Interdependence of Parkin-Mediated Mitophagy and Mitochondrial Fission in Adult Mouse Hearts

Moshi Song, Guohua Gong, Yan Burelle, Åsa B. Gustafsson, Richard N. Kitsis, Scot J. Matkovich, Gerald W. Dorn II

Rationale: The role of Parkin in hearts is unclear. Germ-line Parkin knockout mice have normal hearts, but Parkin is protective in cardiac ischemia. Parkin-mediated mitophagy is reportedly either irrelevant, or a major factor, in the lethal cardiomyopathy evoked by cardiac myocyte–specific interruption of dynamin-related protein 1 (Drp1)-mediated mitochondrial fission.

Objective: To understand the role of Parkin-mediated mitophagy in normal and mitochondrial fission–defective adult mouse hearts.

Methods and Results: Parkin mRNA and protein were present at low levels in normal mouse hearts, but were upregulated after cardiac myocyte–directed Drp1 gene deletion in adult mice. Alone, forced cardiac myocyte Parkin overexpression activated mitophagy without adverse effects. Likewise, cardiac myocyte–specific Parkin deletion evoked no adult cardiac phenotype, revealing no essential function for, and tolerance of, Parkin-mediated mitophagy in normal hearts. Concomitant conditional Parkin deletion with Drp1 ablation in adult mouse hearts prevented Parkin upregulation in mitochondria of fission-defective hearts, also increasing 6-week survival, improving ventricular ejection performance, mitigating adverse cardiac remodeling, and decreasing cardiac myocyte necrosis and replacement fibrosis. Underlying the Parkin knockout rescue was suppression of Drp1-induced hyper-mitophagy, assessed as ubiquitination of mitochondrial proteins and mitochondrial association of autophagosomal p62/sequestosome 1 (SQSTM1) and processed microtubule-associated protein 1 light chain 3 (LC3-II). Consequently, mitochondrial content of Drp1-deficient hearts was preserved. Parkin deletion did not alter characteristic mitochondrial enlargement of Drp1-deficient cardiac myocytes.

Conclusions: Parkin is rare in normal hearts and dispensable for constitutive mitophagic quality control. Ablating Drp1 in adult mouse cardiac myocytes not only interrupts mitochondrial fission, but also markedly upregulates Parkin, thus provoking mitophagic mitochondrial depletion that contributes to the lethal cardiomyopathy. (Circ Res. 2015;117:346-351. DOI: 10.1161/CIRCRESAHA.117.306859.)

Key Words: cardiomyopathies ■ mice ■ mitochondria ■ mitochondrial degradation ■ mitochondrial dynamics ■ Parkin protein

Mitochondria of adult cardiac myocytes are seemingly static, without apparent organelle fission and fusion. Nevertheless, interruption of mitochondrial fusion or fission pathways in cardiac myocytes provokes lethal cardiomyopathies. To resolve this paradox, we proposed functioning of mitochondrial fusion and fission factors in mitophagy. Thus, mitofusin 2 comediates mitochondrial fusion with mitofusin 1, but is also a mitochondrial receptor for the mitophagy effector, Parkin. A role for mitochondrial fission in mitophagy is less well defined. Three independent reports of cardiac myocyte–specific ablation of profission dynamin-related protein 1 (Drp1) described cardiomyopathy and abnormal mitophagy, but the underlying mechanisms are controversial; mitophagy was variously described as decreased, increased, or initiated independent of Parkin, but interrupted. Conclusions about

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Original received May 14, 2014; revision received May 28, 2015; accepted June 2, 2015. In May 2015, the average time from submission to first decision for all original research papers submitted to Circulation Research was 15.49 days.

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The online-only Data Supplement is available with this article at http://circres.ahajournals.org/lookup/suppl/doi:10.1161/CIRCRESAHA.117.306859/-/DC1.

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Circulation Research is available at http://circres.ahajournals.org

DOI: 10.1161/CIRCRESAHA.117.306859
Parkin-mediated mitophagy in the heart are uncertain because the germ-line Parkin knockout mouse has no baseline cardiac phenotype.9–11

A different approach is required to define Parkin’s role in normal and mitochondrial fission–defective hearts. Here, we determined how positive and negative regulation of cardiac myocyte Parkin affects the in vivo mouse heart and interrogated the role of Parkin-mediated mitophagy in the cardiomyopathy provoked by cardiac myocyte–specific ablation of Drp1. In normal hearts, Parkin-stimulated mitophagy is well tolerated and largely dispensable. However, Parkin is upregulated, stimulates mitophagy, and contributes to loss of mitochondria and cardiomyopathy in hearts defective in mitochondrial fission. These results reveal Parkin to be a stress-inducible factor with the potential to help or hurt the heart, depending on functional integrity of the mitophagy-mitochondrial dynamism interactome.

Methods

Detailed Methods are available in the Online Data Supplement.

Results

Parkin Is Upregulated in Mitochondrial Fission–Defective Mouse Hearts

Interrupting mitochondrial fission by cardiac myocyte–specific Drp1 ablation induces cardiomyopathy with loss of mitochondria (Online Figure I).8 Parkin localization to, and mitophagy of, mitochondria in Drp1-deficient cultured mouse embryonic fibroblasts (MEFs)8 suggested a role for Parkin. However, Parkin protein was not abundant in normal hearts (Online Figure II)4 and Parkin mRNA levels equate to <1 transcript per cell (Figure 1A). These low levels of Parkin challenge the notion that Parkin causes hyper-mitophagy in cardiac Drp1-deficiency unless Parkin is upregulated therein. Indeed, we observed ≈5-fold greater Parkin mRNA levels (Figure 1B), and anti-Parkin immunoblotting revealed massively increased myocardial Parkin (Figure 1C) after Drp1 ablation. Thus, myocardial Parkin protein increases out of proportion to Parkin-specific mRNA in hearts rendered defective for mitochondrial fission.
Activating Parkin-Mediated Mitophagy Is Not Intrinsically Damaging to Normal Mouse Hearts

To determine how increased Parkin affects hearts, we transgenically expressed human Parkin in mouse cardiac myocytes. As Parkin evokes mitophagy by translocating to, and polyubiquitinating membrane proteins of, mitochondria, we measured mitochondrial Parkin content and protein ubiquitination. Parkin was plentiful in the mitochondria-enriched 10,000g pellet fraction of Parkin transgenic hearts, and associated with mitochondrial protein polyubiquitination (Figure 2A). The autophagosomal protein microtubule-associated protein 1 light chain 3 (LC3-II) and its docking protein p62/sequestosome 1 (SQSTM1) were likewise increased in mitochondria, indicating activation of mitophagy (Figure 2A). When followed to 30 weeks of age, there was no evidence for cardiac enlargement, contractile dysfunction (Figure 2B and 2C; Online Figure III), or any adverse mitochondrial effect of Parkin (Figure 2D and 2E). Although heart phenotypes may emerge over greater time, increasing Parkin-activated mitophagy without provoking pathology in otherwise normal young adult mouse hearts.

Parkin Ablation Has Minimal Effects on Adult Mouse Hearts

The above results are consistent with observations that Parkin overexpression in other cell types is tolerated, even protective. Accordingly, Parkin upregulation cannot be the exclusive cause of dilated cardiomyopathy after cardiac myocyte Drp1 ablation. To test if Parkin upregulation is a contributory factor, while minimizing the potential for compensatory up-regulation of Parkin-independent mitophagy pathways that occurs with germ-line Park2 deletion, we created cardiac myocyte–specific Parkin knockout mice.

Mice with Lox-P sites flanking exon 7 of the Park2 (Parkin) gene were obtained from Lexicon Pharmaceuticals and bred to myh6-driven MER-Cre-MER (Figure 3A). Tamoxifen was administered to 8-week-old mice for cardiac myocyte–specific Park2 recombination. Cardiac Parkin-deficient mice exhibited no change in cardiac size, heart weight corrected for body weight, or left ventricular contractile function over 20 weeks (Figure 3B and 3C; Online Figure IV). Mitochondrial appearance, respiration, and abundance were normal (Figure 3D and 3E; Online Figure V). Likewise, flow cytometry of cardiac...
mitochondria revealed normal organelle size (forward scatter), polarization status (tetrarmethylrhodamine ethyl ester fluorescence), and reactive oxygen species (ROS) production (MitoSOX fluorescence; Figure 3E). Whereas absence of cardiac Parkin could prove to be detrimental with increasing age, within the current 20-week study it was not.

**Concomitant Ablation of Parkin Delays the Cardiac Phenotype Caused by Drp1 Deficiency**

As cardiac Parkin ablation had no deleterious effects on otherwise normal hearts, we assessed the scale of Parkin up-regulation in Drp1 deficiency by concomitantly deleting the Drp1 and Parkin genes in adult mouse hearts (Figure 4A). As reported,8 Drp1 ablation caused lethal-dilated cardiomyopathy. Concurrent Parkin ablation increased 6-week survival of cardiac Drp1-deficient mice, improving contractile function and protecting against adverse ventricular remodeling (Figure 4B and 4C; Online Figure VI), likely by suppressing cardiac myocyte necrosis and replacement fibrosis (Figure 4D). Parkin ablation moderated hyper-mitophagy in Drp1-deficient hearts as assessed by mitochondrial protein ubiquitin content and mitochondria association of p62/SQSTM1 and LC3-II (Figure 4E). The loss of mitochondria seen late after Drp1 ablation was consequently ameliorated (Figure 4F). As previously described,8 mitochondrial function was normal in Drp1-deficient hearts and was unaffected by concomitant Parkin deletion (Online Figure VII).

**Discussion**

These results help resolve ambiguous results relating to mitochondrial quality control in mammalian hearts. Parkin-mediated mitophagy acts as a stress-reactive pathway to remove damaged mitochondria as originally proposed by Huang et al,18 but seems unnecessary to routinely maintain mitochondrial quality. Absence of a housekeeper function can explain normal hearts in mice systemically lacking Parkin.11 Our work shows that Parkin-mediated hyper-mitophagy can contribute to cardiomyopathy, here induced by interrupting Drp1-mediated mitochondrial fission. In a perinatal cardiac Drp1-deficient model, Kageyama et al8 observed mitophagy that was not prevented by germ-line Parkin deletion. Our results do not address Parkin’s role in perinatal hearts, but...
strongly implicate Parkin in mitophagy induced by interrupting mitochondrial fission in adult hearts. The cardiac myocyte-specific Parkin-deficient and -overexpressing models developed for this project should prove useful for rigorous interrogations of Parkin-mediated mitophagy and mitochondrial clearance after other forms of cardiac stress, helping to better define the roles of Parkin-dependent and ROS-stimulated Parkin-independent mitochondrial quality control pathways.

These results provide in vivo support for Shirihai’s concept that mitophagy and Drp1 are inextricably linked through the mechanism of asymmetrical mitochondrial fission.19 This has ramifications for ongoing therapeutic efforts directed toward inhibiting mitochondrial fission in cardiac disease,20 as inhibition of Drp1 may concomitantly stimulate Parkin-mediated mitochondrial removal, ultimately reducing mitochondrial content.

Acknowledgments
Parkin floxed allele mice were obtained from, and used under an MTA with, Lexicon Pharmaceuticals, Inc.

Sources of Funding
This work was supported by National Institutes of Health HL59888 (G.W. Dorn) and HL128071 (G.W. Dorn and R.N. Kitsis) and an American Heart Association predoctoral fellowship award (M. Song).
Parkin-mediated mitophagy is induced and contributes to cardiomyopathy in mitochondrial fission–deficient hearts. This is supported by the observation that Parkin is highly expressed in hearts, but is not constitutively expressed at high levels in normal hearts, and is induced in mitochondrial fission–deficient hearts. However, it is not clear whether Parkin is constitutively expressed at high levels in normal hearts, or whether it is induced in response to stress.

References


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Circ Res. 2015;117:346-351; originally published online June 2, 2015; doi: 10.1161/CIRCRESAHA.117.306859

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SUPPLEMENTAL MATERIAL

Detailed Methods

Mouse Generation and Phenotypic Analyses
Drp1\textsuperscript{loxP/loxP} mice and Parkin\textsuperscript{loxP/loxP} mice (obtained from Lexicon Pharmaceuticals \textsuperscript{2}), individually and in combination, were bred with mice carrying a myh6 promoter-driven nuclear-directed modified estrogen receptor (MER)-Cre transgene \textsuperscript{3} to achieve tamoxifen-inducible gene deletion. Tamoxifen was administered to 8 week old adult mice as previously described \textsuperscript{4}. Cardiomyocyte-specific human Parkin overexpression was achieved using the myh6 promoter-driven doxycycline-suppressible (“tet-off”) bi-transgeic system \textsuperscript{5}; doxycycline was never administered to dama or pups, inducing transgene expression after birth \textsuperscript{6}. M-mode echocardiography was performed on unsedated mice \textsuperscript{4}; controls were age-matched Drp1\textsuperscript{loxP/loxP} littermates, Drp1\textsuperscript{loxP/loxP} + Parkin\textsuperscript{loxP/loxP} littermates with or without tamoxifen administration and tetracycline-controlled transactivator protein (tTA) transgenic mice. All experimental procedures were approved by the Washington University Institutional Animal Care and Use Committee.

RNA Expression Analysis
mRNA abundance of Parkin and associated mitophagy factors, mitochondrial dynamics factors, mitochondrial biogenesis factors, and representative cardiac-expressed genes were obtained by analyzing RNA sequence data on 25 normal adult FVB mouse hearts generated from this laboratory, available at NCBI GEO GSE55792 \textsuperscript{7}. Parkin mRNA abundance was also measured by quantitative real-time PCR. Total RNAs were extracted from snap-frozen cardiac tissues with TRIzol (Invitrogen, 15596-026); single strand cDNA was generated using high-capacity cDNA reverse transcription kit with RNase inhibitor (Invitrogen, 4374966) following the manufacturer’s protocol. RNA expression analysis was performed using Taqman qRT-PCR 2x master mix (Invitrogen, 4440038) with predesigned primer/probes sets for mouse Park2 (Mm00450187_m1) and GAPDH (Hs02758991_g1).

Western Blotting
Cardiac tissues were collected, snap-frozen in liquid nitrogen, and homogenized in either tissue extraction reagent (Invitrogen, FNN0071) or a homogenizing buffer (10 mM HEPES, 320 mM sucrose, 3 mM MgCl\textsubscript{2} and 1mM DTT), with protease inhibitor (Roche, 05892970001) and phosphatase inhibitor (Roche, 04906837001) as previously described \textsuperscript{4}. Myocardial homogenates were collected from the supernatant after centrifugation at 3,800 \textit{g}, myocardial mitochondrial fractions were pelleted and resuspended after centrifugation at 10,000 \textit{g}, and myocardial cytosolic fractions were collected from the supernatant after centrifugation at 100,000 \textit{g}. Proteins were size-separated by 4-15% pre-cast gradient SDS-PAGE (Bio-Rad, 456-1084 and 456-1086), transferred to PVDF membranes (GE Healthcare, 10600021), and blocked with phosphate-buffered saline (PBS, Invitrogen, 70011-069) containing 0.1% Tween-20 (Promega, H5152) plus 5% nonfat dry milk (Bio-Rad, 170-6404) and/or 5% bovine serum albumin (BSA, Santa Cruz Biotech, 9048-46-8) at room temperature for 1 hour, incubated with primary antibodies at room temperature for 2 hours followed by incubation with horseradish peroxidase (HRP) conjugated secondary antibodies at room temperature for 1 hour, and visualized using the ECL chemiluminescence reagent (Bio-Rad, 170-5060).

Antibodies
For western blotting, primary antibodies against Drp1 (1:1000, ab56788), GAPDH (1:3000, ab8245), p62 (1:1000, ab56416), LC3 I/II (1:1000, ab128025), COX IV (1:1000, ab14744) were from Abcam. Primary antibody against ubiquitin (1:100, sc-8017) was from Santa Cruz Biotechnology. Primary antibody against Parkin was from Cell Signaling Technology (1:1000, #2132). Horseradish peroxidase (HRP)
conjugated secondary antibodies anti-mouse IgG (1:3000, cs7076) and anti-rabbit IgG (1:3000, cs7074) were from Cell Signaling Technology.

**Histological Studies**
Mouse heart tissues were fixed with 4% formaldehyde solution in PBS with or without prior transcardial perfusion. Paraffin-embedded tissues were sectioned at a thickness of 5 µm on a Leica RM2255 rotary microtome. The sections were de-paraffinized in xylene, rehydrated with a gradient (100-50%) of ethanol, and washed in distilled water. Masson’s trichrome stain (Sigma, HT15, HT10132 & 34256) was performed according to the manufacturers’ protocol. For Evans blue studies, mice were intraperitoneally administered 1% Evans blue dye (Sigma, E2129) solution in PBS at 1% volume relative (ml) to body mass (g) 20-24 h prior to tissue sampling. The sections were stained with FITC conjugated-wheat germ agglutinin (Invitrogen, W834) at room temperature for 30 min before nuclear counterstaining with DAPI (Vector Laboratories, H-1200). Evans blue positive cardiomyocytes fluoresce in red.

**Transmission Electron Microscopy**
Mouse heart tissues were fixed with EM fixation buffer (4% paraformaldehyde, 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4). A Jeol electron microscope (JEM-1400, JEOL, Tokyo, Japan) at 800x - 8,000x direct magnifications was used for ultrastructural examination of osmium tetroxide/uranyl acetate stained mouse heart thin sections (90 nm). Mitochondrial content (% of the areas taken by mitochondria compared to those of the cardiomyocytes) and individual mitochondrial size and aspect ratio were measured using ImageJ (NIH) on transmission electron microscopic images.

**Isolation of Cardiac Mitochondria**
Isolation of cardiac mitochondria used a modification of a published protocol. Briefly, mouse hearts were minced, incubated with trypsin and then homogenized with a glass/teflon Potter Elvehjem homogenizer. The homogenates were centrifuged at 800 xg at 4 °C for 10 min. The supernatants were centrifuged at 8,000 xg at 4 °C for 10 min and the resulting supernatants were discarded. The pellet containing the mitochondria was washed and centrifuged at 8,000 xg at 4 °C for 10 min before resuspension for functional analyses. Mitochondrial protein concentration was colorimetrically measured using Bio-Rad protein assay dye reagent concentrate (Bio-Rad, 500-0006).

**Mitochondrial Respiration**
Respiratory activities of isolated mitochondria were assessed using a micro Clark-type electrode in a closed and magnetically stirred glass chamber as previously described. Non-stimulated (-ADP) respiration (state 2) and ADP (Sigma, A2754)-stimulated respiration (state 3) were measured. Oligomycin (Sigma, 75351) was added to inhibit ATP synthesis.

**Flow Cytometric Analyses of Isolated Mitochondria**
Isolated mitochondria were stained with 200 nM MitoTracker Green (Invitrogen, M-7514), 2 µM of tetramethylrhodamine, ethyl ester (TMRE, Invitrogen, T-669), or 2.5 µM MitoSOX red (Invitrogen, M36008) at room temperature for 20 min and washed twice with PBS. Flow cytometric analyses of mitochondrial size (forward scatter, FSC), mitochondrial membrane potential (TMRE fluorescence intensity detected by PE channel), or mitochondrial superoxide level (MitoSOX red fluorescence intensity detected by PE channel) were performed on a BD LSR II Flow Cytometer (Becton Dickinson, San Jose, CA). Data are presented as histograms for, and as bar graphs of average signal intensity of ~50,000 ungated events.

**Statistical Analysis**
Data are reported as mean ± SEM, unless otherwise stated. Unpaired Student’s t test or ANOVA with Tukey’s comparison were used for paired and group comparisons, respectively. P<0.05 was defined as significant.
Online Figure I. Ultrastructural examination of cardiomyocyte mitochondria in Drp1 null hearts. Representative transmission electron microscopic images showing loss of cardiomyocyte mitochondria 6 weeks after conditional cardiac Drp1 deletion in an 8 week old mouse. Original magnification is 1,000x (upper left) and 3,000x (upper right). For clarity, the areas in the black squares of the upper right panel are enlarged in the lower panels.
Online Figure II. Multi-antibody immunoblot analysis of Parkin in the 100,000 xg supernatant myocardial fraction of normal, cardiac Parkin-deficient, and cardiac Drp1-deficient mouse hearts. GAPDH is loading control; arrows indicate predicted position of Parkin or GAPDH protein.
Online Figure III. Serial studies of myh6-Parkin cardiac transgenic mice. A. Schematic depiction of experimental design. B-C. Serial quantitative group echocardiographic data showing left ventricular (LV) mass, LV end-diastolic dimension (EDD), LV end-systolic dimension (ESD) and fractional shortening (FS) from 4 to 30 weeks. Ctrl (nontransgenic littermates, n=5) are open circles; Parkin transgenic mice (n=5) are closed squares. D. Representative transmission electron microscopic images showing cardiomyocyte mitochondria of 30 week old mouse hearts. Original magnification is 3,000x. Quantitative metrics for mitochondria are to the right. Data are shown as mean ± SEM. No statistically significant differences are present.
Online Figure IV. Serial studies of conditional cardiac Parkin knockout mice. A. Schematic depiction of the experimental design. B. Quantitative group echocardiographic data showing LV EDD, ESD and FS before, and up to 20 weeks after, cardiac Parkin ablation. Controls (Ctrl, mice with floxed alleles only, n=12) are open circles; cardiac Parkin knockout mice (n=10) are closed squares; data are shown as mean ± SEM. No statistically significant differences are present.
Online Figure V. Ultrastructural examination of cardiomyocyte mitochondria in cardiac Parkin-deficient hearts. Representative transmission electron microscopic images showing cardiomyocyte mitochondria of 14 week old mouse hearts, 6 weeks after conditional cardiac Park2 ablation. Controls (Ctrl) are mice with floxed alleles only. Original magnification is 3,000x (upper panel) and 5,000x (lower panel).
Online Figure VI. Survival and cardiac function of mice after conditional cardiomyocyte-specific Drp1, Parkin, and combined Drp1/Parkin ablation. A. Survival of mice after tamoxifen-mediated gene recombination at 8 weeks of age. B. Quantitative group serial echocardiographic data showing LV ESD and the ratio of LV end-diastolic radius (r) to wall thickness (h) before, and up to 6 weeks after, Drp1, Parkin, and combined Drp1/Parkin gene ablation. Controls (Ctrl, floxed alleles without Cre, n=16) are open circles; cardiac Parkin knockout mice (n=5) are open squares; cardiac Drp1 knockout mice (n=16 for the time points before 6 weeks; n=9 for the 6-week time point) are closed circles; concomitant cardiac Drp1 and Parkin knockout mice (n=9) are closed squares. The dead mouse icons indicate that cardiac function at 6 weeks reflects only live mice, and not the ~half of cardiac Drp1 KO mice that died between 5 and 6 weeks after gene recombination. Data are shown as mean ± SEM; * p<0.05 vs Ctrl and # p<0.05 vs Drp1 KO mice.
Online Figure VII. Functional studies of cardiac Drp1 KO, cardiac Parkin KO, and cardiac Drp1/Parkin KO mouse heart mitochondria. A. Isolated cardiac mitochondrial respiration at 6 weeks after gene ablation; inset is quantitative group data for state 3 (ADP stimulated)/state 2. B. Flow cytometry analyses of isolated cardiac mitochondrial transmembrane electrical potential measured with TMRE. C. O$_2$ measured with MitoSOX Red. Insets for B and C are quantitative group data. White bar (black line) is Ctrl, grey is Parkin KO, blue is Drp1 KO, and red is Drp1 KO/Parkin KO. Data are mean ± SEM of indicated number of experiments. There are no statistically significant differences.
Supplemental References