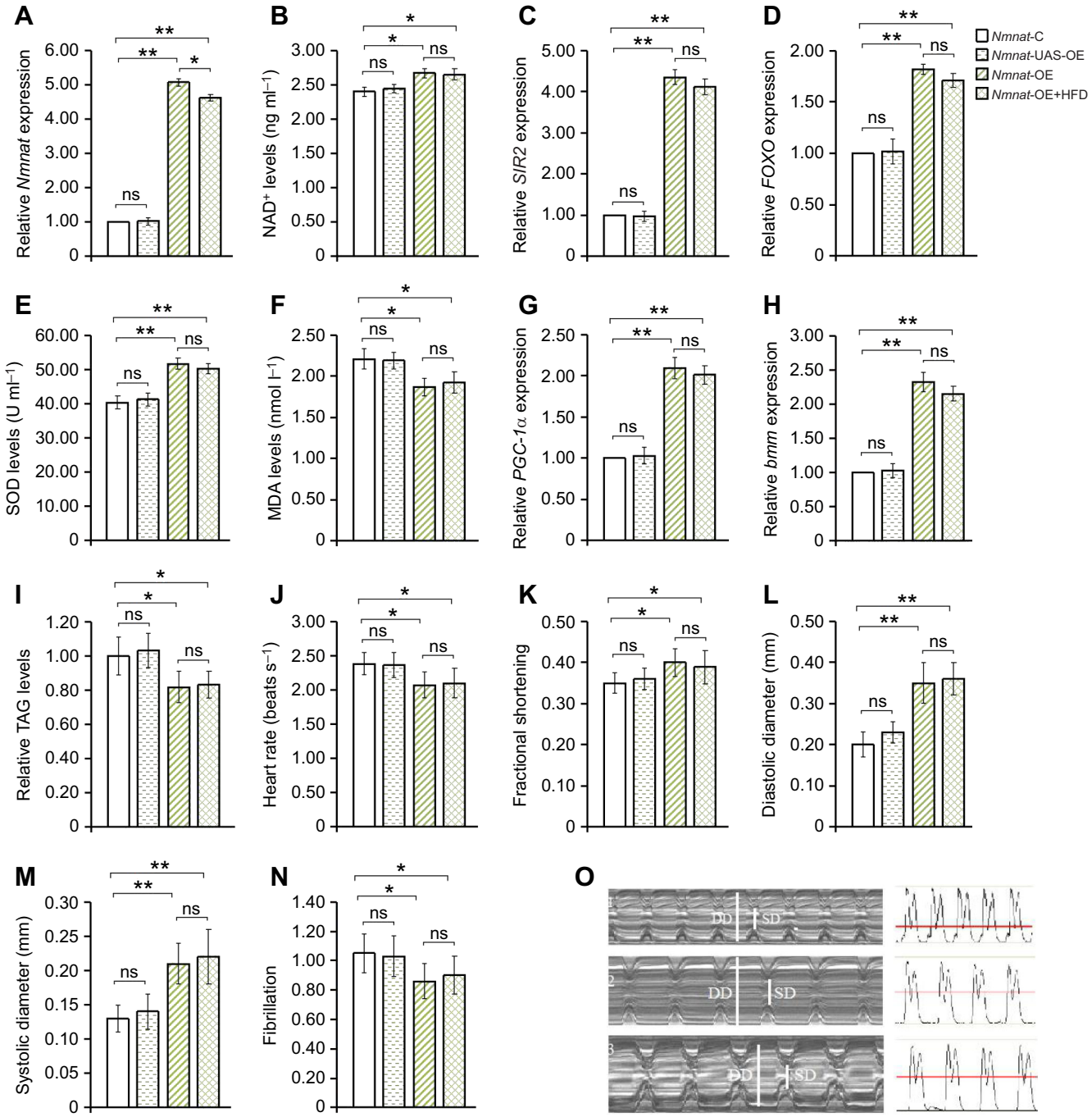

Fig. 7. Effect of cardiac *Nmnat* overexpression and HFD on heart function. (A) Heart relative *Nmnat* expression. (B) Heart NAD^+ levels. (C) Heart relative *SIR2* expression. (D) Heart relative *FOXO* expression. (E) Heart SOD activity levels. (F) Heart MDA levels. (G) Heart relative *PGC-1α* expression. (H) Heart relative *bmm* expression. (I) Heart relative TAG levels. (J) Heart rate. (K) Fractional shortening. (L) Cardiac diastolic diameter. (M) Cardiac systolic diameter. (N) Fibrillation. (O) Qualitative differences in heart function parameters (3 s): fractional shortening and arrhythmia index: (1) *Nmnat*-C, (2) *Nmnat*-OE and (3) *Nmnat*-OE+HFD. The genotype of *Nmnat*-C group flies is *hand-Gal4>w*^{1118}. The genotype of *Nmnat*-UAS-OE group flies (which control for insertion of the UAS) is *y*^{1} *w*; P{UAS-Nmnat.Z}2/CyO*. The genotype of *Nmnat*-OE group flies is *hand-Gal4>y*^{1} *w*; P{UAS-Nmnat.Z}2/CyO*. These experimental data were measured on the last day of the fifth week of age of flies. Data are presented as means±s.e.m. **P*<0.05, ***P*<0.01.

cardiac *Nmnat*-KD group flies and cardiac *Nmnat*-C group flies (log-rank test, $P<0.05$ or $P<0.01$) (Fig. 8E). Moreover, the lifespan of cardiac *Nmnat*-OE group flies was longer than that of cardiac *Nmnat*-C group flies (log-rank test, $P<0.01$), and the lifespan of cardiac *Nmnat*-OE+HFD group flies was shorter than that of cardiac *Nmnat*-OE group flies and cardiac *Nmnat*-C group flies (log-rank test, $P<0.01$) (Fig. 8F). These results suggest that cardiac *Nmnat* knockdown reduced the climbing ability of older flies and lifespan, but endurance exercise could completely reverse the adverse effects on climbing ability and lifespan induced by cardiac *Nmnat* knockdown. Cardiac *Nmnat* overexpression increased the climbing ability of older flies and lifespan, but cardiac *Nmnat* overexpression did not ameliorate the impairment of exercise ability and loss of lifespan that a high-fat diet induced.

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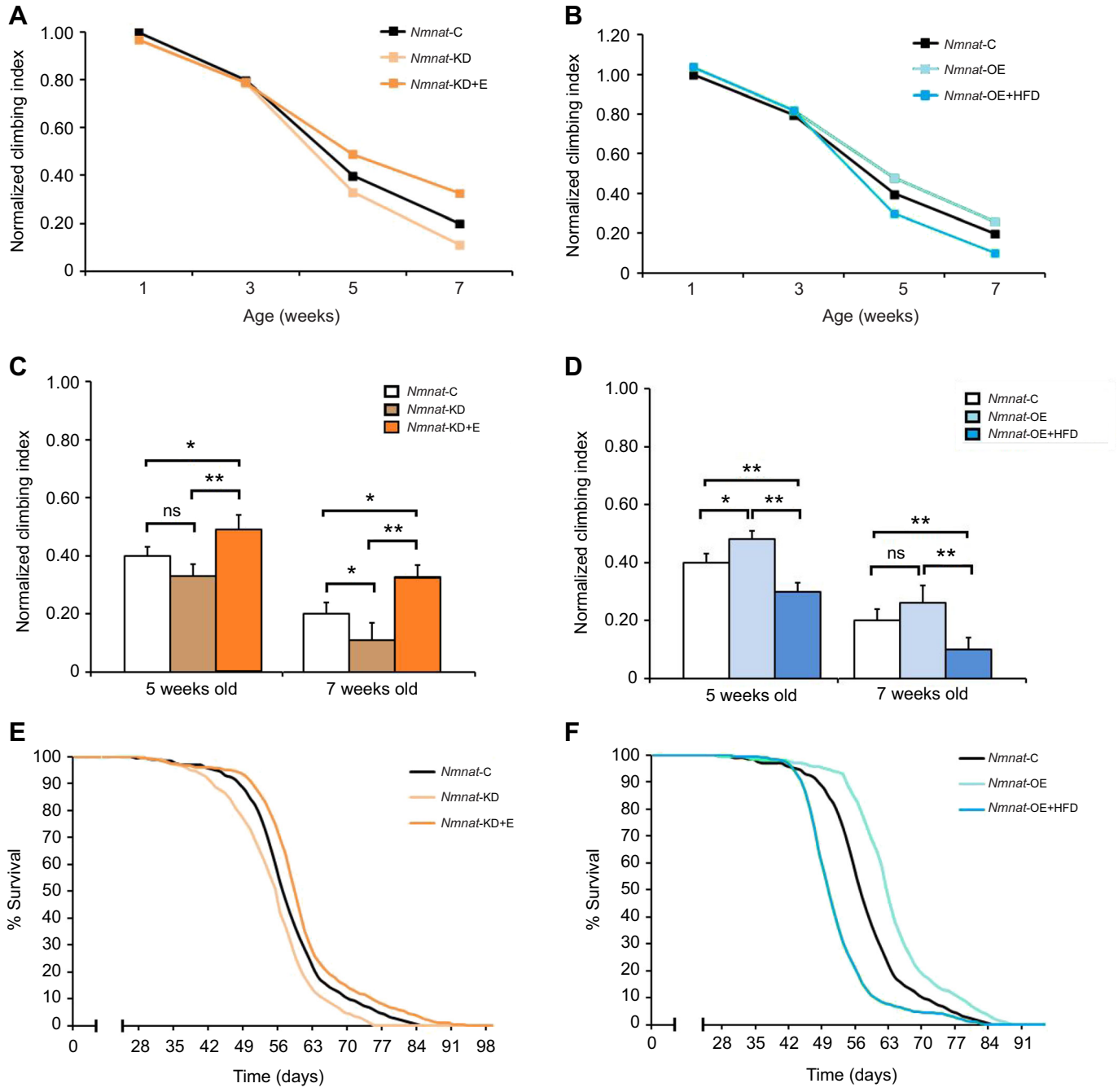

Fig. 8. Effect of cardiac *Nmnat*, exercise and HFD on mobility and lifespan. (A) Relative climbing index changes with age in cardiac *Nmnat* knockdown flies. (B) Relative climbing index changes with age in cardiac *Nmnat* overexpressing flies. (C) Relative climbing index in cardiac *Nmnat* knockdown flies. (D) Relative climbing index in cardiac *Nmnat* overexpressing flies. (E) Percentage survival in cardiac *Nmnat* knockdown flies. (F) Percentage survival in cardiac *Nmnat* overexpressing flies. The sample size for climbing index was 100–120 flies for each group, and data are presented as means±s.e.m. **P*<0.05, ***P*<0.01. The sample size for lifespan was 200–220 flies for each group; data were analyzed using a non-parametric followed by a log-rank test.

DISCUSSION

In both mammals and fruit flies, a HFD causes obesity and cardiac defects. For example, a HFD causes cardiac lipid accumulation, reduction in cardiac contractility and fractional shortening, conduction blocks and severe structural pathologies (Birse et al., 2010; Mdaki et al., 2016). Additionally, a HFD can induce changes in expression of some key genes, such as a decrease in heart *bmm*, *FOXO* and *PGC-1α* expression and an increase in heart *TOR* and *FAS* expression (Birse et al., 2010; Diop et al., 2015; Wen et al., 2018). In this study, the results again confirmed previous research. We also found that a HFD reduced cardiac Nmnat/NAD^+/SIR2 pathway activity, by reducing cardiac *Nmnat* expression, NAD^+ levels, *SIR2* expression, *FOXO* expression and *PGC-1α* expression. As overexpression of Sir2 deacetylase or increasing NAD^+ levels activates transcriptional activity of PGC-1α and FOXO (Dabrowska et al., 2016; Lan et al., 2017; Wen et al., 2019a,b), cardiac Nmnat/NAD^+/SIR2/PGC-1α and Nmnat/NAD^+/SIR2/FOXO activity were suppressed by a HFD. FOXO is an upstream regulator of SOD, and SOD is an oxygen free radical-scavenging enzyme. So, a HFD may increase oxidative damage to cardiac cells by decreasing Nmnat/

NAD^+/SIR2/FOXO/SOD activity and increasing MDA levels. Moreover, deacetylation of PGC-1α has been shown by several studies to be dependent on Sirt1 and NAD^+ activity, which increases the transcriptional activity of PGC-1α (Dominy et al., 2010; Jang et al., 2012; Ma et al., 2020). PGC-1α is a key transcriptional regulator of mitochondrial biogenesis and function, and decreasing PGC-1α expression causes mitochondrial loss and defective mitochondrial function (Li and Susztak, 2018). So, a HFD decreased cardiac *PGC-1α* expression by decreasing Nmnat/NAD^+/SIR2 activity, which led to a decreased mitochondrial content in myocardial cells. Increasing evidence indicates that HFD-induced obesity decreases the levels of NAD^+ in several ways. For example, HFD-induced increases in oxidative stress reduce NAD^+ levels via PARP-1 activation-mediated cell death (Horton et al., 2005; Konecny and Kristeleit, 2016; Massudi et al., 2012; Morales et al., 2014; Pang et al., 2015). So, a HFD causes a severe lipid accumulation in heart. At the same time, a HFD reduces cardiac Nmnat/NAD^+/SIR2 pathway activity by reducing NAD^+ levels, which causes more severe oxidative stress and mitochondrial dysfunction. Changes in these molecular pathways as a result of a HFD led to decreased contractility in cardiac myocytes.

Although our previous results indicated that exercise resistance to HFD-induced lipotoxic cardiomyopathy is associated with activation of cardiac Nmnat/NAD^+/SIR2 pathways, it remained unclear whether the cardiac Nmnat/NAD^+/SIR2 pathways could regulate the formation of HFD-induced lipotoxic cardiomyopathy. To confirm this, loss-of-function and gain-of-function cardiac *Nmnat* gene constructs were built using the UAS/hand-Gal4 system. In the fly heart, accumulating evidence confirmed that loss of function of some key genes in the heart causes cardiac dysfunction and lipid accumulation, such as cardiac *bmm*, *PGC-1α* and *FOXO*, similar to lipotoxic cardiomyopathy. In contrast, gain of function of these genes protects the heart from HFD-induced lipotoxic cardiomyopathy (Birse et al., 2010; Diop et al., 2015). So, cardiac *PGC-1α*, *FOXO* and *bmm* have been identified as key antagonists of HFD-induced lipotoxic cardiomyopathy in flies. These studies also suggest that gene gain or loss of function has become a classic method to confirm gene function. It has been reported that the *Nmnat* gene loss of function in brain causes axon degeneration, Wallerian degeneration and neurodegeneration, and these can be prevented by *Nmnat* gene gain of function (Ocampo et al., 2013; Rallis et al., 2013; Yahata et al., 2009). In this study, we found that knockdown of heart *Nmnat* decreased cardiac Nmnat/NAD^+/SIR2 pathway activity and SOD activity, increased cardiac fat accumulation and MDA levels, reduced cardiac contractility and elevated cardiac fibrillation. These changes induced by cardiac *Nmnat* knockdown were similar to HFD-induced lipotoxic cardiomyopathy. As *Nmnat* reversibly catalyzes the important step in the biosynthesis of NAD from ATP and NMN (Schweiger et al., 2001), the loss of cardiac *Nmnat* decreased heart NAD^+ levels and eventually caused lipotoxic cardiomyopathy via inhibition of cardiac Nmnat/NAD^+/SIR2/PGC-1α and Nmnat/NAD^+/SIR2/FOXO activity (Koh et al., 2012; Li et al., 2016; Zhang et al., 2009). Conversely, overexpression of heart *Nmnat* up-regulated cardiac Nmnat/NAD^+/SIR2 pathway activity and SOD activity, decreased cardiac fat accumulation and MDA levels, enhanced cardiac contractility and reduced cardiac fibrillation. These results suggest that activation of cardiac Nmnat/NAD^+/SIR2 pathways reduced the incidence of lipotoxic cardiomyopathy. However, we did not yet know whether overexpression of heart *Nmnat* could effectively resist HFD-induced lipotoxic cardiomyopathy. To further investigate this, heart *Nmnat*-overexpressing flies were fed a HFD. The results showed that a HFD did not reduce cardiac Nmnat/NAD^+/SIR2 pathway activity and SOD activity, and did not increase cardiac fat accumulation and MDA levels, decrease cardiac contractility or elevate cardiac fibrillation in cardiac *Nmnat*-overexpressing flies. Therefore, we assert that cardiac Nmnat/NAD^+/SIR2 pathways are important antagonists of HFD-induced lipotoxic cardiomyopathy in flies.

Many studies have confirmed that appropriate endurance exercise is a healthy and economical way to prevent and cure obesity, and it is also considered a good way to improve heart function in obese or old individuals (Stanley et al., 2019). For instance, exercise training strengthens the heart's ability to use fatty acids to provide energy by increasing the activity of related enzymes, which prevents excessive lipid accumulation in the heart (Wang and Xu, 2017). Additionally, exercise training improves heart function, such as cardiac contractility, and reduces heart failure in obese individuals (Goit, 2017; Kwak, 2013; May et al., 2016; Voulgari et al., 2013). Moreover, it has been reported that exercise increases cardiac SOD and FOXO activity and enhances the ability of myocardial cells to resist oxidative stress (Ahmadian and Roshan, 2018; Li et al., 2017). Finally, although exercise increases muscle and neuronal NAD^+ levels, which activates transcriptional activity of *PGC-1α* and increases mitochondrial density in muscles and neurons (Dabrowska et al., 2016; Lan et al., 2017), it was unclear whether exercise can prevent HFD-induced lipotoxic cardiomyopathy via up-regulation of cardiac Nmnat/NAD^+/SIR2 pathway activity. In this study, we found that endurance exercise prevented HFD-induced cardiac fat accumulation, heart contractility reduction and fibrillation increase, consistent with previous studies (Birse et al., 2010; Wen et al., 2018). In addition, we found that endurance exercise resisted HFD-induced cardiac lipid accumulation by increasing *bmm* expression, which promoted the catabolism of fatty acids. Moreover, we found that endurance exercise could prevent cardiac Nmnat/NAD^+/SIR2 pathway inhibition induced by a HFD, which may be the molecular regulation mechanism by which exercise promotes resistance to HFD-induced lipotoxic cardiomyopathy.

To further confirm this hypothesis, the cardiac *Nmnat* knockdown flies were also subjected to endurance exercise. We found endurance exercise improved lipotoxic cardiomyopathy induced by cardiac *Nmnat* knockdown, and increased cardiac NAD^+ levels, but it did not increase the expression of cardiac *Nmnat.* In the cell, there are two major pathways contributing to NAD synthesis: *de novo* synthesis and salvage from precursors. The *de novo* pathway of NAD synthesis converts tryptophan to quinolinic acid via the kynurenine pathway. The salvage pathways recycle NMN, nicotinamide riboside, nicotinamide and nicotinic acid in various cellular compartments including the nucleus and mitochondria (Covarrubias et al., 2021; Garten et al., 2015). There are three main approaches that researchers and drug developers are exploring to increase NAD^+ levels: supplementation with NAD^+ precursors, activation of NAD biosynthetic enzymes and inhibition of NAD^+ degradation (Covarrubias et al., 2021; Garten et al., 2015). For example, overexpression of *CG9940* and *Nmnat* increases NAD^+ levels, and exercise training increases NAD^+ levels by moderately up-regulating the *CG9940* gene (Jayaram et al., 2011; Liu et al., 2018; Wen et al., 2016). In this study, cardiac *Nmnat* knockdown decreased Nmnat protein levels, and this decreased heart NAD^+ levels. However, exercise training may enhance the *de novo* synthesis of NAD^+ via increasing NAD synthase activity, and this could also increase heart NAD^+ levels and NAD^+/SIR2 pathway activity in *Nmnat* knockdown flies. This hypothesis needs to be confirmed by further experiments. Current evidence suggests that activation of cardiac Nmnat/NAD^+/

SIR2 pathways is an important molecular mechanism of endurance exercise resistance against lipotoxic cardiomyopathy.

We also explored the effects of cardiac *Nmnat*, endurance exercise and a HFD on the mobility and lifespan of fruit flies. In humans, heart disease is a major cause of death in elderly individuals (Masriadi et al., 2016). Heart function is an important factor in determining exercise ability as it is closely related to the rate of oxygen transport in cells and tissues (Cohen-Solal et al., 1996; Nolte et al., 2014; Zhu et al., 2019). It has been reported that whole-body *Nmnat* overexpression can improve health/lifespan by enhancing stress resistance and locomotor activity in aging *Drosophila* (Liu et al., 2018). In this study, we found knockdown of cardiac *Nmnat* induced locomotor impairment of older flies, and it notably reduced lifespan. In youth and adulthood, the heart shows a strong ability to contract (Barry et al., 1987; Simkhovich et al., 2007). So, cardiac *Nmnat* knockdown may not significantly reduce mobility by decreasing oxygen transport. However, cardiac *Nmnat* knockdown accelerated heart dysfunction with aging as NAD^+ is an important factor in the regulation of cellular senescence (Chaturvedi and Tyagi, 2018; Fang et al., 2017), and this eventually reduced the climbing ability of flies. Our results also showed that endurance exercise improved climbing ability and lifespan in cardiac *Nmnat* knockdown flies, and the climbing ability and lifespan in cardiac *Nmnat* knockdown and exercised flies were better than in cardiac *Nmnat* control flies. The reason may be that exercise improved not only heart function but also skeletal muscle, brain and other vital organs. In addition, we found cardiac *Nmnat* overexpression increased the climbing ability of older flies and lifespan, but it did not prevent the adverse the effects on climbing ability and lifespan induced by a HFD. It has been reported that a HFD causes obesity in both flies and other animals, which increases the incidence of diabetes, fatty liver, stroke and hypertension (Finzi et al., 1959; Song et al., 2017; Ting et al., 2017). This may be the reason why cardiac *Nmnat* overexpression did not prevent HFD-induced locomotor impairment and lifespan reduction.

In summary, our results confirm that the cardiac *Nmnat* gene plays an important role in regulating the formation of lipotoxic cardiomyopathy. Overexpression of cardiac *Nmnat* activated cardiac Nmnat/NAD^+/SIR2 pathways, which resisted HFD-induced lipid accumulation, cardiac dysfunction and oxidative stress, but did not reverse HFD-induced lifespan shortening and locomotor impairment of older flies. In contrast, a HFD or cardiac *Nmnat* knockdown both induced lipid accumulation, cardiac dysfunction and oxidative stress. However, endurance exercise resisted HFD-induced or *Nmnat* knockdown-induced lipotoxic cardiomyopathy via activation of cardiac NAD^+/*SIR2* pathways (Fig. 9).

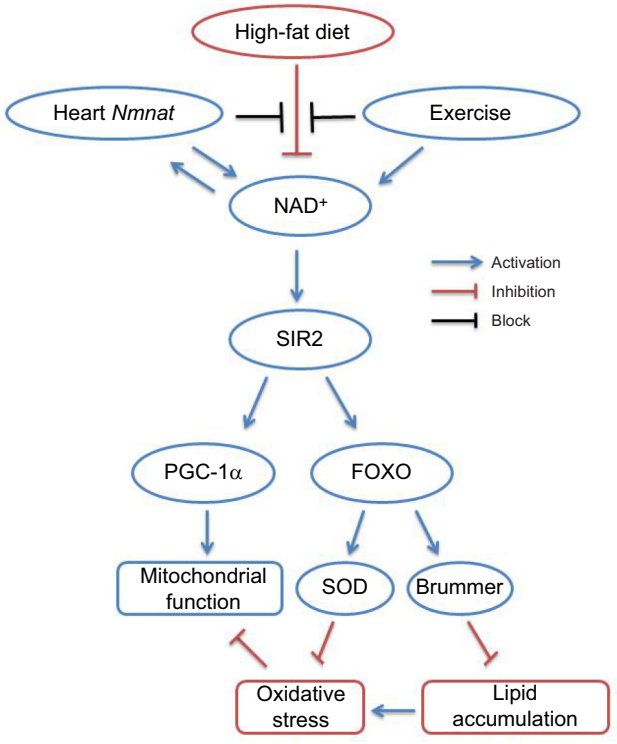

Fig. 9. Relationship between exercise, HFD and Nmnat/NAD^+/SIR2 pathways. Current results confirmed that cardiac Nmnat/NAD^+/SIR2 pathways are important antagonists of HFD-induced lipotoxic cardiomyopathy. Cardiac Nmnat/NAD^+/SIR2 pathway activation is an important underlying molecular mechanism for the effect of endurance exercise and cardiac *Nmnat* overexpression on lipotoxic cardiomyopathy in *Drosophila*.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Methodology: D.-t,W., K.L.; Resources: D.-t.W., L.Z.; Data curation: W.-q.H.; Writing - review & editing: D.-t.W.

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