Endogenous Drp1 Mediates Mitochondrial Autophagy and Protects the Heart Against Energy Stress

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Rationale: Both fusion and fission contribute to mitochondrial quality control. How unopposed fusion affects survival of cardiomyocytes and left ventricular function in the heart is poorly understood.

Objective: We investigated the role of dynamin-related protein 1 (Drp1), a GTPase that mediates mitochondrial fission, in mediating mitochondrial autophagy, ventricular function, and stress resistance in the heart.

Methods and Results: Drp1 downregulation induced mitochondrial elongation, accumulation of damaged mitochondria, and increased apoptosis in cardiomyocytes at baseline. Drp1 downregulation also suppressed autophagosome formation and autophagic flux at baseline and in response to glucose deprivation in cardiomyocytes. The lack of lysosomal translocation of mitochondrially targeted Keima indicates that Drp1 downregulation suppressed mitochondrial autophagy. Mitochondrial elongation and accumulation of damaged mitochondria were also observed in tamoxifen-inducible cardiac-specific Drp1 knockout mice. After Drp1 downregulation, cardiac-specific Drp1 knockout mice developed left ventricular dysfunction, preceded by mitochondrial dysfunction, and died within 13 weeks. Autophagic flux is significantly suppressed in cardiac-specific Drp1 knockout mice. Although left ventricular function in cardiac-specific Drp1 heterozygous knockout mice was normal at 12 weeks of age, left ventricular function decreased more severely after 48 hours of fasting, and the infarct size/area at risk after ischemia/reperfusion was significantly greater in cardiac-specific Drp1 heterozygous knockout than in control mice.

Conclusions: Disruption of Drp1 induces mitochondrial elongation, inhibits mitochondrial autophagy, and causes mitochondrial dysfunction, thereby promoting cardiac dysfunction and increased susceptibility to ischemia/reperfusion. (Circ Res. 2015;116:264-278. DOI: 10.1161/CIRCRESAHA.116.303356.)

Key Words: autophagy ■ Drp1 protein, mouse ■ heart ■ ischemia/reperfusion injury ■ mitochondria

The heart muscle is characterized by a large volume of mitochondria because of its high energy demand. Mitochondria produce ATP primarily by using the electrochemical gradient formed by electron transfer via the electron transport chain located on the inner mitochondrial membrane. However, electron leakage from the electron transport chain and production of $\text{O}_2^-$ and $\text{H}_2\text{O}_2$, which arises from dismutation of $\text{O}_3^-$, occur constantly as byproducts of ATP synthesis, making mitochondria a major source of reactive oxygen species in cardiomyocytes. Although reactive oxygen species at physiological levels act as signaling molecules to induce adaptive responses, dysregulated reactive oxygen species production in response to stress damages mitochondrial proteins, stimulating a feed-forward mechanism for reactive oxygen species production, mitochondrial dysfunction, and cell death, including apoptosis triggered by cytochrome c release and necrosis triggered by mitochondrial permeability transition pore (mPTP) opening. To protect against these catastrophic events, cells have intrinsic quality control mechanisms to maintain the overall health of mitochondria, including fusion, fission, and mitochondrial autophagy.

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Mitochondria are dynamic organelles that constantly undergo fusion and fission to adapt to changes in the cellular environment. Although mitochondrial fusion allows mitochondria to maintain membrane potential by fusing depolarized mitochondria to intact ones, fission allows the segregation of unrecoverable mitochondrial proteins so that they can be eliminated by autophagy or mitophagy, a specialized form of autophagy. Mitochondrial fusion is critically important to maintain the structural and functional integrity of the organelle. In contrast, mitochondrial fission is critical for maintaining mitochondrial quality and eliminating damaged mitochondria.
Nonstandard Abbreviations and Acronyms

<table>
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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>Ad-shDrp1</td>
<td>adenovirus harboring Drp1 shRNA</td>
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<td>Ad-shScr</td>
<td>adenovirus harboring scramble shRNA</td>
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<tr>
<td>α-MHC</td>
<td>alpha myosin heavy chain</td>
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<td>Drp1</td>
<td>dynamin-related protein 1</td>
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<td>Drp1-CKO</td>
<td>cardiac-specific conditional Drp1 knockout</td>
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<td>Drp1-hetCKO</td>
<td>cardiac-specific heterozygous Drp1 knockout</td>
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<td>EM</td>
<td>electron microscopic/microscopy</td>
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<td>GD</td>
<td>glucose deprivation</td>
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<td>Keima-MLS</td>
<td>Keima with mitochondrial localization signal</td>
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<tr>
<td>I/R</td>
<td>ischemia/reperfusion</td>
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<td>LV</td>
<td>left ventricular</td>
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<td>mPTP</td>
<td>mitochondrial permeability transition pore</td>
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<td>OCR</td>
<td>oxygen consumption rate</td>
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Drp1 Downregulation Stimulates Apoptosis in Cardiomyocytes

To evaluate the role of endogenous Drp1 in regulating mitochondrial morphology in cardiomyocytes, we constructed adeno- virus harboring Drp1 shRNA (Ad-shDrp1) and confirmed that Ad-shDrp1 decreases Drp1 in cardiomyocytes compared with adenovirus harboring scramble shRNA (Ad-shScr; Online Figure IA). To observe the morphology of mitochondria, cultured cardiomyocytes were cotransduced with adeno- virus harboring mitochondrially targeted DsRed2. Ninety-six hours after transduction, mitochondria in Ad-shDrp1–transduced cardiomyocytes were elongated compared with those in Ad-shScr–transduced cardiomyocytes (Figure 1A). The proportion of cardiomyocytes with elongated mitochondria, as defined by an average mitochondrial length >2 sarcomere units (Online Figure IB), was significantly greater in Ad-shDrp1–transduced cardiomyocytes than in Ad-shScr–transduced cardiomyocytes (Figure 1A). On the contrary, cardiomyocytes with foreshortened mitochondria, as defined by an average mitochondrial length <1 sarcomere unit (Online Figure IB), were markedly reduced in Ad-shDrp1–transduced cardiomyocytes. These results suggest that Drp1 is required for mitochondrial foreshortening in cardiomyocytes at baseline.

Transduction with Ad-shDrp1 significantly increased the number of terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)-positive cardiomyocytes (Figure 1B) and the amount of cleaved caspase 3 compared with transduction with Ad-shScr (Figure 1C), suggesting that endogenous Drp1 is essential in protection against apoptosis in cardiomyocytes. To exclude the possibility that our timing prevented observation of a period during which Drp1 downregulation-induced fusion is protective, we evaluated cardiomyocyte viability at various time points after transduction of Ad-shDrp1.

**Results**

**Statistical Analysis**

Data are expressed as mean±SEM. The difference in means between 2 groups was evaluated using the t-test. One-way ANOVA was used to compare multiple groups. Post hoc comparisons of considered pairs were performed using the Bonferroni post hoc test. P values of <0.05 were considered statistically significant. In figure legends, n indicates the number of experiments.

**Methods**

An expanded Methods section is available in the online Data Supplement.
and Ad-shScr. Viability time-dependently decreased between 0 and 96 hours after transduction of Ad-shDrp1 into cardiomyocytes and was significantly lower in Ad-shDrp1–transduced cardiomyocytes than in Ad-shScr–transduced cardiomyocytes after 72 hours (Figure 1D), indicating that Drp1 downregulation is persistently detrimental.

Endogenous Drp1 Mediates Autophagy and Mitochondrial Quality Control

We next examined the role of Drp1 in cardiomyocyte autophagy. Drp1 downregulation with Ad-shDrp1 significantly reduced LC3-II and increased p62/sequestosome 1 (SQSTM1), a protein degraded by autophagy (Figure 2A). To evaluate autophagic flux, cardiomyocytes were treated with chloroquine, which inhibits fusion of autophagosomes with lysosomes.20 Ad-shDrp1 significantly depressed chloroquine-induced accumulation of LC3-II, and accumulation of p62/SQSTM1 did not change significantly after chloroquine treatment in the presence of Ad-shDrp1 (Figure 2A), suggesting that Drp1 downregulation suppresses autophagic flux in cardiomyocytes at baseline.

To further evaluate the effect of Drp1 downregulation on autophagosome formation, cardiomyocytes were transduced with Ad-GFP-LC3. The number of GFP-LC3 dots was low at baseline, and there was no statistically significant difference between Ad-shScr– and Ad-shDrp1–transduced cardiomyocytes (Figure 2B). However, there was significantly less chloroquine-induced accumulation of GFP-LC3 dots in Ad-shDrp1–transduced cardiomyocytes than in Ad-shScr–transduced cardiomyocytes (Figure 2B), further supporting the idea that Drp1 downregulation suppresses autophagic flux at baseline.

There were significantly more mitochondria, as evaluated with real-time polymerase chain reaction of cytochrome b DNA and immunoblotting of cytochrome c oxidase subunit IV, in Ad-shDrp1–transduced cardiomyocytes than in control cardiomyocytes (Figure 2C and 2D), suggesting that suppression of autophagy caused by Drp1 downregulation leads to accumulation of mitochondria. Peroxisome proliferator-activated receptor-γ coactivator 1α expression was not significantly altered in Ad-shDrp1–transduced cardiomyocytes (Online Figure IC), suggesting that mitochondrial biogenesis was not affected. Because suppression of autophagy may impair mitochondrial quality control, we evaluated the effect of Drp1 downregulation on mitochondrial function.

Mitochondrial ATP production was significantly lower in cardiomyocytes transduced with Ad-shDrp1 than in those with Ad-shScr (Figure 2E). The effect of Drp1 downregulation on mitochondrial membrane potential was evaluated with JC-1. Drp1 knockdown led to the appearance of green JC-1 staining, indicating depolarization of the mitochondrial membrane potential in cardiomyocytes (Figure 2F). Furthermore, decreases in absorbance at 540 nm in mitochondrial swelling assays, indicative of mPTP opening, were significantly greater in cardiomyocytes with Drp1 knockdown than in control cardiomyocytes (Figure 2G), suggesting that mPTP opening is accelerated by Drp1 downregulation. Cyclosporin A attenuated cardiomyocyte death as evaluated with CellTiter Blue assays, suggesting that mPTP opening contributes to cardiomyocyte death in response to Drp1 downregulation (Figure 2H).

We also evaluated the rate of oxidative phosphorylation in cardiomyocytes, using a Seahorse analyzer (Online Figure IIA). We normalized the oxygen consumption rate (OCR) with either mitochondrial DNA content or cell viability to compensate for potential cell loss caused by cell death. The basal OCR was significantly lower in Drp1-downregulated cardiomyocytes than in control cardiomyocytes (Online Figure IIB). The OCR-linked ATP synthesis, as evaluated with oligomycin treatment, and the maximum respiratory rate, as determined by FCCP (trifluorocarbonyl cyanide phenylhydrazone) uncoupling, were also significantly lower in Drp1-downregulated cardiomyocytes than in control cardiomyocytes (Online Figure IIC and IID). The level of proton leak, determined by subtracting OCR-linked ATP synthesis from basal OCR, did not significantly differ between Drp1-downregulated and
control cardiomyocytes (Online Figure IIE). Together, these data indicate that Drp1 downregulation induces accumulation of mitochondria accompanied by mitochondrial dysfunction in cardiomyocytes.

Drp1 Mediates Mitochondrial Foreshortening in Response to Glucose Deprivation

We next investigated the involvement of Drp1 in mitochondrial dynamics in response to energy stress. Drp1 was localized...
primarily in the cytosol in control cardiomyocytes (Figure 3A). Glucose deprivation (GD), which is known to affect mitochondrial dynamics in other cell types,12,13 induced modest mitochondrial accumulation of Drp1 in cultured cardiomyocytes within 4 hours (Figure 3A), accompanied by a slight decrease in cytosolic Drp1, although the reduction did not reach statistical significance. GD-induced mitochondrial expression of Drp1 was also observed with anti-Drp1 immunostaining in mitochondrially targeted DsRed2 expressing cardiomyocytes (Figure 3B). These results suggest that GD increases Drp1 translocation from the cytosol to mitochondria in cardiomyocytes.

After 1 hour of GD, the proportion of cardiomyocytes with elongated mitochondria was increased, but that of cardiomyocytes with foreshortened mitochondria was also increased slightly in Ad-shScr–transduced cardiomyocytes (Figure 3C). A similar result was obtained in cardiomyocytes transduced with adenovirus harboring LacZ (Ad–LacZ; not shown). More than 50% of Ad-shDrp1–transduced cardiomyocytes exhibited foreshortened mitochondria after 1 hour of GD. After 4 hours of GD, however, >15% of Ad-shScr– or Ad-LacZ–transduced cardiomyocytes exhibited foreshortened mitochondria, whereas >50% still showed elongated mitochondria and <1% exhibited foreshortened mitochondria in Ad-shDrp1–transduced cardiomyocytes (Figure 3C). Thus, although GD induces transient mitochondrial elongation followed by foreshortening, Drp1 downregulation induces persistent increases in elongation irrespective of GD. These results suggest that Drp1 plays an essential role in mitochondrial foreshortening at baseline and during GD. Transduction with Ad-shDrp1 significantly increased TUNEL-positive cardiomyocytes after 1 and 4 hours of GD compared with transduction with Ad-shScr (Figure 3D), suggesting that endogenous Drp1 protects cardiomyocytes against apoptosis during GD.

We evaluated the role of endogenous Drp1 in mediating autophagy in response to GD. Four hours of GD significantly increased the number of GFP-LC3 dots in Ad-shScr–transduced cardiomyocytes, but this increase was significantly attenuated in Ad-shDrp1–transduced cardiomyocytes (Figure 3E). To evaluate autophagic flux, cardiomyocytes were cotransduced with adenovirus harboring tandem fluorescent mRFP-GFP-LC3.21 mRFP retains its fluorescence, whereas GFP loses its fluorescence in the acidic environment of lysosomes. In merged images, the red puncta that overlay green puncta and appear yellow indicate autophagosomes, whereas free red puncta indicate autolysosomes. After 4 hours of GD, the numbers of both yellow and free red dots were increased in Ad-shScr–transduced cardiomyocytes, indicating stimulation of autophagic flux. However, the GD-induced increases were attenuated in Ad-shDrp1–transduced cardiomyocytes (Figure 3F), suggesting that Drp1 downregulation inhibits GD-induced autophagic flux. Atg7 increases autophagic flux in cardiomyocytes.22,23 Drp1 downregulation significantly reduced Atg7-induced increases in autophagosomes and autolysosomes at baseline and in response to GD in cardiomyocytes (Figure 3G). Together, the data indicate that endogenous Drp1 plays an essential role in mediating mitochondrial foreshortening, autophagy, and cell survival during GD in cardiomyocytes.

Because Drp1 physically interacts with Bcl-xL in neurons24 and Bcl-xL inhibits autophagy through its binding to Beclin1, we investigated the involvement of Bcl-xL in the suppression of autophagy by Drp1. Using coimmunoprecipitation assays, we confirmed that Drp1 physically interacts with Bcl-2 and Bcl-xL in cardiomyocytes in the presence of Drp1 overexpression (Online Figure IIIA). Increased expression of Drp1 inhibited, whereas downregulation of Drp1 augmented the physical interaction between Beclin1 and Bcl-2 or Bcl-xL (Online Figure IIIB). Downregulation of Drp1 decreased the number of GFP-LC3 dots at baseline and in response to 4 hours of GD. However, the number of GFP-LC3 dots increased significantly when Drp1 was downregulated in the presence of Bcl-xL, downregulation with or without chloroquine20 (Online Figure IIIC and IIID). These results suggest that downregulation of Drp1 inhibits autophagy through a Bcl-xL–dependent mechanism, most likely by enhancing interaction between Beclin1 and Bcl-xL.

**Prolonged Treatment With Mdivi-1 Mimics the Effect of Drp1 Downregulation**

Because previous studies showed that suppression of Drp1 by mdivi-1 protects cardiomyocytes from cell death,11 we investigated the effect of mdivi-1 on mitochondrial morphology and cell death. Single treatment with mdivi-1 at 50 or 100 μmol/L for 1 hour significantly increased the number of cardiomyocytes with elongated mitochondria at baseline. Mdivi-1 at 100 μmol/L also prevented foreshortening of mitochondria after 4 hours of GD (Online Figure IVA). To compare the effects of Drp1 downregulation and mdivi-1 on survival of cardiomyocytes side-by-side, cardiomyocytes were treated with chelerythrine (10 μmol/L), an inducer of apoptosis,25 in the presence or absence of either mdivi-1 or Ad-shDrp1. Although Ad-shDrp1 transduction for 96 hours decreased cardiomyocyte survival at baseline and in response to chelerythrine, mdivi-1 treatment for 1 hour increased cardiomyocyte survival at baseline and in response to chelerythrine compared with vehicle treatment (Online Figure IVB). Mdivi-1 treatment at 50 μmol/L did not significantly affect cardiomyocyte viability in response to GD but the treatment at 100 μmol/L significantly reduced it (Online Figure IVC). Taken together, these results suggest that a single treatment with mdivi-1 has direct cell-protective effects on cardiomyocytes independent of Drp1. However, a higher dose of mdivi-1 partially mimics the effect of Drp1 downregulation even after a single application.

Treatment of cardiomyocytes with 50 μmol/L mdivi-1 every 24 hours for 1 week induced elongation of mitochondria at baseline and inhibited foreshortening of mitochondria even after 4 hours of GD (Online Figure IVD). Prolonged treatment with mdivi-1 time-dependently decreased cardiomyocyte viability compared with vehicle treatment (Online Figure IVE) and significantly suppressed GD-induced autophagic flux as evaluated with mRFP-GFP-LC3 (Online Figure IVF). Thus, prolonged treatment with mdivi-1 mimics the effect of Drp1 downregulation.

**Drp1 Mediates Autophagic Removal of Mitochondria**

We investigated whether clearance of mitochondria is regulated by Drp1 using mitochondria-targeted Keima fluorescence.19 Keima has a bimodal excitation spectrum peaking at
Figure 3. Dynamin-related protein 1 (Drp1) mediates mitochondrial fission, autophagy, and cell survival during glucose deprivation (GD). A, Immunoblots for Drp1, cytochrome c oxidase subunit IV (COX IV), and α-tubulin in cardiomyocyte mitochondrial and cytosolic fractions. *P<0.01 vs control (Ctr; n=3 per group). B, Immunohistochemistry for Drp1 and mitochondrialy targeted DsRed2 (mt-DsRed2). Green: Drp1, Red: mt-DsRed2. Scale bar, 50 µm. C, Assessment of mitochondrial morphology using mt-DsRed2. Insets show representative mitochondria. Gray bar: cells with elongated/total cell number, white bar: cells with foreshortened/total cell number, black bar: cells with foreshortened in adenovirus harboring scramble shRNA (Ad-shScr) at baseline; #P<0.01 vs foreshortened in Ad-shDrp1 at baseline; †P<0.01 vs foreshortened in adenovirus harboring scramble shRNA (Ad-shScr) at baseline; #P<0.01 vs foreshortened in Ad-shDrp1 at baseline; †P<0.01 vs foreshortened in Ad-shDrp1 at baseline. D, TUNEL staining of cardiomyocytes with Drp1 knockdown. *P<0.01 vs shScr Ctr; †P<0.01 vs adenovirus harboring Drp1 shRNA (Ad-shDrp1) Ctr; †P<0.01 vs Ad-shDrp1 at 1 hour GD; ††P<0.01 vs foreshortened in adenovirus harboring scramble shRNA (Ad-shScr) at baseline; #P<0.01 vs foreshortened in Ad-shDrp1 after 4 hours GD; **P<0.05 vs elongated in Ad-shScr at baseline. E, Representative images of GFP-LC3 puncta. Scale bar, 50 µm. F, Representative images of mRFP-GFP-LC3 puncta. Free red puncta indicate autolysosomes per cell. *P<0.01 vs Ad-shScr Ctr; #P<0.01 vs Ad-shDrp1 Ctr; †P<0.01 vs Ad-shDrp1 after 4 hours GD (n=5 per group). Scale bar, 200 µm. F, Representative images of GFP-LC3 puncta. Scale bar, 50 µm. G, Representative images of mRFP-GFP-LC3 puncta. Scale bar, 50 µm. Bar graph indicates mean number of autophagosomes per cell. *P<0.01 vs Ad-shScr Ctr; #P<0.01 vs Ad-shDrp1 Ctr; †P<0.01 vs Ad-shDrp1 after 1 hour GD; ††P<0.01 vs foreshortened in Ad-shScr after 1 hour GD; †††P<0.01 vs elongated in Ad-shScr at baseline; *P<0.01 vs control (Ctr; n=3 per group). H, Representative images of DsRed2-GFP-LC3 puncta. Scale bar, 50 µm. I, Representative images of mRFP-GFP-LC3 puncta. Scale bar, 50 µm. Bar graph indicates mean number of autophagosomes per cell. *P<0.01 vs Ad-shScr Ctr; †P<0.01 vs Ad-shDrp1 at baseline; †P<0.01 vs Ad-shDrp1 after 4 hours GD; **P<0.01 vs Ad-shDrp1 at baseline; ††P<0.01 vs Ad-shDrp1 at baseline; †††P<0.05 vs foreshortened in Ad-shScr at baseline; #P<0.01 vs Ad-shDrp1 at baseline; #P<0.01 vs Ad-shDrp1 at baseline; †P<0.01 vs Ad-shDrp1 after 4 hours GD (n=3 per group). G, Representative images of green fluorescent protein; RFP, red fluorescent protein; and TnI indicates troponin-I.
438±12 and 550±15 nm, corresponding to neutral and acidic pH states, respectively. Because fusion of autophagosomes with lysosomes exposes the autophagosome contents to acidic pH, the maturation of autophagosomes to autolysosomes can be monitored by measuring Keima fluorescence. We confirmed that Keima-MLS is expressed in cardiomyocyte mitochondria (Figure 4A). Puncta with a high ratio of excitation at 560/440 nm (high 560/440) colocalized with Alexa 488 Dextran colocalized with puncta with a high ratio of red to green, detected at 560 nm and 440 nm, respectively (n=3 per group). Scale bar, 20 μm. C and D, Representative images of Keima-MLS. Puncta with high ratio of 560/440 indicate mitochondrial autophagy. The ratio of the area of puncta with high ratio of 560/440 vs the total cell area is shown. C, Cardiomyocytes were transduced with Ad-Keima-MLS and then with adenovirus harboring scramble shRNA (Ad-shScr) or adenovirus harboring Drp1 shRNA (Ad-shDrp1). Some then underwent 4 hours of GD. *P<0.01 vs Ad-shScr control (Ctr); #P<0.01 vs Ad-shScr with GD (n=5 per group). Scale bar, 20 μm. D, Cardiomyocytes were transduced with Ad-Keima-MLS and Ad-shDrp1, followed by Ad-LacZ or adenovirus harboring Atg7 (Ad-Atg7). Scale bar, 20 μm. E, Cardiomyocytes were transduced with Ad-shScr or Ad-shDrp1 followed by Ad-LacZ or Ad-Atg7. Cell viability was evaluated with the CellTiter Blue assay. *P<0.01 vs Ad-shScr without Ad-Atg7; †P<0.01 vs Ad-shScr with Ad-Atg7 (n=3 per group). F, Representative electron microscopic images of cardiomyocytes in vitro. Asterisks indicate elongated mitochondria; open arrows, autophagic vacuoles without mitochondria; and closed arrows, autophagic vacuoles containing mitochondria. Scale bar, 2 μm. G, Left, The number of mitochondria per cell. *P<0.01 vs Ad-shScr without GD. Right, Relative mitochondrial mass per cell. Mitochondrial mass in cardiomyocytes transduced with Ad-shScr at baseline is expressed as 1. *P<0.01 vs Ad-shScr at baseline; †#P<0.01 vs Ad-shScr with GD. H, Left, The mean number of autophagic vacuoles per cell. *P<0.01 vs Ad-shScr without GD; †P<0.01 vs Ad-shScr with GD; †#P<0.01 vs Ad-shDrp1 without GD. Right, The number of autophagic vacuoles containing mitochondria per cell. *P<0.01 vs Ad-shScr with GD. COX IV indicates cytochrome c oxidase subunit IV.

Figure 4. Dynamin-related protein 1 (Drp1) mediates mitochondrial autophagy during glucose deprivation (GD). A, Cardiomyocytes were transduced with adenovirus harboring LacZ (Ad-LacZ) or adenovirus harboring Keima with mitochondrial localization signal (Ad-Keima-MLS), and cytosolic and mitochondrial fractions were analyzed by immunoblot (n=3 per group). B, Cardiomyocytes were treated with cyanide 3-chlorophenylhydrazone (CCCP; 25 μmol/L) for 16 hours. Representative images of Keima-MLS. Lysosomes visualized with Alexa 488 Dextran colocalized with puncta with a high ratio of red to green, detected at 560 nm and 440 nm, respectively (n=3 per group). Scale bar, 20 μm. C and D, Representative images of Keima-MLS. Puncta with high ratio of 560/440 indicate mitochondrial autophagy. The ratio of the area of puncta with high ratio of 560/440 vs the total cell area is shown. C, Cardiomyocytes were transduced with Ad-Keima-MLS and then with adenovirus harboring scramble shRNA (Ad-shScr) or adenovirus harboring Drp1 shRNA (Ad-shDrp1). Some then underwent 4 hours of GD. *P<0.01 vs Ad-shScr control (Ctr); #P<0.01 vs Ad-shScr with GD (n=5 per group). Scale bar, 20 μm. D, Cardiomyocytes were transduced with Ad-Keima-MLS and Ad-shDrp1, followed by Ad-LacZ or adenovirus harboring Atg7 (Ad-Atg7). Scale bar, 20 μm. E, Cardiomyocytes were transduced with Ad-shScr or Ad-shDrp1 followed by Ad-LacZ or Ad-Atg7. Cell viability was evaluated with the CellTiter Blue assay. *P<0.01 vs Ad-shScr without Ad-Atg7; †P<0.01 vs Ad-shScr with Ad-Atg7 (n=3 per group). F, Representative electron microscopic images of cardiomyocytes in vitro. Asterisks indicate elongated mitochondria; open arrows, autophagic vacuoles without mitochondria; and closed arrows, autophagic vacuoles containing mitochondria. Scale bar, 2 μm. G, Left, The number of mitochondria per cell. *P<0.01 vs Ad-shScr without GD. Right, Relative mitochondrial mass per cell. Mitochondrial mass in cardiomyocytes transduced with Ad-shScr at baseline is expressed as 1. *P<0.01 vs Ad-shScr at baseline; †#P<0.01 vs Ad-shScr with GD. H, Left, The mean number of autophagic vacuoles per cell. *P<0.01 vs Ad-shScr without GD; †P<0.01 vs Ad-shScr with GD; †#P<0.01 vs Ad-shDrp1 without GD. Right, The number of autophagic vacuoles containing mitochondria per cell. *P<0.01 vs Ad-shScr with GD. COX IV indicates cytochrome c oxidase subunit IV.
Forced Drp1 Overexpression Induces Apoptosis in Cardiomyocytes

Adenovirus-mediated overexpression of Drp1, which is higher than the level induced by GD, induced foreshortening of mitochondria in cardiomyocytes at baseline by 5-fold (Online Figure VIA and VIB). Under this condition, increases in apoptosis, decreases in mitochondrial DNA, and decreases in mitochondrial membrane potential were observed (Online Figure VIC–VIE). These results suggest that persistent and high-level expression of Drp1 induces mitochondrial dysfunction and apoptosis in cardiomyocytes. Drp1 overexpression significantly increased Keima-MLS puncta with high 560/440 in cardiomyocytes both at baseline and after 4 hours of GD (Online Figure VIF), indicating stimulation of lysosomal removal of mitochondria. Interestingly, suppression of autophagy by Ad-shAtg7 attenuated the increased cell death induced by Drp1 overexpression (Online Figure VIG), suggesting that excessive activation of autophagy by Drp1 may induce cell death.

Basal Characterization of Drp1-CKO Mice

To evaluate the role of endogenous Drp1 in vivo, we used loss-of-function mouse models. No homozygous mice were born during attempts to generate Drp1-CKO mice using αMHC-MCM mice. Therefore, to examine the effect of Drp1 on cardiac function in adult mice in vivo, we generated Drp1-CKO mice by crossing Drp1 flox homo (fl/fl) and αMHC-MCM mice, and Drp1 expression was downregulated in a tamoxifen-dependent manner. We used Drp1-CKO without TI and Drp1 fl/fl with or without TI as controls. Fifteen-week-old male mice were subjected to TI (20 mg/kg, IP) for 5 days. Four and 8 weeks after TI, we measured cardiac function and performed biochemical and histological analyses (Online Figure VIIA). Immunoblot analyses confirmed that cardiac Drp1 levels were significantly lower in Drp1-CKO mice than in control mice (Figure 5A) and that Drp1 was downregulated in a heart-specific manner in Drp1-CKO mice (Online Figure VIIB). Cardiac levels of other proteins involved in mitochondrial dynamics, such as mitofusin 1, mitofusin 2, OPA1, and fission 1, were unaltered in Drp1-CKO mice compared with control mice (Online Figure VIII). Drp1-CKO mice started to die 8 weeks after TI and all died by 13 weeks after injection, whereas no control mice died during the observation period of 16 weeks after TI. Kaplan–Meier analysis revealed that the survival rate was significantly lower in Drp1-CKO mice than in control mice (Online Figure VIID). Four weeks after TI, the hearts of Drp1-CKO mice were enlarged compared with control hearts (Figure 5B). Postmortem assessment showed that both left ventricular (LV) weight/tibial length, an index of LV hypertrophy, and lung weight/tibial length, an index of lung congestion, were significantly greater in Drp1-CKO than in control mice 4 and 8 weeks after TI (Online Tables I and II). Wheat germ agglutinin staining of LV sections 4 and 8 weeks after TI showed that cardiomyocyte cross-sectional area was significantly greater in Drp1-CKO than in control mice (Figure 5C; Online Figure VIIIE). Myocardial fibrosis, as evaluated with Picric Acid Sirius Red and Masson’s Trichrome staining, was also significantly greater in Drp1-CKO mice than in control mice (Figure 5D; Online Figure VIIIF and VIIIG). Echocardiographic measurements 4 and 8 weeks after TI showed that the LV diastolic dimension was significantly greater in Drp1-CKO mice than in control mice (Online Figures III and IV). Hemodynamic measurements at 4 weeks after TI showed that LV +dP/dt was decreased, whereas LV end-diastolic pressure was significantly elevated in Drp1-CKO compared with control mice (Online Table II). We confirmed that αMHC-MCM alone did not influence cardiac function or histology in either the presence or absence of tamoxifen (Online Figure VIII–VIIJ). Taken together, these results suggest that Drp1 downregulation induces LV dysfunction and cardiac hypertrophy at baseline.

488 Dextran, reflecting increased lysosomal localization of Keima-MLS, after treatment with 25 μmol/L of cyanide 3-chlorophenylhydrazone, a mitochondrial uncoupler, for 16 hours to induce mitochondrial autophagy (Figure 4B; Online Figure VA), confirming that Keima-MLS works as expected in cardiomyocytes. Puncta with high 560/440 in cardiomyocytes transduced with Ad-shScr, but not in cardiomyocytes transduced with Ad-shDrp1 (Figure 4C). This increase was abolished in the presence of Ad-shBeclin1-mediated Beclin1 downregulation (Online Figure VB), suggesting that it is mediated by autophagy and that Drp1 is necessary for stimulating autophagic mitochondrial degradation. Interestingly, downregulation of Beclin1 did not affect GD-induced increases in mitochondrial foreshortening (Online Figure VC) but significantly increased GD-induced cell death (Online Figure VD). Thus, although evidence suggests that unopposed fusion of mitochondria alone can induce cell death, suppression of autophagy alone may also induce cardiomyocyte death even when mitochondrial foreshortening is not affected.

Atg7 overexpression, which is known to stimulate autophagy, failed to increase puncta with high 560/440 in Drp1-downregulated cardiomyocytes (Figure 4D), even though it increased autophagosomes and autolysosomes in this condition (Figure 3F and 3G), nor did it inhibit Drp1 knockdown-induced cell death (Figure 4E).

To elucidate the role of endogenous Drp1 in autophagy further, cardiomyocytes were subjected to GD in the presence or absence of Drp1 knockdown and electron microscopic (EM) analyses were conducted (Figure 4F). Drp1 downregulation significantly reduced the number of mitochondria and increased relative mitochondria mass at baseline (Figure 4G). Drp1 downregulation also decreased the total number of autophagosomes at baseline and in response to GD and decreased the number of autophagosomes selectively containing mitochondria (Figure 4H). These results suggest that endogenous Drp1 is important in mediating both general autophagies, including mitochondrial autophagy.
Drp1 Downregulation Induces Mitochondrial Elongation and Dysfunction

To examine how Drp1 deletion affects mitochondrial morphology in the heart, EM analysis was performed. At baseline, mitochondria in control mice hearts were primarily rectangular or spherical in shape, whereas tubular mitochondria were observed less frequently. On the contrary, mitochondria in Drp1-CKO mice were mostly elongated/enlarged 4 and 8 weeks after TI (Figure 6A; Online Figure VIIIA). After 48 hours of fasting, mitochondria in control mice hearts became smaller and spherical. In contrast, mitochondria in Drp1-CKO mice remained elongated even after fasting (Figure 6A). Autophagosomes containing mitochondria were observed in control mice hearts after 48 hours of fasting, but not in Drp1-CKO mice hearts (Figure 6A). Quantitative analysis revealed that mitochondrial mass was significantly greater in Drp1-CKO mice hearts than in control mice hearts at baseline and after fasting (Figure 6A). These results suggest that endogenous Drp1 plays an essential role in mediating mitochondrial foreshortening at baseline and during fasting in mouse heart in vivo.

Four or 8 weeks after TI, Drp1 depletion increased the cytochrome c oxidase subunit IV protein level (Figure 6B; Online Figure VIIIB), and mitochondrial DNA content, evaluated with real-time polymerase chain reaction of cytochrome b DNA, was significantly greater in Drp1-CKO mice than in control mice (Figure 6C). These results suggest that the mitochondrial content is increased by Drp1 downregulation. Mitochondrial biogenesis was evaluated by immunoblot analyses of proliferator-activated receptor-gamma coactivator-1α and mitochondrial transcription factor A. Cardiac protein expressions of proliferator-activated receptor-gamma coactivator-1α and mitochondrial transcription factor A did not differ between Drp1-CKO and control mice 4 and 8 weeks after TI, suggesting that Drp1 depletion did not affect mitochondrial biogenesis (Figure 6D; Online Figure VIIIC). However, mitochondrial ATP production was significantly attenuated in Drp1-CKO mice hearts 4 and 8 weeks after TI, suggesting that Drp1 depletion did not affect mitochondrial biogenesis (Figure 6D; Online Figure VIIID). The activity of mitochondrial complexes I, II + III, and IV was also significantly attenuated in Drp1-CKO mice hearts 8 weeks after TI, compared with control mice hearts (Online Figure VIIIE). The extent of mPTP opening, as evaluated by the decrease in absorbance at 540 nm in mitochondrial swelling assays, was significantly greater in Drp1-CKO mice hearts 4 weeks after TI than in controls (Figure 6F), suggesting that mPTP opening is accelerated in Drp1-CKO mice. The cardiac levels of 4-hydroxynonenal, a marker of oxidative stress, and mitochondrial production of H₂O₂, evaluated with Amplex Red assays, were also significantly higher in Drp1-CKO mice hearts 4 weeks after TI than in control mice (Figure 6G and 6H). Thus, Drp1 depletion results in mitochondrial dysfunction and oxidative stress in the heart.

Because the initial assessment of mitochondrial function was conducted using hearts harvested 4 to 8 weeks after TI, when both hypertrophy and LV dysfunction are obvious in Drp1-CKO mice, mitochondrial dysfunction could be secondary to arteriosclerosis. Therefore, the initial assessment of mitochondrial function was performed 4 weeks after TI, when mitotic activity is obvious in Drp1-CKO mice. Therefore, the initial assessment of mitochondrial function could be secondary to arteriosclerosis.
pathological hypertrophy. We, therefore, also investigated an earlier time point. Echocardiographic analyses revealed no significant difference in LV ejection fraction between control and Drp1-CKO mice 10 days after TI (Online Table V), nor was there a significant difference in cardiomyocyte cross-sectional area or cardiac fibrosis (Online Figure VIIIIf and VIIIf), confirming that this time point precedes the development of pathological hypertrophy. Nevertheless, mitochondrial function, as assessed by ATP production and mitochondrial swelling assays, was already severely attenuated in Drp1-CKO mice compared with control mice 10 days after TI (Figure 6E and 6F). Together with the observation that Drp1 downregulation directly induces mitochondrial dysfunction in cultured cardiomyocytes (Figure 2E to 2H), these results suggest that Drp1 depletion induces mitochondrial dysfunction in the heart even before manifestation of hypertrophy and LV dysfunction.

We investigated whether Drp1 downregulation in the heart affects apoptosis. There were significantly more TUNEL-positive nuclei in Drp1-CKO mice hearts than in controls 4 and 8 weeks after TI (Figure 7A; Online Figure VIIIIf). Cleaved caspase-3 and cytochrome c release into the cytosolic fraction were also significantly elevated in Drp1-CKO mice hearts 4 weeks after TI (Figure 7B), as was the serum HMGB1 (high mobility group box 1) level, an indicator of necrosis (Online Figure VIIIIf). These results suggest that endogenous Drp1 is required for protection against the mitochondrial mechanisms of apoptosis and necrosis in cardiomyocytes.

**Autophagy Is Inhibited in Drp1-CKO Mice**

We next investigated the role of Drp1 in mediating autophagy in the heart in vivo. There was significantly less LC3-II and significantly more p62 in Drp1-CKO mouse hearts than in controls, 4 weeks after TI (Figure 7C). To examine whether Drp1 downregulation attenuates autophagic flux, we evaluated the effect of chloroquine injection on autophagosome accumulation. LC3-II accumulation was suppressed even in the presence of chloroquine, whereas p62/SQSTM1 accumulation did not change significantly after chloroquine treatment in the Drp1-CKO mouse heart (Figure 7D). To further evaluate the level of autophagic flux in cardiomyocytes in vivo, we crossed Drp1-CKO and Drp1 fl/fl with cardiac-specific mRFP-GFP-LC3 (tf-LC3) transgenic mice. Both Drp1-CKO × tf-LC3 and Drp1 fl/fl × tf-LC3 (control tf-LC3) were injected with tamoxifen for 5 days. Fasting increased the number of LC3 dots with both green and red color (appearing yellow in merged images), representing autophagosomes, as well as the number of dots with only red color, representing autolysosomes, in control tf-LC3 mice, indicating increased autophagic flux (Figure 7E). In contrast, the number of yellow and free red dots did not increase in response to fasting in Drp1-CKO × tf-LC3 mice (Figure 7E). Taken together, these results suggest that Drp1 downregulation suppresses autophagic flux at baseline.
Drp1 Depletion Induces Stress Intolerance and Enhances I/R Injury

Although mitochondrial fission and fusion are essential for maintaining mitochondrial quality control, their role in cardiac development and stress resistance remains unknown. To address this question, we crossed Drp1 flox/lox mice with αMHC-MCM mice. Although no mice with Drp1-CKO were born, mice with Drp1-hetCKO were viable at 12 weeks, suggesting that Drp1 is required for normal prenatal development but that 1 functional allele is sufficient during this period. Cardiac Drp1 expression was 40% lower in Drp1-hetCKO mice than in control (Drp1 flox/+) mice (Figure 8A). LV function, assessed by LV ejection fraction, was significantly lower in 12-week-old Drp1-hetCKO mice (Figure 8C), suggesting that mitochondrial dysfunction develops before histological and hemodynamic changes in Drp1-hetCKO mice. The fact that LV function is maintained at 12 weeks of age in Drp1-hetCKO mice allowed us to use these mice to examine the role of Drp1 during stress in the heart.

Mitochondrial Drp1 was significantly increased in response to 48-hour fasting or I/R but not in Drp1-hetCKO mice (Figure 8D). To evaluate the role of endogenous Drp1 in protection against stress in vivo, 12-week-old Drp1-hetCKO and control mice underwent 48-hour fasting. The LV ejection fraction was significantly lower in Drp1-hetCKO mice than in control mice after fasting, suggesting that endogenous Drp1 acts to preserve LV function during fasting (Figure 8E). Similar results were observed in Drp1-CKO mice with tamoxifen treatment (Online Figure IXC). To evaluate the role of endogenous Drp1 in protection against I/R, 12-week-old Drp1-hetCKO and control mice...
Figure 8. Cardiac-specific heterozygous dynamin-related protein 1 (Drp1) knockout (Drp1-hetCKO) mice develop cardiac dysfunction and are more susceptible to ischemia/reperfusion (I/R) injury. A, Immunoblots for Drp1 and α-tubulin. *P<0.01 vs control (Ctr; n=3 per group). B, Left ventricular (LV) ejection fraction (LVEF) at 12 weeks of age. There was no significant difference in LVEF between control and Drp1-hetCKO mice (n=4 per group). C, Relative ATP production. *P<0.01 vs Ctr (n=3 per group). D, Immunoblots for Drp1, cytochrome c oxidase subunit IV (COX IV), and α-tubulin in mitochondrial and cytosolic fractions. I/R: 30-minute ischemia and 24-hour reperfusion. *P<0.01 vs Ctr at baseline; #P<0.01 vs Ctr at baseline and Drp1-hetCKO at baseline and after fasting (Fat); †P<0.01 vs Ctr sham; *P<0.01 vs Ctr sham and Drp1-hetCKO sham and after Fat (n=3 per group). E, LVEF, as assessed by echocardiography. *P<0.01 vs Ctr at baseline, Ctr after Fat and Drp1-hetCKO at baseline (n=3 per group). F, Electron microscopic images of Drp1-hetCKO and control mice hearts. The inset shows mitochondrial autophagy seen only in control mice hearts after I/R. Asterisks indicate elongated mitochondria. Mitochondrial mass in control mice hearts without I/R is expressed as 1. *P<0.01 vs Ctr without I/R; #P<0.01 vs Ctr with I/R (n=3 per group). Scale bar, 2 μm. G, Immunoblots for LC3, p62, and α-tubulin. *P<0.01 vs Ctr without I/R; #P<0.01 vs Ctr with I/R (n=3 per group). H, Representative images of tetrazolium chloride/Alcian Blue staining of LV sections after I/R. Statistical analyses of % area at risk (AAR) and myocardial infarct/AAR are shown. *P<0.05 vs control (n=3 per group).

were subjected to 30 minutes of myocardial ischemia followed by 24 hours of reperfusion. EM analyses showed that I/R increased the number of smaller and spherical mitochondria in control mice, suggesting that mitochondrial fission was induced. However, these changes were significantly attenuated in Drp1-hetCKO mice (Figure 8F), suggesting that endogenous Drp1 mediates mitochondrial fission after I/R. Autophagosomes containing mitochondria were observed in control mice hearts but not in Drp1-hetCKO mice hearts after I/R (Figure 8F). There was also significantly less LC3-II and more p62 in Drp1-hetCKO mice hearts than in control hearts at baseline and after I/R (Figure 8G), suggesting that autophagy is suppressed by heterozygous Drp1 downregulation. The infarct size/area at risk after I/R, as evaluated with Alcian Blue and tetrazolium chloride staining, was not affected by αMHC-MCM alone (Online Figure 1XD) but was significantly greater in Drp1-hetCKO mice than in control mice (55.2±3.0% versus 40.2±1.6%; P<0.05; n=3 per group; Figure 8H). Similar results were observed in Drp1-CKO mice with tamoxifen treatment (Online Figure IXE). Taken together, these results suggest that inhibition of mitochondrial fission through Drp1 downregulation enhances myocardial injury in response to I/R.

We also evaluated the effect of mdivi-1 on I/R injury. One-time treatment with mdivi-1 just before I/R significantly reduced the infarct size/area at risk (Online Figure XA), confirming previous observations by others. However, the same treatment also reduced the infarct size/area at risk in Drp1-hetCKO mice, suggesting that short-term treatment with mdivi-1 protects the heart through Drp1-independent mechanisms (Online Figure XB). Although repetitive applications of mdivi-1 (1.2 mg/kg per day for 7 days) did not significantly reduce LV systolic function (Online Figure XC and Table VII), it significantly increased mitochondrial mass, as determined by EM (Online Figure XD), reduced mitochondrial function to a similar extent as heterozygous Drp1 downregulation, as determined by mitochondrial swelling assays and ATP production (Online Figure XE and XF), and significantly enhanced the infarct size/area at risk after I/R (Online Figure XG), thereby mimicking the effect of Drp1-hetCKO. Thus, although the effects of long-term treatment with mdivi-1 are similar to those of Drp1 downregulation with regard to I/R injury enhancement, albeit weaker, 1-time treatment with mdivi-1 seems to have protective effects, which are most likely independent of Drp1.
Discussion

Our results suggest that endogenous Drp1 induces mitochondrial foreshortening at baseline and in response to stress in the heart and the cardiomyocytes therein. Contrary to previous reports, downregulation of endogenous Drp1 in cardiomyocytes induces mitochondrial dysfunction and apoptosis, despite significant induction of mitochondrial elongation, thereby inducing cardiac dysfunction at baseline and exacerbating myocardial injury in response to I/R. Using Keima-MLS, we show that Drp1 plays an essential role in mediating lysosomal removal of mitochondria in cardiomyocytes. Thus, our results suggest that endogenous Drp1 contributes to mitochondrial quality control.

Although it is generally thought that fused mitochondria function better, Drp1 downregulation significantly increased the number of cardiomyocytes with depolarized mitochondria even at baseline. Although Drp1 is localized primarily in the cytosol in unstimulated cardiomyocytes, a low level of mitochondrial turnover mediated by Drp1 seems essential to maintain mitochondrial function in cardiomyocytes. Given that even heterozygous Drp1 downregulation induces mitochondrial dysfunction and heart failure in mice, it seems that endogenous Drp1 plays an essential role in mitochondrial quality control in the heart in vivo as well.

Whether mitochondria undergo fusion or fission during stress may depend on cell type and stress. In MEF cells, fasting induces mitochondrial fusion induced by phosphorylation of Drp1 at Ser637 by protein kinase A and translocation of Drp1 to the cytoplasm, which allows mitochondria to maintain ATP synthesis and escape autophagic destruction. On the contrary, in HL1 cells in vitro and cardiomyocytes in the heart in vivo, fasting and hypoxia stimulate mitochondrial fission. Regardless of whether fusion or fission is stimulated by stress, these studies showed that suppression of fission and stimulation of fusion through Drp1 downregulation, expression of dominant-negative Drp1, mdivi-1, or expression of mitofusin 1/2 promotes ATP production and cell survival. Here, we show that mitochondria in cardiomyocytes transiently undergo elongation during GD, but that the number of mitochondria with foreshortening also increases thereafter, accompanied by accumulation of Drp1 in mitochondria. Drp1 downregulation in this scenario blunted foreshortening of mitochondria and exacerbated cell death, suggesting that the induction of foreshortening is adaptive in cardiomyocytes.

Our results suggest that endogenous Drp1 is important in mediating autophagy in cardiomyocytes. Drp1 controls autophagic flux at least at the level of autophagosome formation because there were fewer GFP-LC3 puncta when Drp1 was downregulated in the presence of chloroquine, an inhibitor of autophagosome–lysosome fusion or autophagic flux. The suppressive effect of Drp1 downregulation upon global autophagy, rather than its specific effect on mitochondria-specific autophagy, was unexpected. We here show that Drp1 physically interacts with Bcl-2/Bcl-xL, and that downregulation of Drp1 promotes interaction between Beclin1 and Bcl-2/Bcl-xL. Because Bcl-2 and Bcl-xL are endogenous inhibitors of Beclin1 (Pattingre), increased interaction between Beclin1 and Bcl-2/Bcl-xL in the presence of Drp1 downregulation should lead to suppression of autophagy. In fact, the suppression of general autophagy by Drp1 downregulation was rescued by downregulation of Bcl-xL, indicating the critical role of the Bcl-2 family proteins in this process.

We here show that a GD-induced increase in lysosomal localization of Keima-MLS is attenuated in the presence of Drp1 downregulation. Given the mitochondrial localization of Keima-MLS and that Keima-MLS puncta with high 560/440, indicating acidic pH, are localized in lysosomes and are abolished when Beclin1 is downregulated, increases in Keima-MLS puncta with high 560/440 presumably reflect autophagic degradation of mitochondria. Thus, the significant reduction in lysosomal Keima-MLS puncta, together with EM images showing a significant reduction in autophagosomes primarily containing mitochondria, in Drp1 knockdown cardiomyocytes indicates that endogenous Drp1 plays an essential role in mediating GD-induced increases in mitochondrial autophagy. The Keima-MLS analysis was not sensitive enough to demonstrate a reduction in lysosomal removal of mitochondria at baseline when Drp1 is downregulated. However, given that dysfunctional mitochondria accumulate in Drp1-downregulated cardiomyocytes, it is likely that Drp1 also mediates autophagic mitochondrial degradation at baseline.

In this work, we used the term “mitochondrial autophagy” to describe the clearance of mitochondria by autophagy. Although our results suggest that Drp1 regulates mitochondrial clearance through general autophagy, whether or not Drp1 also affects mitochondria-selective autophagy, namely mitophagy, could not be evaluated because of technical limitations. To this end, specific assays to accurately evaluate the presence of mitophagy and specific interventions to modulate mitophagy seem essential.

Conditional Drp1 downregulation leads to decreases in cardiac function within 4 weeks, and all animals died within 13 weeks caused by heart failure. Histological analyses showed that the Drp1 deficiency induces hypertrophy and fibrosis in the heart and increases cardiomyocyte apoptosis. The fact that conditional cardiac-specific combined downregulation of mitofusin 1 and mitofusin 2 also leads to rapid development of cardiac dysfunction within 2 weeks indicates that both unopposed fission and unopposed fusion of mitochondria may cause cardiac dysfunction and suggests the critical importance of mitochondrial remodeling in the heart.

There are some differences between the cardiac phenotypes of Drp1-CKO and cardiac-specific combined downregulation of mitofusin 1 and mitofusin 2 knockout mice. For example, neither cardiac hypertrophy nor the increased cardiomyocyte apoptosis observed in Drp1-CKO were apparent in cardiac-specific combined downregulation of mitofusin 1 and mitofusin 2 knockout mice. This suggests that ATP depletion may be more profound in the absence of fission than in the absence of fission.

The reason for the opposite effects of Drp1 downregulation by genetic deletion and Drp1 suppression with mdivi-1 in response to I/R remains to be elucidated. One possibility is that our shRNA treatment may have induced stronger, more prolonged suppression of mitochondrial fission than a single dose of mdivi-1 at 50 μmol/L, the concentration used by others.
We observed modest cell death–promoting effects when cardiomyocytes were treated with mdivi-1 at a higher concentration (100 μM) or multiple times. A second possibility is that Drp1 downregulation may induce more potent suppression of general autophagy to even below physiological levels than mdivi-1. Although suppression of excessive autophagy may be salutary, suppression below physiological levels may be harmful. Third, mdivi-1 may more strongly suppress cell death than Drp1 downregulation by directly acting on apoptosis mechanisms. Mdivi-1 blocks Bax/Bak-dependent release of both Smac/Diablo and cytochrome c in HeLa cells, and we found that mdivi-1 inhibited chelerythrine-induced apoptosis in cardiomyocytes, which Drp1 downregulation did not. Furthermore, 1-time treatment with mdivi-1 reduced I/R injury even in Drp1-hetCKO mice, suggesting that mdivi-1 most likely has a Drp1-independent antiapoptotic function. Along this same line, mdivi-1 affects other molecules besides Drp1, including delayed rectifier K+ channels, raising the issue of specificity of the chemical inhibitor. Fourth, mitochondrial localization of Drp1 is positively regulated by protein kinase A, calcineurin, PUMA, Bax/Bak, ceramide, and O-linked-β-N-acetylglucosamine modification, and is negatively regulated by mir-499 and Pim1. Thus, some experimental conditions may induce excessive Drp1 activation/upregulation, which may in turn induce deleterious effects in cardiomyocytes. In fact, Drp1 overexpression in cardiomyocytes above the level caused by GD induced cell death. Drp1 suppression by mdivi-1 may be protective under such experimental conditions.

We have shown previously that Beclin1 haploinsufficiency inhibits I/R injury and suppresses autophagy. Here, we show that Drp1 haploinsufficiency exacerbates I/R injury but is also accompanied by suppression of autophagy. At present, mechanisms explaining the difference remain to be clarified. Drp1 downregulation may have a more pronounced effect on general autophagy and mitochondrial autophagy than Beclin1 downregulation, thereby suppressing autophagy below physiological levels. Another possibility is that Drp1 downregulation may more globally affect mitochondrial quality control mechanisms, including inducing unopposed mitochondrial elongation and suppression of global autophagy, rather than being limited to suppression of autophagic mitochondrial degradation. Further investigation is required to address this issue.

In summary, persistent Drp1 downregulation inhibits clearance of mitochondria by autophagy and causes mitochondrial dysfunction and consequent cell death in the heart and in the cardiomyocytes therein, both at baseline and under stress conditions. Drp1 plays an important role in mediating mitochondrial foreshortening and autophagic mitochondrial degradation in cardiomyocytes.

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**Disclosures**

None.

**References**


Dynamin-related protein 1 (Drp1) is a GTPase that mediates mitochondrial fission, causes mitochondrial fragmentation and dysfunction and heart failure in mice.

Dynamin-related protein 1 (Drp1) is a GTPase that mediates mitochondrial fission in noncardiac cells.

Pharmacological suppression of Drp1 with mdivi-1 attenuates myocardial injury in response to ischemia/reperfusion.

What New Information Does This Article Contribute?

Chronic downregulation of Drp1 induces elongation of mitochondria, mitochondrial dysfunction, heart failure and premature death in mice.

Downregulation of Drp1 inhibits general autophagy and movement of mitochondrial proteins into lysosomes in cardiomyocytes.

Chronic downregulation of Drp1 enhances myocardial injury in response to ischemia/reperfusion.

Mitochondria have the ability to remove damaged parts through the process of fission and fusion and consequent degradation through autophagy. Using a loss-of-function mouse model, we show that chronic downregulation of Drp1, a GTPase known to induce mitochondrial fission, causes mitochondrial dysfunction, myocardial cell death, heart failure, and the death of the animal. In vitro analyses show that genetic downregulation of Drp1 directly inhibits general autophagy in cardiomyocytes through Bcl-xL–dependent mechanisms. Furthermore, using mito-Kéima, a pH-sensitive protein, we show that endogenous Drp1 is essential for mitochondrial autophagy in response to glucose starvation in cardiomyocytes. Downregulation of Drp1 in turn causes accumulation of dysfunctional mitochondria and increased cell death. Furthermore, downregulation of Drp1 exacerbates ischemia/reperfusion injury in the mouse heart in vivo. These results suggest that endogenous Drp1 plays an important role in mediating mitochondrial autophagy and maintaining mitochondrial function in response to stress.
Endogenous Drp1 Mediates Mitochondrial Autophagy and Protects the Heart Against Energy Stress
Yoshiyuki Ikeda, Akihiro Shirakabe, Yasuhiro Maejima, Peiyong Zhai, Sebastiano Sciarretta, Jessica Toli, Masatoshi Nomura, Katsuyoshi Mihara, Kensuke Egashira, Mitsuru Ohishi, Maha Abdellatif and Junichi Sadoshima

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Supplemental Materials and Methods

Primary culture of neonatal rat ventricular myocytes

Primary cultures of ventricular cardiomyocytes (CMs) were prepared from 1-day-old Crl:(WI) BR-Wistar rats (Harlan). A CM-rich fraction was obtained by centrifugation through a discontinuous Percoll gradient as described.1

Glucose deprivation

To obtain starvation conditions, CMs were washed three times with phosphate-buffered saline (PBS) and incubated in glucose-free serum-free DMEM (11966-025, Invitrogen) at 37 °C as described.¹

Chloroquine treatment

To inhibit autophagic flux in vivo, chloroquine was injected (10 mg/kg) intraperitoneally as previously described.² Four hours later, animals were euthanized for immunoblot detection of autophagy markers. To inhibit autophagic flux in vitro, cultured CMs were treated with 10 mM chloroquine for 4 hours.

Adenovirus construction and transduction

Adenoviruses harboring GFP-LC3 (Ad-GFP-LC3),² tandem fluorescent mRFP-GFP-LC3 (Ad-tf-LC3),² mt-DsRed2,³ wild-type Atg7,³ LacZ (Ad-LacZ),³ hemagglutinin (HA)-tagged Drp1,⁴ Beclin1 short hairpin (sh) RNA (Ad-shBeclin1),¹ flag-tagged Beclin1,⁵ Bcl-xL shRNA (Ad-shBcl-xL),⁵ and Scramble shRNA (Ad-shScr)¹ have been described. Adenoviruses harboring shRNA for Drp1 (Ad-shDrp1) and for Atg7 (Ad-shAtg7) were generated using the Admax system (Microbix) as previously described¹ using the following hairpin-forming oligos: 5’-

CGGCAATTGAGCTAGCATATTTCAAGAGAATGCTAGCTCAATGCGCTTTTTTA-3’ for
Ad-shDrp1 and 5’-
CGCGTCACAGCCCTGCCATATTCAAGAGATATGGCAGGGCTGTGACGCTTTTTA-3’
for Ad-shAtg7. Transductions with Ad-Drp1, Ad-Keima-MLS, Ad-mt-DsRed2, Ad-Atg7,
Ad-tf-LC3, Ad-GFP-LC3, and Ad-LacZ were carried out for 48 hours. Knockdown
adenoviruses, including Ad-shScr, Ad-shDrp1, Ad-shBeclin1, and Ad-shAtg7, were
transduced for 96 hours. Adenoviruses were transduced at 15 MOI.

**Evaluation of fluorescent LC3 puncta**

The method of evaluating tandem fluorescent LC3 puncta using Ad-tf-LC3 has been
described previously.2 CMs cultured on cover slips were transduced with Ad-GFP-LC3
or Ad-tf-LC3 at 15 MOI. Forty-eight hours after adenovirus transduction, the cells were
washed with PBS, fixed with 4% paraformaldehyde (PFA), mounted with a reagent
containing 4’,6-diamidino-2-phenylindole (DAPI) (Vectashield, Vector Laboratories), and
viewed under a fluorescence microscope (Nikon Eclipse E800). The number of GFP
and mRFP dots was determined by manual counting of fluorescent puncta from at least
4 different myocyte preparations with a 60X objective. At least 50 cells were scored in
each experiment. The nuclear number was evaluated by counting the number of DAPI-
stained nuclei in the same field. The number of dots/cell was obtained by dividing the
total number of dots by the number of nuclei in each microscopic field. For *in vivo*
determination of the number of fluorescent LC3 dots, fresh heart slices were embedded
in Tissue-Tek OCT compound (Sakura Finetechnical Co.) and frozen at -80°C. Sections
10 μm thick were obtained from the frozen tissue samples using a cryostat (CM3050 S;
Leica), air-dried for 30 min, fixed by washing in 95% ethanol for 10 min, mounted using
a reagent containing DAPI, and viewed under a fluorescence microscope.
Histological analysis

Histological analysis was performed as described. In brief, heart specimens were fixed with formalin, embedded in paraffin, and sectioned at 6 μm thickness. Interstitial fibrosis was evaluated by Masson’s Trichrome and Picric Acid Sirius Red (PASR) staining. The myocyte cross-sectional area was measured from images captured from wheat germ agglutinin (WGA)-stained sections. The outlines of 100–200 myocytes were traced in each section using NIH ImageJ.

Immunohistochemistry

The method of immunostaining has been described. CMs were stained with anti-Drp1 mouse monoclonal antibody (BD Transduction, 611112), anti-Troponin I rabbit polyclonal antibody (Santa Cruz, 15368), Alexa Fluor 488-conjugated goat anti-mouse IgG (Invitrogen), Alexa Fluor 488-conjugated goat anti-rabbit IgG (Invitrogen), and Vectashield mounting medium with DAPI (Vector Laboratories). Analyses were performed using fluorescence microscopy (Zeiss).

Electron microscopy

Conventional electron microscopy was performed as described previously. In brief, CMs were fixed in Karnofsky’s fixative and then postfixed in 1% osmium tetraoxide, dehydrated in a graded series of acetone concentrations, and embedded in Sparr resin. Sections of 98 nm thickness were placed on copper grids that were double-stained with uranyl acetate and lead citrate. Discs were examined with a JEOL 1200 electron microscope. Mitochondrial mass was analyzed using Image J. The average mitochondrial mass was calculated from 50 mitochondria per slide on three different slides.
Evaluation of mitochondrial morphology

Mitochondrial morphology was examined according to the modified methods described previously. At least 100 CMs transduced with mitochondria-targeted DsRed2 and immunostained with Troponin I were examined using confocal microscopy. Mitochondria whose length is shorter than one sarcomere (the distance between consecutive Z-bands) are defined as foreshortened, those whose length is longer than 2 sarcomeres are defined as elongated, and those whose length is longer than one sarcomere and shorter than 2 sarcomeres are defined as intermediate (mid). Cells displaying either predominantly (>50%) elongated or (>50%) foreshortened mitochondria were classified as cells with elongated or foreshortened mitochondria, respectively. Cells containing <50% elongated and <50% foreshortened mitochondria were classified as intermediate (mid).

Quantitative real-time PCR for mitochondrial DNA

Total DNA was extracted from mouse hearts using the Quick-gDNA MiniPrep kit (ZYMO RESEARCH) according to the manufacturer’s protocol. The mtDNA content was quantified by real-time PCR of cardiac DNA as described. Primer sequences used for cytochrome b and β-actin are as follows: 5’-CCACTTCATCTTTACCATATTATCGC-3’ (forward primer) and 5’-TTTTATCTGCATCTGAGTTTAA-3’ (reverse primer) for cytochrome b, and 5’-CTGCCTGACGGCCAGG-3’ (forward primer) and 5’-CTATGGCCTCAGGAAGTTTTGTC-3’ (reverse primer) for genomic β-actin.

Subcellular fractionation

Mitochondrial and cytosolic fractions were purified through a previously described procedure. Briefly, isolated mouse hearts were homogenized in 10 volumes of ice-cold
Buffer A [200 mM mannitol, 50 mM sucrose, 10 mM KCl, 1 mM EDTA, 10 mM Hepes-KOH (pH 7.4), 0.1% BSA, and a mixture of protease inhibitors]. Homogenates were centrifuged at 600 × g for 5 min at 4 °C. Supernatants were then centrifuged at 3,500 × g for 15 min at 4 °C. The pellets were resuspended in Buffer A and centrifuged at 1,500 × g for 5 min. The supernatants were centrifuged at 5,500 × g for 10 min at 4 °C, and then the pellets were suspended as the mitochondrial fraction in PBS containing protease inhibitors. The supernatant was further centrifuged at 100,000 × g for 60 min, and the resultant pellet and supernatant were used as microsomal and cytosolic fractions, respectively.

**ATP production assay**

The mitochondrial fraction of mouse hearts was prepared as described above. ATP production was measured with an ATP Bioluminescent Assay kit (Sigma). 25mg of mitochondria is incubated with ATP assay mix and MSH buffer containing 625 μM ADP and substrate (10mM pyruvate and 10mM malate).

**Mitochondrial complex activity assay**

The mitochondrial fraction was prepared from mouse hearts as described above. Electron transport chain complex activities were measured, using MitoCheck Complex I, II-III and IV Activity Assay Kit (Cayman Chemical Company, USA) according to the method described previously. Briefly, complex I was assayed by monitoring the rotenone-sensitive ubiquinone-1 (Q1)-stimulated NADH oxidation, complex II+III by measuring the rate of reduced cytochrome c formation using succinate as substrate, and complex IV by measuring ferrocytochrome c oxidation with or without KCN. Mitochondrial complex activities were normalized by the weight of mitochondria.
Mitochondrial swelling assay

The mitochondrial swelling assay was performed as described.\textsuperscript{7} In brief, 50 µg of isolated mitochondria from mice or 30 µg of isolated mitochondria from CMs were suspended in a swelling buffer [250 mM sucrose, 10 mM MOPS, 5 µM EGTA, 2 mM MgCl\textsubscript{2}, 5 mM KH\textsubscript{2}PO\textsubscript{4}, 5 mM pyruvate, and 5 mM malate] and incubated with 150 µM calcium chloride (CaCl\textsubscript{2}) in a final volume of 200 µL in a 96-well plate for 20 min. Absorbance was read at 540 nm.

Evaluation of mitochondrial membrane potential

In order to evaluate mitochondrial membrane potential/integrity, cultured CMs were stained with tetramethylrhodamine ethyl ester (TMRE) and 5,5',6,6'- tetrachloro-1,1',3,3'-tetraethylbenzimidazolocarbocyanine iodide (JC-1) using MitoPT® TMRE and MitoPT® JC-1 (ImmunoChemistry Technologies), respectively, according to the manufacturer’s instructions.

Mitochondrial flux analyses using the Seahorse system

To measure the rate of oxidative phosphorylation in intact CMs, a Seahorse XF24 Extracellular Flux Analyzer (Seahorse Bioscience, Billerica, MA, USA) was used according to the methods described previously.\textsuperscript{9} CMs were plated at a density of 120,000 cells/well in 24-well Seahorse assay plates. CMs were transduced with Ad-shScr or Ad-shDrp1 for 96 hours prior to measurement. One hour prior to the beginning of measurements, the medium was replaced with XF medium supplemented with 17.5 mM glucose and 1 mM pyruvate and incubated for 1 hour in a 37°C incubator without CO\textsubscript{2}. Oxygen consumption rate (OCR) was measured three times at baseline, followed by injection with oligomycin (1 µM) to measure the ATP-linked OCR. Carbonylcyanide-
p-trifluoromethoxyphenylhydrazone (FCCP, 3 μM), an uncoupler, was used to
determine maximal respiration, and rotenone (1 μM) and antimycin A (1 μM) were
injected to determine the non-mitochondrial respiration. Three independent experiments
were performed. OCR was normalized by the amount of mtDNA or the rate of CM cell
viability in each well.

**H₂O₂ measurement**

H₂O₂ production was measured with an Amplex Red H₂O₂ assay kit (Molecular Probes;
Invitrogen) as described.¹⁰

**Assays for measurement of oxidative stress**

Cardiac tissue homogenates were assessed for 4-Hydroxynonenal (4-HNE) content (Cell
Biolabs, Inc, San Diego, CA, USA) as described.¹¹

**Cell viability**

Cell viability was measured by CellTiter-Blue (CTB) assays (Promega) as described.¹ In
brief, CMs (1 X 10⁵ per 100 μl) were seeded onto 96-well dishes. After 24 hours, the
cells were incubated with complete medium or glucose-free medium in the presence or
absence of chelerythrine chloride (10 mM) or adenovirus vectors. Viable cell numbers
were measured on the indicated days by the CTB assay. The CTB assays were
performed according to the supplier's protocol. The experiments were conducted in
triplicate at least three times.

**Evaluation of apoptosis**

DNA fragmentation was detected *in situ* with the use of terminal deoxynucleotidyl
transferase dUTP nick end labeling (TUNEL), as described.¹ Nuclear density was
determined by manual counting of DAPI-stained nuclei in 6 fields for each animal with
the 40x objective, and the number of TUNEL-positive nuclei was counted by examining the entire section with the same power objective.

**Immunoblot analysis**

The methods used for preparation of cell lysates from *in vitro* and *in vivo* samples and for immunoblot analyses have been described previously.² For *in vitro* samples, protein lysates were prepared from myocytes cultured in 6 cm culture dishes using boiled (95 °C for 2 min) 2X SDS sample buffer containing 4% SDS, 20% glycerol, 120 mM Tris-HCl (pH 6.8), 0.01% bromophenol blue, and 5% beta-mercaptoethanol. The protein samples were immediately boiled again at 95 °C for 3 min. Heart tissue homogenates were prepared using RIPA buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1% SDS, 1% Igepal CA-630, and 0.5% sodium deoxycholate with protease inhibitors (Sigma, P8340) at a 1:400 dilution. The antibodies used include Drp1 (BD Transduction, 611112), Mfn1 (abcam, ab57602), Mfn2 (Sigma, M6319), OPA1 (BD Transduction, 612608), Fis1 (Santa Cruz, sc98900), LC3 (BML, M186-3), p62 (ORIGENE, TA307334), PGC-1α (Santa Cruz, sc-13067), TFAM (Sigma, SAB1401383), COX IV (Cell Signaling, 4844S), cytochrome c (Cell Signaling, 4272S), cleaved caspase 3 (Cell Signaling, 9664S), Keima (BML, M182-3), and α-tubulin (Sigma, T6199). Densitometric analyses were performed using Scion Image software (Scion).

**Immunoprecipitation**

Immunoprecipitation was performed according to the methods described previously.⁵ In brief, CMs were lysed with IGEPAL CA-630 buffer (50 mM Tris-HCl (pH 7.4), 1% IGEPAL CA-630, 10 mM EDTA, 150 mM NaCl, 50 mM NaF, 1 μM leupeptin and 0.1 μM aprotinin). Primary antibody was covalently immobilized on protein A/G agarose using
the Pierce Crosslink Immunoprecipitation Kit according to the manufacturer’s instructions (Thermo Scientific). Samples were incubated with immobilized antibody beads for at least 2 h at 4 °C. After immunoprecipitation, the samples were washed with TBS five times. They were then eluted with glycine-HCl (0.1 M, pH 3.5) and the immunoprecipitates were subjected to immunoblotting using specific primary antibodies and a conformation-specific secondary antibody that recognizes only native IgG (Cell Signaling).

**Hemodynamic analysis**

Echocardiography and measurement of LV +dP/dt were performed as described, using ultrasonography (Acuson Sequoia C256; Siemens Medical Solutions) and a high-fidelity microtip pressure transducer catheter (1.4 Fr, Model SPR-839; Millar Instruments, Houston, TX), respectively.

**I/R surgery and assessment of area at risk and infarct size**

Myocardial I/R was achieved by temporarily occluding the left anterior descending coronary artery (LAD) and then releasing the occlusion as described. The duration of ischemia was 30 min, and that of reperfusion was 24 hours. To demarcate the ischemic area at risk (AAR), Alcian blue dye (1%) was perfused into the aorta and coronary arteries. Hearts were excised, and LVs were sliced into 1 mm thick cross sections. The heart sections were then incubated with a 1% triphenyltetrazolium chloride solution at 37 °C for 10 min. The infarct area (pale), the AAR (not blue), and the total LV area from both sides of each section were measured using Adobe Photoshop (Adobe Systems Inc.), and the values obtained were averaged. The percentage of area of infarction and AAR of each section were multiplied by the weight of the section and then totaled from
all sections. AAR/LV and infarct area/AAR were expressed as a percentage as previously reported.¹

**Mdivi1 treatment**

CMs or mice were administered with mdivi1 (Sigma) as described.⁶
References


2012;303:H979-88.
**Online table I.** Measurement of organ weight parameters in Drp1-CKO mice 4 weeks after tamoxifen injection

<table>
<thead>
<tr>
<th></th>
<th>Drp1 flox/flox</th>
<th>αMHC-MerCreMer</th>
<th>Tamoxifen ip</th>
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<td>+</td>
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<tr>
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<td>4</td>
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<td>4</td>
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<tr>
<td>BW (g)</td>
<td>26.0±3.4</td>
<td>28.2±3.9</td>
<td>24.1±0.5</td>
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<td>Tibia length (mm)</td>
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<td>19.6±0.4</td>
<td>19.5±0.6</td>
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<tr>
<td>LV weight (mg)</td>
<td>84±4</td>
<td>86±3</td>
<td>83±2</td>
<td>105±13 *</td>
</tr>
<tr>
<td>LV weight/Tibia length (mg/mm)</td>
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<td>4.4±0.2</td>
<td>4.2±0.2</td>
<td>5.4±0.8 *</td>
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<tr>
<td>RV weight (mg)</td>
<td>15±3</td>
<td>14±3</td>
<td>12±3</td>
<td>21±4 *</td>
</tr>
<tr>
<td>RV/Tibia length (mg/mm)</td>
<td>0.8±0.2</td>
<td>0.7±0.1</td>
<td>0.6±0.1</td>
<td>1.1±0.2 *</td>
</tr>
<tr>
<td>Lung weight (mg)</td>
<td>166±4</td>
<td>162±12</td>
<td>163±16</td>
<td>191±16 *</td>
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<tr>
<td>Lung weight/Tibia length (mg/mm)</td>
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<td>9.8±0.8 *</td>
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<tr>
<td>Liver weight (mg)</td>
<td>983±111</td>
<td>945±73</td>
<td>935±123</td>
<td>1089±294</td>
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<tr>
<td>Liver weight/Tibia length (mg/mm)</td>
<td>50.3±5.8</td>
<td>48.8±3.7</td>
<td>47.9±6.7</td>
<td>56.3±16.5</td>
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</tbody>
</table>

BW: Body weight, LV: Left ventricle, RV: Right ventricle. * p<0.01 vs. Drp1 flox/flox without Tamoxifen ip, Drp1 flox/flox with Tamoxifen ip or Drp1 flox/flox X αMHC-MerCreMer without Tamoxifen ip.
**Online table II.** Measurement of organ weight parameters in Drp1-CKO mice 8 weeks after tamoxifen injection

<table>
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<th>Drp1-CKO</th>
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<tbody>
<tr>
<td>Drp1 flox/flox</td>
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</tr>
<tr>
<td>αMHC-MerCreMer</td>
<td>-</td>
<td>+</td>
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<tr>
<td>Tamoxifen ip</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>n</strong></td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td><strong>BW (g)</strong></td>
<td>26.3±2.6</td>
<td>27.5±2.3</td>
</tr>
<tr>
<td><strong>Tibia length (mm)</strong></td>
<td>20.2±0.9</td>
<td>20.4±0.4</td>
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<tr>
<td><strong>LV weight (mg)</strong></td>
<td>87.5±7.6</td>
<td>112.3±5.3</td>
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<tr>
<td><strong>LV weight/Tibia length (mg/mm)</strong></td>
<td>4.34±0.44</td>
<td>5.51±0.32 #</td>
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<tr>
<td><strong>RV weight (mg)</strong></td>
<td>13.0±2.4</td>
<td>25.0±5.1 #</td>
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<tr>
<td><strong>RV/Tibia length (mg/mm)</strong></td>
<td>0.64±0.09</td>
<td>1.23±0.26 #</td>
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<tr>
<td><strong>Lung weight (mg)</strong></td>
<td>158.0±13.9</td>
<td>199.0±21.0 *</td>
</tr>
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<td><strong>Lung weight/Tibia length (mg/mm)</strong></td>
<td>7.9±1.0</td>
<td>9.8±1.1 *</td>
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<tr>
<td><strong>Liver weight (mg)</strong></td>
<td>1079±141</td>
<td>1340±91 *</td>
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<tr>
<td><strong>Liver weight/Tibia length (mg/mm)</strong></td>
<td>53.6±8.7</td>
<td>65.7±4.1 *</td>
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</table>

BW: Body weight, LV: Left ventricle, RV: Right ventricle. * p<0.05 vs. Ctr, # p<0.01 vs. Ctr.
Online table III. Measurement of hemodynamic parameters in Drp1-CKO mice 4 weeks after tamoxifen injection

<table>
<thead>
<tr>
<th></th>
<th>Drp1 flox/flox</th>
<th>αMHC-MerCreMer</th>
<th>Tamoxifen ip</th>
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<table>
<thead>
<tr>
<th>Parameter</th>
<th>Drp1 flox/flox</th>
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<th>Tamoxifen ip</th>
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<tbody>
<tr>
<td>n</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>HR (beat/min)</td>
<td>495±34</td>
<td>495±34</td>
<td>488±30</td>
</tr>
<tr>
<td>LVEDP (mmHg)</td>
<td>2.5±1.0</td>
<td>2.5±1.0</td>
<td>3.5±1.9</td>
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<tr>
<td>LVSP (mmHg)</td>
<td>88.0±5.7</td>
<td>89.5±4.4</td>
<td>88.5±4.1</td>
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<tr>
<td>dP/dt Max (mmHg/sec)</td>
<td>7250±100</td>
<td>7500±890</td>
<td>7400±520</td>
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<tr>
<td>dP/dt Min (mmHg/sec)</td>
<td>7200±650</td>
<td>7500±380</td>
<td>7500±500</td>
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<tr>
<td>LVDd (X10^-2mm)</td>
<td>308±28</td>
<td>328±39</td>
<td>317±21</td>
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<tr>
<td>LVDs (X10^-2mm)</td>
<td>169±26</td>
<td>179±22</td>
<td>176±11</td>
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<tr>
<td>LVEF (%)</td>
<td>83.7±3.3</td>
<td>82.0±1.9</td>
<td>82.8±1.9</td>
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<tr>
<td>IVS (X10^-2mm)</td>
<td>80.5±1.3</td>
<td>79.5±1.7</td>
<td>79.8±1.7</td>
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<tr>
<td>LVPW (X10^-2mm)</td>
<td>79.8±1.7</td>
<td>80.5±2.1</td>
<td>80.0±0.8</td>
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</table>

HR: Heart rate, LVEDP: Left ventricular end diastolic pressure, LVSP: Left ventricular systolic pressure, LVDd: Left ventricular diastolic dimension, LVDs: Left ventricular systolic dimension, LVEF: Left ventricular ejection fraction, IVS: Interventricular septum, LVPW: Left ventricular posterior wall. * p<0.01 vs. Drp1 flox/flox without Tamoxifen ip, Drp1 flox/flox with Tamoxifen ip or Drp1 flox/flox X αMHC-MerCreMer without Tamoxifen ip.
**Online table IV.** Measurement of hemodynamic parameters 8 weeks after tamoxifen injection

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<td>Drp1 flox/flox</td>
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<td>+</td>
</tr>
<tr>
<td>αMHC-MerCreMer</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Tamoxifen ip</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>n</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>HR (beats/min)</td>
<td>498±79</td>
<td>493±54</td>
</tr>
<tr>
<td>LVDd (X10⁻²mm)</td>
<td>319±15</td>
<td>409±11 *</td>
</tr>
<tr>
<td>LVDs (X10⁻²mm)</td>
<td>175±15</td>
<td>339±24 *</td>
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<tr>
<td>LVEF (%)</td>
<td>83.2±4.4</td>
<td>41.9±12.9 *</td>
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</table>

HR: Heart rate, LVDd: Left ventricular diastolic dimension, LVDs: Left ventricular systolic dimension, LVEF: Left ventricular ejection fraction. * p<0.01 vs. Ctr.
**Online table V.** Measurement of hemodynamic parameters 10 days after tamoxifen injection

<table>
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<td>αMHC-MerCreMer</td>
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<td>+</td>
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<td>Tamoxifen ip</td>
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<td>+</td>
</tr>
<tr>
<td><strong>n</strong></td>
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<tr>
<td><strong>HR (beats/min)</strong></td>
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<tr>
<td><strong>LVDs (X10^-2mm)</strong></td>
<td><strong>178±15</strong></td>
<td><strong>177±16</strong></td>
</tr>
<tr>
<td><strong>LVEF (%)</strong></td>
<td><strong>81.9±4.8</strong></td>
<td><strong>82.0±5.9</strong></td>
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</tbody>
</table>

HR: Heart rate, LVDd: Left ventricular diastolic dimension, LVDs: Left ventricular systolic dimension, LVEF: Left ventricular ejection fraction.
**Online table VI.** Measurement of organ weight parameters in Drp1-hetCKO mice

| Drp1 flox/+ | + | + |
| αMHC-Cre   | - | + |
| Age (Week) | 12 | 12 |
| n          | 4  | 4  |
| BW (g)     | 22.1±2.4 | 22.4±2.5 |
| Tibia length (mm) | 18.2±0.3 | 18.2±0.6 |
| LV weight (mg) | 73.5±5.7 | 74.0±9.5 |
| LV weight/Tibia length (mg/mm) | 4.04±0.27 | 4.06±0.40 |
| RV weight (mg) | 13.0±3.8 | 13.0±3.8 |
| RV/Tibia length (mg/mm) | 0.71±0.21 | 0.73±0.26 |
| Lung weight (mg) | 147.0±7.4 | 146.5±7.0 |
| Lung weight/Tibia length (mg/mm) | 8.1±0.5 | 8.0±0.2 |
| Liver weight (mg) | 882±88 | 883±94 |
| Liver weight/Tibia length (mg/mm) | 48.5±4.9 | 48.4±3.9 |

BW: Body weight, LV: Left ventricle, RV: Right ventricle.
Online table VII. Measurement of organ weight parameters in mdivi-1-treated mice

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</tr>
<tr>
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<td>4</td>
<td>4</td>
</tr>
<tr>
<td>BW (g)</td>
<td>26.4±1.3</td>
<td>25.1±2.3</td>
</tr>
<tr>
<td>Tibia length (mm)</td>
<td>18.3±0.2</td>
<td>18.8±0.6</td>
</tr>
<tr>
<td>LV weight (mg)</td>
<td>88.8±7.7</td>
<td>88.0±2.7</td>
</tr>
<tr>
<td>LV weight/Tibia length (mg/mm)</td>
<td>4.84±0.40</td>
<td>4.69±0.23</td>
</tr>
<tr>
<td>RV weight (mg)</td>
<td>18.3±5.1</td>
<td>18.3±3.8</td>
</tr>
<tr>
<td>RV/Tibia length (mg/mm)</td>
<td>1.00±0.28</td>
<td>0.97±0.18</td>
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<tr>
<td>Lung weight (mg)</td>
<td>145.0±18.6</td>
<td>1461.5±11.5</td>
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<td>Lung weight/Tibia length (mg/mm)</td>
<td>7.9±1.0</td>
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<tr>
<td>Liver weight (mg)</td>
<td>858124</td>
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<td>Liver weight/Tibia length (mg/mm)</td>
<td>48.3±5.1</td>
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BW: Body weight, LV: Left ventricle, RV: Right ventricle.
Online Figure I

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<td>α-tubulin</td>
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Relative Drp1 Expression

B

Foreshortened mitochondria
Elongated mitochondria

TnI  mt-DsRed2 Merge

C

PGC1-α

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*
Online Figure I. Experimental validations. A, Construction of Ad-shDrp1. Representative immunoblots for Drp1 and α-tubulin are shown. * p<0.01 vs. Ad-shScr. The experiment was repeated 3 times. B, Definition of foreshortened and elongated mitochondria. Mitochondria whose length is shorter than one sarcomere (the distance between consecutive Zbands) are defined as foreshortened, those whose length is longer than 2 sarcomeres are defined as elongated, and those whose length is longer than one sarcomere and shorter than 2 sarcomeres are defined as intermediate (mid). TnI: Troponin-I, mt-DsRed2: mitochondria-targeted DsRed2. C, Immunoblot for PGC-1α and α-tubulin in CMs transduced with Ad-LacZ or Ad-shDrp1. The experiment was repeated 3 times.
Online Figure II. Downregulation of Drp1 inhibits mitochondrial function in CMs as assessed with a Seahorse system. **A**, Representative changes in OCR in CMs in response to oligomycin, FCCP, and rotenone plus antimycin A. Each data point represents the mean of 4 replicates. **B-E**, Bar graphs showing basal OCR (B), ATP-linked OCR (C), maximum OCR (D) and proton leak (E). Experimental data were normalized with mtDNA (middle columns) or cell viability (left columns). In all graphs, data from CMs transduced with Ad-shScr is expressed as 1. *p<0.05, #p<0.01 vs. Ctr. The experiment was repeated 3 times.
Online Figure III

A

Input

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IP: HA

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B

Input

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IP: Flag

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<tr>
<td>Ad-HA-Drp1</td>
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</tr>
<tr>
<td>Ad-sh-Drp1</td>
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C

shScr  | shDrp1  | shBcl-xL  | shBcl-xL  |
|-------|---------|-----------|-----------|

GD (-)  

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</table>

D

shScr  | shDrp1  | shBcl-xL |
|-------|---------|----------|

Ctr  

Chl 10μM 4Hr  

Number of GFP-LC3 dots / cell

Baseline  

GD4Hr  

Chl

-  +  -  +  

shScr  

shDrp1  

shBcl-xL
Online Figure III. Drp1 physically interacts with Bcl-2/Bcl-xL, thereby inhibiting interaction between Beclin1 and Bcl-2/Bcl-xL. Downregulation of Drp1 inhibits autophagy by stimulating interaction between Beclin1 and Bcl-2/Bcl-xL. A, CMs were transduced with Ad-HA-Drp1 or Ad-LacZ. Forty-eight hours after transduction, lysates were extracted for immunoprecipitation with HA antibody, followed by probing with Bcl-2 or Bcl-xL antibodies. Representative images are shown. B, CMs were transduced with Ad-Flag-Beclin1 either in the absence or presence of Ad-HA-Drp1 or Ad-sh-Drp1. Seventy-two hours after transduction, lysates were extracted for immunoprecipitation with Flag antibody, followed by probing with Bcl-2 or Bcl-xL antibodies. Representative images are shown. In A and B, experiments were repeated 3 times. C, Representative images of GFP-LC3 puncta. Scale bar: 50 μm. Bar graph indicates mean number of autophagosomes per cell. * p<0.01 vs. shScr without GD, # p<0.01 vs. shDrp1 without GD, † p<0.01 vs. shDrp1 with GD. D, Representative images of GFP-LC3 puncta in CMs incubated with chloroquine (10 μM) or vehicles for 4 hours. Scale bar: 50 μm. Chl: chloroquine. Bar graph indicates mean number of autophagosomes per cell. * p<0.01 vs. shScr without Chl, # p<0.01 vs. shScr with Chl, † p<0.01 vs. shDrp1+shBcl-xL without Chl. In C and D, 50 myocytes per group were evaluated in each experiment and experiments were repeated 5 times.
Online Figure IV. GD induces mitochondrial fission in CMs treated with mdivi-1.

A, Assessment of mitochondrial morphology using mt-DsRed2. CMs were treated with mdivi-1 (50 μM) or DMSO (vehicle) as control. Insets show typical mitochondrial morphology. Gray bar: cells with elongated/total cell number; black bar: cells with foreshortened/total cell number; white bar: cells with intermediate (mid)/total cell number. Base: baseline, Ctr: DMSO (control). a p<0.01 vs. Ctr foreshortened at baseline, b p<0.01 vs. Ctr foreshortened after 1 hour GD, c p<0.01 vs. Ctr foreshortened after 4 hours GD, d p<0.01 vs. mdivi-1 foreshortened at baseline, e p<0.01 vs. mdivi-1 after 1 hour GD, f p<0.01 vs. mdivi-1 foreshortened after 4 hours GD, g p<0.01 vs. Ctr elongated at baseline, h p<0.01 vs. Ctr elongated after 4 hours GD (n=4/group). Scale bar: 20 μm.

B, Upper panel: Cell viability of CMs with DMSO as a control or 50 μM mdivi-1. * p<0.01 vs. DMSO without chelerythrine, † p<0.01 vs. DMSO with 10 μM chelerythrine (n=4/group). Lower panel: Cell viability of CMs in Ad-shScr- or Ad-shDrp1-transduced CMs. * p<0.01 vs. Ad-shScr without chelerythrine, # p<0.01 vs. Ad-shScr with 10 μM chelerythrine, † p<0.01 vs. Ad-shDrp1 without chelerythrine (n=4/group). C, Cell viability of CMs with DMSO as a control or mdivi-1 at 2 different doses (50 μM and 100 μM). *p<0.05 vs Ctr or 50 μM mdivi-1 with GD. D, Assessment of mitochondrial morphology using mt-DsRed2. CMs were treated with mdivi-1 or the same volume of DMSO as control for 4 days. Insets show representative mitochondria. Gray bar: cells with elongated/total cell number; black bar: cells with foreshortened/total cell number; white bar: cells with intermediate (mid)/total cell number. a p<0.01 vs. Ctr foreshortened at baseline, b p<0.05 vs. Ctr foreshortened at baseline, c p<0.01 vs. Ctr foreshortened after 1 hour GD, d p<0.01 vs. Ctr foreshortened after 4 hours GD, e p<0.01 vs. Ctr elongated at baseline, f p<0.05 vs. Ctr elongated at baseline, g p<0.01 vs. Ctr elongated after 1 hour GD, h p<0.05 vs. Ctr elongated after 1 hour GD, i p<0.01 vs. Ctr elongated after 4 hours GD (n=4/group). Scale bar: 20 μm.

E, Time course of cell viability in CMs treated daily with DMSO or 50 μM mdivi-1, as evaluated with the CellTiter Blue assay (n=4/group). * p<0.01 vs. Ctr 72 hours after transduction, # p<0.01 vs. Ctr 96 hours after transduction.

F, Representative images of mRFP-GFP-LC3 puncta. Scale bar: 50 μm. Bar graph indicates mean number of autophagosomes and autolysosomes per cell. a p<0.01 vs. Ctr at baseline, b p<0.01 vs. Ctr with 4 hours GD, c p<0.01 vs. mdivi-1 at baseline, d p<0.01 vs. Ctr at baseline, e p<0.01 vs. Ctr with 4 hours GD, f p<0.01 vs. mdivi-1 at baseline (n=3/group).
Online Figure V

A

B

C

D

Relative cell viability

0 0.2 0.4 0.6 0.8 1.0 1.2

* 

Cells with elongated, foreshortened / total cell number (%)

Elongated
Mid Foreshortened

shScr shBeclin1

Relative cell viability

shScr + GD 4Hr

shBeclin1 + GD 4Hr
Online Figure V. Detection of CCCP-induced mitophagy using mitochondria-targeted Keima. A, Representative images of fluorescent Keima puncta in CMs with or without 25 μM CCCP treatment after transduction with adenovirus harboring mitochondria-targeted Keima (Ad-Keima-MLS). Inset shows punctum with high 560/440 ratio. Scale bar: 20 μm. B, CMs were transduced with Ad-Keima-MLS and either Ad-shScr or Ad-shBeclin1. Some were then subjected to 4 hours of GD. Inset shows foreshortened mitochondria. * p<0.01 vs. Ad-Scr with GD (n=5/group). Scale bar: 20 μm. C, Assessment of mitochondrial morphology using mt-DsRed2. CMs were transduced with Ad-shBeclin1 or Ad-shScr as control for 4 days. Insets show representative mitochondria. Gray bar: cells with elongated/total cell number; black bar: cells with foreshortened/total cell number; white bar: cells with intermediate (mid)/total cell number. a p<0.01 vs. shScr foreshortened at baseline, b p<0.01 vs. shScr foreshortened after 1 hour GD, c p<0.01 vs. shScr foreshortened after 4 hours GD, d p<0.01 vs. shBeclin1 elongated after 4 hours GD, e p<0.01 vs. shScr elongated at baseline, f p<0.01 vs. shScr elongated after 1 hour GD, g p<0.01 vs. shScr elongated after 4 hours GD, h p<0.01 vs. shBeclin1 elongated at baseline (n=4/group). Scale bar: 20 μm. D, Cell viability of CMs transduced with either Ad-shScr or Ad-shBeclin1 for 4 days followed by 4 hours GD. * p<0.01 vs. shScr with 4 hours GD (n=4/group).
Online Figure VI

A

B

C

D

E

F

G

Relative Drp1 Expression

LacZ  Drp1

α-tubulin

LacZ  Drp1

% TUNEL positive nuclei

LacZ  Drp1

TUNEL

DAPI

Green fluorescent cell number / total cell number (%)

LacZ  Drp1

Cells with elongated, foreshortened / total cell number (%)

LacZ  Drp1

Relative mt DNA content

LacZ  Drp1

Relative cell viability

shAtg7  

LacZ  Drp1

High (560/440) signal area / cell area (%)

LacZ  Drp1

Ratio

440 nm

560 nm

Ratio
Online Figure VI. Forced overexpression of Drp1 induces mitochondrial dysfunction and apoptosis in CMs. 

A, Construction of Ad-Drp1. Representative immunoblots for Drp1 and α-tubulin are shown. * p<0.01 vs. Ad-LacZ. 

B, Assessment of mitochondrial morphology using mt-DsRed2. The proportions of CMs with elongated and foreshortened mitochondria were quantitated. TnI staining indicates CM. Gray bar: cells with elongated /total cell number; black bar: cells with foreshortened /total cell number; white bar: cells with intermediate (mid)/total cell number. * p<0.01 vs. foreshortened in Ad-LacZ, # p<0.01 vs. elongated in Ad-LacZ (n=4/group). Scale bar: 20 μm. 

C, TUNEL staining of CMs with overexpression of Drp1. * p<0.01 vs. Ad-LacZ (n=3/group). Scale bar: 200 μm. 

D, Mitochondrial membrane potential was evaluated with JC-1. Red color indicates mitochondria in which membrane potential is maintained, whereas green color indicates depolarized mitochondria. Quantification of CMs with depolarized mitochondria is shown. * p<0.01 vs. Ad-LacZ (n=3/group). Yellow scale bar: 500 μm; white scale bar: 100 μm. 

E, Relative mitochondrial DNA content in CMs with Drp1 overexpression, as evaluated by PCR for cytochrome b. * p<0.01 vs. Ad-LacZ (n=3/group). 

F, Representative images of Keima fluorescent puncta after transduction with Ad-Keima-MLS. A high 560/440 ratio indicates mitophagy. Ctr: control. The proportions of the high ratio (560/440) signal area to the total cellular area are shown. * p<0.01 vs. Ctr of Ad-LacZ, # p<0.01 vs. GD of Ad-LacZ, † p<0.01 vs. Ctr of Ad-Drp1 (n=5/group). Scale bar: 20 μm. 

G, Cell viability in Ad-LacZ- or Ad-Drp1-transduced CMs, as evaluated with the CellTiter Blue assay. * p<0.01 vs. Ad-LacZ without Ad-shAtg7, # p<0.01 vs. Ad-LacZ with Ad-shAtg7, † p<0.01 vs. Ad-Drp1 without Ad-shAtg7 (n=8/group).
**Online Figure VII**

**A**

Drp1-CKO mice

Control mice

15 weeks old

Tamoxifen (20mg / ip)

for 5 days

16 weeks old

4 weeks

20 weeks old

4 weeks

24 weeks old

Measured hemodynamics and harvested tissue samples

**B**

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<th>Kidney</th>
<th>Heart</th>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-tubulin</td>
<td></td>
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Drp1 ~ 80kDa

α-tubulin ~ 50kDa

**C**

Mfn-1

Mfn-2

OPA1

Fis1

α-tubulin

Tamoxifen - + - +

Drp1 fl/fl + + + +

α-MHC MCM - - + +

Ctr

**D**

Survival rate (%)

Drp1-CKO

Control

0 10 20 30 (weeks)

Tamoxifen ip

(15-16 weeks old)

**E**

Ctr

Drp1-CKO

<table>
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<tr>
<th>Drp1 fl/fl</th>
<th>α-MHC MCM</th>
<th>Tamoxifen</th>
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<tr>
<td>+</td>
<td>-</td>
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Drp1 fl/fl × α-MHC MCM

Cross-sectional area (μm²)

Drp1 fl/fl + +

α-MHC MCM - +

Tamoxifen + +

8 weeks after tamoxifen ip

**F**

% Fibrosis

Tamoxifen - + - +

Drp1 fl/fl + + + +

α-MHC MCM - - + +

Ctr
Online Figure VII

**G**

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<tr>
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<td>+</td>
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<tr>
<td>α-MHC MCM</td>
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<td>+</td>
</tr>
<tr>
<td>Tamoxifen</td>
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8 weeks after tamoxifen ip

**H**

**I**

**J**

Cross-sectional area (μm²)

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% Fibrosis

% LVEF (%)

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Online Figure VII. Basal characterization of Drp1-CKO mice. A, A scheme of the experimental protocol. MCM: MerCreMer B, Immunoblots for Drp1 and α-tubulin in Drp1-CKO mice (n=3/group). C, Immunoblots for factors related to mitochondrial dynamics in Drp1-CKO and control mice (n=3/group). D, Kaplan–Meier curve of Drp1-CKO and control mice. p<0.05 vs. control (n=8/group). E, Assessment of CM cross-sectional area in control and Drp1-CKO mice using WGA staining 8 weeks after tamoxifen injection. Scale bar: 200 μm. * p<0.01 vs. Ctr (n=3/group). F, Masson’s trichrome staining of sections from Drp1-CKO and control mice. * p<0.01 vs. controls (n=4/group). Scale bar: 500 μm. G, Assessment of CM fibrosis in control and Drp1-CKO mice using Picric Acid Sirius Red (PASR) staining 8 weeks after tamoxifen injection. Scale bar: 500 μm. * p<0.01 vs. Ctr (n=3/group). H, Assessment of CM size in Tg-αMHC-MerCreMer and wild-type mice with or without tamoxifen using WGA staining. Scale bar: 200 μm. There was no significant difference between the 4 groups of mice (n=4/group). WT: wild-type. I, Assessment of fibrosis in Tg-αMHC-MerCreMer and wild-type mice with or without tamoxifen using PASR staining. Scale bar: 500 μm. There was no significant difference between the 4 groups (n=4/group). J, LV ejection fractions (LVEF), as evaluated with echocardiography 4 weeks after tamoxifen injection (n=4/group). In B, C and F, heart samples were harvested 4 weeks after tamoxifen injection. In E and G, heart samples were harvested 8 weeks after tamoxifen injection.
Online Figure VIII

A  8 weeks after tamoxifen ip

B

C

D

E

F

G

H

I
Online Figure VIII. Hypertrophy and mitochondrial dysfunction in Drp1-CKO mice. 

A, Electron microscope images of Drp1-CKO and control mouse hearts 8 weeks after tamoxifen injection. Asterisks indicate elongated mitochondria. Mitochondrial mass in control mouse hearts is expressed as 1. * p<0.01 vs. Ctr (n=3/group). Scale bar: 2 μm. 

B, Immunoblots for COX IV and α-tubulin in Drp1-CKO and control mice. * p<0.01 vs. Ctr 4weeks after tamoxifen ip, # p<0.01 vs. Ctr 8weeks after tamoxifen ip (n=3/group). 

C, Immunoblot for PGC-1α and α-tubulin in Drp1-CKO and control mice whose hearts were harvested 4 or 8 weeks after tamoxifen injection. 

D, Relative cardiac ATP production in Drp1-CKO and age-matched control mice 8 weeks after tamoxifen injection. * p<0.01 vs. Ctr (n=3/group). 

E, Respiratory chain complex activity in mitochondria from mouse hearts. Relative respiratory chain complex I, II+III, and IV activities are shown. The activity in mitochondria from Ctr mouse hearts is expressed as 1. *p<0.05 vs. Ctr (n=4/group). 

F, Assessment of CM size in control and Drp1-CKO mice using WGA staining 10 days after tamoxifen injection. Scale bar: 200 μm. There was no significant difference between the 2 groups (n=3/group). 

G, Assessment of CM fibrosis in control and Drp1-CKO mice using PASR staining 10 days after tamoxifen injection. Scale bar: 500 μm. There was no significant difference between the 2 groups (n=3/group). 

H, TUNEL staining of hearts in Drp1-CKO and control mice 8 weeks after tamoxifen injection. Arrows indicate TUNEL-positive nuclei. * p<0.01 vs. Ctr. Scale bar: 50 μm. 

I, Serum HMGB1 concentration in Drp1-CKO and age-matched control mice 4 weeks after tamoxifen injection was assessed by ELISA. * p<0.05 vs. Ctr (n=3/group). 

In A-E and H, samples were harvested 8 weeks after tamoxifen injection. In B and I, samples were harvested 4 weeks after tamoxifen injection.
**Online Figure IX. Basal characterization of Drp1-hetCKO mice.**

**B,** PASR staining to assess cardiac fibrosis. (n=3/group). Scale bar: 500 μm.  
**C,** LV ejection fraction, as evaluated with echocardiography, and +dP/dt, as evaluated by hemodynamic measurement. * p<0.01 vs. Ctr at baseline, # p<0.01 vs. Drp1-hetCKO at baseline. Fst: fasting (n=4/group).  
**D,** Representative images of heart sections with I/R injury. WT: wild-type. Statistical analysis of % area at risk (AAR) and ratio of infarct size to AAR are shown. There was no significant difference between Tg-αMHC-Cre and wild-type mice (n=3/group).  
**E,** Representative images of TTC/Alcian Blue staining of LV sections after I/R. All of the mice underwent I/R surgery 10 days after tamoxifen injection. Statistical analyses of AAR and infarct size/AAR are shown. * p<0.05 vs. Ctr (n=3/group).
Online Figure X

A

Ctr (DMSO) mdivi-1 (50μM)

B

Drp1-hetCKO

mdivi-1 - +

C

LVEF (%)

D

Ctr (DMSO 1week) mdivi-1 (1 week) Ctr (Drp1 fl/+)

Drp1-hetCKO

E

OD540nm

Time (min)

F

Relative ATP production

G

Ctr (DMSO, 1W) mdivi-1 (50μM, 1W)

AAR (%) Infarct / AAR (%)

AAR (%)

Infarct / AAR (%)

Relative mitochondrial mass

% Decrease in OD540

% Decrease in OD540

Relative ATP production
Online Figure X. Chronic treatment with mdivi-1 leads to mitochondrial dysfunction and exacerbates I/R injury. A, Representative images of TTC/Alcian Blue staining of LV sections after I/R. Mice were injected with mdivi-1 at 1.2 mg/kg or the same volume of DMSO once 30 minutes prior to myocardial ischemia. Statistical analyses of % area at risk (AAR) and infarct size/AAR are shown. * p<0.05 vs. Ctr (DMSO ip once) (n=3/group). B, Representative images of TTC/Alcian Blue staining of LV sections after I/R. Drp1-hetCKO mice were treated with mdivi-1 at 1.2 mg/kg or the same volume of DMSO once 30 minutes prior to myocardial ischemia. Statistical analyses of AAR and infarct size/AAR are shown. * p<0.05 vs. Drp1-hetCKO without mdivi-1 (n=3/group). C, LVEF of mice treated with mdivi-1 at 1.2 mg/kg or the same volume of DMSO. There was no significant difference in LVEF between control and mdivi-1-treated mice (n=3/group). D, Electron microscope images of hearts from mdivi-1-treated, Drp1-hetCKO and control mice. Mdivi-1 at 1.2 mg/kg or the same volume of DMSO was injected intraperitoneally for 1 week in the mdivi-1-treated and control groups, respectively. Asterisks indicate elongated mitochondria. Mitochondrial mass in control (injected with DMSO) mouse hearts is expressed as 1. * p<0.05 vs. Ctr (DMSO), # p<0.01 vs. Ctr (Drp1 fl/+) (n=3/group). Scale bar: 2 μm. E, Mitochondrial swelling induced by Ca²⁺. Each data curve in the left panel represents the average of 3 individual measurements. Right panel shows the decrease in optical density at 540 nm. * p<0.01 vs. Ctr (DMSO ip for 1 week), # p<0.01 vs. mdivi-1 ip for 1 week, † p<0.01 vs. Ctr (Drp1 fl/+) (n=3/group). F, Relative cardiac ATP production in mdivi-1 treated, Drp1-hetCKO and control mice. Mdivi-1 at 1.2 mg/kg or the same volume of DMSO was injected intraperitoneally for 1 week in the mdivi-1-treated and control groups, respectively. * p<0.01 vs. Ctr (DMSO), # p<0.01 vs. Ctr (Drp1 fl/+) at 12 weeks of age (n=3/group). G, Representative images of TTC/Alcian Blue staining of LV sections after I/R. Mice underwent treatment with mdivi-1 at 1.2 mg/kg or the same volume of DMSO for 1 week. Statistical analyses of % area at risk (AAR) and infarct size/AAR are shown. * p<0.05 vs. Ctr (DMSO ip for 1 week) (n=3/group).