Mitochondria interact via actions of outer and inner membrane fusion proteins. The role of mitochondrial fusion in functioning of the heart, where mitochondria comprise ~30% of cardiomyocyte volume and their intermyofilament spatial arrangement with other mitochondria is highly ordered, is unknown.

Objective: Model and analyze mitochondrial fusion defects in *Drosophila* melanogaster heart tubes with tincΔ4Gal4-directed expression of RNA interference (RNAi) for mitochondrial assembly regulatory factor (MARF) and optic atrophy (Opa)1.

Methods and Results: Live imaging analysis revealed that heart tube–specific knockdown of MARF or Opa1 increases mitochondrial morphometric heterogeneity and induces heart tube dilation with profound contractile impairment. Sarcoplasmic reticular structure was unaffected. Cardiomyocyte expression of human mitofusin (mfn)1 or -2 rescued MARF RNAi cardiomyopathy, demonstrating functional homology between *Drosophila* MARF and human mitofusins. Suppressing mitochondrial fusion increased compensatory expression of nuclear-encoded mitochondrial genes, indicating mitochondrial biogenesis. The MARF RNAi cardiomyopathy was prevented by transgenic expression of superoxide dismutase 1.

Conclusions: Mitochondrial fusion is essential to cardiomyocyte mitochondrial function and regeneration. Reactive oxygen species are key mediators of cardiomyopathy in mitochondrial fusion-defective cardiomyocytes. Postulated mitochondrial–endoplasmic reticulum interactions mediated uniquely by mfn2 appear dispensable to functioning of the fly heart. (Circ Res. 2011;108:12-17.)

Key Words: mitochondrial fusion ■ cardiomyopathy ■ superoxide dismutase

Mitochondria generate energy and play central roles governing cell signaling and programmed death.1,2 Individual mitochondria periodically fuse through the coordinated efforts of 3 small, membrane-bound GTPases. Mitofusins (Mfn)1 and -2 on outer mitochondrial membranes are essential for early mitochondrial tethering and outer membrane fusion. Optic atrophy (Opa)1 on inner mitochondrial membranes mediates late fusion. Mitochondria occupy ~30% of the volume of a cardiac myocyte and are highly organized within these cells. Accordingly, cardiac myocytes are sensitive to abnormalities of mitochondrial number or function.3,4 The role of mitochondrial fusion within the highly ordered mitochondrial stacks of cardiomyocytes, and the consequences of altered mitochondrial fusion or mitochondrial–sarcoplasmic reticulum (SR) tethering in these uniquely structured cells, is unknown.

Fly Stocks and New Transgenic Lines
Fly stocks were obtained from the Bloomington *Drosophila* Stock Center at Indiana University; stock numbers are UAS-GFP (no. 9899), UAS-mitoGFP (no. 8442), and UAS-Sod.A (no. 24750). Rolf Bodmer (Sanford-Burnham Medical Research Institute, La Jolla, California) provided tincΔ4Gal4.5 Ming Guo (University of California, Los Angeles) provided MARF RNAi-UAS and Opa1 RNAi-UAS.6 hMfn1 and hMfn2 transgenic lines were constructed by subcloning their cDNAs into pUAST. Five independent lines each of hMfn1 and hMfn2 flies were examined; 2 of each were selected for studies. Adding a cb5 epitope to green fluorescent protein (GFP) using PCR mutagenesis and subcloning into pUAST created the ER/SR-GFP fly.

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.
Results

As a first step to determine the roles of mitochondrial fusion and mitochondrial-SR tethering on cardiac myocyte function we examined hearts of adult *Drosophila* expressing RNAi for mitochondrial assembly regulatory factor (MARF), a *Drosophila* ortholog of mammalian mitofusins.\textsuperscript{6} MARF expression was suppressed 80% by a ubiquitously expressed MARF RNAi (tubulin-Gal4 driver) in second instar larvae (Figure 1a), but all larvae died before pupation. Therefore, we directed expression of MARF RNAi to cardiomyocytes using tinc\textsuperscript{Δ4}-Gal4. Heart tube–specific MARF RNAi flies were viable with normal longevity (Online Figure I). We examined the consequences of MARF suppression on cardiomyocyte mitochondria using mitochondrial-directed GFP expressed in cardiomyocytes using tinc\textsuperscript{Δ4}-Gal4, and confocal microscopy of live isolated (or for phalloidin staining, formalin-fixed) heart tubes. Wild-type cardiomyocyte mitochondria appeared as relatively homogenous individual organelles arranged longitudinally between myofilaments (Figure 1b through 1d). Mitochondria are motile in neurons and fibroblasts\textsuperscript{7,8} but were immobile in cardiomyocytes, as determined by time-lapse confocal microscopy of living heart tubes (Online Figure II). MARF RNAi cardiomyocyte mitochondria tended to cluster in aggregates that distorted the normal myofibrillar architecture (Figure 1c). As is characteristic of mitofusin 1 and 2 double-deficient murine embryonic fibroblasts,\textsuperscript{9} higher magnification revealed extensive mitochondrial heterogeneity with both

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Figure 1. Suppression of mitochondrial fusion proteins MARF or Opa1 in *Drosophila* cardiomyocytes alters mitochondrial morphogenesis. a, RT-qPCR analysis of Marf and Opa mRNA in Marf RNAi flies. b through h, Live cell (b, d, and f) and fixed heart tube (c, g, and h) confocal analysis of mitochondrial (b, c, d, and f), t-tubule (g), and SR (h) morphometry and subcellular distribution in control (Gal4), MARF RNAi (b through d, g, and h) and Opa1 RNAi (f) cardiomyocytes. *P<0.05 vs Gal4 controls.
mitochondrial fragments and enlarged organelles (Figure 1d). Mean mitochondrial size was decreased $\approx 30\%$ (Figure 1e).

We also generated flies in which expression of the mitochondrial inner membrane fusion protein, Opa1, was suppressed in cardiac myocytes. Mitochondrial morphogenesis of Opa1 RNAi cardiomyocytes showed similar abnormalities, with mitochondrial clustering, fragmentation, and a $\approx 30\%$ decrease in mean mitochondrial size (Figure 1e and 1f).

To determine whether morphological abnormalities induced by suppressing mitochondrial fusion were specific to mitochondria, we examined 2 other subcellular structures that are physically and structurally linked to cardiomyocyte mitochondria, t-tubules and SR.10 T-tubules, which are invaginations of the sarcolemma, were visualized with fluorescein-labeled wheat germ agglutinin and were unaffected by MARF suppression (Figure 1g). Cardiomyocyte SR was visualized by tinc–Gal4–driven expression of a GFP-cb5 fusion protein. Wild-type SR has a fine reticular structure (Figure 1h) that was not disrupted in MARF RNAi hearts. Thus, suppressing Drosophila cardiomyocyte mitochondrial fusion proteins alters mitochondrial, but not SR or t-tubule, morphology.

The functional consequences of suppressing mitochondrial fusion were assessed using optical coherence tomography of unanesthetized adult Drosophila heart tubes.11 Compared to wild-type flies, heart tubes of cardiac MARF RNAi flies were dilated and exhibited impaired shortening (Figure 2a through 2e) with no change in beating rate (Figure 2b and 2f). Remodeling and contractile impairment induced by MARF RNAi affected the entire heart tube (Figure 2a; Online Movies 1 and 2). Opa RNAi heart tubes were also dilated (Figure 2c), but the contractile abnormality and remodeling were more severe (Figure 2a, 2d, and 2e; Online Movie 3). Consistent with the more severe cardiomyopathy, lifespan of Opa1 RNAi flies was reduced by $\approx 25\%$ (Online Figure I). TUNEL staining did not show evidence for cardiomyocyte death (Online Figure III).

These results link mitochondrial structural abnormalities and heart tube dysfunction to deficiency of mitochondrial outer or inner membrane fusion proteins. Whereas Drosophila has only the single outer mitochondrial membrane fusion protein MARF, mammals have 2 structurally similar mitochondrial outer membrane proteins, Mfn1 and Mfn2. Mfn2 is distinguished from Mfn1 by lower GTPase activity.12 Both Mfn1 and Mfn2 induce mitochondrial tethering and outer membrane fusion, but in fibroblasts, Mfn1 uniquely requires Opa1,13 whereas Mfn2 uniquely mediates mitochondrial–endoplasmic reticulum (ER) tethering.14 We explored whether these differences in Mfn1 and Mfn2 impacted the cardiomyopathy of MARF deficiency by expressing human (h)Mfn1 and hMfn2 in Drosophila cardiomyocytes. hMfn1 and hMfn2 had little impact on adult Drosophila heart tube dimension or contractile function in the wild-type background (Online Figure IV). However, both hMfn1 (Figure 3a and 3b) and hMfn2 (Figure 3c and 3d) improved the cardiomyopathy induced by MARF suppression. The Mfn2 “rescue” was complete, whereas that for Mfn1 was line-dependent. These findings demonstrate that Drosophila MARF is functionally analogous to mammalian mitofusins and indicate that the unique properties of mammalian Mfn1 and Mfn2 have little impact on fly cardiomyocyte function.

mRNA sequencing of MARF RNAi heart tubes showed abnormal cardiomyocyte expression of skeletal muscle myosin and actin isoforms, recapitulating isoform-switching characteristic of cardiac hypertrophy in mammalian hearts. Gene Ontology grouping of upregulated Drosophila MARF RNAi heart tube mRNAs (Online Figure V) revealed disproportionately increased gene expression in 3 categories, mitochondrial inner membrane,
mitochondrial envelope, and carbohydrate metabolism, each of which is rich in nuclear-encoded mitochondrial genes. Thus, cardiomyocyte mitochondrial fusion defects stimulated mitochondrial biogenesis.

Mitochondrial biogenesis can be a compensatory response to eroding mitochondrial function. Mitochondrial production of reactive oxygen species is both a cause and consequence of mitochondrial dysfunction. To determine whether mitochondrial reactive oxygen species contributed to MARF RNAi cardiomyopathy, superoxide dismutase (SOD)1 was expressed in heart tubes. SOD1 expression did not alter normal heart tube dimension or contraction (Online Figure IV). Strikingly, however, SOD normalized heart tube dilatation and contractile dysfunction of MARF RNAi flies (Figure 4a) and markedly improved mitochondrial morphometrics (Figure 4b).

Discussion
These studies provide the first evidence that mitochondrial fusion occurs in cardiomyocytes and is essential to mitochondrial and heart function. Mitochondrial organization within Drosophila and mammalian cardiomyocytes is highly ordered, with closely packed mitochondria interspersed in “stacks” between the myofilaments. Spatially enforced intermitochondrial interactions and limited intracellular mitochondrial mobility suggested that mitochondrial tethering proteins, ie, Drosophila MARF and the mammalian mitofusins, might be superfluous in cardiomyocytes. On the other hand, the postulated critical roles in cardiomyocytes for ER/SR–mitochondrial calcium transport through tight interorganelle junctions mediated specifically by Mfn2 suggest an essential role for Mfn2 independent of, or in addition to, mitochondrial fusion. Based on the present data, we propose that mitochondrial fusion is essential in cardiomyocytes notwithstanding mitochondrial packing, but that specific functions of Mfn2, such as tethering of cardiomyocyte mitochondria to SR, are dispensable for normal Drosophila heart tube function.

The finding that the MARF RNAi cardiomyopathy is rescued by both hMfn1 and hMfn2 demonstrates that similar mechanisms regulate mitochondrial fusion and
functional stability in insects and vertebrates, and has 2 additional important implications. First, *Drosophila* MARF is functionally analogous to the mammalian mitofusin proteins. Second, Mfn1 and Mfn2 are largely interchangeable with each other and with MARF in *Drosophila* heart tubes. Thus, the unique properties of mammalian Mfn1 (higher GTPase activity\(^{12}\) and functional interaction with Opa1\(^{13}\)) and Mfn2 (ability to tether mitochondria to ER/SR\(^{14}\)) are not essential to *Drosophila* cardiomyocyte health. This does not indicate that Mfn2-mediated mitochondrial–ER/SR interactions do not occur in cardiomyocytes, or are not important for other aspects of cardiomyocyte biology such as programmed cell death,\(^{18}\) but does suggest that putative mitochondrial–SR interactions mediated by Mfn2 are not essential to normal heart tube function in flies.

**Sources of Funding**

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**Disclosures**

None.

**References**

What Is Known?

- Because of the energy required for life-long cardiac pumping, myocardium is the most mitochondrial-rich tissue.
- Although it has not been studied in cardiac myocytes, in most cell types, normal mitochondrial regeneration requires periodic intermitochondrial exchange of contents.
- This mitochondrial content exchange process requires mitochondrial tethering and outer membrane fusion mediated by mitofusins 1 and 2 and inner membrane fusion mediated by optic atrophy (Opa)1.

What New Information Does This Article Contribute?

- Using the fruit fly heart tube as a model, we show that mitochondrial fusion proteins are necessary to maintain normal cardiac structure and contractile function.
- Genetic complementation to rescue the cardiomyopathy that resulted from suppressing mitochondrial fusion reveals that the fruit fly and human outer mitochondrial membrane fusion proteins are functionally redundant.
- Through a similar genetic complementation strategy, we show that mitochondrial dysfunction and reactive oxygen species production are critical mediators of cardiac dysfunction induced by loss of mitochondrial fusion.

Mitochondrial fusion is observed in many cells, but its occurrence and functional implications are unknown in cardiomyocytes. Here, genetic inhibition of Drosophila melanogaster cardiomyocyte mitochondrial fusion through RNAi suppression of the mitofusin analog Marf, or inner membrane protein Opa1, produced heterogeneity of mitochondrial size, transcriptional evidence for mitochondrial biogenesis, and dilated cardiomyopathies. Human mitofusins 1 or 2 prevented Marf RNAi cardiomyopathy, demonstrating overlapping functions in Drosophila cardiomyocytes. Marf and Opa1 RNAi cardiomyopathies were rescued by cardiomyocyte expression of superoxide dismutase, implicating reactive oxygen species. Thus, mitochondrial fusion in cardiomyocytes is essential for normal mitochondrial function and to prevent dilated cardiomyopathy.
EXPANDED METHODS SUPPLEMENT

**Live imaging of *Drosophila* heart tube contraction.** Optical coherence tomographic imaging of *Drosophila* heart tubes used a modification of a previously described technique ¹ on a Michelson Diagnostics (Maidstone, UK) EX 1301 OCT microscope at 1,300-nm wavelength laser directed transversely and scanning at 4,000 lines per second. Flies anesthetized with CO₂ were positioned ventral side down on a tacky gel-filled petri dish, allowed to recover, and the heart tube imaged at its largest diameter, just caudal to the thoraco-abdominal junction. B-mode images were generated by post-analysis. Measures of internal chamber diameter at end systole (ESD) and end-diastole (EDD) were determined at room temperature in two orthogonal views and averaged to calculate fractional shortening (FS=EDD-ESD/EDD).

Fluorescence imaging of heart tube function in flies expressing cardiomyocyte-directed soluble GFP was performed on similarly prepared flies on a Nikon AZ100 UV fluorescent microscope at 100x magnification. Images were acquired at 50 frames per second using a Photometrics Evolve EMCCD camera.

**mRNA expression profiling.** For RT-PCR, RNA was prepared from twelve 5-day old larvae using TRIzol (Invitrogen) and cDNA was prepared by oligo (dT) priming and reverse transcription. PCR was used the following primers: MARF Forward GGCGAGGCGTATCTTATGAC, Reverse AGCTTCTCCTGGCACAA, Probe CGCCAGTTGTTGATGTTCCACCACAT (145 bp amplicon); Opa1 Forward CTCTGAGCACCAAGCTAT, Reverse GGCGCAACTTGATGTCTA, Probe CAGTCAGGTTCTCAAAATTCCTTCAACA CG (120 bp amplicon), and quantified by real-time qPCR.

For transcriptional profiling of *Drosophila* heart tubes, RNA was prepared from 8-10 heart tubes per sample and pooled. RNA sequencing libraries were prepared and sequenced on an Illumina Genome Analyzer II as described ², except that due to the low starting RNA input, polyA(+)-selection was not performed prior to RNA fragmentation and further processing, and following sequencing adapter ligation, 16-18 cycles of PCR were needed to recover libraries. Sequence reads were aligned to release 3 of the *Drosophila* genome using the bowtie module in Partek Genomics Suite v6.5 (Partek, St. Louis, MO), and expression levels calculated as number of reads per kilobase per million aligned reads (RPKM) ³. Libraries were sequenced to a depth of 2 million aligned reads.

**Live confocal imaging.** Heart tubes were dissected from *Drosophila* expressing mito-GFP using a dissecting scope, maintained in S2 (Schneider’s Drosophila) media (Invitrogen), and positioned on the stage of a Nikon Eclipse Ti laser confocal microscope with a Plan Apo VC 60x/1.40 Oil objective, images at 488 nm. Mitochondrial morphometrics were assessed using Nikon NIS-Elements AR3.0 software in at least four cardiac myocytes from the cranial portion of 10-12 heart tubes per group, and are expressed as arbitrary units generated by pixel area. In some experiments, cardiomyocyte nuclei were visualized with Hoescht dye.

For fixed tissue staining, isolated heart tubes were formalin-fixed prior to staining with Alexa633-conjugated phalloidin (1:1,000; Invitrogen) to visualize sarcomeric elements and DAPI to visualize nuclei; the green channel was used for mito- or ER/SR-GFP or TUNEL staining, as indicated.

**Statistics.** All data are presented as means±s.e.m. unless indicated. Paired studies were analyzed using a two-tailed Student’s t-test. Multiple group comparisons used one-way ANOVA with Tukey’s post-hoc test.
References


SUPPLEMENTAL FIGURES

![Online Figure 1. Decreased longevity of flies with cardiomyopathy caused by Opa1 suppression.](image)

Male Gal4 control, MARF RNAi, and Opa1 RNA flies were collected the day of hatching, placed into vials in groups of 25 each, and aged at 25 C on yeast glucose media. Flies were counted every other day and survivors flipped onto fresh food. Survival curves were compared by Kaplan-Meier analysis and log-rank test. Decreased survival of Opa1 RNAi flies was replicated in two independent studies of ~300 flies each.
Online Figure II. Absence of mitochondrial mobility in live cardiomyocytes from isolated Drosophila heart tubes. Serial confocal imaging of cardiomyocyte from tincΔ4Gal4, mito-GFP fly heart tube. Mitochondria are green, Hoescht-stained nuclei are blue. Images were acquired every 15 minutes. Top, 600x magnification of single cardiomyocyte showing typical pattern of inter-myofibrilar mitochondrial stacking that does not change over 2 hour observation period. Bottom, digital zoom (4x) image from a different cell showing individual mitochondria.
Online Figure III. Absence of TUNEL staining of cardiomyocytes from MARF and Opa1 RNAi heart tubes. Merged confocal micrographs (600x) of fixed heart tubes labeled with TUNEL (green) and counterstained with F-actin phalloidin (red) and nuclear DAPI (blue). DNAase-treated control is shown in lower right panel.

Online Figure IV. Quantitative analysis by optical coherence tomography of heart tube dimensions and contractile performance of fly lines. OCT was performed on 7-day old adult flies on the indicated lines. Experimental n for each group is shown in bars of upper left panel.
**Online Figure V.** RNA profiling and gene-ontology analysis of Drosophila heart tubes. Transcript expression levels were determined by Illumina RNA sequencing of pools of 10 heart tubes per group. Heat map on left shows hierarchical clustering of mRNA expression values. Charts on right show results of GO analyses of up-, down-, and non-regulated transcripts. Asterisks indicate significantly regulated GO category, versus Gal4 control.