BBA - Molecular Basis of Disease 1865 (2019) 1905–1914

Contents lists available at ScienceDirect

BBA - Molecular Basis of Disease

journal homepage: www.elsevier.com/locate/bbadis

Mst1 inhibits Sirt3 expression and contributes to diabetic cardiomyopathy through inhibiting Parkin-dependent mitophagy☆

Shanjie Wang[a,b,1], Zhijing Zhao[a,1], Yanhong Fan[a,1], Mingming Zhang[a,b], Xinyu Feng[a,b], Jie Lin[a,b], Jianqiang Hu[a,b], Zheng Cheng[a,b], Chuang Sun[a,b], Tingting Liu[c], Zhenyu Xiong[a,b], Zhi Yang[a], Haichang Wang[b,*], Dongdong Sun[a,b,**]

[a] Department of Cardiology, Xijing Hospital, Fourth Military Medical University, Xi'an, China
[b] Department of Cardiology, Tangdu Hospital, Fourth Military Medical University, Xi'an, China
[c] Department of Radiology, Xijing Hospital, Fourth Military Medical University, Xi'an, China

ARTICLE INFO

Keywords:
Mst1
Sirt3
Parkin
Mitophagy
Diabetic cardiomyopathy

ABSTRACT

Mitochondrial dysfunction contributes to heart failure induced mortality in approximately 80% of diabetic patients. Mitophagy degrades defective mitochondria and maintains a healthy mitochondrial population, which is essential for cardiomyocyte survival in diabetic stress. Herein, we determined whether Mst1 regulated mitophagy and investigated the downstream signaling pathway in the development of diabetic cardiomyopathy (DCM). Mst1 deficiency promoted elimination of dysfunctional mitochondria in diabetic cardiomyopathy without affecting mitochondrial biogenesis. Enhanced mitophagy was observed in Mst1 interfering cardiomyocytes subjected to high glucose treatment using 3-Methyladenine and Chloroquine. Consistent with these results, in vivo and in vitro loss of function experiments indicated that Mst1 participated in the development of DCM by inhibiting Parkin-dependent mitophagy. Mst1 deficiency alleviated the detrimental phenotype of DCM. Interestingly, the protective effects of Mst1 knockout on DCM were compromised in diabetic Parkin$^{-/-}$ mice. Mechanistically, Mst1 knockdown significantly enhanced Parkin expression and translocation to the mitochondria, as evidenced by immunofluorescence study and Western blot analysis. Furthermore, Sirt3 deletion abolished the detrimental effects of Mst1 on DCM. Collectively, Mst1 inhibits Sirt3 expression thus participates in the development of DCM by inhibiting cardiomyocyte mitophagy. The mechanism is associated with Parkin inhibition.

1. Introduction

Cardiovascular complications of diabetes mellitus (DM) represent the leading cause of morbidity and mortality in diabetic patients [1]. Diabetic cardiomyopathy (DCM) is characterized by left ventricular remodeling and heart failure (HF) in the absence of coronary artery disease [2,3]. According to the statistics, the risk of HF in diabetic patients is 2.4–5-fold higher than in non-diabetic patients [4,5]. Despite significant improvements in the management of diabetes, patients with DCM still has worse clinical outcome [3]. Thus, the remedy for DCM remains to be investigated.

Mitochondria regulate a variety of cellular processes in both physiological conditions and pathological stress insults [2,6,7]. In cardiomyocytes, mitochondria are involved in the contractile function, electrical stability, and cell survival or death [2]. As the energy plant of cardiomyocytes, mitochondria occupy 35–40% of the cytoplasm volume and supply 95% of the ATP for the adult beating heart [8,9]. In diabetic stress, mitochondrial dysfunction may be the central mediator for the development of DCM [2,10,11]. However, a comprehensive understanding of the molecular mechanisms linking mitochondrial dysfunction to DCM is lacking.

It is important to consider that mitochondria are highly dynamic

Abbreviations: CQ, chloroquine; DCM, diabetic cardiomyopathy; DM, diabetes mellitus; HF, heart failure; HG, high glucose; FS, fractional shortening; LVEDD, left ventricular end-diastolic diameter; LVEF, left ventricular ejection fraction; LVESD, left ventricular end-systolic diameter; LTG, lysoTracker Green; Mst1, mammalian sterile 20-like kinase 1; MTR, MitoTracker Red; STZ, streptozotocin; 3-MA, 3-methyladenine

☆ This article is part of a Special Issue entitled: Genetic and epigenetic regulation of aging and longevity edited by Jun Ren & Megan Yingmei Zhang.

* Correspondence to: H. Wang, 569 Xinsi Road, Department of Cardiology, Tangdu Hospital, Fourth Military Medical University, Xi'an, Shaanxi 710038, China.
** Correspondence to: D. Sun, 127 West Changle Road, Department of Cardiology, Xijing Hospital, Fourth Military Medical University, Xi'an, Shaanxi 710032, China.
E-mail addresses: wanghc@fmmu.edu.cn (H. Wang), wintersun3@gmail.com (D. Sun).
[1] Contributed equally to this work.

https://doi.org/10.1016/j.bbadis.2018.04.009
Received 13 January 2018; Received in revised form 27 February 2018; Accepted 10 April 2018
Available online 17 April 2018
0925-4439/

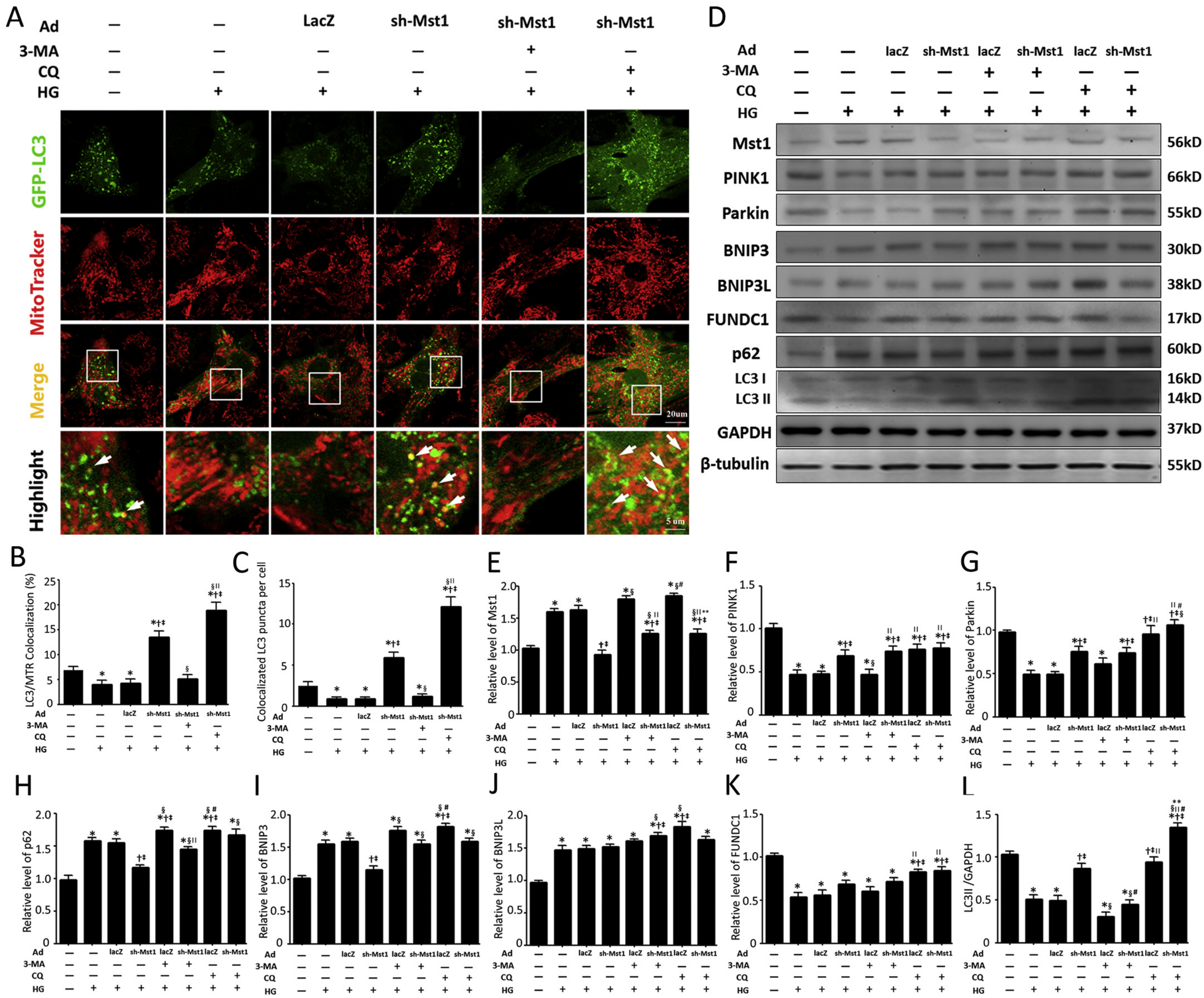

Fig. 1. Mst1 participates in the regulation of mitophagy.

A, Representative colocalization images of GFP-LC3 (green) and mitochondria (MitoTracker Red) (scale bars: upper 3 panels 20 μm, lower panel 5 μm). White arrowheads highlight colocalization of GFP-LC3 and mitochondria. B, Quantitative analysis of GFP-LC3/MitoTracker Red colocalization (percentage of whole cells). C, Quantitative analysis of GFP-LC3 puncta colocalization with mitochondria (MitoTracker Red) per cell. The columns and error bars represent the means and SEM. $^{*}P < 0.05$ vs Con; $^{\dagger}P < 0.05$ vs HG; $^{\ddagger}P < 0.05$ vs HG + Ad-LacZ; $^{\S}P < 0.05$ vs HG + Ad-sh-Mst1; and $^{||}P < 0.05$ vs HG + Ad-sh-Mst1 + 3-MA. D, Protein expression with representative gel blots of Mst1, PINK1, Parkin, BNIP3, BNIP3L, FUNDC1, p62,LC3 I/II, GAPDH (loading control for Mst1, PINK1, Parkin, p62, BNIP3, FUNDC1, and LC3 II), β-tubulin (loading control for BNIP3L). E, Relative level of Mst1. F, Relative level of PINK1. G, Relative level of Parkin. H, Relative level of p62. I, Relative level of BNIP3. J, Relative level of BNIP3L. K, Relative level of FUNDC1. L, Relative level of LC3 II. The columns and error bars represent the means and SEM. $^{*}P < 0.05$ vs Con; $^{\dagger}P < 0.05$ vs HG; $^{\ddagger}P < 0.05$ vs HG + Ad-LacZ; $^{\S}P < 0.05$ vs HG + Ad-sh-Mst1; $^{||}P < 0.05$ vs HG + Ad-sh-LacZ + 3-MA; $^{\#}P < 0.05$ vs HG + Ad-sh-Mst1 + 3-MA; and $^{**}P < 0.05$ vs HG + Ad-sh-LacZ + CQ.

and play critical roles in the pathogenesis of diabetes, as well as aging [12]. There is emerging evidence that defects in mitophagy and mitochondrial hemostasis are common features of aging and DCM [13]. The maintenance of a healthy mitochondrial population is essential for cardiomyocyte survival in diabetic or aging-related stress [14,15]. Damaged mitochondria trigger mitophagy to remove defective mitochondria and recycle their essential components through their encapsulation by a double-membrane structure [16,17]. The proper elimination of damaged mitochondria by upregulating mitophagy protected cardiomyocytes against diabetic injury or aging [2,14].

Sirtuins are a class of protein deacylases with important implications in aging [18]. Seven sirtuins (SIRT1–7) with distinct subcellular localizations and functions have been investigated in cardiovascular system [19]. Among them, SIRT3 is a major mitochondrial deacetylase which regulates mitophagy and has been shown to slow age-associated decline of function and senescence in multiple cell types [20–22]. Since abnormalities of mitophagy results in increased accumulation of damaged mitochondria is a contributing factor leading to diminished cardiac function and senescence [23]. Thus, therapeutic strategies that mitigate age-related changes including mitophagy should prove beneficial in improving cardiac function with advanced age.

Mammalian sterile 20-like kinase 1 (Mst1) is a key component of the "Hippo" signaling pathway [24]. Our previous study demonstrated that Mst1 participates in DCM progression by modulating autophagy and apoptosis through Sirt3 inhibition [25]. However, whether Mst1/Sirt3 signaling regulates mitophagy in the progression of DCM is not clear. The present study was designed to investigate the potential role of Mst1/Sirt3 signaling in regulating mitophagy and the underlying

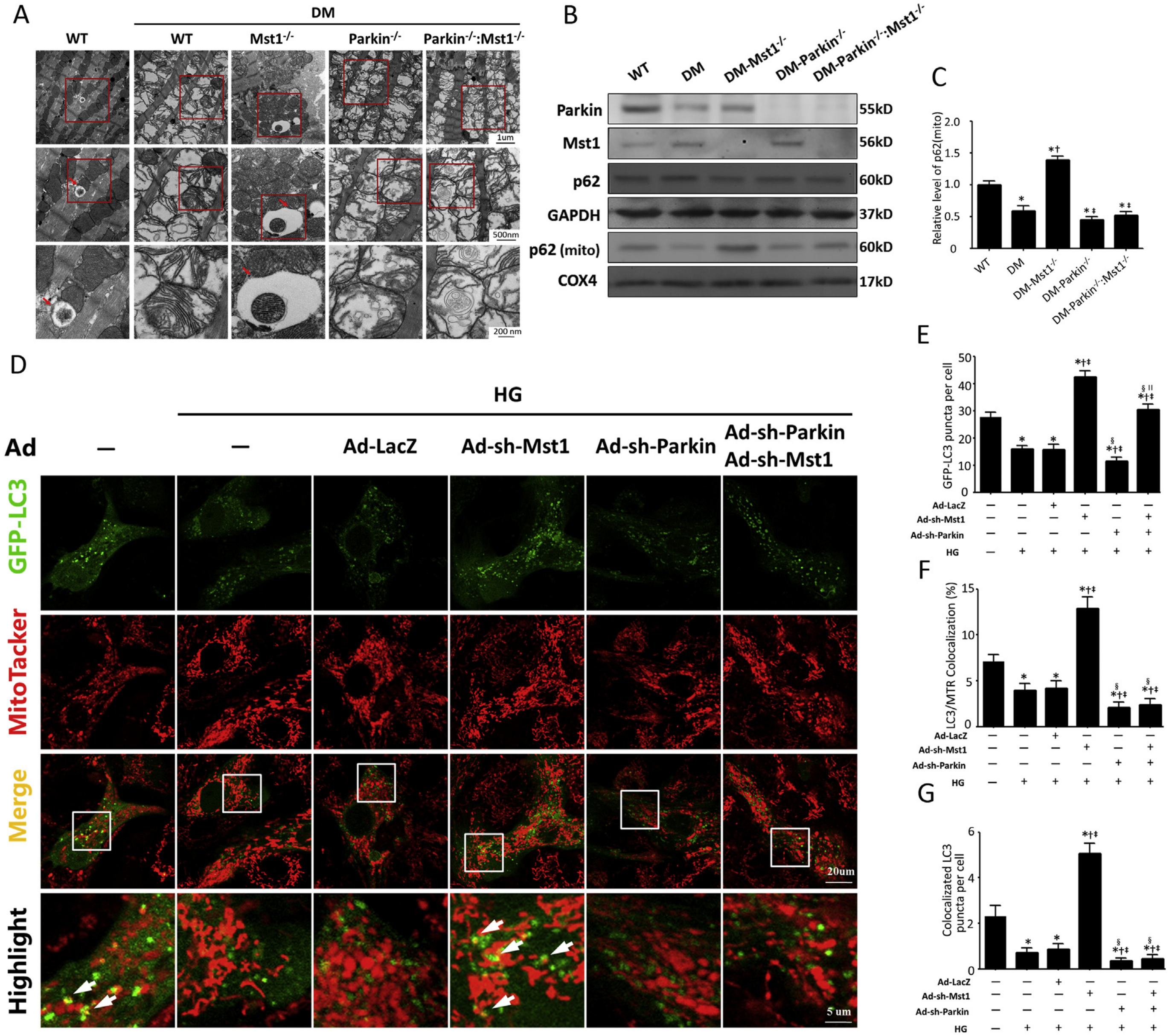

Fig. 2. Mst1 suppresses mitophagy by inhibiting Parkin.
A, Representative colocalization images of GFP-LC3 (green) and mitochondria (MitoTracker Red) (Scale bars: upper 3 panels 20 μm, lower panel 5 μm). White arrowheads highlight colocalization of GFP-LC3 and mitochondria. B, Quantitative analysis of GFP-LC3 puncta per cell. C, Percentage of cells with LC3 and mitochondria (MitoTracker Red, MTR) colocalization. D, Quantitative analysis of GFP-LC3 puncta colocalization with mitochondria (MitoTracker Red) per cell. The columns and error bars represent the means and SEM. *P < 0.05 vs Con; †P < 0.05 vs HG; ‡P < 0.05 vs HG + Ad-LacZ; §P < 0.05 vs HG + Ad-sh-Mst1; and ||P < 0.05 vs HG + Ad-sh-Parkin. E, Representative images of typical autophagosome engulfing damaged mitochondria (scale bars: upper panel 1 μm, medium panel 500 nm, and lower panel 200 nm). F, Protein expression with representative gel blots of Parkin, Mst1, p62, p62 (mito), LC3 I/II, GAPDH (loading control for Mst1, p62, and LC3 I/II) and COX4 (loading control for p62 (mito)). G, Relative level of p62 (mito). The columns and error bars represent the means and SEM. *P < 0.05 vs WT; †P < 0.05 vs DM; and ‡P < 0.05 vs DM-Mst1$^{-/-}$.

mechanisms using the animal model of DCM.

2. Research design and methods

2.1. Animals

Parkin knockout (Parkin$^{-/-}$) mice (C57BL/6 background) were purchased from Jackson Laboratories (No.006582, Bar Harbor, ME, USA). Mst1 knockout (Mst1$^{-/-}$) mice (C57BL/6 background) were purchased from K&D gene technology (WuHan, China). Parkin$^{-/-}$ mice and Mst1$^{-/-}$ mice were crossed to generate Parkin$^{-/-}$:Mst1$^{-/-}$ mice. All animals were identified by Western blot and real-time PCR analysis. The present study was performed according to the NIH guidelines on the use of laboratory animals. All protocols were approved by the Institutional Animal Care in the Fourth Military Medical University.

As previously described [25], 8 weeks old mice were intraperitoneally injected with streptozotocin (STZ, 50 mg/kg, dissolved in 100 mmol/L citrate buffer, pH 4.5) for 5 days to induce diabetic model, and only those with random blood glucose levels ≥16.6 mmol/L twice were labeled as having diabetes. Seven groups were set as follows: (1) Wild type group (WT, n = 30); (2) diabetic group (DM, n = 28); (3) diabetic Mst1$^{-/-}$ group (DM-Mst1$^{-/-}$, n = 27); (4) diabetic Parkin$^{-/-}$ group (DM-Parkin$^{-/-}$, n = 23); (5) Diabetic-Parkin$^{-/-}$:Mst1$^{-/-}$

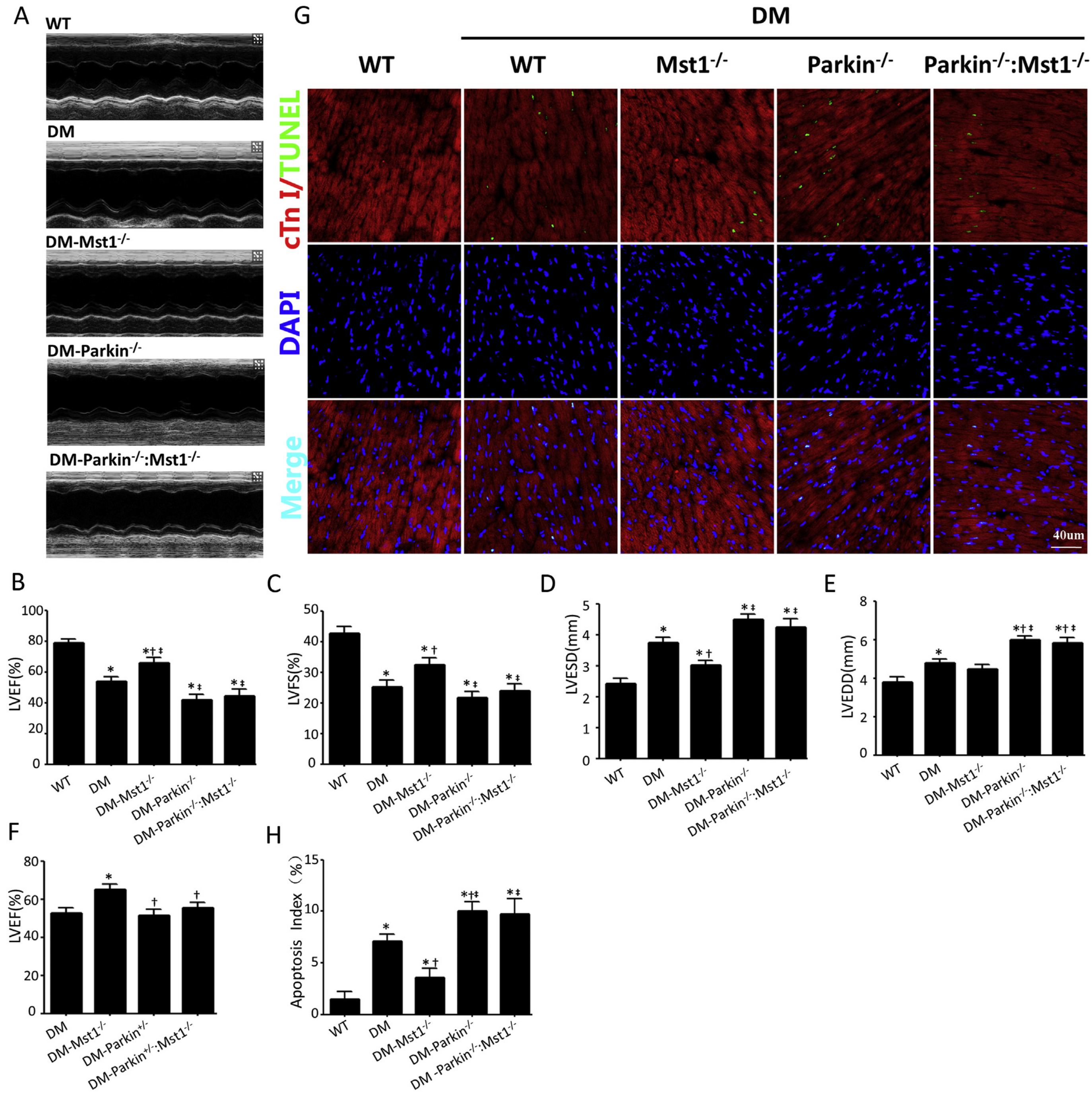

Fig. 3. Mst1 deficiency ameliorates cardiac phenotype of DCM via Parkin.
A, Representative images of echocardiography. B, Measurements of LVEF (%). C, Measurements of LVFS (%). D, Measurements of LVESD (mm). E, Measurements of LVEDD (mm). The columns and error bars represent the means and SEM. *$P < 0.05$ vs WT; †$P < 0.05$ vs DM; and ‡$P < 0.05$ vs DM-Mst1$^{-/-}$. F, Measurements of LVEF (%). The columns and error bars represent the means and SEM. *$P < 0.05$ vs DM; †$P < 0.05$ vs DM-Mst1$^{-/-}$. G, Representative images of TUNEL staining (scale bar: 40 μm). H, Quantitative analysis of the apoptotic index (percentage of TUNEL-positive nuclei, %). The columns and error bars represent the means and SEM. *$P < 0.05$ vs WT; †$P < 0.05$ vs DM; and ‡$P < 0.05$ vs DM-Mst1$^{-/-}$.

group (DM Parkin$^{-/-}$:Mst1$^{-/-}$, n = 19); (6) diabetic Parkin$^{+/-}$ group (DM-Parkin$^{+/-}$, n = 20); (7) diabetic Parkin$^{+/-}$:Mst1$^{-/-}$ group (DM-Parkin$^{+/-}$:Mst1$^{-/-}$, n = 20).

In order to observe the role of Sirt3 in Mst1/Parkin signaling pathway, we applied Sirt3 knockout (Sirt3$^{-/-}$) mice and crossed them with Mst1 Tg mice to generate Sirt3$^{-/-}$:Mst1 Tg mice. Thus, we raised other four groups randomly (n = 20 each): (1) Sirt3$^{-/-}$ group (Sirt3$^{-/-}$); (2) diabetic Sirt3$^{-/-}$ group (DM-Sirt3$^{-/-}$); (3) diabetic Tg-Mst1 group (DM-Tg-Mst1); (4) diabetic Sirt3$^{-/-}$:Tg-Mst1 group (DM-Sirt3$^{-/-}$:Tg-Mst1).

2.2. Primary neonatal mouse cardiomyocyte isolation, culture and treatment

Primary cardiomyocytes were isolated from wild-type C57BL/6 neonatal mice as previously described [25,26]. Isolated cells were cultured in DMEM containing 10% fetal bovine serum (HyClone, Australia) under the condition of 20% O_2, 5% CO_2 and 75% N_2 at 37 °C.

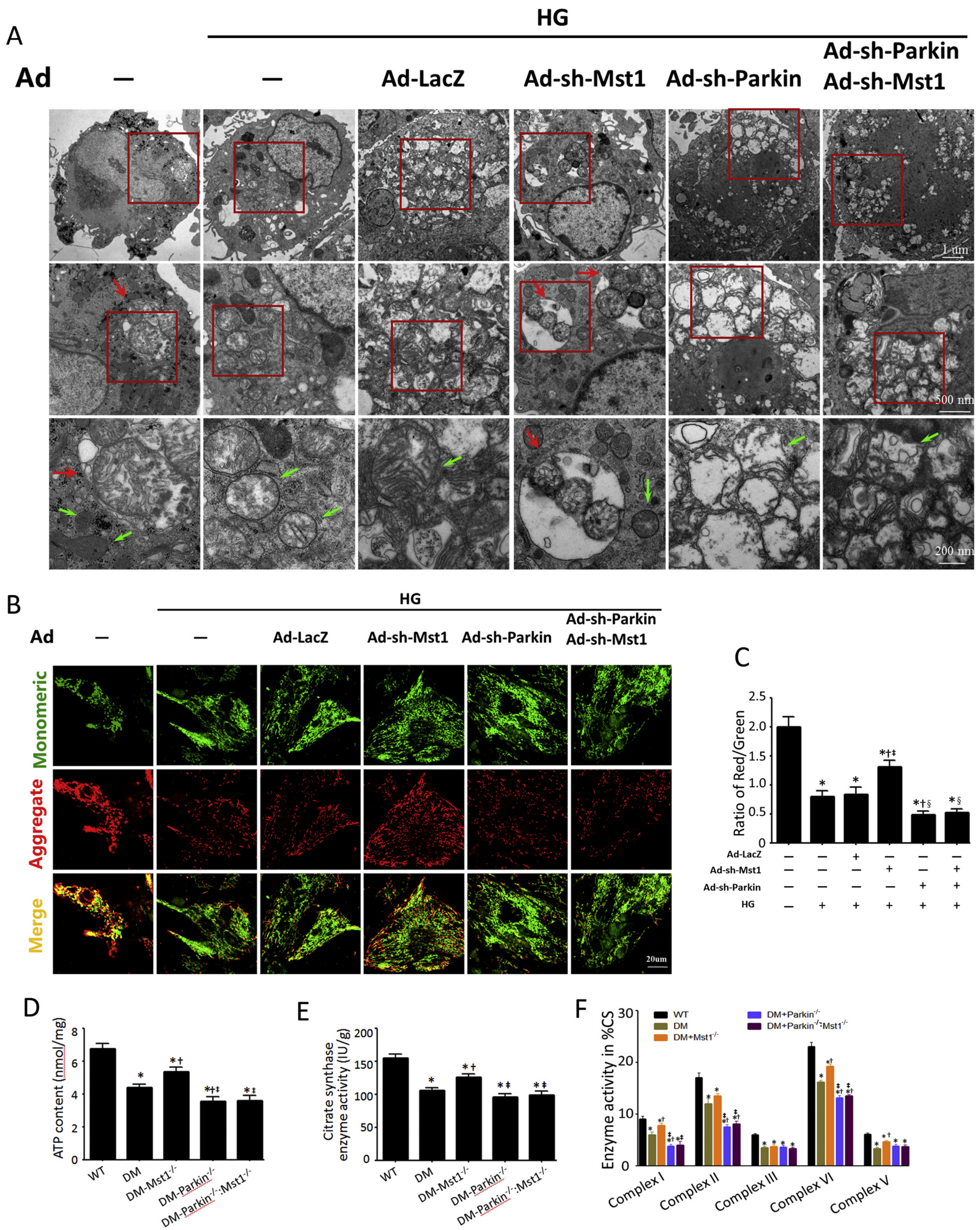

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Fig. 4. Mst1 inhibition improves mitochondrial morphology and bioenergetics in diabetic myocardium via Parkin.
A, Representative images of transmission electron microscopy (TEM) (Scale bars: upper panel 1 μm, medium panel 500 nm, and lower panel 200 nm). B, Representative images of JC-1 fluorescence imaging (scale bar: 20 μm). C, The ratio of aggregated (red) and monomeric (green) JC-1. The columns and error bars represent the means and SEM. *P < 0.05 vs Con; †P < 0.05 vs HG; ‡P < 0.05 vs HG + Ad-LacZ; and §P < 0.05 vs HG + Ad-sh-Mst1. D, Myocardial ATP content. E, Citrate synthase (CS) activity. F, Complex I/II/III/IV/V activities in diabetic heart. The columns and error bars represent the means and SEM. *P < 0.05 vs WT; †P < 0.05 vs DM; and ‡P < 0.05 vs DM-$Mst1^{-/-}$.

The adenoviruses harboring GFP-LC3 (GFP-LC3) were transfected into cardiomyocytes, if necessary. The recombinant adenoviruses with Mst1 shRNA (Ad-sh-Mst1), Parkin shRNA (Ad-sh-Parkin) and control vector (Ad-LacZ) were transduced 24 h after GFP-LC3 transduction. After 36 h, cardiomyocytes were treated by regular medium (5.5 mmol/L) or high glucose medium (33 mmol/L) for 48 h. Finally, 3-methyladenine (3-MA, 10 mmol/L, M9281, Sigma) or chloroquine (CQ, 50 μmol/L, GeneChem) was administered when necessary. All adenoviruses were purchased from Hanbio technology Ltd. (Shanghai, China). The MOI of the adenovirus was 100:1.

2.3. Echocardiography

The cardiac function was evaluated by a M-mode echocardiography system [25] (see Supplementary methods for details).

2.4. Mitophagy measurement by fluorescence imaging

The colocalization of mitochondrion with LC3, lysosome or Parkin was used to evaluate mitophagy [27,28]. Images were captured under the confocal microscope and analyzed in the ImageJ software (see Supplementary methods for details).

2.5. Transmission electron microscopy (TEM)

TEM was applied to visualize the mitochondria morphology and typical autophagosomes engulfing mitochondria, as previously described (see Supplementary methods for details) [20,26].

2.6. Assessment of apoptosis

Cardiomyocyte apoptosis was observed by terminal deoxyribonucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) staining (Roche, Penzberg, Germany) (see Supplementary methods for details) [29,30].

2.7. Mitochondrial and cytosol protein extraction

Mitochondrial and cytosol ingredients were extracted using a Mitochondria Isolation Kit (C3601, C3606, Beyotime) (see Supplementary methods for details) [28,31].

2.8. Western blot

Protein extractions were separated by 10–12% SDS-PAGE (P0012A, beyotime, China), and transferred to 0.45-μm PVDF (Millipore, USA). After being blocked and incubated with antibody, PVDF membranes were scanned by chemiluminescence system [20,25] (see Supplementary methods for details and antibodies).

2.9. Mitochondria biological function

To assess the functional status of mitochondria, ATP level, citrate synthase and electron transport chain complex activities (complexes I/II/III/IV/V) were measured [25] (see Supplementary methods).

2.10. Determination of mitochondrial membrane potential

JC-1, a fluorescent dye that depends on the mitochondrial membrane potential (△Ψm), was used to observe the change in △Ψm (see Supplementary methods) [20].

2.11. Statistical analyses

Continuous variables were expressed as the means ± standard error of means (SEM). Statistical analyses of differences between groups were performed using an unpaired Student's *t*-test, one-way ANOVA followed by a Fisher's post hoc comparison test or a two-way ANOVA with multiple post hoc comparisons. Two-sided tests were used throughout the study, and a P value < 0.05 was considered statistically significant. SPSS software package version 14.0 (SPSS, Chicago, IL) was used for data analysis.

3. Results

3.1. Mst1 knockout promotes dysfunctional mitochondria elimination in diabetic cardiomyopathy

Since DCM was characterized by mitochondrial damage and Mst1 knockout ameliorated the pathophysiological development of DCM in our previous study [10,25], we investigated whether Mst1 mediated mitochondrial quality control in DCM. Compared with wild-type mice, the mitochondrial number of diabetic mice was increased (Fig. S1A). Mst1 knockout reduced mitochondrial number in diabetic mice (Fig. S1A and B). The difference might be underestimated since swelling mitochondria in diabetic mice caused lower mitochondrial number under equivalent fields. Additionally, Mst1 knockout did not significantly change the expression of PGC-1α, NRF-1 and Tfam (Fig. S1C–E). There results indicated that the decreased mitochondrial number in Mst1-deficient diabetic mice was not attributed to inadequate mitochondrial biogenesis. The above data indicated that Mst1 knockout promoted dysfunctional mitochondria elimination during DCM.

3.2. Mst1 inhibition enhances mitophagy in cardiomyocytes under high glucose treatment

Redundant organelles were degraded primarily through mitophagy [32]. We next investigated whether Mst1 mediated mitophagy to remove damaged mitochondria. Cardiomyocytes were incubated with 3-MA or CQ to analyze Mst1-regulated mitophagic activity. Mst1 inhibition increased LC3 colocalization with mitochondria in cardiomyocytes subjected to HG culture (Fig. 1A–C, Fig. S2A). The administration of 3-MA decreased, while CQ increased the LC3 puncta colocalization with mitochondria in Mst1 knockdown cardiomyocytes subjected to HG culture (Fig. 1A–C, Fig. S2A). Next, we observed the colocalization of lysosomes with mitochondria. Mst1 knockdown enhanced the colocalization of lysosomes with mitochondria (Fig. S2B–D). Pretreatment with CQ abolished the effects of Mst1 knockdown on the colocalization (Fig. S2B–D). These results suggested that Mst1 induced mitophagy in cardiomyocytes under HG conditions.

Mitophagy can be regulated via a Parkin-, BNIP3-, BNIP3L- or FUNDC1-dependent pathway [2]. In HG cultured cardiomyocytes, the expression levels of BNIP3 and BNIP3L were increased, whereas those

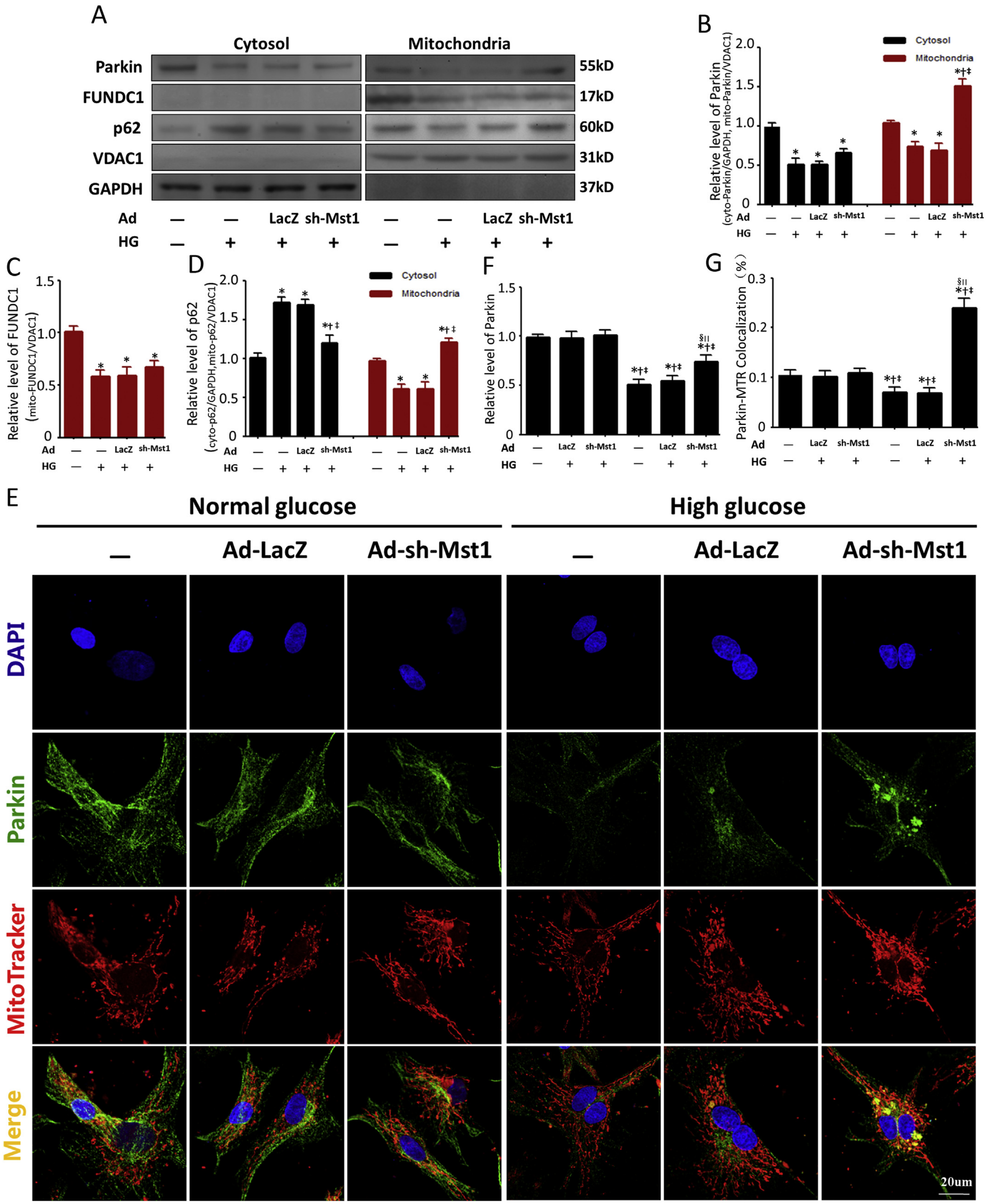

Fig. 5. Mst1 inhibition activates Parkin recruitment to mitochondria.

A, Cytosol and mitochondrial protein expression with representative gel blots of Parkin, FUNDC1, p62, VDAC1 (loading control for mitochondrial protein) and GAPDH (loading control for cytosol protein). B, Relative level of Parkin. C, Relative level of FUNDC1. D, Relative level of p62. The columns and error bars represent the means and SEM. *P < 0.05 vs Con; †P < 0.05 vs HG; and ‡P < 0.05 vs HG + Ad-LacZ. E, Representative colocalization images of Parkin (green) and mitochondria (MitoTracker Red) (scale bars: 20 μm). F, Fluorescent intensity ratio of Parkin/MTR (MitoTracker Red). G, Quantitative analysis of Parkin/MitoTracker Red colocalization (percentage of whole cells). The columns and error bars represent the means and SEM. *P < 0.05 vs Con; †P < 0.05 vs Con + Ad-LacZ; ‡P < 0.05 vs Con + Ad-sh-Mst1; §P < 0.05 vs HG; and ||P < 0.05 vs HG + Ad-LacZ.

of Parkin and FUNDC1 were decreased (Fig. 1D–L). Mst1 knockdown significantly upregulated Parkin expression (Fig. 1D and G). The results indicated that Mst1 inhibition enhanced mitophagy to assimilate redundant mitochondria, which was associated with Parkin pathway.

3.3. Parkin is essential for Mst1 mediated mitophagy

TEM observed typical autophagosomes engulfing mitochondria in the diabetic Mst1$^{-/-}$ heart. However, in diabetic-Parkin$^{-/-}$ and diabetic-Parkin$^{-/-}$: Mst1$^{-/-}$ mice, autophagosomes with mitochondria were rarely seen. These results suggested that Parkin deletion abolished the effects of Mst1 knockout on mitophagy in DCM (Fig. 2A). Western blot analysis demonstrated that the expression of Parkin was upregulated in Mst1 deficient diabetic mice hearts (Fig. 2B, Fig. S5A and B). Mst1 knockout decreased the p62 expression in diabetic mice hearts, thus indicating enhanced autophagic flux. However, Parkin knockout did not obviously change p62 expression in diabetic mice hearts, which could have been due to the diabetic heart already having basal low-level autophagy activity itself (Fig. 2B, Fig. S3C). As far as we know, mitochondrial located p62 is essential for Parkin-dependent mitophagy. Mitochondrial protein analysis revealed that Mst1 knockout increased, while Parkin knockout decreased the mitochondrial p62 level in diabetic hearts (Fig. 2B and C). These results indicated that Mst1 participated in the development of DCM by inhibiting Parkin-dependent mitochondrial elimination. In vitro, Parkin knockdown decreased LC3 colocalization with mitochondria in cardiomyocytes under HG conditions (Fig. 2D–G). Interestingly, Parkin knockdown abolished the effects of Mst1 on LC3 colocalization with mitochondria (Fig. 2D–G).

3.4. Mst1 deficiency ameliorates the cardiac phenotype of DCM through Parkin-dependent mitophagy

We next investigated whether Mst1 knockout ameliorated cardiac function and myocardial injury via Parkin-dependent pathway. Mice with DCM exhibited decreased LVEF and LVFS, as well as increased LVESD and LVEDD compared to the WT mice (Fig. 3A–E). Mst1 knockout alleviated left ventricular dysfunction, as evidenced by decreased LVESD and LVEDD, as well as increased LVEF and LVFS in diabetic mice. By contrast, Parkin knockout aggravated cardiac dysfunction in diabetic mice. Intriguingly, Parkin knockout abolished the protective effects of Mst1 knockout on cardiac dysfunction in the setting of DCM. (Fig. 3A–E) Given that diabetic Parkin$^{-/-}$ mice had a significant deterioration of cardiac function, we used Parkin$^{+/-}$ and Parkin$^{+/-}$: Mst1$^{-/-}$ mice to induce DCM. A similar phenomenon was observed (Fig. 3F). These findings illustrated that Mst1 deficiency improved cardiac function through Parkin dependent pathway.

TUNEL-positive cardiomyocytes were less frequently observed in diabetic Mst1$^{-/-}$ mice (Fig. 3G). Parkin knockout increased the apoptotic index in diabetic heart (Fig. 3H). Mst1 deletion alleviated myocardial apoptosis without rescuing the apoptotic index in Parkin ablation diabetic mice (Fig. 3G and H). These data indicated that Mst1 deficiency ameliorated the cardiac pathological phenotype of diabetic mice via Parkin-dependent pathway.

3.5. Mst1 aggravates mitochondrial injury by inhibiting Parkin-dependent mitophagy in the diabetic myocardium

Next, we investigated whether Mst1 inhibition altered the mitochondrial morphology and bioenergetics in the diabetic myocardium via Parkin-dependent mitophagy. TEM was employed to survey the morphologic injury of mitochondria. Consistent with our previous results, Mst1 knockdown alleviated, while Parkin knockdown aggravated mitochondrial cristae disorganization in cardiomyocytes underwent HG treatment (Fig. 4A). Mst1 inhibition failed to mitigate mitochondrial morphologic injury in HG treated cardiomyocytes with Parkin inhibition (Fig. 4A).

Mst1 knockdown increased, while Parkin knockdown decreased the $\triangle\Psi m$ in HG treated cardiomyocytes, as evidenced by JC-1 fluorescence imaging (Fig. 4B–C). Parkin knockdown abolished the effects of Mst1 inhibition on the cardiomyocyte $\triangle\Psi m$ (Fig. 4B–C). We also surveyed the mitochondrial enzymatic activity and ATP content to assess mitochondrial function. Mst1 deficiency increased, while Parkin knockout decreased the myocardial citrate synthase (CS), complex I/II/III/IV/V activities and ATP content in diabetic hearts (Fig. 4D–F). These results indicated that Mst1 inhibition relieved mitochondrial morphologic and functional injury in diabetic hearts via Parkin dependent pathway.

3.6. Mst1 inhibition enhances mitophagy by activating Parkin recruitment to the mitochondria

Parkin, as an E3 ubiquitin ligase, was located in the cytosol under normal conditions, where Parkin was considered to be catalytically inactive [33]. Parkin's transfer from the cytosol to mitochondrion was essential for initiating Parkin-mediated mitophagy [33,34]. First, we extracted mitochondrial and cytosol proteins and found that Mst1 knockdown upregulated the Parkin level on the mitochondria of HG treated cardiomyocytes, without affecting the expression of mitochondria FUNDC1 (Fig. 5A–C). Additionally, Mst1 inhibition increased mitochondrial p62 expression, while decreased cytosol p62 expression (Fig. 5A and D). Since Parkin recruited p62 to mitochondria during mitophagy, the increased p62 expression on mitochondria induced by Mst1 inhibition also suggested enhanced mitophagy in HG treated cardiomyocytes.

Immunofluorescence analysis was performed to observe the mitochondrial localization of Parkin. Mst1 knockdown significantly enhanced Parkin localization on the mitochondria in HG treated cardiomyocytes (Fig. 5E–G). These results indicated that Mst1 inhibition enhanced cardiomyocyte mitophagy by promoting Parkin translocation to mitochondria.

3.7. Mst1/Sirt3/Parkin signaling pathway involves in the pathogenesis of DCM

Consistent with our previous study, the expression of Sirt3 was upregulated in the Mst1-deficient mice with DCM [29]. On the contrary, Parkin deletion failed to alter the expression of Sirt3. These results indicated that Sirt3 may serve as a downstream regulator of Mst1 as well as an upstream regulator of Parkin (Fig. S4A and B). To further verify this hypothesis, we constructed DCM model with Mst1 Tg: Sirt3$^{-/-}$ mice. Sirt3 knockout significantly impaired cardiac function as evidenced by decreased LVEF and LVFS, as well as increased LVESD and LVEDD (Fig. S4C–F). Interestingly, Mst1 overexpression failed to further aggravate cardiac dysfunction in Sirt3 deletion mice with DCM (Fig. S4C–F). Consistently, the evaluations of cardiac ATP and ROS levels further supported that Mst1 inhibited Sirt3 expression thus impaired Parkin-mediated mitophagy and participated in the pathogenesis of DCM.

4. Discussion

Diabetic patients are predisposed to serious cardiovascular complications, thus making it necessary to elaborate the molecular mechanism of DCM and explore potential therapeutic targets for clinic [1,2]. Here, our works mainly demonstrated that Mst1/Sirt3/Parkin signaling was involved in the pathophysiology of diabetic cardiomyopathy. Concretely, Mst1 inhibition upregulated Sirt3 expression, activated Parkin expression and mitochondrial recruitment, thus enhanced mitophagy and alleviated the phenotype of DCM (Fig. S5).

It is well elucidated that several mechanisms have been used to maintain mitochondria homeostasis in cardiomyocytes. [9] Mitophagy mediates the selective removal of damaged mitochondria that is essential for mitochondrial quality control [7,16,35]. The formation of autophagosomes then triggers the degradation of depolarized mitochondria via lysosomes [36]. Mitochondria are critical organelles for maintaining cardiac function since cardiomyocytes have a high demand for ATP synthesis [2,8]. However, depolarized mitochondrion releases toxic medium, such as reactive oxygen species and cytochrome C, to induce cell death [31]. Mitochondrial homeostasis disorder is a hallmark of cardiomyocyte dysfunction [6,9,37]. Dysregulated mitophagy and mitochondrial dysfunction play critical roles in the pathophysiology of diabetes. Thus, mitochondria homeostasis could be a promising target to treat patients with DCM [6,10,38].

Yu et al. demonstrated that damaged mitochondria were accumulated in the myocardium under diabetic stress and induced subsequent cardiomyocyte energy deficiency and cardiac dysfunction [20]. In the present study, we observed mitochondria morphology, bioenergetics and membrane potential in diabetic mice as well as cardiomyocytes subjected to HG culture. The results indicated that diabetes compromised mitochondrial function, which was attenuated by Mst1 knockout or knockdown. Mst1 deficiency did not rescue the mitochondria injury in Parkin-deficient mice subjected to diabetes stress. These results provide direct evidence that mitochondrial injury plays a key role in diabetes and that Mst1 deteriorates mitochondrial dysfunction by inhibiting Parkin.

As described by Heiko Bugger and colleagues, mitochondrial mass was upregulated in diabetic hearts [39]. We observed that Mst1 deficiency reduced the number of mitochondria in diabetic mice via TEM. The protein analysis of PGC-1α, NRF-1 and Tfam showed no difference between diabetic group and diabetic Mst1$^{-/-}$ group. The present study thus excluded the hypothesis that Mst1 affected the mitochondrial number by inhibiting mitochondrial biogenesis.

Parkin, BNIP3, BNIP3L and FUNDC1 are well-known mitophagy mediators [2]. Presently, we observed that Mst1 suppressed mitophagy via Parkin inhibition, which was independent of FUNDC1, BNIP3, and BNIP3L. Although previous experiments had illustrated that BNIP3 and BNIP3L induced mitophagy in hypoxia situations [40,41]. The present study also observed increased expression of BNIP3 and BNIP3L in diabetic heart. However, Mst1 knockout induced mitophagy was independent of FUNDC1, BNIP3, and BNIP3L. Since BNIP3 and BNIP3L are also serve as pro-apoptosis regulators, which participate in cell death during cancer and cardiac diseases [2,42,43]. Hence, we inferred that the pro-apoptosis activity of BNIP3 and BNIP3L may be dominant in the diabetic heart rather than just regulating mitophagy.

Parkin mediates mitophagy in a large proportion of cells and is widely expressed in brain, heart or liver [17,44]. Parkin deletion results in the failure of selective mitochondrial clearance [45]. Several studies reported that Parkin knockout mice lacked apparent cardiac phenotype under baseline conditions [46,47]. In the present study, Parkin knockout impaired cardiac function as indicated by decreased LVEF and LVFS in diabetic mice. Heterozygous Parkin knockout did not alleviate cardiac dysfunction in diabetic mice. Another previous study reported that Parkin had both protective and cytotoxic effects by regulating mitophagy and apoptosis respectively, according to the degree of mitochondrial damage [48]. The present study demonstrated that Parkin served as the downstream regulator of Mst1 in the development of DCM. Further studies should illustrate whether Parkin plays multifunctional roles in diabetic cardiomyopathy.

SIRT3 is crucial to aging and the regulation of the cardiomyocyte energy metabolism, oxidative stress and mitophagy [20]. Cell survival and lifespan are intimately coupled to the homeostatic role of mitophagy. Age-related decline in mitophagy is directly linked to cardiac senescence and cardiac dysfunction [49]. Our present study also illustrated that Sirt3 may serve as the downstream regulator of Mst1 as well as a upstream regulator of Parkin and play a vital role in the development of diabetic cardiomyopathy.

Taken together, we observed that Mst1 inhibition improved mitochondrial biogenetics and alleviated cardiac dysfunction in DCM. Mst1 deficiency enhanced mitophagy and eliminated depolarized mitochondria partly through Sirt3 inhibition thus increasing the expression and mitochondrial translocation of Parkin. Mst1 knockout upregulated Sirt3 expression and activated Parkin-dependent mitophagy to eliminate superfluous and damaged mitochondria could be the potential therapeutic target for DCM.

Funding

This work was supported by the National Natural Science Foundation of China (No. 81770224, No. 81570318, No. 81570361, No. 81670204 and No. 81370194).

Conflicts of interest

None.

Author contributions

S.J.W., Z.J.Z., Y.H.F., H.C.W. and D.D.S. conceived the project, researched data, analyzed data and wrote the manuscript. M.M.Z, X.Y.F., J.L., J.Q.H., Z.C., C.S., T.T.L. and Z.Y.X. researched the data and reviewed and edited the manuscript. D.D.S. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and accuracy of the data analysis.

Transparency document

The http://dx.doi.org/10.1016/j.bbadis.2018.04.009 associated this article can be found, in online version.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbadis.2018.04.009.

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