Published in final edited form as:

J Mol Cell Cardiol. 2015 March ; 80: 71–80. doi:10.1016/j.yjmcc.2014.12.018.

Dissociation of mitochondrial from sarcoplasmic reticular stress in *Drosophila* cardiomyopathy induced by molecularly distinct mitochondrial fusion defects

Poonam Bhandari, Moshi Song, and Gerald W Dorn II*

Center for Pharmacogenomics, Department of Internal Medicine, Washington University School of Medicine, St. Louis, MO U.S.A

Abstract

Mitochondrial dynamism (fusion and fission) is responsible for remodeling interconnected mitochondrial networks in some cell types. Adult cardiac myocytes lack mitochondrial networks, and their mitochondria are inherently “fragmented”. Mitochondrial fusion/fission is so infrequent in cardiomyocytes as to not be observable under normal conditions, suggesting that mitochondrial dynamism may be dispensable in this cell type. However, we previously observed that cardiomyocyte-specific genetic suppression of mitochondrial fusion factors optic atrophy 1 (Opa1) and mitofusin/MARF evokes cardiomyopathy in *Drosophila* hearts. We posited that fusion-mediated remodeling of mitochondria may be critical for cardiac homeostasis, although never directly observed. Alternately, we considered that inner membrane Opa1 and outer membrane mitofusin/MARF might have other as-yet poorly described roles that affect mitochondrial and cardiac function. Here we compared heart tube function in three models of mitochondrial fragmentation in *Drosophila* cardiomyocytes: Drp1 expression, Opa1 RNAi, and mitofusin MARF RNAi. Mitochondrial fragmentation evoked by enhanced Drp1-mediated fission did not adversely impact heart tube function. In contrast, RNAi-mediated suppression of either Opa1 or mitofusin/MARF induced cardiac dysfunction associated with mitochondrial depolarization and ROS production. Inhibiting ROS by overexpressing superoxide dismutase (SOD) or suppressing ROMO1 prevented mitochondrial and heart tube dysfunction provoked by Opa1 RNAi, but not by mitofusin/MARF RNAi. In contrast, enhancing the ability of endoplasmic/sarcoplasmic reticulum to handle stress by expressing Xbp1 rescued the cardiomyopathy of mitofusin/MARF insufficiency without improving that caused by Opa1 deficiency. We conclude that decreased mitochondrial size is not inherently detrimental to cardiomyocytes. Rather, preservation of mitochondrial function by Opa1 located on the inner mitochondrial membrane, and prevention of

© 2014 Elsevier Ltd. All rights reserved.

*Correspondence to: Gerald W. Dorn II, MD, Philip and Sima K Needleman Professor, Washington University Center for Pharmacogenomics, 660 S Euclid Ave., Campus Box 8220 St. Louis, MO 63110, Phone: 314 362-4892. Fax 314 362-8844., gdorn@dom.wustl.edu.

Disclosures: None declared

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

ER stress by mitofusin/MARF located on the outer mitochondrial membrane, are central functions of these “mitochondrial fusion proteins”.

Keywords

Mitochondrial fusion; reactive oxygen species; endoplasmic reticular stress; mitofusin; Optic Atrophy 1; *Drosophila*

Introduction

It is axiomatic that mitochondria are dynamic, meaning they exist as interconnected networks that constantly undergo structural remodeling. The problem is that mitochondria of cardiomyocytes in adult hearts appear structurally homogenous and static, i.e. hypo-dynamic [1]. Yet, accumulation of smaller organelles after genetic suppression of mitochondrial outer and inner membrane fusion proteins in the cardiomyocytes of *Drosophila* is compelling indirect evidence for homeostatic mitochondrial fusion, at least in fly hearts [2]. Likewise, naturally occurring and experimental cardiomyopathies linked to genetic defects in mitochondrial fusion proteins [3, 4] indicate that mitochondrial fusion in cardiomyocytes, however infrequent, is essential to heart health. What is not understood are the roles played by different mitochondrial fusion factors in sustaining normal cardiac performance.

Mitochondrial fusion is a three-step process: Initially, two mitochondria are reversibly tethered, and then the outer membranes irreversibly fuse. Both of these events are mediated by outer membrane mitofusins (Mfn1 and Mfn2 in vertebrates and MARF in *Drosophila*). Subsequent to outer membrane fusion, the two inner mitochondrial membranes fuse, which is mediated by Opa1. The presence of two mitofusins that are functionally redundant for promoting organelle tethering and outer membrane fusion, but not for other mitofusin functions, complicates interpretation of cardiac-specific single and double Mfn2 gene deletion studies in mice [5]. For this reason, we interrogated mitochondrial dynamism in *Drosophila* heart tubes, employing conditional cardiomyocyte-specific expression of RNAi to suppress either inner membrane Opa1 or outer membrane mitofusin (i.e. MARF); we observe cardiac contractile dysfunction after interrupting mitochondrial fusion at either the inner or outer mitochondrial membrane [2, 6]. Our recent findings suggest that cardiomyopathy provoked by mitofusin deficiency cannot primarily be attributed to impaired mitophagy because the cardiac phenotypes caused by *Drosophila* mitofusin- and Parkin-deficiency differ [7]. Indeed, suppressing mitochondrial fusion actually delayed heart tube dysfunction in mitophagy-defective fly hearts [7].

Mitofusins not only form molecular tethers between mitochondria, but mammalian Mfn2 can bridge cardiomyocyte mitochondria and adjacent sarcoplasmic reticulum (SR), thereby facilitating calcium signaling between these two calcium-rich organelles [8–10]. In *Drosophila* heart tubes MARF can perform this same function [11]. Because SR-mitochondrial calcium signaling is an essential component of the ER stress response [12], we posited that the cardiomyopathy caused by interrupting fusion at the step of outer mitochondrial membrane tethering (to SR or other mitochondria) and fusion (by mitofusin/MARF) could result from ER stress instead of, or in addition to, mitochondrial stress [13].

We further hypothesized that the cardiomyopathy induced by interrupting fusion at the step of inner mitochondrial membrane fusion/assembly (by Opa1) could evoke mitochondrial stress without ER stress, because Opa1 is not known to direct mitochondrial-ER/SR interactions [14]. If these notions are correct, then effective therapeutics will need to be appropriately targeted to the specific molecular lesion at the inner or outer membrane, rather than indiscriminately applied to any condition in which fusion-defective (so called “fragmented”) mitochondria are observed.

Materials and Methods

Drosophila strains

w¹¹¹⁸ (#6326), *UAS-mitoGFP* (#8442), *UAS-SOD1* (#33605 and #24750), *UAS-SOD2* (#24494) were obtained from the Bloomington Stock Center. *UAS-MARF* RNAi, *UAS-Opa1* RNAi, and *UAS-Drp1* TG were provided by M. Guo (University of California, Los Angeles, CA) [15]. *UAS-Romo1* RNAi (#v101353 and #v9224) were obtained from the Vienna Drosophila RNAi Center [16]. The strain expressing XBP1 (*XBP1^{d08698}*) was obtained from Exelixis collection at Harvard. UAS transgenes were expressed in *Drosophila* cardiomyocytes using the *tinc* 4-Gal4 driver provided by Rolf Bodmer (Sanford-Burnham Medical Research Institute, La Jolla, CA) [17].

Drosophila heart function

Optical coherence tomography (OCT) heart tube images of two week adult flies were acquired using a Michelson Diagnostics (Maidstone, UK) EX 1301 OCT microscope as described previously [2, 18]. Image J was used to analyze B-mode images to measure the internal chamber diameter at end-systole (ESD) and end diastole (EDD). % Fractional Shortening (FS) was calculated as (EDD-ESD/EDD).

Confocal microscopy

Fly heart tubes were dissected and mounted in haemolymph as described [11] for live confocal imaging. A Nikon Eclipse Ti confocal system or Carl-Zeiss LSM510-Meta Laser Scanning confocal Microscope with Plan Apo VC 60x/1.40 Oil objective and 4X digital zoom were used to image mitochondria in *Drosophila* cardiomyocytes expressing *Tinc* 4-Gal4 driven *UAS-mitoGFP*.

Mitochondrial dimensions were measured using Image J (19.5 pixels/um). Size distribution curves were generated by grouping the data into .25 um² bins (range: 0 to 1.5um²). Median mitochondrial area was determined by averaging ten different samples with 150–300 mitochondria per sample.

Tetramethylrhodamine ethyl ester (TMRE) fluorescence was used to assess mitochondrial membrane potential. Heart tubes were dissected and incubated in TMRE (250nM) (Life Technologies) for 20 min, and then washed for 10 min in Hank's balanced salt solution (HBBS) prior to confocal imaging. The numbers of orange and green mitochondria were determined by manual counting from ten separate heart tube images with a sample of at least 300 mitochondria from each image. To assess cardiomyocyte mitochondrial ROS

production, dissected heart tubes were incubated in MitoSOX (2.5mM) (Life Technologies) in PBS for 20 minutes at 25°C, washed for 10 min with PBS, and visualized by confocal fluorescent microscopy. The mitoSOX (red) to mitotracker (green) ratio was determined by comparing the red/green fluorescence intensity in the confocal images using Image J.

Genetic crosses for the generation of flies with various genotypes

1. tinc 4-Gal4>MARF RNAi, SOD1—The tinc 4-Gal4 females were crossed to the 2nd and 3rd balancer chromosome SM5 and TM3 males, and balanced F1 progeny self-crossed to generate tinc 4-Gal4/ tinc 4-Gal4 with 2nd chromosome balancer SM5. Similarly UAS-SOD1 (#33605) was crossed to the 2nd and 3rd balancer chromosome SM5 and TM3 and balanced F1 progeny self-crossed to generate UAS-SOD1/ UAS-SOD1 with 3rd chromosome balancer TM3. +/-SM5; tinc 4-Gal4/ tinc 4-Gal4 females were then crossed to UAS-SOD1/ UAS-SOD1; +/-TM3 males. Males with the genotype UAS-SOD1/SM5; tinc 4-Gal4/ TM3 were crossed to MARF RNAi/ MARF RNAi females to generate female UAS-SOD1/+; tinc 4-Gal4/ MARF RNAi flies for studies.

2. tinc 4-Gal4>MARF RNAi, SOD2—UAS-SOD2 (#24494) was crossed to the 2nd and 3rd balancer chromosome SM5 and TM3, and balanced F1 progeny self-crossed to generate UAS-SOD2/ UAS-SOD2 with 3rd chromosome balancer TM3. +/-SM5; tinc 4-Gal4/ tinc 4-Gal4 females were crossed to UAS-SOD2/ UAS-SOD2; +/-TM3 males and resulting males with the genotype UAS-SOD2/SM5; tinc 4-Gal4/ TM3 crossed to MARF RNAi/ MARF RNAi females to generate UAS-SOD2/+; tinc 4-Gal4/ MARF RNAi for studies.

3. tinc 4-Gal4>Opa1 RNAi, SOD1—UAS-SOD1 (#33605) was crossed to the 2nd and 3rd balancer chromosome SM5 and TM3 and balanced F1 progeny self-crossed to generate UAS-SOD1/ UAS-SOD1 with 3rd chromosome balancer TM3. +/-SM5; tinc 4-Gal4/ tinc 4-Gal4 females were crossed to UAS-SOD1/ UAS-SOD1; +/-TM3 males producing males with the genotype UAS-SOD1/SM5; tinc 4-Gal4/ TM3 that were crossed to Opa1 RNAi/ Opa1 RNAi females to generate UAS-SOD1/ Opa1 RNAi; tinc 4-Gal4/+ females for studies.

4. tinc 4-Gal4>Opa1 RNAi, SOD2—/+SM5; tinc 4-Gal4/ tinc 4-Gal4 females were crossed to UAS-SOD2/ UAS-SOD2; +/-TM3 males. The progeny were screened for UAS-SOD2/SM5; tinc 4-Gal4/ TM3 males, which were crossed to Opa1 RNAi/ Opa1 RNAi females to generate UAS-SOD2/ Opa1 RNAi; tinc 4-Gal4/ + females for studies.

5. tinc 4-Gal4>UAS-mito-GFP, MARF RNAi, SOD1—MARF RNAi/ MARF RNAi females were crossed to the 2nd and 3rd balancer chromosome SM5 and TM3 males and balanced F1 progeny were self-crossed to generate MARF RNAi/ MARF RNAi with 2nd chromosome balancer SM5. Similarly, UAS-SOD1 (#33605) was crossed to the 2nd and 3rd balancer chromosome SM5 and TM3. The F1 balanced progeny were self-crossed to generate UAS-SOD1/ UAS-SOD1 with 3rd chromosome balancer TM3. +/-SM5; MARF RNAi/ MARF RNAi males were crossed to UAS-SOD1/ UAS-SOD1; +/-TM3 females to generate UAS-SOD1/ SM5; MARF RNAi /TM3 males, which were crossed to UAS-mito-GFP/CyO; tinc 4-Gal4/TM6 females. The resulting progeny were screened for UAS-SOD1/ UAS-mito-GFP; MARF RNAi/ tinc 4-Gal4 females, which were studied.

6. tinc 4-Gal4>UAS-mito-GFP, Opa1, SOD1—Opa1 RNAi/ Opa1 RNAi females were crossed to the 2nd and 3rd balancer chromosome SM5 and TM3 males, and balanced F1 progeny self-crossed to generate Opa1 RNAi/ Opa1 RNAi with 3rd chromosome balancer TM3. Similarly, UAS-SOD1 (#24730) was crossed to the 2nd and 3rd balancer chromosome SM5 and TM3. The F1 balanced progeny were self-crossed to generate UAS-SOD1/ UAS-SOD1 with 2nd chromosome balancer SM5. Opa1 RNAi/ Opa1 RNAi; +/-TM3 males were crossed to +/-SM5; UAS-SOD1/ UAS-SOD1 females to generate Opa1 RNAi / SM5; UAS-SOD1/TM3 males, which were crossed to UAS-mito-GFP/CyO; tinc 4-Gal4/TM6 females to generate Opa1 RNAi / UAS-mito-GFP; UAS-SOD1/ tinc 4-Gal4 females for studies.

7. tinc 4-Gal4>UAS-mito-GFP, MARF RNAi, Romo1 RNAi—Romo1 RNAi (#v101353) was crossed to the 2nd and 3rd balancer chromosome SM5 and TM3; F1 balanced progeny were self crossed to obtain Romo1 RNAi / Romo1 RNAi with 3rd chromosome balancer TM3. +/-SM5; MARF RNAi/ MARF RNAi males were crossed with Romo1 RNAi / Romo1 RNAi; +/-TM3 females to generate Romo1 RNAi / SM5; MARF RNAi /TM3 males, which were crossed to UAS-mito-GFP/CyO; tinc 4-Gal4/TM6 females to generate Romo1 RNAi / UAS-mito-GFP; MARF RNAi/ tinc 4-Gal4 female progeny used in OCT and confocal studies.

8. tinc 4-Gal4>UAS-mito-GFP, Opa1 RNAi, Romo1 RNAi—Romo1 RNAi (#v9224) was crossed to the 2nd and 3rd balancer chromosome SM5 and TM3. The F1 balanced progeny was self crossed to generate the strain Romo1 RNAi / Romo1 RNAi with 2nd chromosome balancer SM5. +/-SM5; Romo1 RNAi / Romo1 RNAi males were crossed to Opa1 RNAi/ Opa1 RNAi; +/-TM3 females to generate Opa1 RNAi / SM5; Romo1 RNAi /TM3 males, which were crossed to UAS-mito-GFP/CyO; tinc 4-Gal4/TM6 females. Female Opa1 RNAi / UAS-mito-GFP; Romo1 RNAi/ tinc 4-Gal4 progeny were used for OCT and confocal studies.

9. tinc 4-Gal4>UAS-mito-GFP, MARF RNAi, UAS-Xbp1—UAS-Xbp1 was crossed to the 2nd and 3rd balancer chromosome SM5 and TM3. F1 balanced progeny were self-crossed to generate UAS- UAS-Xbp1 / UAS-Xbp1 with 3rd chromosome balancer TM3. +/- SM5; MARF RNAi/ MARF RNAi males were crossed to UAS-Xbp1 / UAS-Xbp1; +/-TM3 females to generate UAS-Xbp1/ SM5; MARF RNAi /TM3 males, which were crossed to UAS-mito-GFP/CyO; tinc 4-Gal4/TM6 females. The progeny were screened for UAS-SOD2/ UAS-mito-GFP; MARF RNAi/ tinc 4-Gal4 females, which were studied.

10. tinc 4-Gal4> Opa1 RNAi, UAS-Xbp1—+/-SM5; tinc 4-Gal4/ tinc 4-Gal4 females were crossed to UAS-Xbp1 / UAS-Xbp1; +/-TM3 males, the progeny screened for UAS-Xbp1/SM5; tinc 4-Gal4/ TM3 males, which were crossed to the Opa1 RNAi/ Opa1 RNAi females. Resulting females progeny with the genotype UAS-Xbp1/ Opa1 RNAi; tinc 4-Gal4/ + were studied.

11. tinc 4-Gal4> UAS-mito-GFP, UAS-Drp1 (TG)—UAS-mito-GFP/CyO; tinc 4-Gal4/TM6 females were crossed UAS-Drp1 (TG) males. From the F1 progeny UAS-mito-GFP/+; tinc 4-Gal4/ UAS-Drp1 (TG) females were studied.

Statistical methods

Graphs were generated using Microsoft Excel or Graphpad Prism. Data are presented as mean $\pm$ SEM. Groups were compared by One way ANOVA with Bonferroni correction. Significance was defined at $P < 0.05$.

Results

Mitochondrial fragmentation provoked by cardiomyocyte Drp1 overexpression does not compromise mitochondrial fitness or heart tube function

Inhibition of mitochondrial fusion in fruit flies promotes mitochondrial “fragmentation” (the presence of abnormally small mitochondria presumably as a consequence of unopposed fission) and heart tube dysfunction [2]. We asked if the change in organelle morphometry was the direct cause of the cardiac defect (rather than other events mediated by fusion proteins) by inducing mitochondrial fragmentation in a manner that should not interfere with organelle fusion: overexpression of the mitochondrial fission protein, Drp1. Cardiomyocyte mitochondria were indeed reduced in size in cardiac Drp1 transgenic fly heart tubes (Figures 1A). Even though mitochondrial size was decreased by ~half (Figure 1B) and heart tube Drp1 levels were markedly increased (Figure 1C), heart tube contractile function was not adversely affected (Figure 1D). Likewise, mitochondrial polarization status assessed by TMRE fluorescence was not adversely impacted by Drp1-induced fragmentation (Figure 1E). Thus, so-called mitochondrial fragmentation is not intrinsically detrimental to either cardiomyocyte mitochondrial fitness or *Drosophila* heart tube function.

Structural cardiac and mitochondrial abnormalities in Opa1-deficient hearts, and benefits of ROS suppression

Dissociation of mitochondrial fragmentation and cardiac dysfunction in Drp1 cardiac transgenic flies prompted us to re-evaluate our original finding of heart tube abnormalities in *Drosophila* with cardiac-specific suppression of mitochondrial fusion factors Opa1 and mitofusin/MARF [2]. We asked what these two factors are doing to heart mitochondria in addition to regulating their size? Indeed, there is accumulating evidence that defects in inner and outer membrane mitochondrial fusion protein evoke distinct effects on parent cell biology. To learn more about the consequences of interrupting fusion of inner mitochondrial membranes on cardiac biology we again created flies with cardiomyocyte specific suppression of Opa1, and carefully examined them using the more advanced techniques we have developed in the interim. Consistent with our previous report [2], RNAi-mediated Opa1 suppression impaired contractility, and provoked dilatation, of fly heart tubes (Figures 2A, 2B). The mitochondrial size distribution curve, measured by quantitative analysis of fly heart tubes carrying a cardiomyocyte-specific mitochondrial-targeted GFP transgene [18], was shifted to the left; median mitochondrial area shrunk by ~50% (Figure 2C).

Opa1 not only regulates inner mitochondrial membrane fusion, but also mitochondrial respiratory chain complexes [14]. Accordingly, Opa1 deficiency can evoke mitochondrial respiratory dysfunction and pathological production of reactive oxygen species (ROS) [4]. We determined that ROS contributes to cardiac contractile dysfunction provoked by Opa1 deficiency using genetic complementation with a cardiomyocyte-specific superoxide

dismutase (SOD) 1 transgene, which decreased heart tube dilatation, improved ejection performance (Figure 2B, **left panels**), and modestly attenuated mitochondrial the fragmentation (Figure 2C) that is provoked by Opa1 suppression. Transgenic overexpression of the mitochondrial-localized SOD2 isoform likewise attenuated heart tube remodeling and systolic dysfunction (Figure 2B, **right panels**). (Because the mito-GFP and SOD2 transgenes are on the same fly chromosome we could not measure the specific effects of SOD2 on mitochondrial morphometry in these studies.) These results demonstrate that Opa1 suppression in cardiac myocytes causes mitochondrial fragmentation (as expected from shifting the balance away from mitochondrial fusion and toward mitochondrial fission) and a cardiomyopathy. The observation that SOD1 and SOD2 improve this cardiomyopathy implicates mitochondrial ROS production in the cardiac contractile abnormalities resulting from Opa1 deficiency.

Structural cardiac and mitochondrial abnormalities in mitofusin/MARF-deficient hearts, and lack of sustained improvement with ROS suppression

As noted above, in our initial *Drosophila* heart tube studies we reported that the similar cardiac and mitochondrial abnormalities from inhibiting cardiomyocyte mitochondrial fusion by suppressing either Opa1 or mitofusin/MARF were overtly similar, and therefore focused on MARF [2]. Subsequently, we have observed that the modest benefits conferred by SOD1 expression we originally observed in mitofusin/MARF-deficient heart tubes 1 week after pupal eclosure are not sustained [7]. For this reason, and because of definite and sustained SOD-induced improvement observed in the Opa1 insufficiency model (see Figure 1), we re-examined SOD effects on the cardiomyopathy induced by cardiac-expressed mitofusin/MARF RNAi. As initially reported [2], mitofusin/MARF suppression provokes heart tube dilatation and reduced fractional shortening (Figures 3A, 3B) that is similar to Opa1 suppression. Likewise, the reduction in mitochondrial size was comparable in these two models of fusion factor suppression (Figure 3C; compare to Figure 2C). Remarkably however, neither SOD1 nor SOD2 expressed using the same transgenic system used in the cardiac Opa1-deficient model (and previously for cardiac Parkin deficiency [7]) improved mitofusin/MARF-deficient heart tube size or contractile function two weeks after eclosure (Figures 3A and 3B). Because SOD1 and SOD2 rescue of the fly cardiomyopathy induced by cardiomyocyte-specific Parkin deficiency has revealed a mechanistic link between intrinsic mitochondrial dysfunction, ROS cardiotoxicity, and cardiomyopathy [7], absence of any sustained benefit conferred by SOD expression in mitofusin/MARF-deficient *Drosophila* hearts points to a different etiology of cardiac dysfunction. Whereas mitotoxicity seems to be playing a central role in Opa1 deficiency, a different mechanism is operating in mitofusin/MARF insufficiency.

SOD rescues mitochondrial depolarization in fragmented mitochondria of Opa1-deficient, but not mitofusin/MARF-deficient heart tubes

We considered that therapeutic efficacy of SOD in one model of fusion-defective cardiomyocyte mitochondria, but not the other, could represent prevention of ROS-mediated cytotoxicity in Opa1 RNAi, but not mitofusin/MARF RNAi, heart tubes; we call this the “distal therapeutic effect hypothesis”. Alternately, SOD could be positively affecting mitochondrial fitness of Opa1-deficient, but not mitofusin/MARF-deficient cardiomyocytes;

the “proximal therapeutic effect hypothesis”. We previously observed a proximal direct mitochondrial benefit of SOD expression in Parkin-deficient fly hearts [7]. Therefore, we assessed mitochondrial fitness in mito-GFP expressing Opa1- and mitofusin/MARF-deficient heart tubes using the voltage-dependent dye TMRE. TMRE fluoresces red in fully polarized mitochondria, but not in mitochondria in which the inner membrane electrochemical gradient has dissipated. Accordingly, healthy cardiomyocyte mitochondria in our studies are orange (red+green) and depolarized mitochondria are green. Compared to normal controls, it is immediately apparent that a substantial proportion of both Opa1- and mitofusin/MARF-deficient cardiomyocyte mitochondria are depolarized (Figures 4A, 4B); quantitative analysis indicated a similar extent (~40%) of depolarized mitochondria in both models. Strikingly, analysis of mitochondrial polarization status as a function of mitochondrial size revealed that depolarization (green staining) was disproportionately a characteristic of the smaller “fragmented” mitochondria in both Opa1- and mitofusin/MARF-deficient heart tubes, whereas the larger more normal sized organelles exhibited predominately normal polarization (orange) (Figures 4A–D).

Equally noteworthy was the different effect of SOD on mitochondrial ROS production and polarization status in these two genetically distinct models of interrupted mitochondrial fusion. We had expected that mitochondrial ROS (measured with MitoSOX) would increase in parallel with mitochondrial depolarization in Opa1- and mitofusin/MARF-deficient heart tubes, which it did (Figures 4A, 4B, right panels). Surprisingly however, SOD reduced both ROS and the proportion of depolarized mitochondria only in Opa1-deficient hearts; there was no effect of cardiomyocyte-expressed SOD on either ROS or mitochondrial polarization status in mitofusin/MARF-deficient hearts (Figure 4B, right panel), just as there had been no benefit on heart tube remodeling or contractile function (see Figure 2). These findings provide additional support for the notion that the cardiac defect induced by Opa1 suppression derives from a primary mitochondrial abnormality, whereas both mitochondrial and cardiac degeneration engendered by mitofusin/MARF suppression is at least partially attributable to extra-mitochondrial factors.

Mitochondria are the source of damaging ROS in Opa1-deficient, but not mitofusin/MARF-deficient cardiomyocytes

Mitochondrial-derived ROS can be generated by electrons leaking from respiratory chain complexes [19, 20] or through the actions of dedicated mitochondrial enzymes, such as ROS modulator 1 (ROMO1) [21–23]. We therefore examined the role of ROMO1-mediated ROS production in the cardiotoxicity manifested by *Drosophila* heart tubes having fusion-defective mitochondria. ROMO1 was suppressed specifically in fly cardiomyocytes using transgenically expressed RNAi as previously described [7]. ROMO1 suppression alone had no effect on mitochondrial morphometry, heart tube structure, or contractile function (Figures 5A, 5B **left panels** and unpublished observations), but ROMO1 RNAi prevented the cardiomyopathy induced by Opa1 suppression, without impacting (either positively or negatively) the cardiomyopathy induced by mitofusin/MARF RNAi (Figures 5A, 5B **right panels**). Moreover, ROMO1 suppression partially normalized mitochondrial size in Opa1-deficient heart tubes (Figure 5C), without affecting mitochondrial fragmentation in mitofusin/MARF deficient cardiomyocytes (Figure 5D). The modest favorable effect of

ROMO1 suppression (Figure 5C) and forced SOD1 expression (Figure 2C) on mitochondrial size in Opa1 deficient heart tubes likely reflects interruption ROS-induced mitochondrial damage [24]. These results appear to validate the idea that intrinsic mitochondrial dysfunction provokes cardiomyopathy in cardiomyocyte Opa1 deficiency, but not in mitofusin/MARF deficiency.

Cardiomyocyte mitofusin/MARF deficiency, but not Opa1 suppression, induces ER-stress that causes cardiac dysfunction

We previously observed that cardiomyocyte-specific mitofusin/MARF suppression alters SR calcium handling in fly hearts, consistent with a conserved role for *Drosophila* MARF and mammalian Mfn2 as tethers that bridge mitochondria and ER [11]. Because ER/SR calcium signaling to and from mitochondria can mediate a stress response, we posited that the non-mitochondrial cause of the cardiomyopathy produced mitofusin/MARF suppression might reflect increased ER stress [12]. We tested this idea by transgenically expressing Xbp1 in our fusion-deficient hearts. Xbp1 is a transcription factor that activates genes encoding proteins important for both protein folding and clearance of misfolded proteins [25]; Xbp1 expression protects cells from ER stress [26, 27]. Cardiac-specific expression of Xbp1 alone had no effect on fly heart tube size or contractile function (Figures 6A and 6B). Likewise, Xbp1 had no effects on the cardiomyopathy induced by Opa1 suppression (Figures 6A and 6B, **left panels**). Remarkably however, Xbp1 nearly normalized heart tube size and contractile performance in mitofusin/MARF deficient hearts (Figures 6A and 6B, **right panels**). The benefits from Xbp1 are not attributable to modifying the underlying defect in mitochondrial fusion, as mitochondrial fragmentation in mitofusin/MARF-deficient heart tubes was completely unaffected (Figure 6C).

Finally, given that ROMO1 RNAi improved only the Opa1-deficiency fly cardiomyopathy (see Figure 5), and Xbp1 expression improved only the mitofusin/MARF deficiency fly cardiomyopathy (see Figure 6), we examined mitochondrial dysfunction in the respective “rescued” heart tubes. As shown in Figure 7, ROMO1 suppression markedly improved mitochondrial polarization status in Opa1-deficient, but not in mitofusin/MARF insufficient, heart tubes. In contrast, neither ROMO1 suppression nor XBP1 expression improved mitochondrial depolarization in mitofusin/MARF deficient heart tubes. Since XBP1 normalized cardiac function in the same fly hearts, these results are consistent with extra-mitochondrial ER stress being the principal cellular lesion in mitofusin/MARF insufficiency.

Discussion

Here, in what we believe to be the first side-by-side detailed comparison of cardiomyopathies provoked by interrupting fusion of either the outer or inner mitochondrial membranes, we have identified distinct cellular mechanisms for the different molecular lesions. RNAi-mediated suppression of outer mitochondrial membrane mitofusin/MARF and inner mitochondrial membrane Opa1 provoked similar overt phenotypes: in both models mitochondrial size was approximately halved, the proportion of depolarized (sick) mitochondria increased to ~40%, mitochondrial ROS production was comparably greater (~50%), and the heart tubes exhibited similar reductions in fractional shortening. However,

the cardiac defect caused by Opa1 deficiency was readily corrected by attacking the disease at the level of mitochondrial ROS production, through SOD expression or ROMO1 suppression. Indeed, mitochondrial structural and functional abnormalities were also improved by these genetic maneuvers, suggesting that they interrupted a vicious cycle of ROS-induced mitochondrial degeneration [24, 28] provoked by Opa1 insufficiency. Thus, interrupting endogenous mitochondrial ROS production greatly abrogated both the mitotoxicity and the cytotoxicity evoked by Opa1 suppression. This suggests a central role for mitochondrial degeneration in the Opa1-deficient fly heart model and, by extension, other heart diseases caused by defective inner mitochondrial membrane fusion proteins [3, 4].

Whereas mitochondrial size and polarization status are similarly impaired in mitofusin/MARF insufficient heart tubes, neither of the mitochondrial-targeted interventions directed at reducing ROS, both of which rescued Opa1 deficient hearts, improved the cardiomyopathy induced by mitofusin/MARF deficiency. Indeed, whereas transgenic expression of SOD1 or SOD2 has normalized both ROS and heart tube function in Parkin-deficient heart tubes [7] and Opa1-deficient heart tubes (current study), we found it remarkable that SOD failed to improve ROS levels in the mitofusin/MARF deficient heart tubes (see Figure 4b). Together with the original observation of transient heart tube functional improvement with SOD1 [2], these observations suggest that there is “ROS escape” in the mitochondrial fusion impaired model that confers resistance to SOD expression or ROMO1 suppression. Instead, heart tube function was normalized *without improving either mitochondrial structural or functional abnormalities* by genetically enhancing the cardiomyocytes’ ability to handle ER stress through XBP1 expression. While we were frankly stunned at the magnitude of the nearly complete XBP1 rescue of mitofusin/MARF insufficient heart tubes, these results are consistent with an essential role for mitofusin-mediated mitochondrial-ER/SR cross-talk in managing the ER stress response as proposed by Ken Walsh in mouse hearts [29] and others in other tissues [30, 31].

The consequences of Opa1 deficiency on *Drosophila* eye phenotypes have been rescued using SOD1 [32], in accordance with the current findings. However, while we were finalizing these investigations, Debattisti et al [31] described the results of studies having some similar, and some markedly different, results. Their work in *Drosophila* neurons and skeletal muscle also supports an important role for mitofusins, but not Opa1, as modulators of ER stress. However, they report that only human Mfn2, and not human Mfn1, can correct abnormalities induced by mitofusin/MARF suppression in flies. This contrasts with our findings that cardiac-specific expression of either human Mfn1 or Mfn2 will fully correct cardiomyopathy induced by cardiomyocyte-specific MARF RNAi [2]. Furthermore, Debattisti et al describe ER dysmorphology in their MARF-deficient fly tissues, which we did not detect in MARF-deficient heart tubes [2]. It is likely either that the heart has different requirements for mitofusins and ER/SR morphology than the tissues studied by Debattisti, or that the powerful *tinman* gene promoter we used for cardiomyocyte-specific gene manipulation confers different expression characteristics to the heart models. Either way, the overall conclusions regarding a role of ER stress in mitofusin deficiency are in agreement.

Cardiomyocyte mitochondria are the Oompa-Loompas of the heart (with apologies to Roald Dahl [33]): They are diminutive, structurally homogenous, and frequently overlooked despite toiling endlessly behind the scenes to keep the place running. Research has tended to focus on the mitochondrial work product (cardiac metabolism) and the means by which the general mitochondrial population is sustained (through biogenesis), rather than the fate of individual organelles. Indeed, individual cardiomyocyte mitochondria seem hardly worthy of observation, being monotonously similar in appearance and lacking the morphometric remodeling or intra-cellular mobility that has sparked detailed investigations (and visually engaging movie clips) in other cell types [34]. The results presented herein emphasize that (for mitochondria as well as Oompa-Loompas) size is not the critical determinant of function; it is literally what is inside that counts. Accordingly, we should eschew generalizations and extrapolations of mitochondrial status and dysfunction based strictly on morphometry.

Acknowledgments

Supported by NIH/NHLBI HL059888 (GWD) and an American Heart Association predoctoral fellowship award (MS).

References

1. Dorn GW 2nd. Mitochondrial dynamics in heart disease. *Biochim Biophys Acta*. 2013; 1833:233–41. [PubMed: 22450031]
2. Dorn GW 2nd, Clark CF, Eschenbacher WH, Kang MY, Engelhard JT, Warner SJ, et al. MARF and Opa1 control mitochondrial and cardiac function in *Drosophila*. *Circ Res*. 2011; 108:12–7. [PubMed: 21148429]
3. Owczarek-Lipska M, Plattet P, Zipperle L, Drogemuller C, Posthaus H, Dolf G, et al. A nonsense mutation in the optic atrophy 3 gene (OPA3) causes dilated cardiomyopathy in Red Holstein cattle. *Genomics*. 2011; 97:51–7. [PubMed: 20923700]
4. Chen L, Liu T, Tran A, Lu X, Tomilov AA, Davies V, et al. OPA1 mutation and late-onset cardiomyopathy: mitochondrial dysfunction and mtDNA instability. *J Am Heart Assoc*. 2012; 1:e003012. [PubMed: 23316298]
5. Dorn GW 2nd. Mitochondrial dynamism and cardiac fate--a personal perspective. *Circ J*. 2013; 77:1370–9. [PubMed: 23615052]
6. Chen Y, Dorn GW 2nd. PINK1-phosphorylated mitofusin 2 is a Parkin receptor for culling damaged mitochondria. *Science*. 2013; 340:471–5. [PubMed: 23620051]
7. Bhandari P, Song M, Chen Y, Burelle Y, Dorn GW 2nd. Mitochondrial contagion induced by Parkin deficiency in *Drosophila* hearts and its containment by suppressing mitofusin. *Circ Res*. 2014; 114:257–65. [PubMed: 24192653]
8. de Brito OM, Scorrano L. Mitofusin 2 tethers endoplasmic reticulum to mitochondria. *Nature*. 2008; 456:605–10. [PubMed: 19052620]
9. Dorn GW 2nd, Scorrano L. Two close, too close: sarcoplasmic reticulum-mitochondrial crosstalk and cardiomyocyte fate. *Circ Res*. 2010; 107:689–99. [PubMed: 20847324]
10. Dorn GW 2nd, Maack C. SR and mitochondria: calcium cross-talk between kissing cousins. *J Mol Cell Cardiol*. 2013; 55:42–9. [PubMed: 22902320]
11. Chen Y, Csordas G, Jowdy C, Schneider TG, Csordas N, Wang W, et al. Mitofusin 2-containing mitochondrial-reticular microdomains direct rapid cardiomyocyte bioenergetic responses via interorganelle Ca(2+) crosstalk. *Circ Res*. 2012; 111:863–75. [PubMed: 22777004]
12. Bravo R, Vicencio JM, Parra V, Troncoso R, Munoz JP, Bui M, et al. Increased ER-mitochondrial coupling promotes mitochondrial respiration and bioenergetics during early phases of ER stress. *J Cell Sci*. 2011; 124:2143–52. [PubMed: 21628424]

13. Dorn GW 2nd, Song M, Walsh K. Functional implications of mitofusin 2-mediated mitochondrial-SR tethering. *J Mol Cell Cardiol.* 2014 Epub ahead of print.
14. Cogliati S, Frezza C, Soriano ME, Varanita T, Quintana-Cabrera R, Corrado M, et al. Mitochondrial cristae shape determines respiratory chain supercomplexes assembly and respiratory efficiency. *Cell.* 2013; 155:160–71. [PubMed: 24055366]
15. Deng H, Dodson MW, Huang H, Guo M. The Parkinson's disease genes pink1 and parkin promote mitochondrial fission and/or inhibit fusion in *Drosophila*. *Proc Natl Acad Sci U S A.* 2008; 105:14503–8. [PubMed: 18799731]
16. Dickson, B.; Dietzl, G.; Keleman, K. RNAi construct and insertion data. Vienna *Drosophila* RNAi Center; 2014. VDRC project members (2007.7.18); p. FBrf0200327
17. Lo PC, Frasch M. A role for the COUP-TF-related gene seven-up in the diversification of cardioblast identities in the dorsal vessel of *Drosophila*. *Mech Dev.* 2001; 104:49–60. [PubMed: 11404079]
18. Eschenbacher WH, Song M, Chen Y, Bhandari P, Zhao P, Jowdy CC, et al. Two rare human mitofusin 2 mutations alter mitochondrial dynamics and induce retinal and cardiac pathology in *Drosophila*. *PLoS One.* 2012; 7:e44296. [PubMed: 22957060]
19. Murphy MP. How mitochondria produce reactive oxygen species. *Biochem J.* 2009; 417:1–13. [PubMed: 19061483]
20. Chen YR, Zweier JL. Cardiac mitochondria and reactive oxygen species generation. *Circ Res.* 2014; 114:524–37. [PubMed: 24481843]
21. Chung YM, Kim JS, Yoo YD. A novel protein, Romo1, induces ROS production in the mitochondria. *Biochem Biophys Res Commun.* 2006; 347:649–55. [PubMed: 16842742]
22. Chung YM, Lee SB, Kim HJ, Park SH, Kim JJ, Chung JS, et al. Replicative senescence induced by Romo1-derived reactive oxygen species. *J Biol Chem.* 2008; 283:33763–71. [PubMed: 18836179]
23. Chung JS, Park S, Park SH, Park ER, Cha PH, Kim BY, et al. Overexpression of Romo1 promotes production of reactive oxygen species and invasiveness of hepatic tumor cells. *Gastroenterology.* 2012; 143:1084–94. e7. [PubMed: 22749933]
24. Zorov DB, Juhaszova M, Sollott SJ. Mitochondrial reactive oxygen species (ROS) and ROS-induced ROS release. *Physiol Rev.* 2014; 94:909–50. [PubMed: 24987008]
25. Yoshida H. Unconventional splicing of XBP-1 mRNA in the unfolded protein response. *Antioxid Redox Signal.* 2007; 9:2323–33. [PubMed: 17979529]
26. Lin JH, Li H, Yasumura D, Cohen HR, Zhang C, Panning B, et al. IRE1 signaling affects cell fate during the unfolded protein response. *Science.* 2007; 318:944–9. [PubMed: 17991856]
27. Casas-Tinto S, Zhang Y, Sanchez-Garcia J, Gomez-Velazquez M, Rincon-Limas DE, Fernandez-Funez P. The ER stress factor XBP1s prevents amyloid-beta neurotoxicity. *Hum Mol Genet.* 2011; 20:2144–60. [PubMed: 21389082]
28. Zorov DB, Filburn CR, Klotz LO, Zweier JL, Sollott SJ. Reactive oxygen species (ROS)-induced ROS release: a new phenomenon accompanying induction of the mitochondrial permeability transition in cardiac myocytes. *J Exp Med.* 2000; 192:1001–14. [PubMed: 11015441]
29. Ngoh GA, Papanicolaou KN, Walsh K. Loss of mitofusin 2 promotes endoplasmic reticulum stress. *J Biol Chem.* 2012; 287:20321–32. [PubMed: 22511781]
30. Schneeberger M, Dietrich MO, Sebastian D, Imbernon M, Castano C, Garcia A, et al. Mitofusin 2 in POMC neurons connects ER stress with leptin resistance and energy imbalance. *Cell.* 2013; 155:172–87. [PubMed: 24074867]
31. Debattisti V, Pendin D, Ziviani E, Daga A, Scorrano L. Reduction of endoplasmic reticulum stress attenuates the defects caused by *Drosophila* mitofusin depletion. *J Cell Biol.* 2014; 204:303–12. [PubMed: 24469638]
32. Yarosh W, Monserrate J, Tong JJ, Tse S, Le PK, Nguyen K, et al. The molecular mechanisms of OPA1-mediated optic atrophy in *Drosophila* model and prospects for antioxidant treatment. *PLoS Genet.* 2008; 4:e6. [PubMed: 18193945]
33. Dahl R. *Charlie and the Chocolate Factory.* 1985
34. Archer SL. Mitochondrial fission and fusion in human diseases. *N Engl J Med.* 2014; 370:1074. [PubMed: 24620884]

Highlights

1. Mitochondrial fragmentation was genetically induced in *Drosophila* heart tubes.
2. Forcing mito fission by Drp1 overexpression was benign for mitochondria and hearts.
3. Interrupting fusion by either Opa1 or MARF suppression provoked cardiomyopathy.
4. Opa1 RNAi phenotypes were rescued by ROS scavenging; not by reducing ER stress.
5. MARF RNAi phenotypes were rescued by reducing ER stress; not by ROS scavenging.

Figure 1. Cardiomyocyte-specific expression of Drp1 induces mitochondrial fragmentation without heart tube dysfunction

A. Mitochondrial size in representative control (tinc 4-Gal4/+; Ctrl) and cardiac Drp1 transgenic (TG) cardiomyocytes by confocal analysis of cardiac-expressed mito-GFP fluorescence. White scale bar is 20 μm. **B.** Group histogram data for mitochondrial area (left graph), cumulative distribution curve (inset) and comparison of the median mitochondrial area (bar graph) of control (Ctrl) and Drp1 overexpressing (Drp1 TG) flies. **C.** Drp1 expression in isolated heart tubes. Left, mRNA by RT-qPCR; right, immunoblot analysis. **D.** Optical coherence tomography (OCT) of heart tubes. Group quantitative data for end-systolic dimension (ESD) and fractional shortening are to the right. **E.** Representative images and quantitative data (right) for tinc 4-Gal4-driven mito-GFP (green) and TMRE (red) individual and merged confocal micrographs.

Figure 2. Expression of transgenic SOD1 and SOD2 rescue the cardiac dysfunction evoked by Opa1 knockdown

A. OCT of heart tube contractions in Ctrl (tinc 4-Gal4/+), and strains with cardiac specific expression of Opa1 RNAi, transgenic SOD1, transgenic SOD2, Opa1 RNAi along with SOD1(TG) and Opa1 RNAi along with SOD2 (TG), driven by tinc 4-Gal4. **B.** Bar graphs showing group mean OCT data for ESD and percent fractional shortening for the groups in A. **C.** Mitochondrial size as per Figure 1.

Figure 3. Cardiac dysfunction evoked by MARF knockdown is unaffected by over-expression of transgenic SOD1 or SOD2

A. and B. OCT and **C.** mitochondrial size studies as in Figure 2, except for cardiomyocyte-specific mitofusin/MARF deficiency.

Figure 4. Expression of transgenic SOD alleviates the cardiomyocyte mitochondrial membrane potential loss and suppresses ROS production in Opa1 but not in MARF knockdowns

A and B. Representative images of 4-Gal4-driven mito-GFP (green) and TMRE (red) double stained images of Opa1 RNAi +/- SOD1 (**A**) and MARF RNAi +/- SOD1 (**B**). To the right are group quantitative data from TMRE images (left panels) and for the corresponding ROS studies (right panels). **C and D.** Mitochondrial polarization status as a function of organelle size in Opa1 (**C**) and mitofusin/MARF (**D**) knockdown heart tubes. Insets show individual data points (n=3 hearts per group; * $P < 0.05$). White scale bar is 12 μm .

Figure 5. Decreasing mitochondrial ROS improves cardiac function in Opa1 RNAi, but not mitofusin/MARF RNAi, flies

A. Representative images from OCT studies of contracting heart tubes. **B.** Group mean OCT data. **C. and D.** Group mean histogram data for mitochondrial area (left), cumulative distribution curve (middle) and median mitochondrial area (right) for MARF RNAi (C) and Opa1 RNAi (D) with and without ROMO1 RNAi.

Figure 6. ER stress response gene Xbp1 suppresses cardiac dysfunction induced by mitofusin/MARF knockdown, but not by Opa1 knockdown
A. Representative OCT images. **B.** Group mean OCT data. **C.** Mitochondrial morphology as in Figure 4.

Figure 7. Fusion factor-specific restoration of mitochondrial membrane potential by cardiomyocyte-specific expression of ROMO1 RNAi or Xbp1 transgene

A. Representative double stained images of tinc 4-Gal4–driven mito-GFP (green) and TMRE (red) showing depolarized mitochondria (with less red staining). MARF and Opa1 knockdowns strains show increased number of mitochondria which are not stained by TMRE. Expression of the transgenic Romo1 RNAi decreases the number of depolarized mitochondria in the Opa1 but not in the MARF knockdown. **B.** Group quantitative data of stained mitochondria in heart specific expression of Romo1 RNAi in the Opa1 knockdown. **C.** Group quantitative data of heart specific expression of Romo1 RNAi or Xbp1 (TG) in the MARF knockdown flies.

*=P<0.05; ns=non-significant.