



Mitophagy coordinates the mitochondrial unfolded protein response to attenuate inflammation-mediated myocardial injury

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ABSTRACT

Mitochondrial dysfunction is a fundamental challenge in septic cardiomyopathy. Mitophagy and the mitochondrial unfolded protein response (UPR^{mt}) are the predominant stress-responsive and protective mechanisms involved in repairing damaged mitochondria. Although mitochondrial homeostasis requires the coordinated actions of mitophagy and UPR^{mt}, their molecular basis and interactive actions are poorly understood in sepsis-induced myocardial injury. Our investigations showed that lipopolysaccharide (LPS)-induced sepsis contributed to cardiac dysfunction and mitochondrial damage. Although both mitophagy and UPR^{mt} were slightly activated by LPS in cardiomyocytes, their endogenous activation failed to prevent sepsis-mediated myocardial injury. However, administration of urolithin A, an inducer of mitophagy, obviously reduced sepsis-mediated cardiac depression by normalizing mitochondrial function. Interestingly, this beneficial action was undetectable in cardiomyocyte-specific FUNDC1 knockout (FUNDC1^{CKO}) mice. Notably, supplementation with a mitophagy inducer had no impact on UPR^{mt}, whereas genetic ablation of FUNDC1 significantly upregulated the expression of genes related to UPR^{mt} in LPS-treated hearts. In contrast, enhancement of endogenous UPR^{mt} through oligomycin administration reduced sepsis-mediated mitochondrial injury and myocardial dysfunction; this cardioprotective effect was imperceptible in FUNDC1^{CKO} mice. Lastly, once UPR^{mt} was inhibited, mitophagy-mediated protection of mitochondria and cardiomyocytes was partly blunted. Taken together, it is plausible that endogenous UPR^{mt} and mitophagy are slightly activated by myocardial stress and they work together to sustain mitochondrial performance and cardiac function. Endogenous UPR^{mt}, a downstream signal of mitophagy, played a compensatory role in maintaining mitochondrial homeostasis in the case of mitophagy inhibition. Although UPR^{mt} activation had no negative impact on mitophagy, UPR^{mt} inhibition compromised the partial cardioprotective actions of mitophagy. This study shows how mitophagy modulates UPR^{mt} to attenuate inflammation-related myocardial injury and suggests the potential application of mitophagy and UPR^{mt} targeting in the treatment of myocardial stress.

Abbreviations: UPR^{mt}, Mitochondrial unfolded protein response; LPS, lipopolysaccharide; (PBS), PBS, phosphate buffered saline; DMSO, dimethyl sulfoxide; DMEM/F-12, Dulbecco's modified Eagle's medium/F-12 nutrient mixture; FS, Fractional shortening; LVEF, left ventricular ejection fraction; LVDD, left ventricular diastolic; siRNAs, small interfering RNAs, 4,6-diamidino-2-phenylindole; LDH, lactate dehydrogenase; EM, electron microscopy.

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1. Introduction

Septic cardiomyopathy is characterized by decreased left ventricular dilatation and an impaired ejection fraction resulting from severe sepsis syndrome [1]. In contrast to myocardial infarction, septic cardiomyopathy is a reversible form of myocardial depression. Reportedly, oxidative stress, cytokine overproduction, microvascular damage, and cardiomyocyte ATP metabolism are potential molecular mechanisms underlying sepsis-induced myocardial damage [2–5]. Anti-inflammatory agents and myocardial metabolic therapies have been identified as effective strategies for the treatment of septic cardiomyopathy [6,7]. As the mitochondria are regulators of cardiomyocyte metabolism and inflammation response, their dysfunction is a key event in myocardial depression [8,9]. Mitochondrial dysfunction plays an important role in inducing oxidative stress and promoting energy crisis; these alterations are always followed by cardiomyocyte death through either apoptosis or necrosis [10,11]. Irreversible cardiomyocyte death is a key molecular mechanism in activating the inflammatory response. Despite extensive research focused on understanding the complex impact of mitochondrial damage on sepsis-related myocardial depression, the pathological alterations in the mitochondria in cardiomyocytes are poorly understood.

In order to maintain a functional mitochondrial network, the mitochondria develop specific repair pathways, including mitophagy and the mitochondrial unfolded protein response (UPR^{mt}) [12,13]. UPR^{mt} prevents abnormal protein accumulation within the mitochondria via normalization of mitochondrial protein folding and degradation [14]. Mitophagy selectively removes damaged mitochondria via ubiquitination degradation [15]. In contrast to UPR^{mt}, mitophagy reduces the mitochondrial population, an alteration that is occasionally associated with shortages in ATP and cell death [16]. Unlike mitophagy, UPR^{mt} dynamically controls mitochondrial protein import or export and fine-tunes mitochondrial behaviors [17]. Our previous studies described the features of mitophagy in cardiovascular disorders, such as myocardial infarction, cardiac ischemia-reperfusion injury, and diabetic cardiomyopathy [18–23]. Once moderately activated, mitophagy exerts cardioprotective effects by sustaining mitochondrial quality; however, excessive induction of mitophagy is fatal because most mitochondria are degraded and ATP production is therefore suppressed [24]. Moreover, according to our recent findings, the role of mitophagy in deciding cell fate is also controlled by various mitophagic adaptors, such as FUNDC1, Parkin, and Bnip3 [18,24,25].

Recently, the role of UPR^{mt} in myocardial stress has been reported [26,27]. In a mouse model of chronic pressure overload-mediated cardiac hypertrophy [27], the markers of UPR^{mt}, such as *Atf5*, *CHOP*, *mtDNAj*, *ClpP*, and *LonP1*, were significantly elevated at the mRNA level, suggesting that UPR^{mt} is an adaptive response to myocardial stress. Interestingly, further activation of UPR^{mt} through nicotinamide riboside supplementation has been associated with normalized mitochondrial respiration and decreased cardiomyocytes *in vitro* [27]. Moreover, in response to nicotinamide riboside, cardiomyocyte apoptosis is reduced and cardiac function is improved, suggesting the cardioprotective action of UPR^{mt} activation [27]. In a mouse model of cardiac ischemia-reperfusion (I/R) injury [26], the genes related to UPR^{mt} were also significantly upregulated, as identified using RNA-Seq analysis. Interestingly, genetic ablation of *Atf5* has no influence on baseline cardiac I/R injury [26]. However, administration of the UPR^{mt} inducer reduced I/R-mediated myocardial injury in wild-type (WT) mice, but not in *Atf5*-knockout mice [26]. This result demonstrates that I/R-mediated UPR^{mt} activation is insufficient to protect the heart against acute stress, and that additional induction of UPR^{mt} is a promising target for the treatment of cardiac I/R injury. However, the role of UPR^{mt} in septic cardiomyopathy has not yet been elucidated. Moreover, as the mitochondria repair pathways, the relationship between mitophagy and UPR^{mt} remains unclear, although several studies report that mitophagy inhibition is followed by UPR^{mt} activation [28]. The aim of this study was to explore the role of UPR^{mt} in septic cardiomyopathy and to clarify

the interactive effects of UPR^{mt} and mitophagy.

2. Materials and methods

2.1. Animal care

FUNDC1^{f/f} mice and cardiomyocyte-specific FUNDC1 knockout (FUNDC1^{CKO}) mice were generated as described in our previous study [25]. These mice were bred and maintained at the University of Wyoming Institutional Animal Center. All procedures related to animal treatment and surgery were approved by the University of Wyoming Institutional Animal Use and Care Committee (Laramie, WY, USA). In our study, 8-week-old mice were injected intraperitoneally with a single dose of phosphate buffered saline (PBS) or lipopolysaccharide (LPS) (5 mg/kg) to induce septic cardiomyopathy. The animals were euthanized via pentobarbital 48 h after LPS injection. Heart tissues were then isolated and used for subsequent experiments. To induce UPR^{mt} *in vivo*, mice were intraperitoneally injected with oligomycin (500 µg/kg) in sterile saline with 0.1% dimethyl sulfoxide (DMSO). To activate mitophagy, urolithin A (UA; 30 mg/kg) was intraperitoneally injected in sterile saline with 0.1% DMSO.

2.2. Cell culture and treatments

The AC16 human ventricular cardiomyocyte cell line was maintained in Dulbecco's modified Eagle's medium/F-12 nutrient mixture (DMEM/F-12; Invitrogen, Carlsbad, CA) medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37 °C and 5% CO₂ [29]. After treating AC16 cells with 1 µM human insulin (Santa Cruz Biotechnology, Inc.) for 1 h, they were harvested for protein purification [30]. To induce sepsis-related cardiomyocyte damage, AC16 cells were treated with filtered (0.2 µ syringe filter; Corning) DMEM/F12 complete medium containing 10 µg/mL of LPS (Sigma-Aldrich).

2.3. Mitochondrial membrane potential and mt-Keima assays

As previously described [31], AC16 cells were incubated with JC-1 dyes to observe changes in the mitochondrial membrane potential. Mitophagy activity was observed using the mt-Keima assay, which was performed using a mitochondria-targeted mKeima-Red expression plasmid (pMT-mKeima-Red, #AM-V-251, MBL Medical & Biological Laboratories, Co., Ltd., Woburn, MA), as previously described [32,33].

2.4. MTT cell viability assay

The MTT cell viability assay was performed using an MTT assay kit (Abcam, ab211091), according to the manufacturer's protocol [34,35].

2.5. Echocardiography

Operators blinded to the study groups performed echocardiography on conscious animals using a 20 MHz ultrasound transducer interfaced with a VisualSonics Vevo 2100 imaging system [36]. Two-dimensionally directed M-mode images were obtained from long-axis views. Comprehensive M-mode and two-dimensional imaging were used to determine fractional shortening (FS), wall thickness estimated wall mass, cardiac function, volume, ejection fraction, and cardiac output [37]. Doppler flow imaging was used to assess systolic and diastolic functions [38]. Echocardiographic measurements were performed on three consecutive cardiac cycles using the leading edge-to-leading edge method. Left ventricular ejection fraction (LVEF), left ventricular diastolic dimension (LVDd), and FS were measured or calculated with software [39].

2.6. Quantitative and semi-quantitative

To perform semi-quantitative real-time (RT) PCR, total RNA was extracted from cardiomyocytes using TRIzol (Invitrogen); DNase 1 (Promega) was used to remove genomic DNA. RNA (1 μ g) was reverse transcribed into cDNA using the SuperScript IV First-Strand Synthesis System (Invitrogen) with random hexamer primers; 80 μ L of ddH₂O was added following the reaction at room temperature, and the final cDNA concentration was adjusted to 10 ng/ μ L [40,41]. A 20 μ L regular PCR reaction was performed using human GAPDH primers with 2 μ L of cDNA template (20 ng). The following PCR program was used: 95 °C for 20 s (denaturation), 60 °C for 20 s (annealing), 72 °C for 50 s (extension), for a total of 35 cycles. Next, 7 μ L of the PCR product was loaded into a 1.5% agarose gel with 1X TAE buffer. Band intensity was digitized and quantified using ImageJet software [42,43].

2.7. Cardiomyocyte transfection

Small interfering RNAs (siRNAs) for FUNDC1 and Atf6 and the negative control (Mission, Sigma-Aldrich Corp., St Louis, MO) were used according to the manufacturer's instructions [44,45]. Following isolation, cardiomyocytes were cultured for 48 h prior to transfection with siRNAs at a concentration of 50 nM using Lipofectamine® RNAiMAX (Invitrogen, Carlsbad, CA) [46,47]. Following 6 h of incubation in Optimem®, the medium was removed and replaced with 5% FBS in DMEM/M199. Changes in FUNDC1 and Atf6 protein and/or transcript levels were assessed 48 h post-transfection [48].

2.8. Immunofluorescence

Cardiomyocytes were seeded onto sterile, acid-treated, 18-mm #1.5 glass coverslips in 24-well plates. They were fixed in 2% paraformaldehyde in PBS for 10 min [49]. Permeabilization was performed by incubating the cells in PBS containing 0.1% Triton X-100 for 5 min at 37 °C. Next, cells were incubated with Tom20 (1:1,000, Abcam, #ab186735) primary antibodies for 1 h at room temperature, followed by three washes to remove unbound antibodies. Next, the cells were incubated with secondary antibodies for 1 h. DNA was stained with 4, 6-diamidino-2-phenylindole (DAPI, Sigma, cat no. D9564) at a dilution of 1:5000 for 2 min [50]. Cells were then imaged using a Yokogawa CSU-W1 spinning disk scan head with a 50- μ m pinhole disk mounted on a Nikon Ti inverted microscope (Nikon Ti) [51]. Signals from FITC and rhodamine channels were collected using Chroma ET 525/36 and ET 605/52 emission filters, respectively [52].

2.9. ATP and mitochondrial reactive oxygen species (ROS) measurements

The ATP concentration in cells was measured using the Cell Titer-Glo Luminescent Viability Assay (Promega, Madison, WI). Briefly, CellTiter-Glo reagent was added to each well (100 μ L); cellular contents were mixed for 2–3 min on an orbital shaker [35]. Next, the plate was incubated for 10 min to stabilize the luminescent signal, and luminescence was read using a Tecan Infinite 200 PRO plate reader (Männedorf, Switzerland) [53]. The mitochondrial ROS concentration in cells was measured using the cell-permeant MitoSOX Red mitochondrial superoxide indicator (Molecular Probes, USA) [54].

2.10. Enzyme-linked immunosorbent assay (ELISA)

ELISA-based assays were performed using kits for lactate dehydrogenase (LDH), troponin T, and CK-MB from InvivoGen (San Diego, CA, USA). Colorimetric and luminescent signals from ELISA kits were measured using a Tecan Infinite 200 PRO plate reader (Männedorf, Switzerland), according to the manufacturer's instructions [55,56].

2.11. Statistics

All data are presented as the mean \pm standard error of the mean (SEM). Independent biological replicates are denoted by an 'n' in the figure legends. Statistical analysis was performed in GraphPad (Prizm) using the Student's *t*-test (two-tailed). Statistical significance was set at *P* < 0.05.

3. Results

3.1. Activation of FUNDC1-associated mitophagy attenuates sepsis-induced myocardial injury

First, to observe the cardioprotective role of mitophagy in regulating septic cardiomyopathy, UA, a mitophagy inducer, was administered to mice prior to LPS treatment. Compared to sham-operated control mice, LPS-mediated sepsis significantly elevated the levels of cardiac injury markers, such as LDH, troponin T, and CK-MB (Fig. 1A–C). In contrast, administration of UA to activate mitophagy suppressed myocardial injury biomarker levels (Fig. 1A–C). In addition, echocardiography of cardiac function also showed that LVEF, LVDD, and FS were disrupted by LPS injection (Fig. 1D–F). However, normalized cardiac function, including LVEF, LVDD, and FS, were observed in mice pretreated with UA (Fig. 1D–F). To determine whether UA-mediated cardioprotection is dependent on FUNDC1, cardiomyocyte-specific FUNDC1^{CKO} mice were used in our subsequent experiments. Interestingly, UA failed to suppress cardiac injury biomarker levels in FUNDC1^{CKO} mice (Fig. 1A–C). Consistent with this finding, LPS-induced cardiac dysfunction could not be markedly reversed by UA in FUNDC1^{CKO} mice (Fig. 1D–F). To validate whether UA and/or FUNDC1 are involved in mitophagy activation in cardiomyocytes, mitophagy activity was detected using an mt-Keima assay *in vitro*. Our results showed that mitophagy was moderately induced by LPS and robustly activated by UA (Fig. 1G–H). However, deletion of FUNDC1 largely prevented mitophagy activation in the presence of UA (Fig. 1G–H). Taken together, we reasoned that activation of FUNDC1-associated mitophagy confers cardioprotection in a mouse model of septic cardiomyopathy.

Loss of FUNDC1-associated mitophagy promotes cardiomyocyte death and mitochondrial dysfunction.

Mitochondrial dysfunction, acting as the downstream of inflammation response, is an essential inducer of septic cardiomyopathy through promoting the death of functional cardiomyocytes [1]. Thus, cardiomyocyte death and mitochondrial function were measured. Firstly, cardiomyocyte viability, as evaluated using an MTT assay, was reduced by LPS, reversing to near-normal levels following UA treatment (Fig. 2A). However, in FUNDC1-deleted cardiomyocytes, UA failed to improve cardiomyocyte viability (Fig. 2A). Similar to this finding, analysis of caspase-3 activity demonstrated that UA prevented LPS-mediated caspase-3 activation in WT cardiomyocytes, but not in FUNDC1-depleted cardiomyocytes (Fig. 2B). Similarly, the LPS-dissipated mitochondrial membrane potential could be stabilized via UA; this effect required FUNDC1 in cardiomyocytes (Fig. 2C–D). Moreover, mitochondrial ROS production levels were also suppressed by UA; this action was detectable in WT cardiomyocytes but not in FUNDC1-depleted cardiomyocytes (Fig. 2E–F). Similar results were also observed in changes to ATP production (Fig. 2G). Moreover, mitochondrial dynamics appeared to be disrupted by LPS, as evidenced by the increased number of fragmented mitochondria (Fig. 2H–J). Furthermore, UA treatment was associated with an elongation of mitochondrial length, resulting in a decreased ratio of fragmented mitochondria; however, these beneficial effects were imperceptible if FUNDC1 was knocked out in cardiomyocytes (Fig. 2H–J). Overall, activation of FUNDC1-associated mitophagy prevented mitochondrial damage and cardiomyocyte death during septic cardiomyopathy.

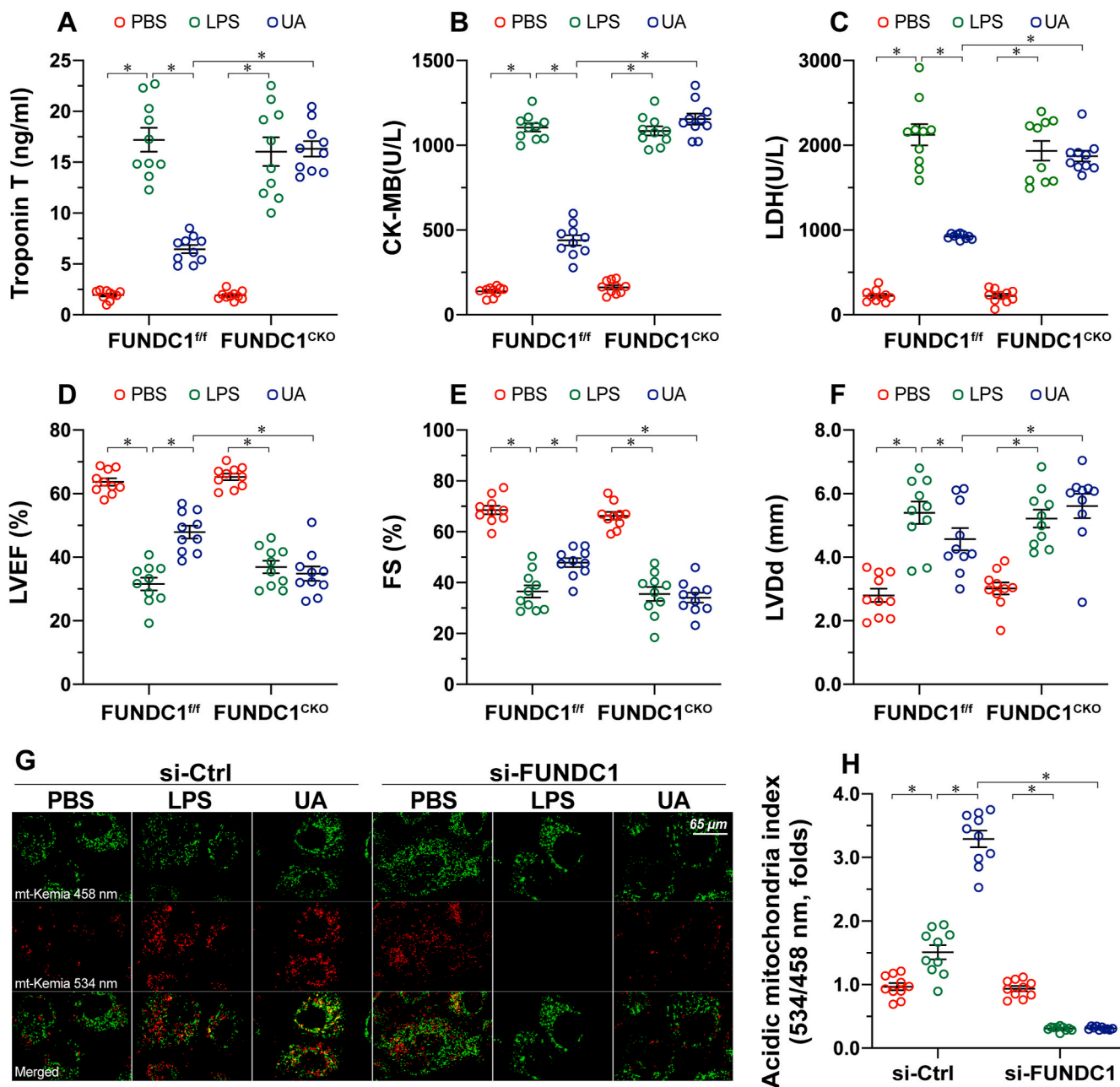


Fig. 1. Activation of FUNDC1-associated mitophagy attenuates sepsis-induced myocardial injury. FUNDC1^{ff} mice and cardiomyocyte-specific FUNDC1 knockout (FUNDC1^{CKO}) mice were injected intraperitoneally with a single dose of PBS or LPS (5 mg/kg) to induce septic cardiomyopathy. Blood and heart samples were isolated 48 h after LPS injection. To activate mitophagy, urolithin A (UA, 30 mg/kg) was injected intraperitoneally in sterile saline with 0.1% DMSO. **A-C.** ELISA analysis of the concentration of cardiac injury markers, including LDH, troponin T, and CK-MB. **D-F.** Echocardiographic data showing left ventricular ejection fraction (LVEF), left ventricular diastolic dimension (LVDD), and fractional shortening (FS) in FUNDC1^{CKO} and FUNDC1^{ff} mice in the presence of LPS. **G-H.** The AC16 human ventricular cardiomyocyte cell line was treated with 10 μ g/mL of LPS to induce sepsis-related cardiomyocyte damage. siRNA against FUNDC1 (si-FUNDC1) was transfected into AC15 to inhibit mitophagy activation. UA, an inducer of mitophagy, was used to culture AC16 cells. Next, mitophagy activity was observed using the mt-Keima assay. A yellow signal highlights increased mitophagic flux within cardiomyocytes. Data are presented as mean \pm SEM, normalized per 1000 cardiomyocytes. n = 6 per group. *P < 0.05. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.2. UPR^{mt} is activated in response to FUNDC1 deficiency

To illustrate the relationship between mitophagy and UPR^{mt}, we measured alterations in UPR^{mt} markers in response to mitophagy activation or inactivation. The qPCR assay demonstrated that the transcription of UPR^{mt} markers, including *Atf5*, *mtDNAj*, *ClpP*, *LonP1*, *CHOP*, *Hsp10*, and *Hsp60*, was moderately upregulated in LPS-treated heart tissue (Fig. 3A–G), suggesting that UPR^{mt} is induced by the inflammatory response. Interestingly, UA treatment appeared to partly prevent

UPR^{mt} activation in LPS-treated mice, suggesting that mitophagy activation is followed by the slight inhibition of UPR^{mt}. In contrast, loss of FUNDC1 significantly upregulated the transcription of UPR^{mt} markers (Fig. 3A–G), indicating that it may act as a compensatory mechanism in response to mitophagy inactivation.

Activation of UPR^{mt} partly reduces sepsis-induced myocardial injury and mitochondrial dysfunction in FUNDC1-knockout mice.

To understand whether UPR^{mt} is a compensatory mechanism during mitophagy inactivation, loss- and gain-of-function assays were

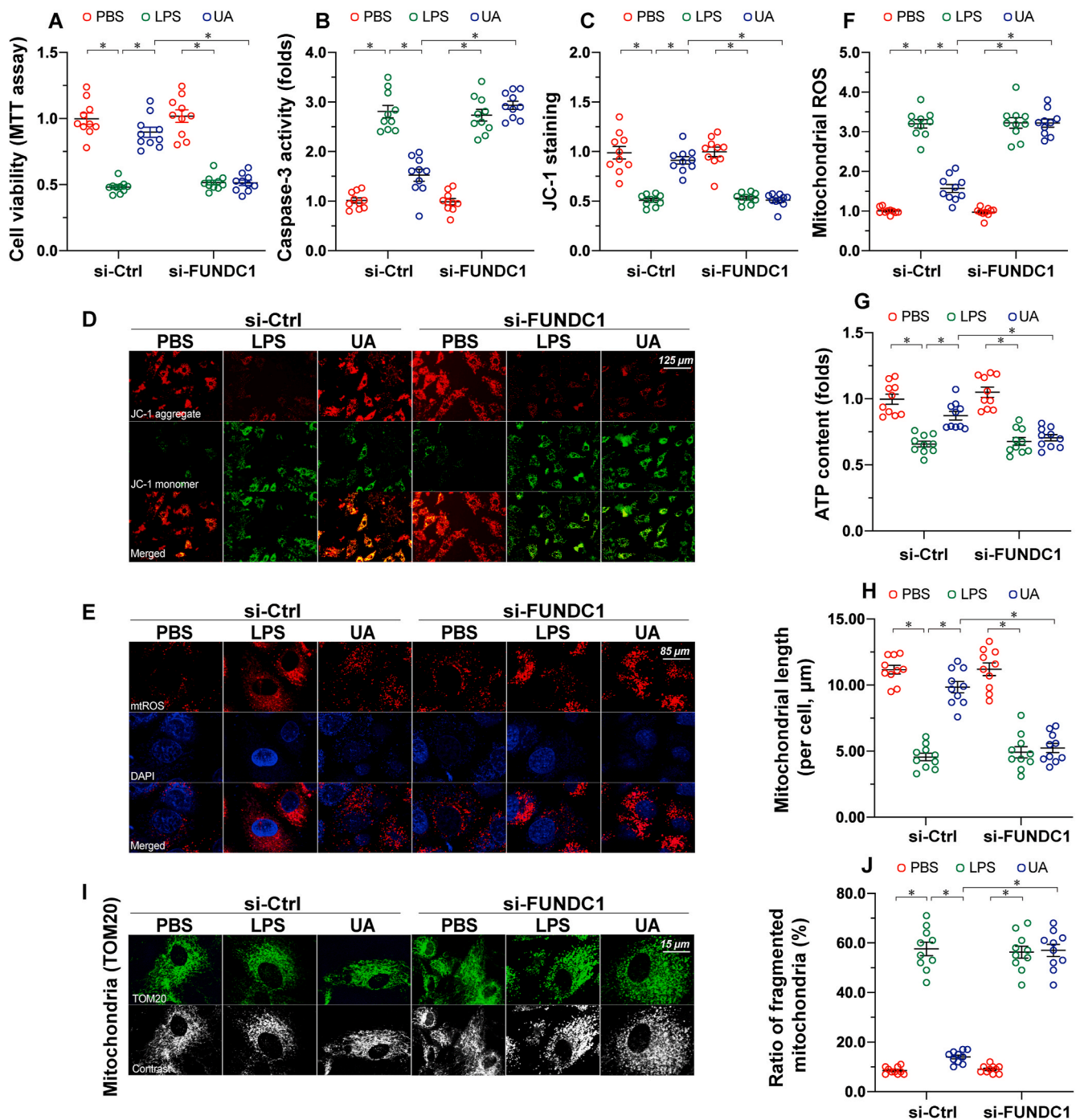


Fig. 2. Loss of FUNDC1-associated mitophagy promotes cardiomyocyte death and mitochondrial dysfunction. The AC16 human ventricular cardiomyocyte cell line was treated with 10 $\mu\text{g}/\text{mL}$ of LPS to induce sepsis-related cardiomyocyte damage. UA, an inducer of mitophagy, was used to culture AC16 cells. siRNA against FUNDC1 (si-FUNDC1) was transfected into AC15 to inhibit mitophagy activation. **A**. Cell viability was determined using an MTT assay. **B**. ELISA analysis of caspase-3 activity. **C-D**. AC16 cells were stained with JC-1 to observe changes in the mitochondrial membrane potential. **E-F**. AC16 cells were stained with the MitoSOX red mitochondrial superoxide indicator to show changes in mitochondrial ROS. **G**. Total ATP production was determined using the Cell Titer-Glo Luminescent Viability assay. **H-J**. Mitochondrial morphology was revealed using confocal immunofluorescence. TOM20 was used to show the shape of mitochondria in response to LPS or FUNDC1 deletion. Data are presented as mean \pm SEM, normalized per 1000 cardiomyocytes. * $P < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

performed. First, oligomycin was injected into mice before the induction of septic cardiomyopathy. Compared to LPS-treated mice, oligomycin treatment improved cardiomyocyte function, as indicated by normalized LVEF, LVDD, and FS indices (Fig. 4A–C). In addition, cardiac injury biomarkers were also partly reduced in response to oligomycin treatment (Fig. 4D–F). At the sub-cellular level, the mitochondrial membrane potential was stabilized (Fig. 4G–H), whereas mitochondrial ROS

production (Fig. 4I–J) was reduced in the presence of oligomycin, which was associated with increased cardiomyocyte viability. Taken together, these results illustrate that UPR^{mt} activation protects the heart and mitochondria against inflammation injury.

To understand whether UPR^{mt} acts as a downstream signal of FUNDC1-associated mitophagy, we further examined whether UPR^{mt} -mediated cardioprotection was detectable in FUNDC1^{CKO} mice.

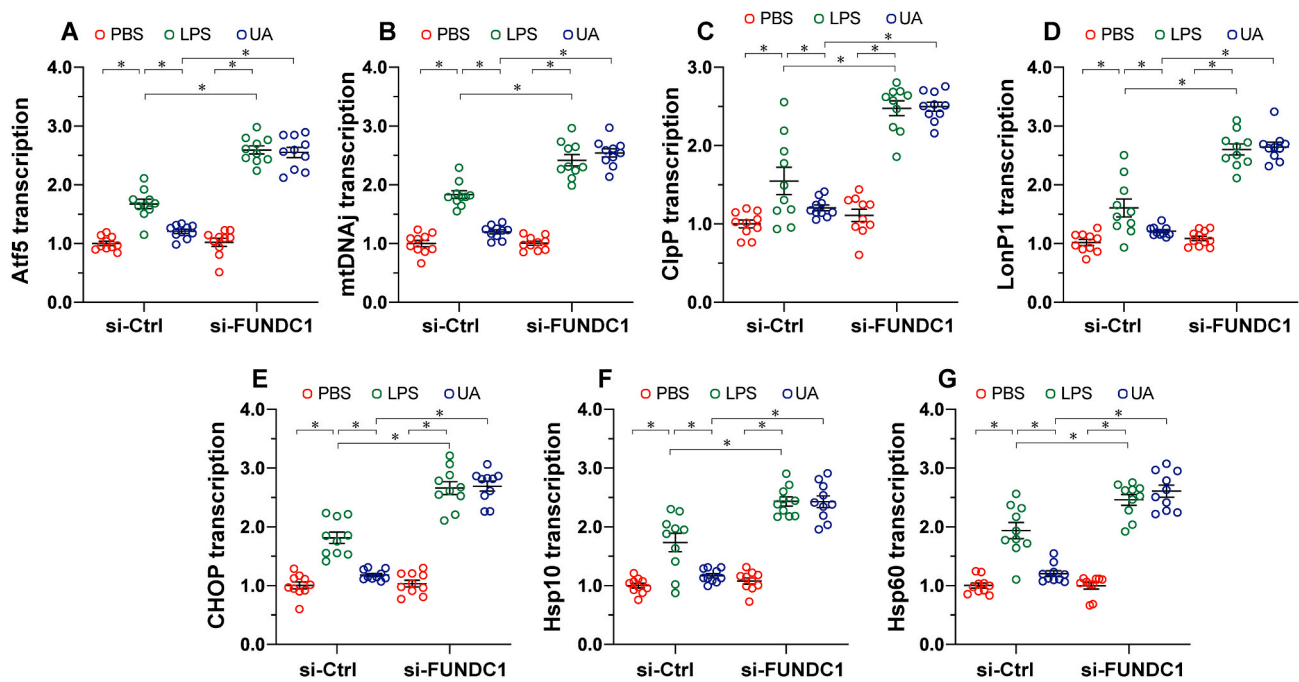


Fig. 3. UPR^{mt} is activated in response to FUNDC1 deficiency. *FUNDC1^{fl/fl}* and cardiomyocyte-specific FUNDC1 knockout (*FUNDC1^{CKO}*) mice were injected intraperitoneally with a single dose of PBS or LPS (5 mg/kg) to induce septic cardiomyopathy. Blood and heart samples were isolated 48 h after LPS injection. To activate mitophagy, urolithin A (UA, 30 mg/kg) was injected intraperitoneally in sterile saline with 0.1% DMSO. A-G. Total RNA was isolated from the ventricular tissue of *FUNDC1^{CKO}* and *FUNDC1^{fl/fl}* mice. RT-PCR was conducted to assess genes involved in UPR^{mt}. Data are presented as mean \pm SEM. n = 6 per group. *P < 0.05.

Interestingly, in the absence of FUNDC1, oligomycin was able to maintain cardiac function and reduce the levels of cardiac injury biomarkers (Fig. 4A–F). More importantly, mitochondrial function was also partly normalized by oligomycin administration in *FUNDC1^{CKO}* mice (Fig. 4G–J). Moreover, no statistical significance was observed regarding myocardial injury markers (Fig. 4A–C), cardiac function (Fig. 4D–F) and mitochondrial performance (Fig. 4G–J) between WT and *FUNDC1^{CKO}* mice in response to oligomycin, suggesting that UPR^{mt} works independently of mitophagy. Therefore, UPR^{mt} serves as a compensatory mechanism to confer cardioprotection in the case of mitophagy inactivation. Lastly, to exclude the influence of UPR^{mt} on mitophagy, the mt-Keima assay was performed. As shown in Fig. 4K–L, although LPS slightly increased mitophagy levels, administration of oligomycin had no effect on mitophagy.

3.3. Inhibition of UPR^{mt} partly abolishes the cardioprotective effect of mitophagy activation

Next, experiments were conducted to analyze the effect of UPR^{mt} inhibition in the presence of mitophagy activation. *In vitro*, siRNA against Atf6 (si-Atf6) and/or control siRNA (si-Ctrl) was transfected into cardiomyocytes to prevent UPR^{mt} activation. In cardiomyocytes transfected with either si-Atf6 or si-Ctrl, UA was capable of activating mitophagy in the presence of LPS (Fig. 5A–B), confirming that mitophagy activation is independent of UPR^{mt}. However, compared to cardiomyocytes transfected with si-Ctrl, UA-mediated cardiomyocyte survival was partly reduced in cardiomyocytes with si-Atf6 (Fig. 5C). Although UA was able to prevent LPS-mediated caspase-3 activation (Fig. 5D), this action was markedly mitigated in si-Atf6-transfected cardiomyocytes. Similar to these observations, the mitochondrial membrane potential could be improved by UA under LPS challenge (Fig. 5E–F); however, this protective action was weakened in si-Atf6-transfected cardiomyocytes. LPS-induced mitochondrial oxidative stress was mostly suppressed by UA in si-Ctrl-transfected cardiomyocytes (Fig. 5G–H); however, UA partly removed mitochondrial ROS in si-Atf6-transfected cardiomyocytes. Besides, ATP production

could be normalized by UA in the presence of LPS treatment, although this effect was partly attenuated by Atf6 knockout (Fig. 5I). Considering mitochondrial structural alterations, mitochondrial fragmentation was rapidly induced by LPS in cardiomyocytes (Fig. 5J–L). Although UA treatment was correlated with an obvious decline in the number of fragmented mitochondria, this protective action was partly retained in si-Atf6-transfected cardiomyocytes (Fig. 5J–L). Collectively, without UPR^{mt}, the cardioprotective action of mitophagy was partly compromised.

4. Discussion

Inflammation-mediated myocardial damage is a key feature of septic cardiomyopathy. Although the incidence of septic cardiomyopathy ranges from 18 to 29% in patients diagnosed with sepsis [57], the mortality rate is high, and effective therapies are lacking. The pathophysiology of septic cardiomyopathy is reportedly associated with the following mechanisms, cardiotoxic factors (such as TNF- α , CRP, IL-6, complement, and endotoxin), overproduction as a result of inflammation, catecholamine toxicity due to sympathetic nerve activation, decreased calcium sensitivity due to ATP shortage, and microvascular spasm in response to anaerobic metabolite accumulation [58]. The molecular biological mechanisms associated with septic cardiomyopathy include oxidative stress, calcium overload, ATP shortage, autophagy inactivation, metabolic reprogramming, mitochondrial malfunction, endoplasmic reticulum stress, and induction of apoptosis and necrosis [57,58]. In the present study, our data showed that sepsis-mediated myocardial depression is characterized by mitochondrial membrane potential reduction, mitochondrial ROS overloading, and cardiomyocyte apoptosis activation. These alterations contribute to myocardial contractility decline and myocardial injury marker elevation. In addition to functional damage, the structure of the myocardium is disrupted by LPS-induced sepsis. These results are in accordance with previous findings, suggesting that mitochondria could be considered a potential target for the treatment of septic cardiomyopathy.

Damaged mitochondria can be fixed through a complex mechanism

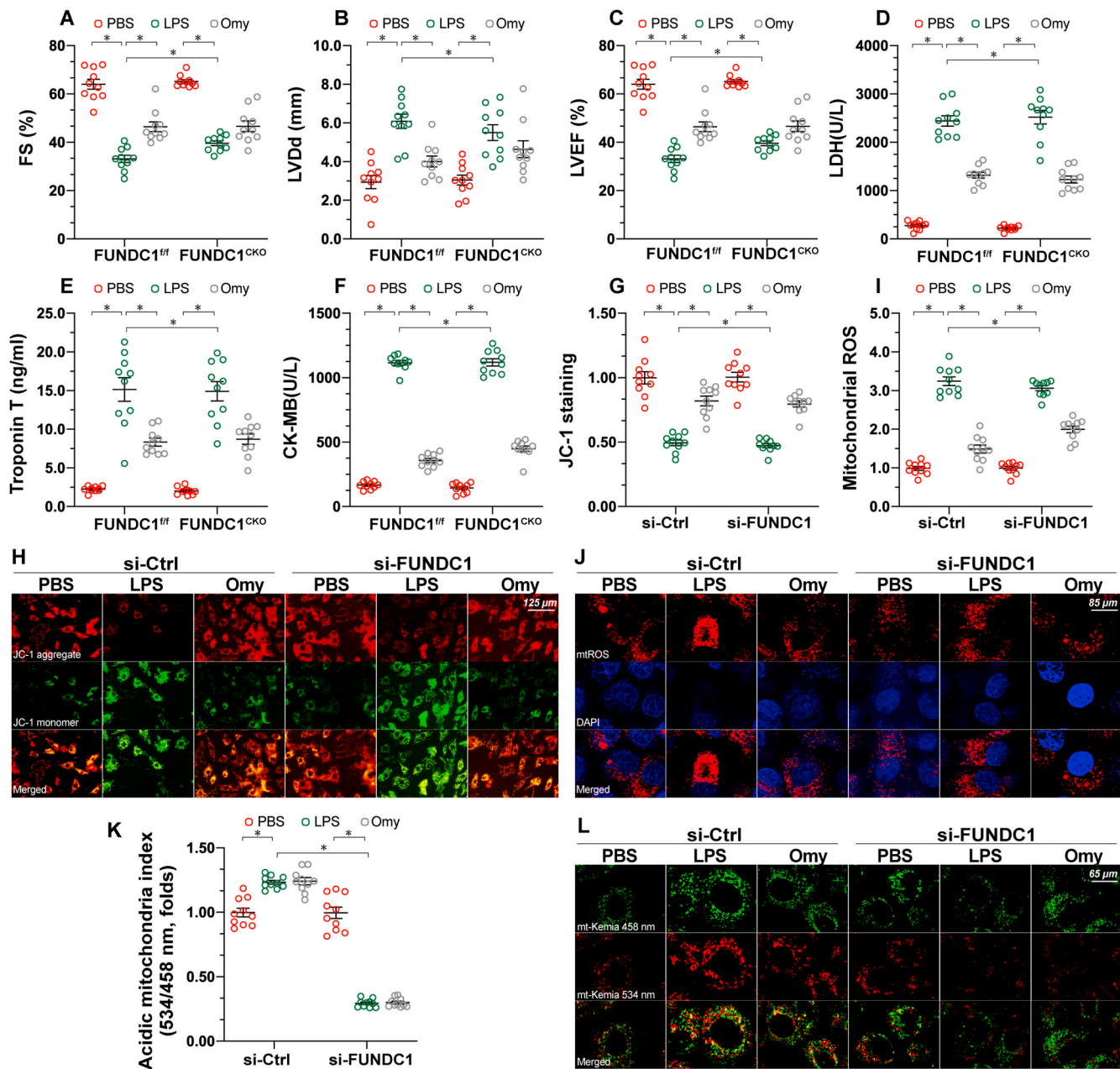


Fig. 4. Activation of UPR^{mt} partly reduces sepsis-induced myocardial injury and mitochondrial dysfunction in FUNDC1-knockout mice. FUNDC1^{fl/fl} and cardiomyocyte-specific FUNDC1 knockout (FUNDC1^{CKO}) mice were injected intraperitoneally with a single dose of PBS or LPS (5 mg/kg) to induce septic cardiomyopathy. Blood and heart samples were isolated 48 h after LPS injection. To induce UPR^{mt} *in vivo*, mice were injected with oligomycin (500 µg/kg) intraperitoneally in sterile saline with 0.1% DMSO. **A-C.** Echocardiographic data showing left ventricular ejection fraction (LVEF), left ventricular diastolic dimension (LVEDd), and fractional shortening (FS) in FUNDC1^{CKO} and FUNDC1^{fl/fl} mice in the presence of LPS or oligomycin. **D-F.** ELISA analysis of the concentration of cardiac injury markers, including LDH, troponin T, and CK-MB. **G-H.** The AC16 human ventricular cardiomyocyte cell line was treated with 10 µg/mL of LPS to induce sepsis-related cardiomyocyte damage. siRNA against FUNDC1 (si-FUNDC1) was transfected into AC15 to inhibit mitophagy activation. Oligomycin, an inducer of UPR^{mt}, was used to culture AC16 cells. Next, cells were stained with JC-1 to observe changes in the mitochondrial membrane potential. **I-J.** AC16 cells were stained with the MitoSOX red mitochondrial superoxide indicator to show changes in mitochondrial ROS. **K-L.** Mitophagy activity was observed using the mt-Keima assay. A yellow signal highlights increased mitophagic flux within cardiomyocytes. Data are presented as mean ± SEM, normalized per 1000 cardiomyocytes. n = 6 per group. *P < 0.05. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

involving a series of adaptive responses, including mitochondrial fission/fusion, mitophagy, UPR^{mt}, and mitochondrial biogenesis [59]. These biological events are termed mitochondrial quality control, and have been carefully reviewed in our previous studies [60,61]. Increased mitochondrial quality control activates UPR^{mt} to optimize mitochondrial protein import and export by upregulating gene transcription or promoting protein degradation [62,63]. If UPR^{mt} cannot completely repair the mitochondrial damage, mitochondrial fission is induced to

isolate the area of damage from the healthy mitochondrial network [64, 65]. Next, mitophagy is employed as a scavenger to eliminate structurally compromised mitochondria [25,66]. As mitophagy-mediated mitochondrial removal is usually followed by a decline in the number of mitochondria, mitochondrial biogenesis is always activated by mitophagy to complement the population and reinforce ATP synthesis [67,68]. Therefore, UPR^{mt} and mitophagy can be considered as distinct mitochondrial repair pathways; the former controlling mitochondrial

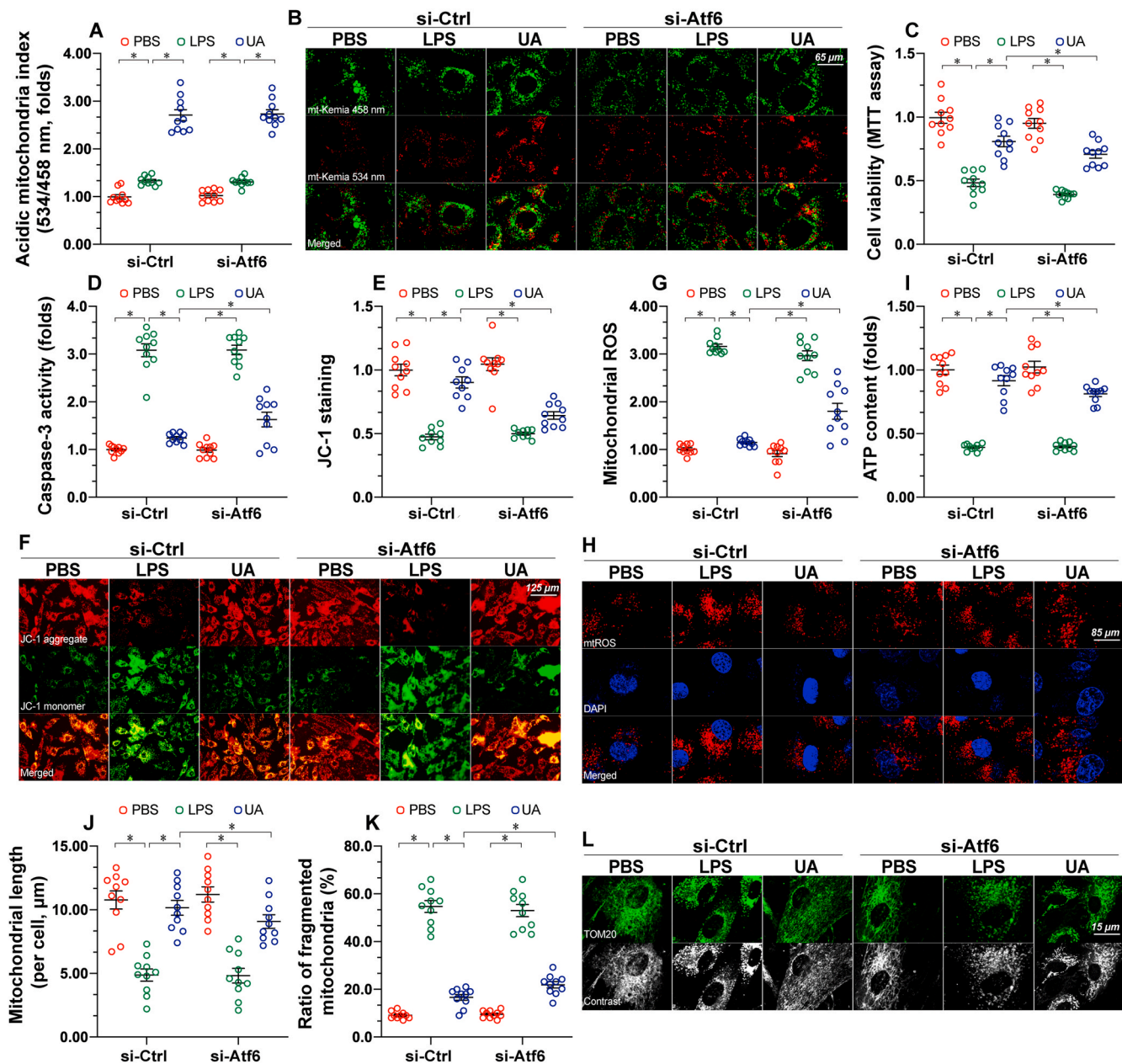


Fig. 5. Inhibition of UPR^{mt} partly abolishes the cardioprotective effect of mitophagy activation. The AC16 human ventricular cardiomyocyte cell line was treated with 10 $\mu\text{g}/\text{mL}$ of LPS to induce sepsis-related cardiomyocyte damage. UA, an inducer of mitophagy, was used to culture AC16 cells. siRNA against FUNDC1 (si-FUNDC1) and Atf6 (si-Atf6) were transfected into AC15 to inhibit the activation of mitophagy and UPR^{mt} , respectively. **A-B.** Mitophagy activity was observed using the mt-Keima assay. A yellow signal highlights increased mitophagic flux within cardiomyocytes. **C.** Cell viability was determined using an MTT assay. **D.** ELISA analysis of caspase-3 activity. **E-F.** AC16 cells were stained with JC-1 to observe changes in the mitochondrial membrane potential. **G-H.** AC16 cells were stained with the MitoSOX red mitochondrial superoxide indicator to show changes in mitochondrial ROS. **I.** Total ATP production was determined using the Cell Titer-Glo Luminescent Viability assay. **J-L.** Mitochondrial morphology was revealed using confocal immunofluorescence. TOM20 was used to show the shape of mitochondria in response to LPS or FUNDC1 deletion. Data are presented as mean \pm SEM, normalized per 1000 cardiomyocytes. * $P < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

proteomics and the latter modifying the mitochondrial number. The origin, regulation, and pathophysiological action of mitophagy in cardiovascular disease are well understood [60,61]. Moderate activation of mitophagy attenuates myocardial stress, whereas abnormal mitophagy induction is unexpectedly linked to cardiomyocyte death due to a sharp drop in residual mitochondria and intracellular ATP. In the present study, we explored the involvement of mitophagy in septic cardiomyopathy. Our data demonstrated that activation of FUNDC1-associated mitophagy protected the heart against LPS-induced sepsis by preserving mitochondrial function and structure. Loss of FUNDC1 repressed

mitophagy, resulting in mitochondrial dysfunction and cardiomyocyte death; this finding further develops the role of FUNDC1-dependent mitophagy in myocardial stress. Based on this finding, designing and developing approaches targeting FUNDC1-dependent mitophagy may be a promising tactic through which to improve cardiac performance during sepsis.

Unlike mitophagy, evidence of UPR^{mt} in cardiovascular pathophysiology is lacking. Previous studies have observed the cardioprotective action of UPR^{mt} in chronic cardiac hypertrophy and acute cardiac ischemia-reperfusion injury [26,27], concluding that baseline deletion

of UPR^{mt}-related genes, such as Atf6, has no significant impact on myocardial function and structure [26]. In response to stress, UPR^{mt} activity rapidly increases, and further enhancement of UPR^{mt} confers cardioprotection against chronic cardiac hypertrophy and acute cardiac ischemia-reperfusion injury [26,27]. These findings suggest that UPR^{mt}, as an adaptive response, could be induced by myocardial stress; although the extent of this activation appears to fail in preventing damage to the myocardium. Administration of a UPR^{mt} chemical agonist further augments UPR^{mt} activity, also exerting additional cardioprotective actions [27]. This finding highlights that endogenous UPR^{mt} is insufficient to benefit the myocardium; similar results were observed in the present study. Moreover, UPR^{mt} was slightly induced by LPS-associated sepsis; further augmentation of UPR^{mt} activity showed a protective effect on LPS-treated hearts. Our data, combined with previous evidence, provide novel insights into the endogenous physiological mechanisms of UPR^{mt}, also offering potential therapeutic targets for myocardial stress.

One of the key considerations is the relationship between mitophagy and UPR^{mt} [69,70]. As discussed above, UPR^{mt} appears to be activated prior to mitophagy induction [71]. As endogenous UPR^{mt} cannot completely repair mitochondrial damage, mitophagy activation is an inevitable consequence under stress conditions, regardless of UPR^{mt} activation. In the present study, we found that mitophagy activation had no influence on UPR^{mt} activity. Unexpectedly, inhibiting mitophagy through the genetic ablation of FUNDC1 significantly augmented endogenous UPR^{mt} activity, suggesting that UPR^{mt} may act as a compensatory mechanism in response to mitophagy repression. Inconsistent with our theoretical hypothesis, UPR^{mt} was found to be a downstream event of mitophagy. To validate our findings, we inhibited UPR^{mt} in the presence of a mitophagy inducer. In UPR^{mt}-inhibited cardiomyocytes, the mitophagy activation-mediated protection of mitochondria and cardiomyocytes was partly blunted, reconfirming that mitophagy-conferred cardioprotection, in part, requires UPR^{mt}. Overall, we concluded that: 1) endogenous UPR^{mt} and mitophagy can be slightly activated by myocardial stress, working together to sustain mitochondrial performance and cardiac function; 2) endogenous UPR^{mt}, as a downstream signal of mitophagy, plays a compensatory role in maintaining mitochondrial homeostasis during mitophagy inhibition; 3) although UPR^{mt} activation had no influence on mitophagy, its inhibition compromised the partial cardioprotective action of mitophagy.

This study has several limitations. First, as we only observed an association between FUNDC1-dependent mitophagy and UPR^{mt}, it remains unknown whether mitophagy mediated by other adaptors also acts as an upstream inducer of UPR^{mt}. Second, the UPR^{mt} loss-of-function assay was only performed *in vitro*; animal studies are necessary to further support our results. Third, although we determined the relationship between mitophagy and UPR^{mt}, the regulatory mechanism through which mitophagy controls UPR^{mt} has not been elucidated [17, 72].

Author contributions

H.Z. and X.C. involved in conception and design, performance of experiments, data analysis and interpretation, and manuscript writing; Y.-W., H.J. and S.T. involved in the development of methodology, D.M. and X.C. involved in the data acquisition, Y.-W., H.Z. and X.C. involved data analysis and interpretation; H.Z. involved in study supervision and final approval of manuscript.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declaration of competing interest

All authors declare that they have no conflict of interest.

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