Mitochondria Are Important Because: We Need Them to Live but They Can Kill Us

The heart is the most mitochondrial-rich mammalian organ. Therefore, hearts are exquisitely dependent on, and susceptible to, being damaged by mitochondria. Although the basic mechanisms of mitochondrial oxidative phosphorylation and ATP synthesis are conserved throughout the evolution, adult cardiomyocyte mitochondria have morphological and functional properties that distinguish them from mitochondria of other mammalian cell types. Therefore, it is not clear how attributes of mitochondria from fibroblasts, neurons, and other cells relate to the unique biological context of cardiomyocytes. For example, a PubMed search of the term "mitochondrial dynamics" retrieves >3500 scientific articles. Yet, those who have performed live-cell studies understand that mitochondrial dynamism seems oxymoronic when applied to adult hearts: cardiomyocyte mitochondria seem static, neither moving, fusing, nor dividing. Nevertheless, the proteins that mediate mitochondrial fusion and fission are highly expressed in hearts. Why is this? Hearts also possess a robust machinery for mitochondrial-mediated apoptotic and necrotic cardiomyocyte death, despite a limited capacity to regenerate or replace suicidal cardiomyocytes. Again, why?

Here, recent data that have advanced our understanding of noncanonical functioning of mitochondrial dynamics and death factors in cardiomyocytes wherein mitochondrial morphometric remodeling does not normally occur and the ability to recover from cell suicide is severely limited. (Circ Res. 2015;116:167-182. DOI: 10.1161/CIRCRESAHA.116.303554.)

Key Words: apoptosis ■ autophagy ■ mitochondria ■ mitochondrial dynamics

Abstract: Mitochondrial research is experiencing a renaissance, in part, because of the recognition that these endosymbiotic descendants of primordial protobacteria seem to be pursuing their own biological agendas. Not only is mitochondrial metabolism required to produce most of the biochemical energy that supports their eukaryotic hosts (us) but mitochondria can actively (through apoptosis and programmed necrosis) or passively (through reactive oxygen species toxicity) drive cellular dysfunction or demise. The cellular mitochondrial collective autoregulates its population through biogenic renewal and mitophagic culling; mitochondrial fission and fusion, 2 components of mitochondrial dynamism, are increasingly recognized as playing central roles as orchestrators of these processes. mitochondrial dynamism is rare in striated muscle cells, so cardiac-specific genetic manipulation of mitochondrial fission and fusion factors has proven useful for revealing noncanonical functions of mitochondrial dynamics proteins. Here, we review newly described functions of mitochondrial fusion/fission proteins in cardiac mitochondrial quality control, cell death, calcium signaling, and cardiac development. A mechanistic conceptual paradigm is proposed in which cell death and selective organelle culling are not distinct processes, but are components of a unified and integrated quality control mechanism that exerts different effects when invoked to different degrees, depending on pathophysiological context. This offers a plausible explanation for seemingly paradoxical expression of mitochondrial dynamics and death factors in cardiomyocytes wherein mitochondrial morphometric remodeling does not normally occur and the ability to recover from cell suicide is severely limited. (Circ Res. 2015;116:167-182. DOI: 10.1161/CIRCRESAHA.116.303554.)
compact, and structurally homogenous, the need to move and remodel mitochondrial networks in these cells is minimal. However, the requirement for mitochondrial ATP to fuel cardiac excitation contraction coupling is endless, and the necessity to detect and remove potentially toxic senescent or damaged mitochondria is continual. For this reason, we propose that maintaining mitochondrial fitness through vigorous quality control may be a dominant normal function of mitochondrial dynamics and cell death factors in adult hearts.

**Where Do Cardiomyocyte Mitochondria Come From? And Where Do They Go?**

The answers to these questions seem obvious from names applied to the relevant processes. Mitochondrial biogenesis means “creation of new mitochondria”, and mitophagy means “eating mitochondria”. Alpha and omega; done. The quandary is that mitochondria are not born, live, and die in the conventional sense. Rather, our mitochondria are literally immortal, having been passed down from our mothers, and from their mothers, all the way back to the figurative mitochondrial Eve. If one goes even further back in time, mitochondria were initially derived from independent protobacteria that invaded our primitive unicellular ancestors and established permanent residency as endosymbionts. After a billion years, and despite having exported 99% of their genes to their hosts (ie, to our nuclei), mitochondria retain key characteristic of their bacterial ancestors: (1) they have their own circular genomes encoding 13 electron transport complex enzymes; (2) they possess replicative, transcriptional, and translational machinery necessary to sustain normal homeostatic functioning, organelle growth, and proliferation through replicative fission; and (3) they communicate with the nucleus to promote or suppress biogenic gene expression.

Given this context, the answer to the first question is that an adult individual’s cardiomyocyte mitochondria are all descended from his or her embryonic cardiomyocyte progenitor cells. As the embryonic cells grew and proliferated, its resident mitochondria did the same. This represents de facto mitochondrial biogenesis, a perpetual cycle in which mitochondria import nuclear-encoded proteins and synthesize mitochondrial-encoded proteins and genomic components for individual organelle growth; the so-called new organelles are periodically created via replicative fission (Figure 1A). The same biogenic process is used for homeostatic mitochondrial renewal in adult cardiomyocytes and is dynamically regulated by physiological or pathological stress. Much has been learned about the genes that coordinate nuclear gene expression for mitochondrial biogenesis, especially PGC-1α and PPARγ, but the mechanisms by which mitochondria communicate with the nucleus to promote or suppress biogenic gene expression are poorly described.

So, where do mitochondria go? The mitochondrial collective of a given organism is essentially immortal as long as the host is viable, but individual mitochondria will sustain damage or eventually become senescent. Modest organelle damage is repaired through biogenic replacement of damaged components, or by fusion with and complementation by a healthy organelle (Figure 1B). Lethal damage of a magnitude or nature that precludes successful repair places the entire cellular mitochondrial pool at risk for contamination (because the consequence of fusion between a severely damaged and healthy mitochondrion is not a larger healthy organelle, but a larger damaged organelle with the potential to fuse with other healthy mitochondria, damaging them, and so on). We call fusion-mediated contamination of the cellular mitochondrial pool through fusion-mediated exchange of DNA, proteins, and lipids.

**Nonstandard Abbreviations and Acronyms**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>Drp1</td>
<td>dynamin-related protein 1</td>
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<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
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<tr>
<td>LC3</td>
<td>microtubule-associated protein 1A/1B-light chain 3</td>
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<tr>
<td>MARF</td>
<td>mitochondrial assembly regulatory facto</td>
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<td>MPTP</td>
<td>mitochondrial permeability transition pore</td>
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<td>Opa1</td>
<td>optic atrophy 1</td>
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<td>PINK1</td>
<td>PTEN-induced putative kinase 1</td>
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<td>ROS</td>
<td>reactive oxygen species</td>
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<td>SOD</td>
<td>superoxide dismutase</td>
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pool mitochondrial contagion. To prevent mitochondrial contagion, cells use mitophagy to identify, functionally sequester, and remove severely damaged mitochondria (Figure 1B).

We have begun to unravel the cellular decision process to either repair or remove a damaged mitochondrion. Consider that the extent of mitochondrial damage will inevitably range across a continuum from mild to severe, but the decision to retain or remove a damaged organelle is categorical (ie, thumbs up or thumbs down). Operationally, the cell must establish a threshold level of mitochondrial damage that will trigger mitophagic removal, while tolerating subthreshold damage. Because subthreshold organelle damage can nevertheless be toxic, mitochondria use the mechanism of asymmetrical fission, integrating mitochondrial fission and mitophagy, to remove functionally compromised organelles before they are sufficiently impaired to do harm to the cell. In this situation, instead of normal replicative fission, the senescent mitochondrion preferentially packages damaged DNA and proteins into 1 daughter organelle, while directing undamaged components into the other. Asymmetrical fission of a sublethally damaged parent mitochondrion thereby produces one healthy daughter and one damaged daughter. Because the dysfunctional components from the parent are enriched in just one of its daughters, this organelle can meet the threshold for removal by mitophagy, thus avoiding cell toxicity (Figure 1B).

These general concepts illustrate how mitochondrial fusion, fission, biogenesis, and mitophagy are operationally interrelated. The molecular mechanisms by which they interact to orchestrate homeostatic mitochondrial regeneration, renewal, and targeted removal are described in detail in the following sections.

Cardiomyocyte Mitochondria, Where Fusion and Fission Have Special Meaning

Before digging into the molecular weeds of cardiac mitochondrial dynamism and quality control, it is worth noting that mitochondria in most mammalian cell types do not resemble the stubby ovoid organelles diagramed in high-school textbooks. In cell types most frequently studied, such as fibroblasts, elongated mitochondria are integrated into a branching and highly interconnected cell-wide reticulum (Figure 2). These mitochondrial networks constantly undergo structural remodeling through fusion and fission; fusion promotes intranetwork organelle communication and complementation/repair, whereas fission can isolate damaged mitochondrial components from the network before their functional sequestration and physical removal (see Figure 1B). Mitochondrial network fission is accelerated before cell mitosis, facilitating equal mitochondria distribution into daughter cells. Dissolution of the mitochondrial network during cell replication may also be necessary to accommodate structural changes in the parent, and then daughter, cells as they divide and reform.

In comparison, in the adult cardiomyocytes of flies, mammals, and organisms that evolutionarily span the 2, mitochondria exist largely as discrete rounded organelles packed together between the myofibrils (Figure 2). Adult cardiomyocyte mitochondria also cluster within the perinuclear and subsarcolemmal regions, but in no instance do they normally form interconnected networks. Thus, when compared with nonmyocytes, individual cardiomyocyte mitochondria appear fragmented.

In considering why cardiomyocyte (and skeletal muscle) mitochondria lack the degree of connectivity that typifies most other cell types, a biomechanical explanation arises: a collection of individual cardiomyocyte mitochondria is intrinsically more deformable than a highly interconnected network. The real-world analogy is the interconnected components of the roll cage of a racing car, a structure specifically designed to resist deformation, compared with a bean bag that can readily and reversibly conform to almost any shape (Figure 2). Thus, mitochondrial fragmentation that is observed in most cells before mitosis not only enables partitioning of mitochondria to daughter cells but also promotes structural malleability necessary to accommodate plasticity in cell size and shape. If mitochondria in these cells maintained their basal interconnected morphometry then, as with a roll cage, cell plasticity would be physically restricted. By extension, if a cardiomyocyte contained highly interconnected mitochondria, it might have

Figure 2. Structural differences between mouse fibroblast and adult cardiomyocyte mitochondria. A, Top, is MitoTracker Green stained filamentous, interconnected mitochondria of a cultured murine embryonic fibroblast; bottom, roll cage of a NASCAR racing car, specifically designed to withstand compressive forces. B, Top, Distinct individual rounded green fluorescent protein–labeled mitochondria on an isolated adult mouse cardiomyocyte; bottom, bean bag (inset shows bean structure), specifically designed to be readily and reversibly deformable.
to fragment and reform mitochondrial interconnections cyclically with each contraction and relaxation cycle, or expend sufficient energy to overcome the resistance produced by this internal elastic component. Consistent with a link between contraction and a fragmented mitochondrial structure, early embryonic cardiomyocytes exhibit a more network-like and interconnected mitochondrial morphometry, but acquire the typical mature individual ovoid structure later in development because the heart is increasingly called on to generate circulatory flow. Therefore, mitochondrial networking is at least dispensable, and likely would be detrimental, to the normal pump functioning of adult cardiac myocytes.

Because mitochondrial networks do not exist in adult cardiac myocytes, and any role for mitochondrial fusion and fission in network remodeling is therefore irrelevant, genetic interdiction of mitochondrial fission and fusion in hearts has uncovered atypical functions of mitochondrial dynamics proteins. Protein mediators of mitochondrial fusion and fission are highly conserved across evolution, and the biomolecular events they evoke are well known. Mitochondrial fission results from recruitment and directed multimerization of a dynamin superfamily GTPase, dynamin-related protein 1 (Drp1). Drp1 oligomerizes in a head to toe configuration around the mitochondrial equator and constricts in a GTP-dependent manner, ligating the parent organelle into 2 daughters. Asymmetrical fission uses the same outer membrane fusion,5 and finally optic atrophy 1 (Opa1)–mediated inner membrane tethering followed by fusion,6,7 Fusion (right) requires initial mitofusin 1 (Mfn1)/Mfn2-mediated outer membrane tethering followed by fusion, and finally optic atrophy 1 (Opa1)–mediated inner membrane fusion.8

Figure 3. Molecular mechanism of mitochondrial fission and fusion. The 3 molecular drivers of fission and fusion are schematically depicted as they would be associated with a normal mitochondrion. Replicative fission (left) is initiated by recruitment of cytosolic dynamin-related protein 1 (Drp1) to the organelle, Drp1 oligomerization, and constriction of the parent into 2 daughters. Asymmetrical fission uses the same mechanism. Fusion (right) requires initial mitofusin 1 (Mfn1)/Mfn2-mediated outer membrane tethering followed by fusion, and finally optic atrophy 1 (Opa1)–mediated inner membrane fusion.9

Mitochondrial fusion is somewhat more complex, requiring 3 distinct steps: tethering, outer membrane fusion, and inner membrane fusion. Tethering is the physical attachment between outer membranes of 2 mitochondria and is a prerequisite to actual membrane fusion. Tethering can be mediated by either (or both) of 2 other dynamin superfamily GTPases, the mitofusins (so named because they also promote outer mitochondrial membrane fusion). If one considers the amino-terminal GTPase domain of a mitofusin protein to be its head, then its carboxyl-terminal tail is comprised of an α helical heptad repeat that can homotypically (mitofusin 1–mitofusin 1 or mitofusin 2–mitofusin 2) or heterotypically (mitofusin 1–mitofusin 2) dimerize in trans (ie, tail to tail) with other mitofusin molecules on adjacent mitochondria. Tethering occurs via transdimerization of the antiparallel coiled coils of 2 mitofusin tails and is GTPase independent10 (Figure 3). The physical nature of this interaction is much like that of Velcro hooks attaching to loops. Because the mitofusin–mitofusin tethering interaction is accomplished by hydrogen bonding, tethering (like Velcro binding) is fully reversible.11

Mitochondrial tethering can be followed by physical fusion between the outer membranes of 2 tethered organelles. Fusion is also mediated by mitofusin 1 or mitofusin 2, but unlike tethering, is dependent on GTP hydrolysis and is irreversible. The product of outer membrane fusion is an organelle having 2 distinct internal matrix structures, described as a double yolk egg12 (Figure 3). This organelle configuration is not commonly observed because outer membrane fusion is normally followed immediately by inner membrane fusion mediated by yet another dynamin superfamily GTPase, Optic Atrophy 1 (Opa1)13,14 (Figure 3).

Mitochondrial fission and fusion occur constantly in cultured fibroblasts and is readily observed using a variety of fluorescent techniques. For those who are interested, static and time-lapse moving images demonstrating typical mitochondrial fission/fusion dynamics in HeLa cells and mouse fibroblasts have been posted on his Web site by Dr David C. Chan, one of the pioneering researchers into mechanisms of mitochondrial fusion (http://www.hhmi.org/research/mitochondrial-dynamics-development-and-disease). Genetic ablation or suppression studies in cells such as these wherein mitochondria are constantly repositioning and mutually interacting have proven that Drp1, mitofusin 1, mitofusin 2, and Opa1 are essential to normal mitochondrial network maintenance and remodeling. However, fluorescent live-cell studies of adult Drosophila heart tubes found no evidence for intracellular mitochondrial movement or interorganelle fusion or fission over several hours.15 The absence of readily observable mitochondrial dynamism in adult cardiomyocytes raises the question of what secondary or ancillary functions mitochondrial dynamics factors might be playing in their hypodynamic mitochondria.

Cardiac Mitochondria and Ducks: Calm on the Surface, but Working Like Mad Underneath

As noted above, fibroblast mitochondria are observably dynamic, whereas cardiomyocyte mitochondria might be characterized as languid. Therefore, it seems incongruous that
mitochondrial network remodeling fission/fusion proteins are abundant in normal adult cardiomyocytes. To define atypical roles of these factors more completely, several groups independently began in vivo genetic dissections of mitochondrial fusion pathways in fly and mouse hearts.

The initial in vivo genetic manipulations of cardiac mitochondrial fusion factors were performed in the heart tubes of *Drosophila* fruit flies.17 Flies have a single mitofusin, called mitochondrial assembly regulatory factor (MARF), which is suppressible in a tissue-specific manner using transgenically expressed RNAi.18 MARF RNAi was transgenically expressed in fly hearts under control of a tinman promoter driver (*tinman* is the *Drosophila* ortholog of the mammalian cardiomyocyte-specific differentiation factor, *nkh2.5*). When compared with controls, MARF-deficient fly cardiomyocytes had smaller mitochondria; cardiac MARF-deficient flies developed enlarged and hypocontractile heart tubes (ie, a cardiomyopathy).17 Suppression of *Drosophila* Opa1 using the same *tinman* RNAi approach induced a somewhat different mitochondrial dysmorphology characterized by greater organelle heterogeneity, but a similar cardiomyopathy.17 These results provided the first evidence that (in flies at least) cardiomyocyte mitochondria must undergo fusion (because they became smaller as a consequence of suppressing MARF), and also demonstrated that MARF- and Opa1-dependent mitochondrial fusion is (for whatever reason) essential to normal heart functioning.

Higher organisms, including mammals, have 2 mitofusin proteins encoded by distinct genes. Germ-line ablation of either mitofusin gene in mice is embryonic lethal, likely because of adverse consequences on placental development.19 However, studies of embryonic fibroblasts derived from mitofusin 1, mitofusin 2, and mitofusin 1/mitofusin 2 double-knockout mice revealed that (1) mitofusin 1 and mitofusin 2 are almost entirely redundant in promoting mitochondrial tethering and outer membrane fusion, each can largely substitute the other;20 (2) mitofusin 2 is unique in its ability to localize to ER and tether this calcium-containing organelle to mitochondria; mitofusin 1 does not share this activity;20 (3) absence of both mitofusins is compatible with cell viability, but mitochondria lacking both mitofusin 1 and mitofusin 2 are severely dysmorphic and partially depolarized.21 David Chan’s group worked around embryonic lethality of germ-line mitofusin 1 and mitofusin 2 ablation by creating the respective floxed allele mice, which have been used by multiple research teams to produce viable tissue-specific mouse genetic knockouts for mitofusin 1 and mitofusin 2. Chan’s studies were the first to prove in vivo functional redundancy between the 2 mitofusins and show that mitofusin activity is essential for normal brain and skeletal muscle development and function.22,23 As detailed below, the same mitofusin 1 and mitofusin 2 floxed allele mouse models have been used in combination with cardiomyocyte-specific Cre lines to ablate mitofusin 1 and mitofusin 2 individually, and both mitofusins in combination, in mouse hearts.

To date, mitofusin 1 and mitofusin 2 have been genetically deleted from mouse hearts using 3 different types of cardiomyocyte-specific Cre mouse lines. The genetic approach that most closely paralleled the *Drosophila* heart tube MARF knockdown study17 used Cre expressed from an *nkh2.5* knockin allele. Like fly *tinman*, the mouse *nkh2.5* gene is activated in early heart development, conferring largely cardi-ac-specific gene ablation beginning in the cardiac crescent (≈E7.5). Combined mitofusin 1 and mitofusin 2 ablation in the early embryonic heart was uniformly lethal, revealing an absolute requirement for mitochondrial fusion in normal cardiac development.24 Perinatal cardiac-specific ablation of both mitofusin 1 and mitofusin 2 using *myh6*-Cre also resulted in early lethality (by P16), with mitochondrial dysmorphology and matrix degeneration, impaired biogenic gene expression, and cardiomyopathy.25

Early lethality with embryonic or perinatal mitofusin 1/mitofusin 2 ablation mandated an alternate approach to uncover the effects of perturbing mitochondrial fusion in adult hearts. Accordingly, the Dorn group conditionally ablated the mitofusin 1 and mitofusin 2 genes using an estrogen receptor–Cre fusion protein expressed as a *myh6*-driven transgene. The estrogen receptor–Cre transgene is activated in cardiomyocytes after birth, but Cre-mediated gene recombination requires administration of tamoxifen (or raloxifien) to induce nuclear Cre translocation. Using this system, mice carrying all 5 gene alleles required for conditional combined cardiac mitofusin 1/mitofusin 2 ablation were grown until 8 weeks, at which time mitofusin 1 and mitofusin 2 gene recombination was induced using pharmacological administration of estrogen receptor ligand. Concomitant ablation of mitofusin 1 and mitofusin 2 in the adult mouse heart induced mitochondrial fragmentation, cardiomyocyte respiratory defects, and rapidly progressive cardiac dilatation with lethal heart failure within 6 to 8 weeks.24 On the basis of half-time for mitochondrial size reduction after combined mitofusin 1/mitofusin 2 ablation, we calculated that a complete mitochondrial fission–fusion cycle takes ≈15 days in the adult mouse heart. The extremely slow rate of fusion and fission explains why cardiomyocyte mitochondria appear static in short-term studies. The conditional mitofusin 1/mitofusin 2 double-cardiac knockout experiments provided the first evidence that mitochondrial fusion occurs in adult cardiomyocytes, and demonstrated that these 2 mitochondrial fusion proteins are necessary for mammalian cardiac health. However, this early work did not define either the essential physiological requirement for cardiac mitofusins or the underlying physiological defect that provokes cardiomyopathy when their expression is interrupted.

**Regulation of Calcium Signaling by Mitofusin 2 and Mitochondrial Death Proteins**

As introduced above, intermitochondrial tethering by mitofusins is mediated by reversible linking of the ends of filamentous structures attached to bodies requiring physical attachment (like Velcro); mitofusins have sticky antiparallel coiled-coils at their C termini (whereas Velcro has plastic hooks and loops). An intrinsic characteristic of this tethering mechanism is that the tethers themselves are agnostic as to what they link: It doesn’t matter whether Velcro is on your ball peen hammer or David Letterman’s Human Fly jump suit,26 it will form attachments with and stick to its opposite number. This idea also applies to mitofusins; they tether mitochondria...
together because they are located on mitochondrial outer membranes. By extension, if mitofusins were present on other organelles, they could tether those organelles to mitochondria. This hypothetical promiscuity in organelle bonding has not been described for mitofusin 1, which is exclusively mitochondrial. However, a fraction of mitofusin 2 has been localized to endoplasmic/sarcoplasmic reticulum (SR) in fibroblasts and cardiomyocytes. Consequently, intermolecular binding between ER/SR-localized mitofusin 2 and mitochondrial-localized mitofusin 1 or mitofusin 2 physically links these calcium-containing and ATP-producing organelles. Tethering of cardiomyocyte mitochondria to SR has functional implications because it creates protected appositional microdomains through which high concentrations of ionic calcium can be exchanged between organelles (without diffusing away into the cytosol). Cardiomyocyte-specific genetic ablation of mitofusin 2 demonstrated the existence of these microdomains and showed how they enhance mitochondrial sensing and uptake of SR-derived calcium. Mitofusin 2–mediated tethering of SR to mitochondria thus constitutes the sensory arm of a rapid response system that minimizes mitochondrial ATP depletion and reactive oxygen species (ROS) production when workload and metabolic demand are acutely increased. Because calcium can also be a potent stimulus for opening the mitochondrial permeability transition pore (MPTP), the downside to physical tethering of SR and mitochondria is increased sensitivity of MPTP opening to SR-derived calcium. Indeed, cardiomyocyte-specific mitofusin 2 ablation protects the heart against several insults linked to calcium-mediated MPTP opening, including ischemia-reperfusion injury. Thus, under pathological conditions, the molecular and functional features of mitofusin 2 that normally promote mitochondrial health (through fusion-mediated complementation) and that facilitate a rapid metabolic response to acute hemodynamic stress (through SR-mitochondrial calcium cross talk) also predispose to MPTP-mediated cardiomyocyte death.

It is interesting that dual localization of factors to mitochondria and adjacent ER/SR, with resulting modulation of SR-mitochondrial calcium cross talk, is a common characteristic of mitochondrial factors implicated in programmed cell death. For example, the cell death promoting mitochondrial Bcl-2 family protein Nix (also known as Bnip3L) originally garnered interest because its transcriptional upregulation in cardiac hypertrophy induces apoptotic cardiomyocyte death. However, Nix also localizes to SR and modulates SR-mitochondrial calcium signaling that can evoke MPTP opening. Indeed, cardiomyocytes undergoing Nix-mediated cell death exhibit characteristics of both apoptosis and necrosis. By genetically targeting Nix either to mitochondria or to ER/SR, the Dorn laboratory showed that Nix organelle localization, and not some intrinsic property of the protein, determines whether Nix provokes cell death via (mitochondria pathway) apoptosis or (ER/SR and MPTP mediated) necrosis. Likewise, the closely related proapoptotic factor Bnip3, which mediates apoptotic cardiomyocyte death and adverse cardiac remodeling after myocardial infarction, contributes to cardiac dysfunction by localizing to reticular structures and increasing SR-mitochondrial calcium transport. This functional duality in factors regulating mitochondrial homeostasis and SR-mitochondrial calcium signaling is a common theme that has emerged from cardiac-specific in vivo molecular dissections of outer membrane mitochondrial fusion and apoptosis proteins.

### Mitochondrial Fusion, Calcium Regulation, and Cardiac Development

Mitochondria are calcium rich; sufficiently so that mitochondrial disruption and release of calcium can produce dystrophic calcification, as observed in necrotic myocardium. Conversely, cardiac mitochondria can act as calcium sponges, taking up and buffering available free cytosolic calcium. As detailed below, a surprising role was recently discovered for mitochondrial calcium buffering in signaling pathways that orchestrate expression of genes directing cardiomyocyte differentiation during embryonic cardiogenesis.

Mitochondrial structure and subcellular organization in embryonic cardiomyocytes and cardiomyocyte progenitor cells resemble those of fibroblasts (ie, long reticular organelles organized into highly interconnected networks), rather than the short round individual organelles observed in fully differentiated adult cardiomyocytes. These developmental differences in mitochondrial morphometry lead to surprising functional interactions between mitochondrial network remodeling, mitochondrial calcium uptake, and embryonic cardiac development. Differentiating embryonic stem cell–derived cardiomyocytes normally have a copious number of perinuclear mitochondria arranged in reticular networks. Inhibiting mitochondrial fusion by suppressing either mitofusin 2 or Opa1 promotes network discontinuity and produces fragmented mitochondria that redistribute away from the nucleus and toward the cell membrane. The atypical subsarcolemmal location of the fragmented mitochondria physiologically positions them to act as buffers for normal transmembrane (outside-in) calcium signaling, paradoxically increasing capacitative calcium entry and activating calcineurin that, in turn, increases notch signaling. Because notch suppression is a requisite for cardiomyocyte differentiation, calcineurin-mediated notch activation in cardiomyocyte progenitor cells with defective mitochondrial fusion impairs their differentiation into cardiomyocytes (Figure 4). We determined the in vivo effect of impaired mitochondrial fusion on cardiomyocyte differentiation by genetically interrupting mitochondrial fusion (combined mitofusin 1 and mitofusin 2 ablation) in cardiomyocytes from the earliest stages of cardiac development (using ntx2.5-Cre). Mitofusin 1/mitofusin 2 double-deficient hearts exhibited depressed Mef2c, Nkx2.5, and SRF-dependent gene expression, interruption of normal myocardial development, and lethal embryonic myocardial hypoplasia. The spectacular effects of induced mitochondrial dysmorphology on developmental cardiac gene expression and heart development provide a striking example of how these semiautonomous cytosolic organelles can commandeer critical nuclear gene expression pathways to determine cell fate, and not just cell viability.

### Mitophagy, Mitofusins, and Mitochondrial Quality Control

A concept that emerges from experimental manipulation of mitochondrial fusion in hearts is that the critical pathophysiological roles of mitofusins derive more from higher order functions of organelle tethering and fusion than from structural mitochondrial
remodeling per se. Thus, tethering of mitochondria to SR by mitofusin 2 is important for transorganelle calcium signaling that normally regulates acute metabolic responsiveness, and under pathological conditions can evoke the mitochondrial permeability transition. Likewise, suppression of mitochondrial fusion provokes increased mitochondrial calcium buffering that can dysregulate developmental cardiac genes. Evidence is accruing that mitochondrial fusion factors also play central roles in mitophagic mitochondrial quality control. Indeed, because mitofusin 1 and mitofusin 2 serve largely redundant roles in mitochondrial mitochondrial quality control. Indeed, because mitofusin 1 and mitofusin 2 serve largely redundant roles in mitochondrial fusion, it is understandable how one of the redundant mitofusins may have evolved additional ancillary functions. Below we make the case that regulating mitophagy is a primary function of mitofusin 2 in the heart.

One of the unexpected findings of early studies was that hearts with genetically engineered mitochondrial fusion defects were ineffective in clearing abnormal mitochondria. Given high mitochondrial density in cardiomyocytes and the importance of removing damaged ROS-producing mitochondria before they become cytotoxic, it was expected that simple interruption of fusion-mediated organelle complementation (without concomitant suppression of biogenic renewal or mitophagic removal pathways) would affect organelle morphometry, but not mitochondrial fitness. However, the decrease in organelle size after complete mitofusin ablation in mouse hearts is associated with markedly increased numbers of degenerated cardiac mitochondria during the early stages of the resulting cardiomyopathy. Ablation of mitofusin 2 alone in mouse hearts did not impede mitochondrial fusion (which was still induced by endogenous mitofusin 1 as evidenced by actual enlargement of mitofusin 2-deficient cardiomyocyte mitochondria), but nevertheless provoked abnormal cardiomyocyte respiration and a slowly progressive chronic cardiomyopathy. The distinct mitochondrial and cardiac phenotypes of combined cardiac mitofusin 1/mitofusin 2 ablation compared with selective mitofusin 2 deficiency reveal different mechanisms for the resulting cardiomyopathies: because abnormal mitochondria accumulated after selective mitofusin 2 ablation and combined mitofusin 1/mitofusin 2 ablation, but not after selective mitofusin 1 ablation, we hypothesized that mitofusin 2 is uniquely important for culling damaged mitochondria.

As introduced above, mitophagy (or “eating mitochondria”) is the central mechanism by which damaged mitochondria are removed to prevent cell damage. Two critical mediators of mitophagy are PTEN-induced putative kinase 1 (PINK1) and Parkin, encoded by genes in which loss of function mutations cause early onset autosomal recessive Parkinson disease. The history of how conventional human genetic studies, gene manipulation in fruit flies and mice, and basic cellular research have all converged to reveal mitophagic dysfunction as the underlying cause of these heritable forms of Parkinson disease is a fascinating one, and the interested reader is referred to recent excellent reviews. For the purpose of understanding mitophagy signaling in the heart, PINK1 is a mitochondrial kinase that accumulates in damaged mitochondria and initiates the signal for translocation of the Parkin cytosolic E3 ubiquitin ligase specifically to damaged organelles, Parkin-mediated ubiquitination of outer membrane proteins on these damaged mitochondria attracts autophagosomes, thus initiating mitophagy (Figure 5). Mitochondria engulfed by an autophagosome are transferred to lysosomes and

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**Figure 4.** Mitochondrial fusion and control of cardiomyocyte differentiation/heart development. Functional interactions between L-type calcium channels (LCC; blue), store-operated calcium channels (purple), mitochondria (green), calcineurin A (yellow), Notch (orange), and developmental gene expression as conceived in cardiomyocyte progenitor cells. **Left,** Normal stem cell with fused perinuclear mitochondria in which LCC calcium signaling is normal and capacitative calcium entry is low. **Right,** How mitochondrial fragmentation and subsarcolemmal redistribution disturbs LCC signaling through mitochondrial calcium uptake (sponge), invoking capacitative calcium entry that activates calcineurin and downstream Notch, repressing developmental gene expression.

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**Figure 5.** The (PTEN)-induced putative kinase 1 (PINK1)-Parkin mechanism of mitophagy. **Left,** Schematic diagram of PINK1-Parkin initiation of mitophagy signaling after asymmetrical mitochondrial fission. **Right,** Confocal fluorescent images showing mcherryParkin (red) translocation from cytosol to mitochondria (MitoTracker green) after mitochondrial depolarization with the uncoupling agent FCCP. Parkin-containing mitochondria appear yellow in the merged image.
degraded, which minimizes cellular toxicity from ROS that damaged mitochondria often produce and release. Interruption of PINK1-Parkin signaling disrupts mitophagy. Accordingly, accumulation of abnormal mitochondria in dopaminergic neurons is the pathological sine qua non of Parkinson disease linked to PINK1 and Parkin gene mutations. It is important to recognize that mitochondria can also be eliminated through nonselective macroautophagy that is transduced through different signaling pathway(s) independent of PINK1 and Parkin. Because the terminal stages of both selective mitophagy and generalized mitochondrial autophagy proceed via autophagosomal mitochondrial engulfment and transfer to lysosomes, it can be challenging to differentiate between these processes.

As Parkin is central to mitochondrial quality control in neurons, skeletal muscle, and perhaps cardiomyocytes,50,51 the Dorn research group examined the integrity of Parkin signaling in mitofusin 2–deficient mouse cardiomyocytes in which abnormal mitochondria appeared not to be properly eliminated, thus testing the hypothesis that genetic deficiency of mitofusin 2 interferes with normal mitophagy. Parkin was abundant in cardiomyocyte sarcoplasm, but did not translocate to depolarized mitochondria of mitofusin 2–deficient cardiomyocytes.45 Parkin translocation to mitochondria of wild-type and mitofusin 1–deficient cardiomyocytes. By contrast, depolarization robustly stimulated Parkin translocation to mitochondria of mitofusin 2–deficient cardiomyocytes.45

Parkin recruitment to and activation at mitochondria are obligatory steps for canonical mitophagy signaling. Relocalization of Parkin from cytosol to mitochondria results in Parkin-mediated ubiquitination of dozens of outer mitochondrial membrane proteins, including mitofusin 1 and mitofusin 2.45 It does not seem that Parkin exhibits much substrate selectivity for accessible mitochondrial proteins, but instead broadly paints the outer mitochondrial membrane with a coat of ubiquitin molecules. If mitofusin 2 plays a central role in the translocation of Parkin to damaged mitochondia, one would expect to observe depressed mitochondrial polyubiquitination in mitofusin 2–deficient cardiomyocytes. This proved to be the case. Mitochondrial polyubiquitination and mitochondrial p62/sequestosome-1 localization (a marker of autophagosomal recruitment) were impaired in mitofusin 2–deficient cardiomyocytes. Again, mitofusin 1 ablation had no effect on these Parkin-dependent mitophagy signaling events. From these results, we concluded that mitofusin 2, but not mitofusin 1, is essential for attracting Parkin to damaged cardiomyocyte mitochondria, and inferred that its absence was interrupting mitophagy signaling.

The initiating signal in Parkin-mediated mitophagy is stabilization of PINK1 kinase.55,56 Like hundreds of other nuclear-encoded mitochondrial proteins, PINK1 kinase is translated in the cytosol and the protein is then imported into mitochondria. Remarkably, on gaining access to the mitochondria, PINK1 is almost immediately degraded (likely by the mitochondrial protease presephin-associated rhomboid-like protein),57,58 preventing PINK1 from having meaningful biological activity as a kinase (Figure 5). For this reason, although PINK1 mRNA is abundant in hearts and other tissues, PINK1 protein levels in normal mitochondria are so low as to test levels of detection. Therefore, it is functionally correct to consider a normal mitochondrion as being PINK1 suppressed or knocked out. However, mitochondrial damage or senescence sufficient to provoke depolarization interrupts normal PINK1 degradation. The consequence of this so-called mitochondrial dead-man switch is that PINK1 protein level increases only in damaged/depolarized mitochondria. Mitochondrial PINK1 activity is the proximate signal for Parkin translocation and activation and is important to heart function.59 In the absence of an experimental manipulation, such as forced overexpression that can overwhelm the normal degradative mechanisms,58 PINK1 stabilization only within depolarized mitochondria therefore recruits Parkin selectively to those damaged organelles.

The mechanism by which PINK1, a mitochondrial-localized kinase, communicates with Parkin, a soluble cytosolic E3 ubiquitin ligase, was unclear. Like most kinases, PINK1 is fairly promiscuous and will phosphorylate any number of accessible proteins, including Parkin itself and ubiquitin.52,53,60,61 We considered that colocalization of PINK1 and mitofusin 2 at the outer mitochondrial membrane provided an opportunity for PINK1 to phosphorylate mitofusin 2, which our studies in mitofusin 2 knockout mice had shown was necessary for mitochondrial Parkin binding. We demonstrated that mitofusin 2 could be a PINK1 phosphorylation substrate by cotransfecting PINK1 and mitofusin 2 in cultured HEK293 cells. PINK1 decreased the electrophoretic mobility of mitofusin 2 in normal gels, and mobility retardation was exaggerated on PhosTag gels. We determined that mitofusin 2 can be a PINK1 substrate using antiphosphoserine antibodies, and further demonstrated that overexpression of kinase active PINK1 (but not a kinase-deficient PINK1 mutant) was sufficient to provoke mitofusin 2-Parkin binding in the absence of mitochondrial depolarization.45

To nail down the functional relevance of mitofusin 2 phosphorylation by PINK1, the Dorn group used site-directed mutagenesis to map PINK1-mediated phosphorylation to mitofusin 2 amino acids to Thr111 and Ser442, located within the mitofusin 2 GTPase domain and just distal (ie, C terminal) to the first heptad repeat, respectively.43 Mutation of either of these 2 amino acids to nonphosphorylatable Ala depressed, but did not eliminate, mitofusin 2-Parkin binding. However, combinatorial mutation of both PINK1 phosphorylation sites to Ala (mitofusin 2 111/442 AA) completely abrogated mitofusin 2-Parkin binding. Finally, we performed the reciprocal experiment by mutating mitofusin 2 Thr111 and Ser442 to Glu (mitofusin 2 111/442 EE), thereby mimicking phosphorylation. The mitofusin 2 111/442 EE mutant was sufficient to bind Parkin in the absence of PINK1 kinase. Thus, pseudo-PINK1 phosphorylated mitofusin 2 is a constitutive mitochondrial Parkin receptor.

The functional interaction among PINK1, mitofusin 2, and Parkin helps explain loss-of-mitochondrial quality control phenotypes induced by cell-type–specific mitofusin 2 ablation in heart, brain, and liver. For example, tissue-specific Cre-mediated mitofusin 2 gene ablation in cardiomyocytes and neurons causes accumulation of abnormal mitochondria linked...
to a Parkin translocation defect, and increased numbers of abnormal mitochondria are also observed after mitofusin 2 deletion from dopaminergic neurons and hepatocytes (although Parkin function was not directly evaluated therein). The insights gained from these studies have implications for future investigations of the PINK1-mitofusin 2-Parkin mitophagy pathway. For example, Parkin can be induced to translocate to the mitochondria of MEFs derived from (embryonic lethal) germ-line mitofusin-deficient (mitofusin 1/mitofusin 2 knockout) mice. We believe that this reflects developmental plasticity and induction of alternate pathway(s) leading to mitochondrial Parkin recruitment when the prototypical mitochondrial Parkin receptor (mitofusin 2) is genetically absent from the germ-line. It will be interesting to determine whether acute conditional mitofusin 2 deletion in tissue culture (eg, using mitofusin 2 floxed allele MEFs with adeno-Cre) might avoid developmental plasticity and experimentally replicate the mitophagy-suppressing effects provoked by in vivo tissue-specific mitofusin 2 ablation. Likewise, conventional overexpression of Parkin does not intrinsically activate mitophagy (because in healthy cells Parkin resides almost entirely in the cytosol), but simply enhances the capacity to respond mitophagically to a mitochondrial damaging insult. It may be possible, however, to use the constitutive Parkin-binding mitofusin 2 T111/S442 EE mutant as a research tool to interrogate the effects of mitochondrial-specific Parkin activation on mitophagy in otherwise normal cells and test whether promiscuous Parkin-mediated mitophagy can have detrimental effects on mitochondria and cell fitness.

**Generalized Mitochondrial Autophagy for When Selective Mitophagy Fails**

Mitophagy might be considered as a smart bomb for eliminating damaged (and potentially damaging) mitochondria. PINK1 phosphorylation of mitofusin 2 acts as the targeting laser for the incoming Parkin/autophagosome bomb. The mitophagy smart bomb approach can eliminate potentially harmful organelles without collateral damage to healthy mitochondria in the same locale. However, if the need to eliminate damaging mitochondria is great, as when mitophagy is interrupted or there is generalized cell-wide mitochondrial dysfunction, the less selective approach of generalized autophagy can be invoked. This is analogous to using carpet bombing when you run out of smart bombs; the harmful mitochondria are removed at a cost of greater collateral damage. As described below, the evidence for compensatory autophagy induction when primary mitophagy fails (either because mitophagy is dysfunctional or is overwhelmed) is inferential, but accumulating data provide some direct support for partial homeostatic redundancy between mitophagy and mitochondrial autophagy.

Before defining the conditions under which mitophagy may be supplemented or supplanted by mitochondrial autophagy, it is worth noting the degree to which the process overlap. Indeed, the differences between mitophagy and autophagy are all at the front end (ie, at target acquisition). Both mitophagy and mitochondrial autophagy proceed through autophagosomal engulfment of the organelle and transfer to a degradative lysosome, followed by breakdown and recycling of constituent components. The downstream effectors of mitophagy and autophagy are, therefore, shared, which can be potentially confounding if assays are not performed and interpreted with care. By way of example, the normally cytosolic microtubule-associated protein 1A/1B-light chain 3 (LC3) protein is conjugated to phosphatidylethanolamine on autophagosomal membranes and forms LC3-II. The conversion of LC3-I to LC3-II is readily detected by a mobility shift on immunoblots and is often used as a marker of ongoing autophagy. However, autophagosomal LC3-II is degraded by lysosomal hydrolases, and so its elimination can reflect increased autophagy. Conversely, increased LC3-II might indicate enhanced stimulation, but failure of autophagy to proceed normally through the lysosomal degradation stage. These vagaries indicate that, under steady-state conditions, immunoreactive LC3 is problematic as even a qualitative metric of autophagosomal flux. The same conundrum exists for other autophagosome-associated proteins like p62/sequestosome-1. Confocal fluorescence studies are helpful to localize LC3 or p62 at specific cellular components, but LC3 or p62 immunoreactivity in unfractionated cell or tissue homogenates does not distinguish between selective mitophagy and nonselective mitochondrial autophagy.

There are some biochemical approaches to specifically detect and (semiquantitatively) assay mitophagy in heart tissue. First, Parkin localization to mitochondria and cell fitness...
polyubiquitination of mitochondrial proteins can be measured in mitochondrial fractions obtained by differential centrifugation of cardiac homogenates. Enrichment of Parkin and increased immunoreactive ubiquitin in cardiac mitochondrial fractions reflects activation of mitophagy signaling, although mitophagy per se will not proceed in the absence of critical downstream effectors. Mitophagy itself, ie, the actual engulfment of mitochondria by an autophagosome, can be detected as the association of autophagosomal proteins, such as p62 and LC3, with mitochondria. Thus, if mitophagy signaling is increased and mitophagy proceeds intact through lysosomal degradation, one will observe increased Parkin, p62, and LC3-II in the mitochondrial-rich subcellular fraction (mitophagy-specific markers) but decreased p62 and LC3-II in cardiac homogenates (because of increased autophagic degradation). Conversely, if autophagy is generally increased without a specific mitophagy component, Parkin, p62, and LC3 levels in mitochondrial protein fractions may be unaltered, but autophagosomal markers will increase in the tissue homogenates. Finally, the visualization of mitochondria within autophagosomes or lysosomes, either by electron microscopy or a confocal visualization of colocalized fluorescent mitochondrial and lysosomal markers, indicates that mitochondria are undergoing autophagosomal engulfment and lysosomal transfer that is the common terminal pathway of organelle disposal for both Parkin-mediated mitophagy and nonspecific mitochondrial autophagy; these imaging techniques do not discriminate between mitophagy and mitochondrial autophagy.

To explore the role of ROS in the upstream processes leading to mitochondrial clearance, the Dorn laboratory examined markers of mitophagy and autophagy in mitofusin 2–deficient hearts. ROS are frequently implicated as injurious factors released by damaged, dysfunctional, or senescent mitochondria and can contribute to various forms of heart disease. Scavenging mitochondrial-derived ROS has, therefore, prevented or ameliorated experimental heart disease linked to mitochondrial dysfunction. Because accumulation of abnormal mitochondria is a hallmark of cardiomyocyte-specific mitofusin deficiency, we anticipated that ROS scavenging would prevent the cardiomyopathy evoked by cardiomyocyte-specific mitofusin ablation. Although this was partially correct, we unexpectedly found that mitochondrial-derived ROS also play an important role in stimulating mitochondrial quality control pathways.

Our initial efforts to test ROS scavenging in cardiomyopathies provoked by mitofusin deficiency used cardiac-specific Drosophila Parkin and MARF RNAi models. Mammalian mitofusin 2 seems functionally synonymous with Drosophila MARF because expression of mitofusin 2 normalizes mitochondrial morphology and heart tube dysfunction induced in the fly by cardiomyocyte-specific expression of MARF RNAi. Accumulation of dysfunctional mitochondria after MARF suppression suggested that inadequate mitophagy might be contributing to the phenotype, as it does after mitofusin 2 ablation in mammalian hearts (vide supra). RNAi-mediated suppression of either MARF or Parkin in fly hearts provoked similar cardiomyocyte mitochondria pathology and heart tube abnormalities, showing that interruption of Parkin signaling is central to both of these fly cardiomyopathies.

Therefore, we reasoned that the mechanistic link between interruption of mitophagy, increased numbers of abnormal mitochondria, and cardiac failure could be increased ROS produced by accumulating damaged organelles; we tested this idea by expressing the potent antioxidant, superoxide dismutase (SOD), in Parkin- and MARF-deficient fly hearts. Consistent with the postulated role of ROS in the mitophagic cardiomyopathy induced by Parkin suppression, both mitochondrial SOD2 and soluble SOD1 completely prevented mitochondrial dysfunction and heart tube dilatation in Parkin RNAi fly hearts. Unexpectedly, SOD was only transiently protective in the cardiomyopathy caused by Drosophila MARF insufficiency. These results supported our hypothesis that mitochondrial ROS-derived cytotoxicity is a major factor leading to cardiomyopathy induced by interdiction of Parkin-mediated mitophagy, but did not support the same role for ROS in cardiac MARF insufficiency. It is possible that fly MARF is important for mitochondrial fitness in ways that are independent of Parkin-mediated mitophagy, eg, by promoting mitochondrial fusion. However, another possibility raised by the absence of cardioprotection afforded by SOD in the cardiomyopathy provoked by fly mitofusin (MARF) insufficiency is that mitochondrial ROS can function as a signaling molecule in compensatory pathways.

Because the single Drosophila protein MARF acts like both mammalian mitofusins, mitofusin 1 and mitofusin 2, the cardiac MARF RNAi fly pathophysiologically resembles the mitofusin 1/mitofusin 2 double-cardiac knockout mouse: both mitochondrial fusion signaling and any related Parkin-mediated mitophagy are interrupted. To better understand the roles that ROS can play within the mitofusin 2–Parkin mitophagy signaling axis the Dorn group returned to studies of the cardiac-specific mitofusin 2 null mouse. As recently reported, we detected increased endogenous catalase levels and observed increased ROS production by mitochondria isolated from mitofusin 2–deficient mouse hearts, constituting evidence for chronically elevated in vivo mitochondria-derived ROS. We determined how mitochondrial ROS affect organelle and organ dysfunction in cardiac-specific mitofusin 2 null mice by transgenically coexpressing mitochondria-directed catalase (mitocatalase, another potent antioxidant enzyme), and examining the compound genetic mice and their parent lines for ROS formation, mitochondrial function and integrity, and the cardiomyopathy that characteristically develops over a 30-week period. We used 2 different mitocatalase mouse lines previously shown to protect against angiotensin II–induced cardiac hypertrophy and Gq-mediated cardiomyopathy: a β-actin–driven catalase transgene expressed in hearts at high levels that we called hi-catalase, and a Cre-activated flox-stopped catalase transgene expressed in hearts at 10-fold lower levels that we called low-catalase. As expected, expression of either hi- or low-catalase alone was totally benign, and low-catalase expression completely rescued organ and organelle dysfunction in mitofusin 2–deficient hearts. This was associated with induction of macroautophagy likely evoked as a secondary mechanism for mitochondrial culling. The primary defect in Parkin-mediated mitophagy...
produced by mitofusin 2 deficiency was not affected by catalase expression.

Given the benefits on the mitofusin 2 knockout mouse heart of ROS suppression with low-catalase, we were astonished that expression of hi-catalase in mitofusin 2–deficient hearts failed to improve either the mitochondrial abnormalities or the cardiomyopathy, despite supersuppressing mitochondrial ROS formation to levels well below normal.76 Failure of hi-catalase to rescue mitofusin 2 hearts was associated with failure to induce macroautophagy, ie, with a breakdown in the putative secondary mitochondrial quality control mechanism. From these results, the Dorn group inferred that interruption of Parkin-mediated mitophagy in mitofusin 2 null mouse hearts can lead to heart failure because damaged mitochondria that would normally be culled instead accumulate and form ROS. This initiates a vicious cycle leading to more mitochondrial damage and activation of compensatory mitochondrial quality control pathways that include generalized autophagy. Preventing toxic accumulation of mitochondria-derived ROS by expressing lower levels of mitochondrial-targeted catalase (low-catalase) interrupts this vicious cycle, improving mitochondrial health and diminishing the load on compensatory autophagy. On the contrary, failure of compensatory autophagy in mitofusin 2 null hearts coexpressing hi-catalase reveals that a threshold level of mitochondrial ROS is necessary to induce macroautophagy, as suggested in a different context (in vitro cardiomyocyte lipopolysaccharide toxicity) by Yuan et al.77 Thus, supersuppression of ROS by hi-catalase impairs the back-up mechanism of autophagic mitochondrial culling which, together with ineffective autophagy, induces 2 hits to the mitochondrial quality control apparatus and accelerates the generalized mitochondrial dysfunction and heart failure (Figure 7).

**Mitoptosis: Connecting Cell Death and Mitochondrial Quality Control**

Cardiomyocytes possess robust cell death programs. Regulated forms of cell death, ie, apoptosis and necrosis, contribute critically to the evolution of myocardial infarction, and to pathological cardiac remodeling and heart failure.78 The recent recognition that some protein mediators of mitochondria-dependent death pathways are also involved in mitochondrial dynamics has raised the intriguing possibility that cell death proteins perform a double-duty as regulators of mitochondria quality control.

Mitochondria are the organelle gatekeepers of the intrinsic pathways of apoptosis and necrosis. Although apoptosis and necrosis signaling at the mitochondria are intertwined, the defining point of no return differs. For apoptosis, it is permeabilization of the outer mitochondrial membrane allowing the release into the cytosol of cytochrome c and other apoptogens that promote formation of the apoptosome and activation of caspases. In contrast, in necrosis, the sentinel event is opening of the MPTP in the inner membrane resulting in dissipation of the proton gradient that drives ATP synthesis and energetic catastrophe.

Bcl-2 proteins are the major regulators of outer mitochondrial membrane permeabilization in apoptosis.79 The BH3-only subclass of this family transduces various death signals from the periphery to Bax and Bak, which then promote outer mitochondrial membrane permeabilization. More precisely, the BH3-only proteins, Bim and truncated Bid (tBid), serve as the direct messengers that bind and conformationally activate Bax and Bak. The other BH3-only proteins are sensitizers that work by displacing Bim and tBid from anti-apoptotic family members, such as Bcl-2 and Bcl-XL, that serve as reservoirs to sequester Bim and tBid (Figure 8A). Conformational activation of Bax sends this protein from the cytosol (where it resides in healthy cells) to the mitochondria, where it inserts tightly into the outer mitochondrial membrane. However, Bak is constitutively localized to the outer mitochondrial membrane, and the direct consequences of its conformational activation are less well understood. Once activated, Bax and Bak homo- and hetero-oligomerize to bring about outer mitochondrial membrane permeabilization, likely through pore formation.

We previously discussed the BH3-only proteins Nix and Bnip3 in connection with their promoting cardiomyocyte death by apoptosis or necrosis depending on whether they are localized at the mitochondria or ER/SR, respectively. The pathophysiological significance of Nix is that it is transcriptionally upregulated in cardiac hypertrophy and provokes cardiomyocyte death that mechanistically contributes to the transition from compensated hypertrophy to decompensated heart failure,33–35 while Bnip3 plays a parallel cell death promoting role in post-ischemic cardiac failure.79,80 Recent evidence has uncovered additional roles for these proteins in mitophagy. Although genetic ablation of Nix or Bnip3 protects hearts against cardiomyocyte dropout and adverse remodeling (after pressure overloading or
ischemia-reperfusion injury respectively), their combined deficiency in mouse hearts induces a progressive cardiomyopathy associated with accumulation of abnormal mitochondria. This observation uncovered a previously unsuspected role for Nix and Bnip3 in cardiomyocyte mitochondrial quality control and pointed to mechanistic connections between proteins involved in mitochondria-mediated cell death and mitophagy.

Consistent with this paradigm, data have emerged demonstrating that Nix and Bnip3 can each play dual roles as mediators of both programmed cell death (via apoptosis or necrosis) and programmed elimination of mitochondria (via mitophagy or autophagy). Nix is recruited specifically to damaged mitochondria and inserts itself into mitochondrial outer membranes. Its pro-apoptotic activity derives from facilitation of Bax/Bak-mediated outer mitochondrial membrane permeabilization that releases cytochrome c and activates the caspase cascade (Figure 8B). In addition, mitochondrial Nix can act as a chaperone to promote autophagosome recruitment by binding to γ-aminobutyric acid type A receptor–associated protein (a.k.a. GABARAP), which is an LC3 homologue critical for autophagosome maturation (Figure 8B).

The dual effects of Nix on apoptosis and mitophagy are perhaps most evident during erythrocyte (red blood cell) development and maturation from bone marrow precursor erythroblasts. Because retained mitochondria in highly oxygenated circulating red blood cells would produce damaging ROS and lead to intravascular hemolysis, the penultimate stage of erythrocyte formation wherein the erythroblast nucleus is extruded is also associated with the elimination of erythroblast mitochondria via developmentally programmed mitophagy. This developmental mitophagy is a Nix-driven and Nix-dependent process: Nix is transcriptionally upregulated during the early stages of erythropoiesis and fine-tunes erythrocyte formation by apoptotically limiting erythroblast abundance. Then, during the terminal stages of erythropoiesis, Nix upregulation mediates mitophagic elimination of erythroblast mitochondria. Thus, within the same cell type Nix promotes either programmed cell or programmed organelle elimination, depending on developmental context. Recent studies have uncovered a similar role for Bnip3 as an LC3-interacting mitophagy effector.

Another example of dual-functionality of a single factor in mitochondrial quality control and mitochondrial-mediated apoptosis pathways is the fission promoting protein, Drp1. Apoptosis is almost universally associated with mitochondrial fragmentation (ie, a disproportionate increase in mitochondrial fission relative to fusion). In asymmetrical mitochondrial fission that is integral to mitophagic quality control (see Figure 5), the central effector of apoptosis-associated mitochondrial fission is Drp1. Pharmacological inhibition of Drp1 with either mdivi or P110 can protect against apoptosis and cardiac failure in ischemically, hemodynamically, or toxicity-damaged hearts. Importantly, recruitment of Drp1 to mitochondria during apoptosis is mediated by Bax and Bak, the same proteins responsible for outer mitochondrial membrane permeabilization in apoptosis. Thus, Bax and Bak provide a molecular link between apoptosis and fission, which in a different context, is a prerequisite for normal mitophagic quality control.

Recent work has unexpectedly extended the role of Bax to the regulation of mitochondrial-mediated necrosis. Deletion of Bax and Bak, or Bax alone in mice reduced infarct size after ischemia-reperfusion and markedly decreased ultrastructural markers of cell necrosis. These effects are of similar magnitude to those resulting from deletion of cyclophilin D. A mitochondrial matrix protein that regulates opening of the MPTP. In fact, the triple knockout of Bax/Bak/cyclophilin D does not result in greater cardioprotection when compared with the double knockout of Bax/Bak, suggesting that Bax/Bak and cyclophilin reside in the same or overlapping pathways. Mechanistic analyses in Bax/Bak null fibroblasts and isolated adult cardiomyocyte mitochondria showed that the absence of Bax decreases the sensitivity of the MPTP to opening in response to Ca2+. Bax produces necrosis through a mechanism distinct from its activation of apoptosis because oligomerization-defective Bax mutants that...
are unable to support apoptosis remain capable of supporting necrosis.99,100

What is the precise mechanism? This is not known with certainty. Some data suggest that Bax on the outer mitochondrial membrane is in complex with MPTP.99 Another mechanism that the Kitsis group has explored is that Bax facilitates necrosis through its previously recognized ability to promote mitochondrial fusion.104,105 Interestingly, oligomerization-deficient Bax mutants, the same ones that support necrosis, are capable of driving mitochondrial fusion.105 Consistent with this model, other genetic manipulations that disable mitochondrial fusion, such as mitofusin 2 deficiency, also desensitize fibroblasts to Ca2+-induced MPTP opening. Conversely, restoration of the fused mitochondrial morphology by an independent means, blocking fission, restores the sensitivity of Ca2+-induced MPTP opening. Conversely, restoration of the fused mitochondrial state, no matter how it is attained, that can sensitize to necrosis.

At present, we do not know with certainty exactly what features of fused mitochondrial are responsible for heightened sensitivity to cell death. There is evidence that fused mitochondria have more efficient diffusion of Ca2+ waves than less interconnected ones, suggesting an explanation for increased sensitivity to MPTP opening.106 Another possibility worthy of consideration is that larger, interconnected mitochondria have deficits in quality control, possibly by virtue of being less susceptible to mitophagy (ie, Bax-driven fusion may be interfering with mitochondria quality control). Although this model requires testing, there are some enticing connections that may come into play. First, Bax is a target for Parkin-mediated ubiquitination, the effect of which is to damp Bax translocation to the mitochondria in response to a death stimulus.107 Second, although the precise mechanism by which Bax promotes fusion is not understood, there seem to be physical and functional interactions between Bax and mitofusin 2,104,105,106 the latter at the nexus of fusion and mitophagy.

These examples illustrate the multifunctionality of Bcl-2 proteins, mitofusin 2, and other fusion and fission factors in the regulation of mitochondrial dynamics, mitophagy, and cell death—apoptotic and necrotic. Whereas the mechanisms that adjudicate outcome are not completely understood, the involvement of the same cast of characters argues for a unified and highly integrated model. One possibility is that outcome is determined more by the quantitative degree to which the players are activated. Thus, mitophagy uses Nix, mitofusin 2, and Drp1 to eliminate certain damaged mitochondria selectively, whereas the same constellation of factors interact cell-wide to initiate apoptosis. This paradigm is consistent with the concept that mitochondria and their host cells developed multiple mechanisms for organelle self-elimination, a concept consistent with so-called “mitoptosis”.108 As originally described, mitoptosis progresses via caspase-independent, ROS-mediated activation of MPTP (ie, programmed necrosis) and is distinct from canonical mitophagy.109 However, molecular cross talk among apoptosis, programmed necrosis, and mitochondrial autophagy/mitophagy signaling at the level of Nix and Bnip3, mitofusin 2, and Drp1 indicates that these pathway distinctions probably lack biological relevance. Interactive signaling between mitochondrial quality control/mitoptosis pathways and programmed cell death pathways does, however, provide a plausible explanation for why apoptosis is so often observed in diseased hearts. We posit that these integrated processes constitute a stress response whose primary objective is to maintain mitochondrial quality control through the elimination of defective organelles. Only when this fails (eg, severely damaging conditions) does the same machinery bring about cell death. One potential implication of this notion is that care must be taken in contemplating therapies to suppress cell death so as not to also disrupt long-term maintenance of normal mitochondrial fitness. Conversely, enhancing mitochondrial quality control is likely to be protective in conditions where programmed cell death contributes to disease progression.67,107,111,112

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Mitochondrial Dynamics-Mitophagy Interactome

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The Mitochondrial Dynamism-Mitophagy-Cell Death Interactome: Multiple Roles Performed by Members of a Mitochondrial Molecular Ensemble

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