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Two close, too close: Sarcoplasmic reticulum-mitochondrial cross-talk and cardiomyocyte fate

Gerald W Dorn II, MD[1] and **Luca Scorrano, MD, PhD**[2,3]

[1] Center for Pharmacogenomics, Department of Internal Medicine, Washington University School of Medicine, St. Louis, MO, USA [2] Department of Cell Physiology and Metabolism, University of Geneva Medical School, Geneva, Switzerland [3] Dulbecco-Telethon Institute, Venetian Institute of Molecular Medicine, Padova, Italy

Abstract

Mitochondria are key organelles in cell life, whose dysfunction is associated with a variety of diseases. Their crucial role in intermediary metabolism and energy conversion makes them a preferred target in tissues, like the heart, where the energetic demands are very high. In the cardiomyocyte, the spatial organization of mitochondria favors their interaction with the sarcoplasmic reticulum, thereby offering a mechanism for Ca^{2+}mediated crosstalk between these two organelles. Recently, the molecular basis for this interaction has started to be unraveled, and we are learning how ER-mitochondrial interactions are often exploited by death signals, like proapoptotic Bcl-2 family members, to amplify the cell death cascade. Here we will review our current understanding of the structural basis and the functional consequences of the close interaction between sarcoplasmic reticulum and mitochondria on cardiomyocyte function and death.

Keywords

Mitochondrial fusion; endoplasmic reticulum; calcium; bcl2 proteins; apoptosis; mitochondrial permeability transition

Introduction

Individually, sarcoplasmic reticulum and mitochondria direct critical functions essential to maintaining myocardial homeostasis and producing cardiac contraction. The sarcoplasmic reticulum (SR) is the major intra-cardiomyocyte storage depot for calcium. During excitation-contraction coupling, calcium influx through plasma membrane L-type voltage - gated calcium channels (LCC) stimulates calcium-induced, ryanodine receptor-mediated release of SR calcium into the cytosol, which induces myofibrillar contraction. In diastole the sarcoplasmic-reticulum calcium ATPase (SERCA) pumps calcium back into the SR, reversing the rise in cytosolic calcium. Functional relationships between cardiomyocyte membrane calcium channels, SR, and myofibrillar elements are facilitated by a highly organized sub-cellular architecture in which deep transverse tubular plasma membrane invaginations (t-tubules) enforce proximity of membrane LCC to intracellular SR located

Corresponding authors: Gerald W. Dorn II, Washington University Center for Pharmacogenomics, Campus Box 8220, 660 S. Euclid Ave., St. Louis, MO 63110, USA. Phone: 314-362-4892. FAX: 314-362-8844. gdorn@dom.wustl.edu or Luca Scorrano. Department of Cell Physiology and Metabolism, University of Geneva, 1 Rue M. Servet, 1206 Geneve, Switzerland, luca.scorrano@unige.ch.

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deep within sarcomeres. SR calcium reuptake in diastole in an energy intensive process, requiring large amounts of mitochondrially-generated ATP. The heart is therefore one of the most mitochondrial-rich organs, with mitochondria occupying approximately 30% of the volume of a ventricular cardiomyocyte. As with the SR, mitochondrial localization within cardiomyocytes is highly ordered.

Consistent with their importance to normal cardiac function, SR and mitochondrial dysfunction are associated with heart failure. Mitochondrial ATP production is impaired in heart failure, which predisposes to cardiomyocyte autophagy. Furthermore, mitochondria are the gatekeepers of apoptotic and necrotic cardiomyocyte death, which is increased in heart failure. SR dysfunction in heart failure is revealed by characteristic prolongation of the calcium transient, mechanistically attributable to impaired SR calcium reuptake. Accordingly, there are ongoing efforts to treat heart failure using gene therapy to increase SERCA or impair the function of its endogenous inhibitor protein, phospholamban.

Accumulating evidence indicates that in addition to their separate functions, SR-mitochondrial cross talk is critically important to cardiac health. Cardiomyocyte ATP production and cardiomyocyte calcium signaling require communications between mitochondrial and SR (reviewed in: [1–3]). Mitochondrial-SR tethering may also contribute to maintaining the highly constrained myofibrillar mitochondrial sub-cellular organization that is characteristic of cardiac myocytes. However, too much of a good thing can have adverse consequences, and this review discusses emerging evidence favoring mitochondrial-SR interactions in programmed cardiac myocyte death. We address the role of mitochondria and ER in the control of cardiac myocyte death and examine the relevance of their interplay for amplification and modulation of noxious signals. Here, we use the term “programmed cell death” to include cell elimination through apoptosis, necrosis, or any other mechanism subsequent to an internal decision or death program. As is described below, apoptosis is distinguished by the essential role of Bcl2 family proteins that target mitochondrial outer membranes to initiate the process, and by its requirement for ATP to fuel the mechanisms of cell death [4–7]. In contrast, necrosis is initiated by calcium signaling to the mitochondria, and results when ATP production is reversed and cellular ATP is insufficient to drive basal metabolism. Because of recent new findings in the field, we discuss in detail the functional implications of specialized, recently discovered protein organelle tethers to mitochondrial-mitochondrial and mitochondrial-ER signaling in the cardiomyocyte.

Mitochondria and cell death: an overview

Mitochondria not only ensure most of the ATP required by the cell, but are central for several signalling cascades. They modulate cytosolic Ca^{2+}transients and participate in anabolic and catabolic reactions essential to normal turnover of essential cellular metabolites. As a byproduct of their respiratory activity, mitochondria produce reactive oxygen species that can act as second messengers. Finally, mitochondria are core components that amplify signals for programmed cell death [8]. In mammalian cells, there are two major downstream apoptotic signalling pathways that culminate in the activation of caspases and are linked in some cells: the death receptor pathway and the mitochondrial pathway [9]. Cleavage of substrates involved in maintenance of cytoskeletal and nuclear integrity, cell cycle progression, and DNA repair by caspase cysteine proteases results in the orderly demise of the cell. Mitochondria participate in the competent activation of caspases by releasing cytochrome *c* (the only soluble component of the respiratory chain) and additional apoptogenic factors (second mitochondrial activator of caspases, HtrA serine peptidase 2, endonuclease G and apoptosis inducing factor) from the mitochondrial intermembrane space into the cytosol. Cytochrome *c* in complex with Apaf -1 activates caspase-9 and other downstream caspases [4]. This process is controlled by Bcl2 family

proteins [10] and is accompanied by mitochondrial dysfunction and a distinct morphological derangement, mitochondrial fission [11].

The role and mechanism of mitochondrial dysfunction during programmed cell death has been extensively addressed in recent reviews. Here we will just remind the reader of two basic tenets of mitochondrial dysfunction that characterizes the initial stage of necrosis and late stages of apoptosis: Permeabilization of the inner mitochondrial membrane to protons, possibly as a consequence of the opening of the high conductance permeability transition pore (PTP); and blockage of electron flow along the respiratory chain as a consequence of cytochrome c dilution following its release into the cytoplasm or of feedback by activated caspases on individual complexes of the respiratory chain. The consequences of these events include decreased mitochondrial ATP production, loss of the driving force for Ca^{2+}uptake, generation of reactive oxygen species, and ultimately, cell death. Thus, dysregulation of Ca^{2+}signalling is one of the key events in the path to necrotic cell death. Additional evidence supports a role for Ca^{2+}in the amplification of apoptosis signalling by a subset of death stimuli [12].

Regulation of mitochondrial morphology

An emerging and long neglected aspect of mitochondrial involvement in apoptotic death is the change in organelle shape, which has profound functional consequences on cell death progression. Mitochondria are morphologically complex. In certain cell types they are organized in networks of interconnected organelles, while in cardiac myocytes and other cells they exist primarily as individual entities [13] capable of undergoing dynamic fusion and fission reactions. From an ultrastructural perspective, mitochondria consist of an outer (OM) and an inner (IM) membrane that are further subdivided into an inner boundary portion and the cristae, bag -like folds of IM connected to OM via narrow tubular junctions [14]. The ultrastructure and the reticular organization of the organelle are determined by mitochondria-shaping proteins that regulate the equilibrium between mitochondrial fusion and fission.

In mammalian cells, mitochondrial fission/division is regulated by Drp1 and Fis1 [15–18]. Drp1 is a cytosolic dynamin-related protein whose inhibition or downregulation result in a highly interconnected mitochondrial network. The same phenotype is caused by downregulation of Fis1 [19], a 16 kDa integral protein of the outer mitochondrial membrane containing a single transmembrane domain and a tetratricopeptide repeat (TPR, involved in protein-protein interaction) domain facing the cytosol [19]. Drp1 is recruited to mitochondria and directly or indirectly interacts with Fis1 to promote constriction of mitochondrial membranes [20](Figure 1). Translocation ofDrp1 to mitochondria occurs in response to cellular and mitochondrial cues, including mitochondrial dysfunction. Mitochondrial depolarization, associated or not with PTP opening, induces a sustained risein cytoplasmic Ca^{2+}that activates the phosphatase calcineurin, promoting dephosphorylation of conserved Ser637 of Drp1 [21];Ser 637 dephosphorylated Drp1 translocates to mitochondria and promotes their fragmentation. Interestingly, Ser 637 is within a protein kinase A phosphorylation domain, functionally linking mitochondrial morphology to another crucial second messenger, cyclic AMP (cAMP) [22, 23]. With mitochondrial dysfunction the phosphorylation status of Ser 637 is dominant over that of Ser 616 [21], a target of cyclin dependent kinase 1 during mitosis [24], providing for distinct mechanisms by which the mitochondrial fission machinery can be regulated during cell stress and cell division.

The Ca^{2+}-dependent kinase CAMKIa may also influence mitochondrial localization of Drp1 through phosphorylation of Ser 600 [25], exerting a pro-fission effect similar to cyclin dependent kinase 1 phosphorylation of Ser 616. CAMKIa activation by Ca^{2+}influx through voltage-dependent Ca^{2+}channels functionally connects mitochondrial fission with

transmembrane Ca^{2+}cycling. Adrenergic stimulation by isoproterenol or physical activity is also associated with phosphorylation of Drp1 at Ser 600, linking Drp1 mitochondrial translocation to cardiac inotropy. However, mutants of Drp1 that mimic phosphorylation at Ser 600 do not constitutively localize to mitochondria, and Ca^{2+}influx promotes their further mitochondrial localization, indicating that phosphorylation at other sites is more important in determining Drp1 translocation. Once Drp1 is localized at the mitochondria, it can be stabilized by SUMOylation [26, 27]. In addition to Fis1 and Drp1, endophilin B1, a member of the endophilin family of fatty acid acyl transferases that participate in endocytosis, is involved in mitochondrial fission [28]. The function of endophilin B1 may be similar to endophilin 1, i.e. lipid modification of dynamin I [29].

Fusion of mitochondrial OM is regulated by two dynamin -related GTPases –mitofusin (Mfn) 1 and 2 (Figure 1). These two highly homologous proteins share structures comprised of a terminal GTPase domain, two hydrophobic heptad repeats (HR), and two transmembrane domains that are inserted in to the mitochondrial OM [30]. Notwithstanding their structural similarities, critical functional differences distinguish the two mitofusins: GTPase activity of Mfn1 is much higher, but affinity for GTP is lower [31]; Mfn1 is responsible for mitochondria l-mitochondrial tethering through its anti-parallel interaction withHR2 of Mfn proteins from adjacent mitochondria [32]; and in fibroblasts, Mfn1 (but not Mfn2)is required for fusion triggered by the inner membrane dynamin related protein Opa1 [33].

The unique functional role of Mfn2 has been some what elusive to delineate. Mfn2can be retrieved in hetero-oligomers with Mfn1 and is suggested to participate in later steps of mitochondrial fusion [34]. In addition, levels of Mfn2 correlate with oxidative metabolism of skeletal muscle [35] and with proliferation of vascular smooth muscle cells, where it sequester s the proto-oncogene Ras [36]. Moreover, mutations in Mfn2 are associated with Charcot-Marie-Tooth type IIa peripheral neuropathy [37]. Finally, as discussed in the final section below, Mfn2 exclusively controls the shape of ER(and in cardiac cells, presumably the sarcoplasmic reticulum)and tethers them to mitochondria [38]. It is conceivable that the relative expression level of these mitochondrial fission and fusion proteins helps to dictate mitochondrial morphology in different cell types and during development, and contributes to the highly ordered mitochondrial organization seen in cardiomyocytes.

Mitochondrial morphology and cell death

During apoptosis mitochondria remodel their inner structure to allow the bulk of cytochrome *c* to be released from the cristae stores, a process christened “cristae remodeling” [39]. Moreover, mitochondria undergo massive and reversible fragmentation prior to the release of cytochrome *c* [40, 41]. Drp1 involvement in mitochondrial fragmentation was revealed by protection against cytochrome c release and cell death by a dominant negative Drp1 mutant [40]. Likewise, Fis1 (the protein partner of Drp1 on mitochondrial membranes) overexpression induces cytochrome *c* release, while its ablation protects against cell death [17, 42]. Importantly, mitochondrial fragmentation is the only known and essential involvement of mitochondria during developmental apoptosis of *C. elegans* [43].

Not only are mitochondrial fission proteins activated during apoptosis, but Mfn1-dependent fusion is impaired [44]. This may occur either by functional inhibition of Mfn1 itself, or of its inner membrane partner Opa1 [33]. The latter possibility is supported by release of Opa1 together with cytochrome *c* early in the course of apoptosis [45]. Also released from mitochondria is TIMMP8a, another intermembrane space protein involved in Drp1translocation from the cytosol to the organelle [46]. Release of pro-fusion proteins and pro-fission cofactors maybe required to trigger mitochondrial fragmentation. However, it remains unclear as to why increased mitochondrial fission accelerates cell death. A unifying

model implicates mitochondrial Ca^{2+}uptake induced by the BH3 -only Bcl-2 family member BIK in cristae remodeling downstream of Drp1 activation[47, 48]. On the other hand, mitochondrial fission is not invariably associated with cell death. For example, *Bax*, *Bak* doubly deficient cells are resistant to apoptosis induced by stimuli that recruit the mitochondrial pathway, yet their mitochondria fragment following treatment with the same stimuli. In addition, a single conservative point mutation in the short inter-membrane space stretch of Fis1 dissociates its pro-fission from its pro-death activity [49]. Conversely, fission by Drp1 can even protect from death induced by Ca^{2+}-dependent apoptotic stimuli that require mitochondria to amplify deadly waves of this second messenger [50]. Taken together, accumulated data show that excessive mitochondrial fission is almost always associated with cell death, but at this time the data are not sufficient to conclude that mitochondrial fission is essential for mitochondrial apoptosis.

Calcium, the link between cardiac contraction and the mitochondrial pathway of cardiomyocyte death

$Ca^{2+}$is a versatile second messenger whose intracellular concentration impacts on a number of integrated cellular functions, including regulation of proliferation and gene transcription, stimulation of ATP production, and muscle contraction. It is therefore not surprising that persistent or very high elevations of intracellular $Ca^{2+}$are detrimental, and that cells expend resources to regulate calcium(if they fail to do so cell death could be a just around the corner). Tight regulation of Ca^{2+}may be even more important in the heart, where phasic high amplitude Ca^{2+}transients are essential for normal minute -by-minute organ function (i.e. contraction). In heart failure, sarcoplasmic reticular (SR) calcium re-uptake is characteristically delayed and SR calcium stores are typically diminished. Although there may be a role for ryanodine receptor leakage in heart failure [51], abnormal calcium cycling is widely attributed to decreased SERCA expression or chronic SERCA inhibition by phospholamban [52]. For this reason, a number of experimental and clinical efforts have aimed to improve cardiac function in heart failure by restoring SR calcium stores [53]. Some mouse models of heart failure have been “rescued” by dis-inhibiting SERCA through genetic ablation of phospholamban [54]. However, there is increasing evidence that enhancing SR calcium cycling and augmenting cardiac myocyte contraction in heart failure can also induce long-term increases in programmed cardiac myocyte death.

Song, et al [55] ablated phospholamban in two murine genetic cardiomyopathy models having calcium cycling abnormalities, the Gαq transgenic mouse [56] and the MYBP -C mutant mouse [57]. As anticipated, SERCA function was improved by phospholamban ablation; cardiomyocyte calcium cycling and contraction were improved, but there was no corresponding improvement in the in vivo cardiomyopathy. In a recent study of similar design, Zhang et al used phospholamban ablation to correct SR calcium cycling defects in Ca^{++}/calmodulin-dependent protein kinase (CaMK)II transgenic mice, but likewise found that the cardiomyopathy phenotype and signs of heart failure were made worse [58]. There are a number of other reports that phospholamban ablation exacerbates murine heart failure and/or induces cardiac myocyte apoptosis [59–61], and it appears increasing SR calcium content by other mechanisms can have a similar detrimental effect on cardiac myocyte viability and heart failure [62–64]. Taken together, the accumulated data support a direct relationship between SR calcium levels and programmed cardiac myocyte death, and suggest that increasing SR calcium beyond normal physiological limits can contribute to *in vivo* heart failure.

The mitochondrial permeability transition in heart failure

Cardiac mitochondria play a role in cardiac myocyte calcium dynamics by interacting with the SR to regulate beat-to-beat phasic calcium cycling [65] (reviewed in [2]). However, the relative contribution of mitochondrial calcium to the phasic calcium transient is small, approximately 1% [66], and the dominant function of mitochondria is ATP production via oxidative phosphorylation. Indeed, mitochondria are the source of ~90% of ATP used for cardiac contraction. As noted above, however, mitochondria regulate critical aspects of cardiac function in addition to energy metabolism and calcium homeostasis: They are the source of reactive oxygen species (ROS) that can either stimulate cell signaling pathways or damage cardiac proteins, and are central regulators of cardiac myocyte apoptotic and necrotic death [67–70].

One of the key aspects of mitochondrial involvement in cell death is control of the PTP. The PTP is a non -selective pore in the mitochondrial inner membrane that opens in response to greatly increased calcium concentrations, initially described in 1979 by Haworth and Hunter [71]. PTP opening permits transmembrane diffusion of molecules smaller than 1.5 kDa down their concentration gradients. Proton diffusion through PTP dissipates the normal pH gradient and membrane potential ($\Delta\psi_m$) that are essential components of the proton motive force used by the ATP synthase for ATP production [72]. Consequently, mitochondria try to compensate, maintaining the electrochemical gradient by reversing the ATP synthase. The net effect is that mitochondria begin consuming ATP. If a sufficient proportion of cardiac myocyte mitochondria undergo this permeability transition, the loss of ATP can be sufficient to initiate necrosis. On the other hand, if cytosolic ATP levels are sufficient to maintain minimal cell functions and to fuel apoptosis effectors, opening of the PTP can activate apoptosis signaling as a consequence of cytochrome c released from ruptured mitochondria and/or cristae remodeling.

Opening the PTP is only one of the Ca^{2+}dependent mitochondrial events. Increased beat-to-beat sarcoplasmic calcium concentrations are reflected by phasic changes in mitochondrial calcium that activate enzymatic production of NADH for the electron transport chain, stimulating ADP production [1]. In cardiac ischemia, calcium overloading combines with ROS production and increased phosphate concentration to induce PTP opening [73], although findings have even suggested that, *in situ*, the PTP is relatively calcium insensitive because of stabilization by cytosolic factors [74]. Thus, although a genetic analysis has proven that calcium triggers apoptosis by opening the PTP [12], calcium may be only one of several factors that interact to initiate mitochondrial permeability transition.

The exact structural components of the PTP are not known at this time. For a detailed discussion of the current state of the field and how individual PTP components interact and relate to the heart, the interested reviewer is referred to two excellent recent reviews [75, 76]. Briefly, the core PTP is believed to consist of a multi-membrane protein complex comprised of the voltage-dependent anion channel (VDAC)in the OM [77–79] and the adenine nucleotide translocator (ANT)in the IM [80–83], which are regulated by cyclophilin D (CyP-D)in the matrix. However, this model has been convincingly refuted by the means of genetic models and techniques that excluded a role for VDAC [84] and for ANT [85] as essential functional components of the PTP. On the other hand, they could both play regulatory effects, as ANT appears to function as phosphate- and calcium-sensitive PTP regulator.

CyP-D was first identified as an ANT-binding protein that mediated the inhibitory effect of cyclosporin A on PTP opening [86]. It is encoded by the peptidyl-prolyl cis-trans isomerase (*Ppif*) gene, and three independent groups have reported that its genetic ablation is sufficient to eliminate the mitochondrial permeability transition, without affecting Bcl2 factor-mediated outer membrane permeabilization and intrinsic pathway apoptosis [87–89].

Accordingly, genetic *Ppif* ablation has been used to demonstrate the role of PTP and death in mouse models of Alzheimer's disease, muscular dystrophy, diabetes mellitus, and heart failure [90–93]. Notwithstanding these compelling results, it has also been suggested that CyP-D is a PTP regulator, rather than an essential component, and that the striking effects observed with its ablation are simply the consequence of PTP inhibition by inorganic phosphate [94, 95].

Bcl-2 protein Nix as a mediator of SR-mitochondrial cross-talk in programmed cardiac myocyte death

As described above, mitochondria are central regulators, the so-called "gatekeepers", of programmed cell death [96]. In part, this is because of PTP opening, and in part because mitochondria are the targets for many actions of Bcl-2 family proteins that regulate apoptosis. Bcl-2 family proteins are classified according to their structural features and function in cell death [97]. Briefly, the "multidomain" proapoptotic proteins, like Bax and Bak, are pore-forming proteins that permeabilize mitochondrial OM, leading to cytochrome c release. Pore-formation by Bax and Bak [98, 99] is facilitated by pro -apoptotic BH3 domain-only factors [100], including cardiac-expressed BNip3 and Nix. BH3-only factors can hetero-dimerize with anti-apoptotic factors like Bcl-2 and Bcl-XL, preventing OM pore formation by Bax and Bak. Dynamic regulation of Bax, Bak, pro-apoptotic, and anti-apoptotic Bcl-2 family proteins is characteristic of heart failure and has been linked with programmed cardiac myocyte death (reviewed in [101]). Particularly detailed information is available for transcriptional upregulation of Nix in cardiac hypertrophy [102, 103]. The mechanisms by which Nix-induced SR-mitochondrial cross-talk contributes to the progression from non-failing hypertrophy to dilated cardiomyopathy through the programmed apoptotic and necrotic loss of cardiac myocytes have only recently been fully elucidated, and point to a key role for Ca^{2+}transfer between SR and mitochondria in Nix -dependent cell death.

Having found by microarray analysis that Nix transcripts are increased in cardiac hypertrophy [102], the Dorn group used transgenesis to determine the *in vivo* consequences of its upregulation, independent of hypertrophy *per se* or of any stimulus thereof. Forced cardiac myocyte Nix expression with the conventional α-myosin heavy chain (αMHC, *MYH6*) promoter produced mice that were normal at birth, but that died of rapidly progressive heart failure after one week. TUNEL staining showed massive cardiomyocyte apoptosis with apoptotic indices of 15–20% [102]. A follow-up study using conditional cardiac-specific Nix overexpressing mice revealed synergy between Nix and surgical pressure overloading for inducing apoptotic heart failure [104], suggesting that Nix can coordinate transcriptional and physiological cues leading to programmed cardiomyocyte death. Accordingly, we hypothesized that elimination of Nix might retard the progression of cardiac hypertrophy to heart failure by preventing programmed cardiac myocyte death. Our approach was to create *Nix* gene knockout mice, subject them to pressure overload or Gq-mediated hypertrophy (in which Nix is normally transcriptionally upregulated), and compare cardiomyocyte apoptosis, ventricular remodeling, and cardiac function between surgically or genetically modeled mice with and without Nix.

Because germ-line *Nix* ablation produces a striking hematological phenotype that could potentially interfere with our cardiac studies [105–107], we employed a Nkx-2.5 Cre-lox strategy to generate mice in which *Nix* was deleted only in cardiac myocytes. The cardiac -specific *Nix* knockout mice then underwent surgical transverse aortic banding to create achronic pressure overload. Whereas pressure overloaded wild-type hearts developed the typical cardiomyocyte apoptosis, ventricular dilatation and cardiac failure, pressure overloaded cardiac *Nix* knockout mice exhibited only half as much cardiomyocyte apoptosis (TUNEL positivity), less late replacement fibrosis, almost no ventricular dilation and wall

thinning, and had preserved systolic function [108]. Germ-line *Nix* ablation provided a similar rescue for the apoptotic peripartum cardiomyopathy that is characteristic of mice with cardiomyocyte-specific overexpression of the alpha subunit of heterotrimeric Gq [108, 109]. These findings established Nix as a critical inducible factor mediating programmed cardiac myocyte death in pressure overload hypertrophy, and linked programmed loss of cardiac myocytes with ventricular remodeling and progression to heart failure. Recently, we have better defined the mechanism for *in vivo* Nix-mediated cardiomyocyte death.

Like all pro-apoptotic Bcl-2 factors, Nix induces caspase-dependent apoptosis by stimulating mitochondrial OM permeabilization. Nix localizes to mitochondria, induces cytochrome c release, caspase activation and oligonucleosomal DNA degradation [102]. However, we recently observed that only ~80% of transfected Nix localizes to mitochondria, and that the remainder is localized to ER and SR reticular structures, depending upon cell type [110]. Furthermore, transcriptional upregulation of Nix in pressure overload hypertrophy preferential increases SR-(not mitochondrial -) associated Nix. We also observed that ER/SR-localized Nix increased cardiomyocyte SR calcium stores, as previously described for Bax [12, 111], while Nix ablation decreased SR calcium. The concordance between Nix ablation preventing Gq-mediated hypertrophy decompensation and cardiomyocyte apoptosis, [108] and also decreasing cardiac myocyte SR calcium stores [110] suggested a direct relationship between SR calcium levels and the programmed cardiomyocyte death that produced cardiomyopathy. We tested this possibility by concomitantly ablating phospholamban (*PLB*) and *Nix* in mice, superimposing the cardiac-specific Gq transgene, and then determining the effects on programmed cardiomyocyte death and cardiomyopathy development in the peripartum state. As anticipated, ablation of phospholamban (that inhibits SERCA -mediated SR calcium uptake) normalized cardiac myocyte SR calcium stores and improved excitation-contraction coupling. Whereas *Nix* ablation had protected hearts against apoptosis, thereby enhancing ventricular function and abrogating peripartum lethality in Gq mice [108, 110], *Nix* null/Gq transgenic mice in whom SR calcium stores were normalized through *PLB* ablation developed an exaggerated cardiomyopathy and increased mortality. These results established a causal link between SR calcium levels, programmed cardiac myocyte elimination, and in vivo cardiomyopathy mediated by pro-apoptotic Bcl-2 family member, Nix.

A number of recent studies have revealed pathophysiological involvement of SR - mitochondrial calcium transfer in heart failure not primarily caused by proapoptotic Bcl -2 family members. In the first such report, Nakayama et al [112] interrogated the mechanism for cardiac myocyte necrosis and heart failure induced by L -type calcium channel-mediated cardiac myocyte calcium overloading. Whereas overexpression of anti-apoptotic Bcl2 failed to rescue the cardiomyopathy of L-type calcium channel overexpression, ablating CyP-D (and thus preventing the mitochondrial permeability transition) normalized cardiac structure, function, and survival.

The second report came from the Heller Brown laboratory, which had previously described cardiomyopathic effects of activating (through overexpression) cardiomyocyte calcium/calmodulin kinase IIδc (CaMKIIδc) signaling [113]. These investigators observed that SR calcium levels were decreased in the CaMKII transgenic mice, which they attributed to ryanodine receptor leak. In a follow-up study [58], the same group used the same phospholamban ablation approach described above as a means to restore SR calcium in the hopes that contractile function would be enhanced [54]. Although *PLB* ablation normalized SR calcium levels in CaMKII transgenic mice, increased SR calcium was associated with worsening (rather than the anticipated improvement) of the cardiomyopathy and increased mortality from heart failure. Elegant cell-based studies demonstrated a link between increased SR calcium stores, increased SR calcium export independent of normal excitation-

contraction coupling (SR calcium “sparks” and leakage), and increased mitochondrial calcium loading. PTP-induced cell death was linked to SR-mitochondrial calcium transport by in vivo studies where CaMKII transgenic/*PLB* knockout cardiac myocytes were rescued from programmed death by cyclosporin A (CyP-D and PTP inhibitor) or RU-360 (mitochondrial calcium uniporter inhibitor).

These studies and other data indicated that calcium transport from sarcoplasmic reticulum to mitochondria through junctional “calcium hot-spots” [114] can be a potent stimulus for programmed cardiac myocyte death. We hypothesized that reticular-mitochondrial cross-talk stimulated by ER/SR-localized Nix might be inducing the necrotic pathway to programmed cell death. If this were the case, then cardiomyocyte “apoptosis” we and others had reported based on evidence of cytochrome c release, caspase activation, and TUNEL positivity might in part be a collateral effect of mitochondrial rupture after PTP opening. To test this notion we created and recombinantly expressed in HEK293 cells [110] or *Nix* null embryonic fibroblasts [93] mitochondria -specific and ER/SR-specific Nix mutants. Mitochondrial-directed Nix produced cell death associated with caspase activation, but with no net decrease in $\Delta\psi_m$, i.e. apoptosis. In contrast, reticular-directed Nix produced cell death preceded by PTP opening, but associated with caspase activation that we attributed to cytochrome c release after outer mitochondrial membrane rupture. Furthermore, we found that pharmacological (cyclosporin A) or genetic (CyP-D, *Ppif* ablation) inhibition of the PTP prevented cell death induced by reticular Nix, but not by mitochondrial Nix [93]. Finally, while death mediated by mitochondrial -directed Nix required Bax or Bak, reticular-directed Nix induced cell death independently of the multidomain pro-apoptotics. We interpreted these results as evidence that mitochondrial-directed Nix stimulates conventional intrinsic pathway apoptosis, whereas reticular-directed Nix induces programmed cell necrosis by increasing reticular calcium concentration and delivery to mitochondria, thereby promoting the mitochondrial permeability pore transition(Figure 2).

Recently, we established the *in vivo* relevance of Nix -mediated activation of dual apoptotic and necrotic programmed cell death pathways to *in vivo* cardiomyocyte death and ventricular remodeling [93]. Conditional, cardiac-specific transgenic expression of wild-type, mitochondrial-directed, or SR-directed Nix induced similar dilated cardiomyopathy phenotypes associated with similar levels of programmed cardiac myocyte death. However, in vivo cardiac myocyte necrosis visualized by anti-complement 9 staining of the membrane attack complex occurred only in mouse hearts expressing Nix that was localized all or in part to the SR(i.e. wild-type Nix or its SR-directed mutant), whereas TUNEL labeling occurred with mitochondrial- or SR-directed Nix. Likewise, ultrastructural evidence for cardiomyocyte PTP opening (mitochondrial swelling, matrix degeneration, and outer membrane disruption) was found only in hearts expressing an SR –localizing Nix. W e demonstrated causality for PTP opening in SR -directed Nix-mediated cardiac myocyte death through concomitant ablation of *Ppif*(encoding CyP-D) with overexpression of Nix or its organelle-directed mutants. CyP-D ablation rescued the cardiomyopathy and cardiomyocyte death only in SR-directed Nix expressing mice, completely eliminated complement 9 staining, and normalized mitochondrial ultrastructure. These findings show that an important aspect of Nix-mediated cell death is programmed necrosis mediated by SR -mitochondrial crosstalk that is a consequence of SR-localized Nix. Since we had previously observed that Nix which is endogenously upregulated during cardiac hypertrophy preferentially localizes to the SR [110], we concluded that MTPT opening stimulated by SR-mitochondrial calcium cross-talk may play a greater role than previously suspected in hypertrophy decompensation and the progression to overt heart failure. Consistent with this notion are a number of recent reports that otherwise implicate cardiac myocyte or SR calcium levels and PTP opening in cardiac injury and heart failure progression [115–117].

Mitofusins in SR -mitochondrial calcium signaling

The implication of SR-mitochondrial calcium transfer through putative high calcium microdomains by Nix and other factors, [110] and observations that a rigidly defined cardiac myocyte subcellular architecture and “mitochondrial packing” are essential to cardiac contractility, [118] suppor t a specific requirement for physical interactions between cardiac SR and mitochondria, suggesting a specific mechanism for mitochondrial-mitochondrial and mitochondrial-ER/SR tethering.

Interactions between organelles are key to spatial organization of cell signaling. The example of mitochondria and ER/SR is prototypical, and is determined by the biophysical properties of the mitochondrial Ca^{2+}uniporter that is responsible for Ca^{2+}uptake in the organelle. This mitochondrial IM channel has a very low affinity for Ca^{2+}, and therefore requires high concentrations of the ion that are not normally achieved in the bulk of cytoplasm following release of $Ca^{2+}$from the RyR or inositol phosphate receptor (IP3R) [119]. However, seminal studies by Rizzuto and Pozzan revealed that following release of $Ca^{2+}$by the IP3R, mitochondria do take it up [120], leading to elaboration of the theory that Ca^{2+}microdomains, hot spots of high [Ca $^{2+}$], are present at the interface between ER and the mitochondria [121]. This theory was corroborated by observations that the two organelles are in close proximity in a variety of cell types, [122] and that release of $Ca^{2+}$from ER triggers activation of mitochondrial dehydrogenases [123] that prolong ATP production [124]. Additional functions of the ER-mitochondria juxtaposition include the transfer of lipids between the former and the latter, where most biosynthethic pathways are lacking [125]. Accumating evidencs suggests that lipid trafficking betweem ER and mitochondria may have a role in PTP-independent cell death [126], generation of autophagosomal membranes [127] and in the above noted pathways to cell death that require inter-oraganelle Ca^{2+}transfer [12]. Artificial zippers between mitochondria and ER further substantiated that cells require this physical interaction for ATP production and death by selected stimuli [128, 129].

Despite the importance of ER/SR-mitochondria connection in cellular pathophysiology, the nature of the physical tether was only recently elucidated. Earlier reports had uncovered roles for the sorting protein PACS2 [130] and for VDAC and IP3 receptor together with the chaperone grp75 [131] in the interaction. However, these proteins did not appear to constitute the physical bridge between the organelles, rather being crucial regulators of the interaction (Figure 3). Trans-organellar bridges are formed by another protein whose primary role lies in the modulation of mitochondrial fusion, Mfn2 [38]. Cytochemical and biochemical analyses revealed that the bulk of Mfn2 is retrieved in mitochondria-associated membranes (MAMs), patches of ER attached to the mitochondrial OM. In addition, a relevant (approx7% of the total) fraction of Mfn2 resides in the endoplasmic reticulum, and Mfn2 ablation alters the structure of this organelle, causing its fragmentation, as substantiated by fluorescence recovery after photobleaching experiments. Selective reconstitution of the endoplasmic reticulum pool of Mfn2 in Mfn2−/−cells completely restored the reticular nature of the organelle, suggesting a role for t he ER-Mfn2 in the modulation of its shape. Finally, cells lacking Mfn2 display an increased average distance between ER and mitochondria, consistent with the localization of the protein in the MAMs. Selective correction of ER and mitochondria using targeted chimeras of Mfn2 and Mfn1 in cells lacking both Mfn1 and Mfn2 supports a model in which ER-Mfn2 engages in homo or hetero interactions with mitochondrial Mfn1 and/or Mfn2 to tether the two organelles (Figure 3). Further support to this model is given by an in vitro assay showing the requirement of ER Mfn2 for co-sedimentation of ER and mitochondria. In fact, cross-linkable, trans complexes of ER-Mfn2 with mitochondrial Mfn1 and Mfn2 exist and are further supported by co-immunoprecipitation assays. The lack of interaction between the two organelles has a major impact on Ca^{2+}transfer between them, and cells without Mfn2 are perfect tools to verify the

Ca $^{2+}$ microdomains theory. Release of $Ca^{2+}$from the ER is coupled with a reduced mitochondrial Ca $^{2+}$ uptake, whose rate is considerably slower in Mfn2−/− cells, compared to wild -type. This is not the consequence of impaired mitochondrial Ca^{2+}uptake, which is unaffected by Mfn2 ablation, but reflects increased distance between the organelles and the resulting limited generation of Ca^{2+} microdomains. Thus, Mfn2−/− cells are lack of function models that, after 15 years, have provided experimental proof for Ca^{2+}microdomains postulated by Rizzuto and Pozzan. Further evidence supports a role for Mfn2 in tissues where the ER-mitochondrial coupling is crucial, like the heart. For example, in cardiomyocytes, during oxidative stress induced apoptosis, upregulation of Mfn2 levels seem to play a crucial role, in a way independent from the effect of Mfn2 on mitochondrial dynamics [132]. During hypertrophy induced by pressure overload, Mfn2 seems to be conversely downregulated in what looks like a compensatory mechanism orchestrated by PPARδ and PGC1β [133, 134].

Recently, a multiprotein complex has been identified using a genetic screen in yeast as responsible for ER-mitochondrial tether. The yeast homologue of Mfn, Fzo1p, was not part of this multiprotein complex that comprised two mitochondrial OM integral proteins, Mdm10 and Mdm34, Mdm12, a cytosolic protein, and Mmm1, which can be retrieved in mitochondria or in the ER (Figure 3) [135]. The deletion strains of these components display growth defects that can be reconstituted using the artificial ER-mitochondrial tether invented by Hajnockzy and colleagues, pointing to a crucial role for these proteins in the establishment of ER-mitochondria interaction. In yeast, this is likely to impact only on phospholipid transfer between the two organelles, since ER is not the main Ca^{2+}stores and yeast mitochondria do not uptake Ca $^{2+}$, as they lack the uniporter. That this complex is a specialized yeast feature would be confirmed by the lack of higher eukaryotes orthologues for these proteins, mitigated by the retrieval of conserved sequences similar to syanptotagmin in at least two components of this complex [136]. Further research will uncover the orthologues of this tethering complex identified in yeast, extending its importance to Ca^{2+}signaling and apoptosis.

In conclusion, the crosstalk between ER and mitochondria is a key feature of the spatial organization of cell signaling. This strict relationship is key to insure proper mitochondrial responses to Ca^{2+}release from the ER, but can also lead to the amplification of cardiac myocyte death by a plethora of different stimuli.

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Non-standard Abbreviations and Acronyms

LCC	L-type calcium channel
ER	Endoplasmic reticulum
SR	Sarcoplasmic reticulum
SERCA	Sarcoplasmic reticular calcium ATPase
ATP	Adenosine tri-phosphate
PTP	Permeability transition pore
TPR	Tetratricopeptide repeat
OM	Mitochondrial outer membrane

IM	Mitochondrial inner membrane
CAMK	Ca^{++}/calmodulin-dependent protein kinase
HR	Heptad repeats
mfn	Mitofusin
Opa1	Optic atrophy 1
Drp1	Dynamin related protein 1
CyP-D	Cyclophilin D
VDAC	voltage-dependentanion channel
ANT	adenine nucleotide translocator
αMHC*MYH6*	α-myosin heavy chain
PLN	Phospholamban
$\Delta\psi_m$	Mitochondrial inner membrane electrical potential
MAM	Mitochondrial-associated membrane
RyR	Ryanodine receptor
IP3R	Inositol-tri-phosphate receptor

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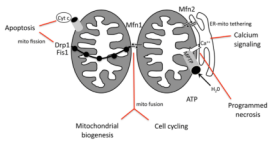

Figure 1. Schematic depiction of the key proteins determining mitochondrial shapeby regulated fission and fusion

Drp1, dynamin-related protein 1; Fis1, fission 1; Mfn1, mitofusin 1; Mfn2, mitofusin 2. MPTP = mitochondrial permeability transition pore.

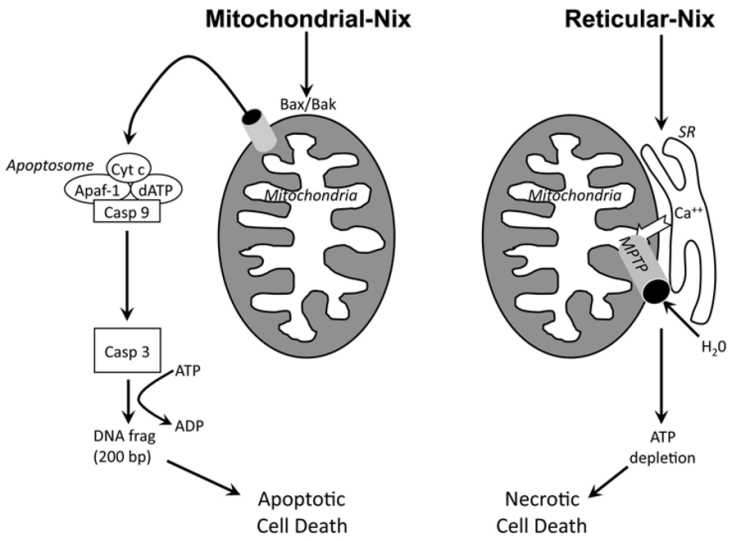

Figure 2. Activation of cell apoptosis and necrosis by Nix, and role of organelle localization

On left is Bax/Bak-dependent apoptosis mediated through mitochondrial Nix; right is mitochondrial permeability transition pore (MPTP)-dependent necrosis mediated through reticular Nix.

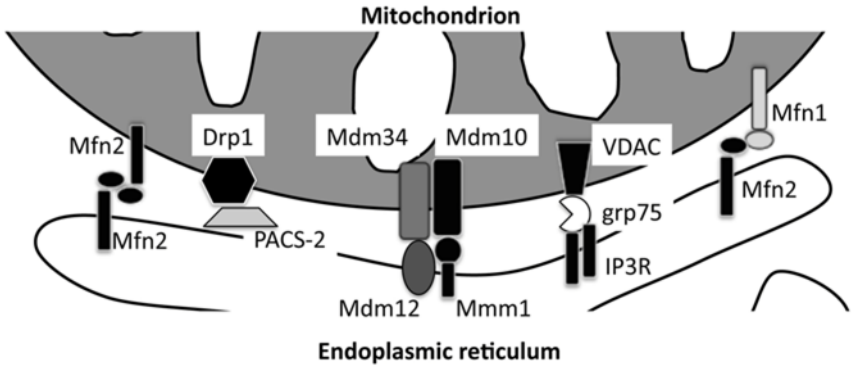

Figure 3.
Schematic depiction of proteins identified to date in yeast and mammals that regulate the juxtaposition between mitochondria and ER.