

Mitochondrial-Shaping Proteins in Cardiac Health and Disease – the Long and the Short of It!

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Abstract Mitochondrial health is critically dependent on the ability of mitochondria to undergo changes in mitochondrial morphology, a process which is regulated by mitochondrial shaping proteins. Mitochondria undergo fission to generate fragmented discrete organelles, a process which is mediated by the mitochondrial fission proteins (Drp1, hFIS1, Mff and MiD49/51), and is required for cell division, and to remove damaged mitochondria by mitophagy. Mitochondria undergo fusion to form elongated interconnected networks, a process which is orchestrated by the mitochondrial fusion proteins (Mfn1, Mfn2 and OPA1), and which enables the replenishment of damaged mitochondrial DNA. In the adult heart, mitochondria are relatively static, are constrained in their movement, and are characteristically arranged into 3 distinct sub-populations based on their locality and function (subsarcolemmal, myofibrillar, and perinuclear). Although the mitochondria are arranged differently, emerging data supports a role for the mitochondrial shaping proteins in cardiac health and disease. Interestingly, in the adult heart, it appears that the pleiotropic effects of the mitochondrial fusion

proteins, Mfn2 (endoplasmic reticulum-tethering, mitophagy) and OPA1 (cristae remodeling, regulation of apoptosis, and energy production) may play more important roles than their pro-fusion effects. In this review article, we provide an overview of the mitochondrial fusion and fission proteins in the adult heart, and highlight their roles as novel therapeutic targets for treating cardiac disease.

Keywords Mitochondrial morphology · Ischemia/reperfusion injury · Mitochondrial fusion · Mitochondrial fission · Mfn1 · OPA1 · Mfn2 · Drp1

Abbreviations

AKAP1	protein A-kinase anchoring protein 1
AMA	antimycin A
AMPK	5' AMP-activated protein kinase
APAF1	Apoptotic protease activating factor 1
Ca ²⁺	calcium
CaM Kinase	calmodulin-dependent protein kinase
Cdk1	cyclin dependent kinase 1
Drp1	dynamain-related protein 1
ER	endoplasmic reticulum
ERK	extracellular-signal-regulated kinase
ESC	embryonic stem cells
ETC	electron transport chain
HFpEF	heart failure with preserved ejection fraction
hFis1	human fission protein-1
HIF	hypoxia-inducible factor
IFM	interfibrillar mitochondria
IMM	inner mitochondrial membrane
INF2	inverted formin-2
iPSCs	induced pluripotent stem cells
IRI	ischemia-reperfusion injury
LV	left ventricular

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LVH	left ventricular hypertrophy
MAPL	mitochondrial-anchored protein ligase
MARCH5	membrane-associated RING-CH
MARF	mitochondrial assembly regulatory factor
Mcl-1	myeloid cell leukemia factor 1
Mff	mitochondrial fission factor
Mfn1 / 2	Mitofusins 1 and 2
MI	myocardial infarction
MiD49/51	mitochondrial dynamics proteins of 49 (MiD49) and 51 kDa (MiD51)
MPTP	mitochondrial permeability transition pore
mtDNA	mitochondrial DNA
NEMO	NF- κ B essential modulator
OMM	outer mitochondrial membrane
OPA1	Optic Atrophy 1
PAH	pulmonary arterial hypertension
PASMC	pulmonary arterial smooth muscle cells
PGC-1 α	peroxisome proliferator-activated receptor gamma coactivator 1-alpha
Pim1	Proto-oncogene serine/threonine-protein kinase Pim-1
PINK1	PTEN-induced putative kinase
PLA	proximity ligation assay
PNM	perinuclear mitochondria
RCS	respiratory chain supercomplexes
RIN1	Rab and Ras Interactor 1
RPC	remote ischemic preconditioning
SOD	superoxide dismutase
SR	sarcoplasmic reticulum
SSM	subsarcolemmal mitochondria
STED	Stimulated emission depletion
SUMO	Small Ubiquitin-like Modifier
TAB	transverse aortic banding
Tfam	mitochondrial transcription factor A
Timm44	translocase of inner mitochondrial membrane 44
VSMC	vascular smooth muscle cells
XIAP	X-linked inhibitor of apoptosis protein

Introduction to Mitochondrial Morphology

Mitochondrial health is critically dependent on the ability of mitochondria to move and change their morphology. By undergoing fission they generate fragmented discrete mitochondria, a process which is regulated by the mitochondrial fission proteins, dynamic-related peptide-1 (Drp1), human fission protein-1 (hFis1), mitochondrial fission factor (Mff) and mitochondrial dynamics proteins 49 and 51 (MiD49 and 51). Mitochondrial fission is essential for cell division and is required to remove damaged mitochondria by mitophagy. In contrast, the fusion of mitochondria generates elongated interconnected networks, a process which is orchestrated by the

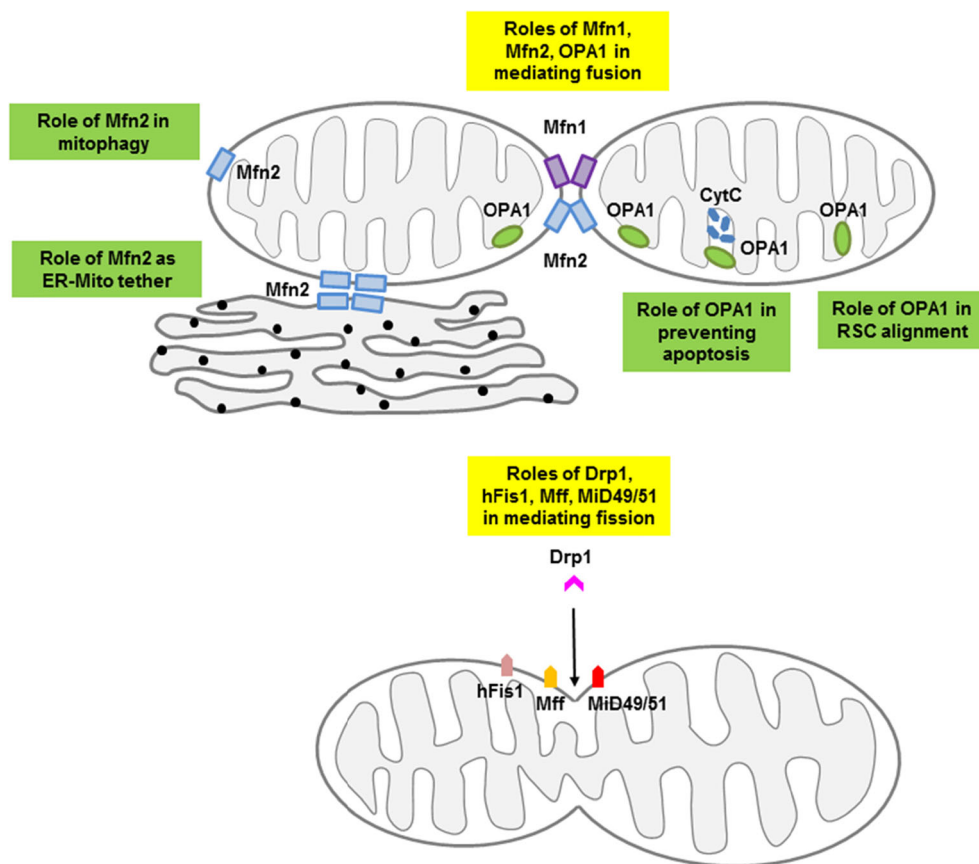
mitochondrial fusion proteins, Mitofusins 1 and 2 (Mfn1 and Mfn2), and optic atrophy protein-1 (OPA1), thereby enabling the replenishment of damaged mitochondrial DNA [1] or facilitation of intracellular energy distribution [2].

Until recently, the investigation of mitochondrial morphology had been largely confined to non-cardiac cells, in which mitochondria have unrestricted movements and are distributed throughout the cytoplasm into tubular networks. In contrast, in the adult heart, mitochondria are relatively immobile, are constrained in their ability to move, and are distributed into 3 distinct subpopulations based on their locality and function (subsarcolemmal, myofibrillar, and perinuclear). Although the mitochondria are arranged differently, emerging data supports a role for the mitochondrial shaping proteins in cardiac health and disease, with their effects on mediating changes in mitochondrial morphology playing a dominant role [3–6]. Interestingly, although the roles of the mitochondrial fission proteins are closely related to their effects on mitochondrial morphology in the adult heart, the mitochondrial fusion proteins appear to have a number of pleiotropic non-fusion roles, which may play more important roles in cardiac health and disease than their pro-fusion effects (see Fig. 1). Mfn2 has been reported to act as a tethering protein between mitochondria and endoplasmic reticulum (ER) [7, 8], and to be a critical mediator of mitophagy [9, 10]. Through its effects on cristae remodeling, OPA1 has been shown to regulate mitochondrial cytochrome C release and propensity to apoptosis, and facilitate mitochondrial energy production via its effects on the respiratory supercomplexes. In this review article, we provide an overview of the mitochondrial shaping proteins in the adult heart, and highlight their roles as novel therapeutic targets for treating cardiac disease. For more detailed general reviews on mitochondrial morphology please refer to the following articles [11–16].

Mitochondrial Fission Proteins

The process of mitochondrial fission requires the activation and dephosphorylation of cytosolic Drp1, which allows the latter to translocate to mitochondria, and bind to docking receptors on the outer mitochondrial membrane (OMM). Here, Drp1 oligomerises and forms a spiral around the mitochondrion, constricting it into two halves [17–20]. The roles the other components of the mitochondrial fission machinery, hFis1, Mff and MiD49/51, play in this process are currently under investigation. The current evidence suggests that Mff [21–25] and MiD49/51 [22, 26–31] act as docking proteins on the OMM for Drp1, and that hFis1 plays a regulatory role in mediating mitochondrial fission [32, 33]. Interestingly, Mff and MiD49/51 have been reported to exert contrasting effects on Drp1 GTPase activity at the OMM, with the former stimulating, and the latter inhibiting Drp1 GTPase activity [22] -

Fig. 1 Diagram depicting interactions of the mitochondrial-shaping proteins. The pro-fusion proteins – Mfn1, Mfn2 and OPA1 function to fuse adjacent mitochondria while Drp1 interacts with the docking proteins – Mff, Fis1 or MiD49/51 to induce mitochondrial fission. In addition to their profusion effect, Mfn2 and OPA1 have pleiotropic non-fusion actions. RSC, respiratory supercomplex; ER, endoplasmic reticulum; Mito, mitochondria



the resultant effect being dependant on the stoichiometric levels of the adaptors as well as the presence of other signals [22]. Another layer of complexity is added with the existence of different subpopulations of Drp1 in the cytosol (dimers or tetramers or high order equilibrium) [34–36] and even multiple splice variants of Drp1 [37], whereby recruitment of specific subpopulations of Drp1 by either Mff or MiD49/51 result in different effects on mitochondrial morphology [23, 38]. A number of other proteins have been shown to regulate mitochondrial fission in non-cardiac cells including COX assembly factor *ccdc56* [39], the OMM protein *FUNDC1* [40–42] and the myeloid cell leukemia factor 1 (*Mcl-1*) protein [43].

The ER and cytoskeleton have recently been demonstrated to contribute to the mitochondrial fission process. Actin polymerisation by the ER-associated inverted formin-2 (*INF2*) [44–46] has been shown to provide the force required for partial constriction of the mitochondria, an event which has been reported to facilitate the translocation of cytosolic Drp1 to these pre-constriction contact sites in the OMM. Drp1 translocation from the cytosol to the OMM has also been demonstrated to be mediated by other components of the cytoskeleton including septins [47]. Transient de novo assembly of F-actin on the OMM has been shown to mediate Drp1-mediated fission [48], in a manner that is synergistic with Mff [49].

Inhibiting actin polymerization, myosin IIA, or the formin *INF2* can also reduce this Drp1/actin interaction [49].

The regulation of Drp1 activity is under the control of a large number of post-translational modifications including SUMOylation [50], phosphorylation [51–53], ubiquitination [54], S-nitrosylation [55], and O-GlcNAcylation [56]. The phosphorylation of Ser-637 by protein kinase A (PKA) [51, 53], calcium (Ca^{2+})/calmodulin-dependent protein kinase (CaM Kinase) [57], and Proto-oncogene serine/threonine-protein kinase Pim-1 (*Pim1*) [58] has been shown to prevent the mitochondrial translocation of Drp1. In contrast, the phosphorylation of Ser-616 by Cdk1/cyclin B (a key mitotic kinase) has been reported to promote mitochondrial fission by Drp1 during mitosis [59, 60]. Under conditions of high cytosolic Ca^{2+} , dephosphorylation of Drp1 at Ser-637 by calcineurin induces mitochondrial fission [51, 52, 61–63]. The ablation of calcineurin in skeletal muscle leads to Ser-637 hyperphosphorylation of Drp1 causing elongation of mitochondria into ‘power-cable’-shaped filaments and increased mitochondrial respiration [64]. In hyperglycemic conditions, O-GlcNAcylation of OPA1 [65] and Drp1 [56] causes dephosphorylation of Ser-637 and the translocation of Drp1 to the OMM.

More recently, it has also been demonstrated that AMPK can phosphorylate Mff to trigger mitochondrial fission in

response to stress thereby initiating mitophagy of the mitochondrial fragments that have extensive damage [66]. Knockdown of the OMM-associated E3 ubiquitin ligase MARCH5 was found to selectively inhibit ubiquitination and proteasomal degradation of MiD49 leading to mitochondrial fission, a phenotype which was reversed by MARCH5 re-expression or MiD49 knockout [67].

Mitochondrial Fission, Apoptosis and Necroptosis

Drp1 has been demonstrated to co-localize with BAX at the OMM in response to apoptotic stimuli [68]. Recent findings have also invoked Drp1-dependent mitochondrial fission via the involvement of MiD49/MiD51 as being pertinent for cristae remodeling during intrinsic apoptosis [30]. Stimulated emission depletion (STED) microscopy has shown that BAX assembles with BAK and Drp1 in the form of a ring to delineate the area required for mitochondrial outer membrane permeabilization (MOMP) [69]. The area enclosed by the BAX ring is devoid of mitochondrial outer membrane proteins such as Tom20, Tom22, and Sam50 [69]. Drp1 is also stabilized by SUMOylation to promote mitochondrial fragmentation, loss of MMP and cytochrome C release [70], although mitochondrial fragmentation does not necessarily equate to cell death [71, 72]. Inhibition of Drp1 slows down rather than fully inhibits apoptosis as other pro-apoptotic proteins may still be released from the mitochondria [63, 73]. The opposite holds true whereby Drp1-dependent mitochondrial fission can occur independently of BAX, BAK and Apoptotic protease activating factor 1 (APAF1) to amplify cell death caused by certain factors such as BID and oxidative stress [74]. Downstream of BAX/BAK, the activation of apoptosis triggers mitochondrial-anchored protein ligase (MAPL/MUL1)-dependent SUMOylation of Drp1, a process requisite for stabilization of ER/mitochondrial contact sites that act as hotspots for mitochondrial constriction, calcium flux, cristae remodeling, and cytochrome c release [75].

Another interesting observation with regard to the role of Drp1 lies in its effects on necroptosis – a pathway of programmed necrosis. Earlier studies suggested the plausible role of Drp1 in mediating mitochondrial fragmentation following recruitment by the RIP3-PGAM5 complex [76–79]. Later studies have however, refuted this claim [80, 81]. Using arenobufagin- and staurosporine -treated HCT116 cells – a human colon cell line, it was reported that PGAM5L, but not PGAM5S constitutes a prerequisite for the activation of BAX and dephosphorylation of Drp1 for intrinsic apoptosis execution [82].

Mitochondrial Fission and Mitophagy

Mitochondrial fission is also essential for the degradation and removal of damaged mitochondria by mitophagy. Down-

regulation of Drp1 induces mitochondrial elongation, impairs mitochondrial autophagy, and prompts mitochondrial dysfunction, leading to cardiac dysfunction and increased susceptibility to ischemia/reperfusion [83]. Nonetheless, the duration of Drp1 downregulation used in this study was a prolonged period (at least 72 h in cardiomyocytes; 4 weeks tamoxifen injection in Drp1-CKO mice; 12-week-old Drp1-hetCKO mice) [83], highlighting the detrimental effects of chronic inhibition of Drp1. Moreover, the PTEN-induced putative kinase (PINK1) serves as a pro-fission signal, independently of Parkin [84]. A reduced level of mitochondrial division still persists even in the absence of Drp1 and is negatively regulated by Parkin [85]. Under normal conditions, the scaffold protein A-kinase anchoring protein 1 (AKAP1) recruits protein kinase A (PKA) to the outer mitochondrial membrane to phosphorylate and inhibit Drp1. Following cellular damage, PINK1 triggers PKA displacement from AKAP1 to ensure fission of damaged mitochondria for organelle degradation [84].

Mitochondrial Fusion Proteins

The fusion of 2 adjacent mitochondria to a single organelle requires the initial fusion of the OMM under the regulation of Mfn1 and Mfn2, followed by the fusion of the inner mitochondrial membrane (IMM) by OPA1 (reviewed in [86]). The mitofusins consist of a mitochondrial GTPase domain, a transmembrane domain, and a coiled-coil domain. The transmembrane domain functions to anchor these proteins to the mitochondrial membranes while the coiled-coil domains face the cytosol and mediate the formation of homotypic (Mfn1–Mfn1, Mfn2–Mfn2 and OPA1–OPA1) or heterotypic (Mfn1–Mfn2) physical connections between adjacent mitochondria [11, 87, 88].

The Mitofusins Mfn1 and Mfn2

The GTPase activity of Mfn1 (which is higher than that for Mfn2 [89]) is regulated by the binding of G-protein $\beta 2$ subunit to Mfn1 which decreases the motility of the latter and facilitates its clustering at specific foci on the OMM, thereby promoting the pro-fusion effects of Mfn1 [90]. Mfn2 expression is regulated by proteins linked to mitochondrial biogenesis such as PGC1- α and PGC1- β [91, 92]. The ubiquitination of Mfn1 and Mfn2 promotes the degradation of these proteins allowing unopposed mitochondrial fission during the selective removal of dysfunctional mitochondria by mitophagy [93, 94]. In addition, other components of the OMM may interact with Mfn1 and Mfn2 to modulate mitochondrial morphology, such as appoptosin, a mitochondrial carrier protein that is located in the IMM [95], and Smad2 which recruits the Rab and Ras Interactor 1 (RIN1) into a

complex with Mfn2 to promote mitochondrial fusion [96]. More recent studies have unravelled the various pleiotropic non-fusion roles of the mitochondrial fusion proteins, which will be described in the following sections and summarised in Table 1.

Mfn1 and Apoptotic Cell Death

Mitochondrial adaptation in mouse embryonic fibroblasts (MEFs) and HeLa cells subjected to oxidative stress (exposed to antimycin A (AMA), an inhibitor of electron transfer at complex III) has been shown to be driven by mitochondrial ubiquitin ligase membrane-associated RING-CH (MARCH5)-dependent quality control of acetylated Mfn1 [97]. In the presence of stress, Mfn1 is rapidly accumulated, inducing mitochondrial hyperfusion. Following that, MARCH5 binding to Mfn1 and its subsequent ubiquitylation of Mfn1 is significantly enhanced. The acetylation status of Mfn1 dictates the process of MARCH5 binding to Mfn1 and its ubiquitylation. Although yet to be shown in the heart, acceleration of Mfn1 degradation by MARCH5 under stress remains an important quality control system that inhibits mitochondrial aggregation and cell death [97].

Pleiotropic Non-fusion Role of Mfn2 as an ER-Tethering Protein

Mfn2 also tethers the ER to the mitochondria for calcium signaling from the ER to mitochondria [8]. This tethering function allows formation of subcellular domains of high calcium concentration close to the mitochondrial calcium uniporter. In the heart, this allows calcium to be efficiently transferred from the sarcoplasmic reticulum (SR) to the mitochondria which is crucial for cardiac contractility [98]. The genetic ablation of Mfn2 in the heart disrupts the SR-mitochondrial tethering and causes an impairment of Ca^{2+} signaling, diminished mitochondrial respiratory function and deterioration in left ventricular (LV) systolic function [99]. Nevertheless, a quantitative electron microscopy (EM) analysis showed an increase in the number of close contacts between the two organelles, in Mfn2^{-/-} MEF cells [100], a finding supported by multiple biochemical, morphological, functional, and genetic data from other studies in subsequent years [101–105]. Whether this discrepancy is cell-specific or reliant on the presence of other specific proteins at the ER-mitochondria interface remains to be further clarified. The advent of more advanced imaging techniques may shed further light on the function of Mfn2 as a tethering protein.

Table 1 Experimental studies implicating pleiotropic non-fusion roles of the mitochondrial fusion proteins

Study	Condition	Cell type	Major non-fusion roles	Other findings
<i>Mfn1</i>				
[97]	oxidative stress (exposed to antimycin A (AMA))	MEFs and HeLa	Mfn1 is rapidly accumulated, inducing mitochondrial hyperfusion. Following that, MARCH5 binding to Mfn1 and its subsequent ubiquitylation of Mfn1 is significantly enhanced.	
<i>Mfn2</i>				
[98–105]	Genetic ablation of Mfn2	MEFs	Reduced contact between mitochondria-ER	Increased contact between mitochondria-ER [101–105]
[106–108]			Mfn2 co-localize with both BAX and BAK in the OMM, impairing the fusion	Increased mPTP formation via the combination of Mfn2, BAX and BAK leads to cell death
[9, 93, 94, 109, 110]	Loss of mitochondrial potential		Removal of Mfn2 via ubiquitination and proteosomal degradation inhibits its pro-fusion activity	Mitochondrial fission occurs leading to removal of the damaged mitochondrion by mitophagy
<i>Opa1</i>				
[113, 115]	Loss of mitochondrial potential	SH-SY5Y and MEFs	OPA1 is up-regulated by the NF- κ B-responsive promoter elements following Parkin recruitment to maintain mitochondrial integrity and protect from cell death [113].	Stress also induces the metalloproteinase OMA1 to degrade the long isoforms of OPA1 and causes mitochondrial fragmentation for removal by mitophagy [115].
[120]	Apoptotic stimuli	MEFs	OPA1 prevents cytochrome c release to inhibit cell death by ‘stapling’ the cristae junctions closed [120]	
[122]		Mice ESCs	OPA1 also regulates formation and stability of respiratory chain supercomplexes (RCS) – components of the electron transport chain (ETC) arranged to facilitate transfer of electrons, via regulation of cristae morphology [122].	

Pleiotropic Non-fusion Role of Mfn2 in Apoptotic Cell Death

Apoptosis or cardiomyocyte cell death has been implicated in both acute and chronic heart diseases. The loss of myocardium constitutes an important pathogenic process in the heart and, hence, targeting the inhibition of apoptosis remains a viable therapeutic option. Mfn2 has been demonstrated to co-localize with both BAX and BAK in the OMM [68, 106]. BAX and BAK are pro-apoptotic proteins and the binding of BAX to Mfn2 impairs its pro-fusion capability [106]. Mfn2 also facilitates formation of the mitochondrial permeability transition pore (MPTP) and decreases stability of the mitochondrial membrane, thus promoting Drp1-mediated mitochondrial fission [107]. The absence of both BAX and BAK rescues the cells from MPTP opening and cell death by necrosis, yet mitochondrial fragmentation ensues [108]. Interestingly, genetic ablation of Mfn2 also prevented MPTP opening, thus suggesting that the combination of BAX-Mfn2 is sufficient to facilitate opening of the MPTP, which is achieved by forming hemifusion-related holes used in the exchange of ions during stress-induced MPTP opening [108]. These findings suggest that the BAX/BAK-Mfn2 interaction may be sufficient to promote MPTP formation and increase susceptibility to cell death. This notion was further supported using the BAX/BAK/cyclophilin D triple knockout mice where MI size was not substantially reduced when compared to the BAX/BAK double knockout mice [108].

Pleiotropic Non-fusion Role of Mfn2 in Mitophagy

Recent data suggested that Mfn2 plays a pivotal role in the removal of damaged mitochondria by mitophagy. Chen et al. [109] found that in damaged mitochondria with loss of mitochondrial membrane potential, Mfn2 is phosphorylated by PINK1 at Thr-111 and Ser-442. This process then facilitates binding of Parkin to the OMM which in turn ubiquitinates Mfn2 [9]. The ubiquitination of Mfn2 inhibits its pro-fusion activity allowing mitochondrial fission to take place, and also recruits p62 to the OMM resulting in the selective removal of the damaged mitochondria by mitophagy [9, 93, 94, 110]. Similarly, stress-induced JNK phosphorylation of Mfn2 leads to recruitment of the ubiquitin ligase (E3) Huwe1/Mule/ARF-BP1/HectH9/E3Histone/Las1 to Mfn2, with the BH3 domain of Huwe1 implicated in this interaction [109]. This results in ubiquitin-mediated proteasomal degradation of Mfn2, leading to mitochondrial fission and enhanced apoptotic cell death [109]. In addition, Wang et al. [10] have demonstrated that Mfn2 plays a crucial role in autophagy as a mediator of the of autophagosome-lysosome fusion, and deficiency of Mfn2 in the heart was found to result in a cardiomyopathy. Furthermore, age-induced reduction in Mfn2 levels in murine skeletal muscle has been reported to impair both mitophagy

and mitochondrial quality, leading to an exacerbated age-related mitochondrial dysfunction [111]. A compensatory mechanism involving a ROS-dependent adaptive signaling pathway through induction of HIF-1 α and BNIP3 is triggered as a result, raising the possibility of a similar feedback mechanism in the heart [111].

OPA1

The activity of the IMM pro-fusion protein OPA1 is regulated by alternative splicing and post-translational modification (for a more detailed review please see [112]). In addition to its pro-fusion effects OPA1 has been demonstrated to display a number of pleiotropic non-fusion effects.

Pleiotropic Non-fusion Role of OPA1 in Mitophagy

Parkin has been shown to be recruited to the linear ubiquitin assembly complex under conditions of stress that subsequently increases linear ubiquitination of NF- κ B essential modulator (NEMO), which is essential for canonical NF- κ B signaling. OPA1 is then up-regulated by the NF- κ B-responsive promoter elements to maintain mitochondrial integrity and protect from cell death. The lack of mitophagy, however, did not hamper the Parkin-induced protection [113]. This finding however, has been confounded by another study which did not find a major role for Parkin in mediating OPA1 regulation [114]. Instead, the authors found that the protective effect of Parkin may rather be related to the ubiquitination of BAX and the limitation of its mitochondrial translocation to the mitochondria [114]. The authors also noted a role of the non-classical NF- κ B pathway in the regulation of mitochondrial dynamics and OPA1 expression by showing that the absence of IKK α induces lower levels of OPA1 and a fragmented mitochondrial network [114]. Stress also induces the metallopeptidase OMA1 to degrade the long isoforms of OPA1 and causes mitochondrial fragmentation for removal by mitophagy [115]. Nevertheless, whether the loss of OPA1 per se will induce impairment of lysosome function in the heart, as can be seen in neurodegenerative models [116], remains to be clarified. Furthermore, in situ proximity ligation assay (PLA) in murine lung epithelial MLE-12 cells and human cervical adenocarcinoma HeLa cells showed that the hexameric intermembrane space protein, NDPK-D (or NM23-H4), binds cardiolipin and facilitates its redistribution to the OMM as a form of mitophagy-induction signal in association with OPA1 [117].

Pleiotropic Non-fusion Role of OPA1 in Cristae Remodeling and Mitochondrial Apoptosis

The anti-apoptotic effects of OPA1 have been attributed to its role in regulating mitochondrial cristae morphology and cytochrome c distribution. The cristae or invaginations of the IMM can be remodeled into either a narrow/tight junction or a

widened gap by tBID. This action is essential for redistribution of cytochrome c from the intra-cristal space into the intermembrane space (IMS) to initiate apoptosis [118–121]. OPA1, conversely, prevents cytochrome c release to inhibit cell death by ‘stapling’ these cristae junctions closed [120].

Pleiotropic Non-fusion Role of OPA1 in Cristae Remodeling and Mitochondrial Respiratory Efficiency

OPA1 also regulates formation and stability of respiratory chain supercomplexes (RCS) – components of the electron transport chain (ETC) arranged to facilitate transfer of electrons, via regulation of cristae morphology [122]. This implicates OPA1 as a regulator of mitochondrial respiration and a potential target for regulating mitochondrial energy production. A genome-wide RNA interference (RNAi) screen identified reactive oxygen species modulator 1 (ROMO1) as a redox-regulated protein that is able to oligomerize OPA1 for mitochondrial fusion and maintenance of normal cristae morphology [123]. The presence of oxidative stress induces the formation of high-molecular weight ROMO1 complexes while knockdown of ROMO1 promoted mitochondrial fission and loss of cristae, causing impaired mitochondrial respiration and increased sensitivity to cell death stimuli [123]. To adapt to cellular metabolic demand, changes in energy substrate availability are sensed by mitochondrial SLC25A transporters, which in turn regulate OPA1 oligomerization [124]. OPA1 oligomerization modulates cristae width and regulates assembly of the ATP synthase, in a mitochondrial fusion-independent manner. A fusion-incompetent form of OPA1(Q297V) rescued OCR, ATP synthase assembly and cell growth of OPA1 KO MEFs in galactose media, which forces mitochondrial respiration for ATP production [124]. Data from T cells supports the link between mitochondrial morphology and metabolism whereby fusion in memory T (TM) cells configures electron transport chain (ETC) complex associations favoring oxidative phosphorylation (OXPHOS) and FAO, while fission in effector T (TE) cells leads to cristae expansion, reducing ETC efficiency and promoting aerobic glycolysis [125].

Mitochondrial Morphology and the Adult Heart

Mitochondria in non-cardiac cells, cardiac cell lines, and neonatal cardiomyocytes are highly mobile and are distributed throughout the cytosol in a filamentous network. In contrast, in the adult cardiomyocyte, mitochondria are relatively static, constrained in their ability to move, are in close contact with each other, and are spatially arranged into 3 distinct subpopulations according to locality and function. The majority of the mitochondria are closely aligned adjacent to the myofibrils, with one or two mitochondria lying alongside each sarcomere – these have been termed the interfibrillar mitochondria (IFM), and are mainly involved in calcium signalling from the

SR to mitochondria and providing the energy required for cardiomyocyte contraction [126, 127] (see Fig. 2). A smaller population of mitochondria are arranged in clusters beneath the subsarcolemmal membrane and are believed to provide the energy required for ion channel function and may be involved with cell signaling – termed subsarcolemmal mitochondria (SSM). The third subpopulation of mitochondria form clusters either side of the nucleus, and are termed perinuclear mitochondria (PNM), and are presumably required to provide energy for transcription [126, 127] (see Fig. 2).

Although relatively immobile due to this characteristic spatial arrangement, emerging data suggest that mitochondria in the adult heart can undergo mitochondrial fusion and fission (see reviews [3–5, 13, 128, 129]). The evidence for this has been provided by electron microscopy [130–134], and using confocal microscopy over a timescale ranging from seconds to minutes to follow the movement of fluorescent marker such as the photoactivatable mitochondrial green fluorescent protein [135, 136], mitochondrial red and green fluorescent proteins [137–140], Renilla luciferase complementation [141] to pH-sensitive fluorescent mitochondrial probes [142, 143] between mitochondria.

Mitochondrial Fusion and Fission Proteins in Cardiac Health and Disease

Cardiac Development and Growth

Mitochondrial Fission Proteins

During early cardiac differentiation, an upregulation of Drp1-mediated mitochondrial fission coincides with removal of mitochondria via SQSTM1 (sequestosome 1)-mediated

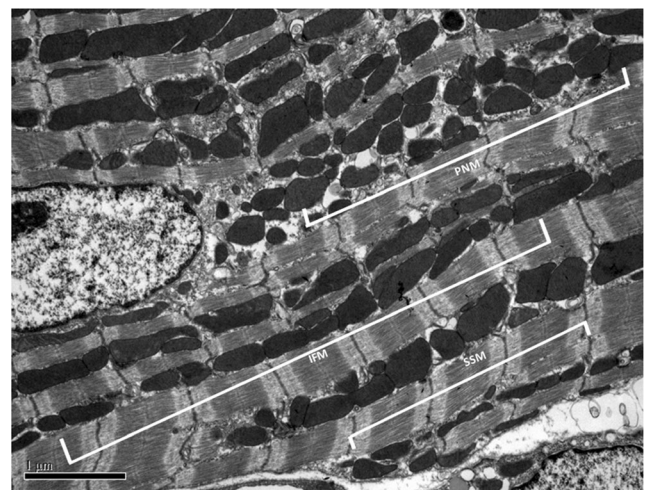


Fig. 2 Electron microscopy image showing the 3 subpopulations of mitochondria in an adult cardiomyocyte. IFM, interfibrillar mitochondria; PNM, perinuclear mitochondria; SSM, subsarcolemmal mitochondria

mitophagy [144]. Mitochondria are then repopulated in the new myotube via PPARGC1A/PGC-1 α (peroxisome proliferator-activated receptor gamma, coactivator 1 alpha)-mediated biogenesis, with a brisk upregulation of OPA1 levels [144]. Genetic ablation of Drp1 has been shown to be embryonically lethal at day E12.5 [145]. In the adult heart, ablation of Drp1 is associated with an impairment of mitophagy which leads to cardiomyopathy [83], accentuating the crucial role of Drp1-mediated fission in maintaining a healthy mitochondrial network. Judging from these findings, the inhibition of Drp1 as a therapeutic approach may not be as straightforward due to the fact that prolonged inhibition of Drp1 may be detrimental to normal cardiac function.

Mitochondrial Fusion Proteins

The importance of mitochondrial fusion in cardiac development has been delineated by death in utero during the mid-gestation period upon genetic ablation of both Mfn1 and Mfn2 [88]. Cardiac-specific ablation of the mitofusins in the embryo causes death at days E9.5–10.5 [146]. Kasahara et al. has demonstrated that the levels of Mfn2 and OPA1 play an essential role in cardiac development, with developmental arrest occurring at E13.5 when Mfn2 and OPA1 were absent. The observation postulates an association between mitochondrial dynamics and cardiac differentiation whereby calcium signaling linking calcineurin and Notch signalling was affected by the ablation of these pro-fusion proteins [147]. In the adult heart devoid of Mfn1, there appears to be a lack of distinct cardiac phenotype [148]. Conditional ablation of cardiac-specific Mfn2 (α -MHC-*Cre*), however, causes mitochondria to become pleomorphic and slightly enlarged while the LV exhibits modest hypertrophy with mild deterioration in systolic function [98, 99]. Mfn2-deficient MEFs exhibited a significantly increased respiration rate which correlated with increased levels of mitochondrial markers (TOM20 and NAO) as well as mitochondrial transcription factor A (TFAM) and peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1 α) protein levels while total ATP content remains unchanged [149]. The adaptive mechanism mediated by increased level of PGC-1 α and TFAM transcription factor prevents an excessive depletion of mtDNA and severe impairment of cell metabolism [149]. Combined ablation of both Mfn1 and Mfn2 in the adult murine heart induces a lethal cardiomyopathy after several weeks, probably due to the mitochondrial fragmentation present in these hearts [146, 150].

Cardiac metabolism changes in line with the growth and maturation occurring in the first few days of post-natal growth. Mitochondrial respiration switches from glycolysis to oxidative phosphorylation during this period. Mitochondria also change shapes from a fragmented phenotype to an elongated shape aligned alongside the myofibrils, based on the increase in mitofusins [150] and reduction in cardiac hypoxia-inducible factor (HIF) signalling. This shift

in HIF signaling also mediates an increase in fusion protein expression from E16.5 in a stepwise manner through to P10.5 with the largest increase taking place between P0.5 and P2.5 in MFN1 and MFN2 and between P2.5 and P10.5 in OPA1 while no changes were detectable in fission protein Drp1 and Fis1 levels over this time period [151]. Ablation of the mitofusins from the heart at the late embryonic stage causes severe mitochondrial dysfunction at 7 days (abnormal mitochondrial structure, down-regulated mitochondrial biogenesis genes, reduced mitochondrial DNA), development of cardiomyopathy, and all died before 14 days old [150]. The association between Mfn1 and PGC-1 α during the postnatal growth of the heart lies in the ability of PGC-1 α to stimulate the transcription of Mfn1 gene by co-activating the orphan nuclear receptor ERR α . Similarly, PGC-1 α is crucial for a high-level expression of nuclear- and mitochondrial-encoded genes involved in mitochondrial energy transduction and OXPHOS pathways, and for full respiratory capacity [152], albeit dispensable for maintenance of mitochondria in the adult heart. Parkin-mediated mitophagy as a form of organelle replacement has been shown to be essential for normal perinatal cardiac mitochondrial and metabolic maturation [153]. Engineered mutant Mfn2 expressed from birth which cannot be PINK1-phosphorylated on Thr111 and Ser442 as required for Mfn2-Parkin binding disrupts developmental perinatal transformation of cardiac metabolism [153].

Stem Cell Differentiation into Cardiomyocytes

Cardiac stem cells differentiating into adult cardiomyocytes experience a change in mitochondrial function and structure to accommodate for the switch from anaerobic glycolysis to oxidative phosphorylation, to support the increased metabolic demands of the differentiated beating cardiomyocyte ([154]; reviewed in [155]). In line with the change in mitochondrial metabolism, mitochondria change from a fragmented state (lacking cristae) in the embryonic stem cells (ESC), to an elongated state (with well-developed cristae), closely aligned with the myofibrils of the differentiated contractile cardiomyocyte [154]. This change in mitochondrial morphology is due to decreased Drp1 and increased Mfn2. The presence of the mitofusins constitutes a barrier to reprogramming whereby removing Mfn1/2 inhibits the p53-p21 pathway and promotes the conversion of somatic cells to a pluripotent state. The ablation of the mitofusins facilitates the glycolytic metabolic transition through the activation of the Ras-Raf and hypoxia-inducible factor 1 α (HIF1 α) signalling [156]. Interestingly, Kim et al. showed in the same year that Drp1 activation promoted differentiation of C2C12 myoblasts induced by serum starvation. Inhibiting mitochondrial fission in the same cell type via *mdiv1-1* or Drp1K38A impairs myogenic maturation further supporting the role of both fission and fusion in cardiomyocyte differentiation [157]. A more recent study in

Drosophila also demonstrated crosstalk between the fusion components (ChChd3, OPA1 and Marf) and the Hippo pathway in regulating cellular proliferation where a loss of fusion impairs proliferation during development [158]. The conflicting observations imply that the role of mitochondrial dynamics in cardiac differentiation is cell-specific and the cellular background (presence of certain mutations) may exert a more prominent role than expected. More recently, it was demonstrated that prohibitin 2 (PHB2) - a pleiotrophic factor mainly localised in mitochondria, is upregulated in undifferentiated mouse ES cells while differentiation causes the downregulation of the level of PHB2. This varying expression of PHB2 affects mitochondrial function via modulation of OPA1 processing, although the effects on OPA1 seem to be separate from the differentiation of ES cells [159].

It is interesting to note that reprogramming a somatic cell into induced pluripotent stem cells (iPSCs) requires an induction of genes involved in mitochondrial biogenesis (TFAM, NRF1), mitochondrial fusion (MFN1, MFN2) and glycine production while able to tolerate a certain degree of OXPHOS defects [160]. Reprogramming MEFs to iPSCs however, also induces a downregulation of the MAP kinase phosphatase Dusp6 to subsequently activate ERK signalling and promote a Drp1-dependent mitochondrial fission pathway that is necessary for pluripotency [161].

Heart Failure

Mitochondrial Fission Proteins

A mutation in the Drp1 gene (C452F) in a mouse mutant, Python, caused an autosomal dominant form of dilated cardiomyopathy. The homozygous mutation was embryonically lethal but the heterozygous form achieved adulthood albeit it developed a severe dilated cardiomyopathy after 11 weeks. This cardiomyopathy was associated with reduced content of mitochondrial respiratory enzymes and ATP. In terms of mitochondrial morphology, mitochondria of heterozygous Python MEFs were abnormal with numerous long tubular mitochondria whilst homozygous Python MEFs had grossly abnormal mitochondria which appeared to be spherical and aggregated [162]. A further study by the same group refined the details by showing that the C452F mutation in mice not only increased Drp1 GTPase activity but also conferred resistance to oligomer disassembly, ultimately leading to impaired mitophagy, mitochondrial depolarization, aberrant calcium handling, impaired ATP synthesis, and activation of sterile myocardial inflammation [163]. Interestingly, a single human mutation in Drp1 (A395D), lying close to the C452F mutation in the middle domain of the protein was also previously described [164]. The mutant A395D protein is however defective for self-assembly and GTP hydrolysis [165]. Mitochondrial fragmentation evoked by enhanced Drp1-mediated fission in *Drosophila*

cardiomyocytes did not adversely impact heart tube function [166]. Likewise, mitochondrial polarization status assessed by TMRE fluorescence was also not adversely impacted by Drp1-induced fragmentation [166]. The discrepancy in these observations for Drp1 mutations may be attributed to various factors, including: (1) species difference; (2) different Drp1 mutations (3) different gene promoters used for cardiomyocyte-specific gene manipulation.

In the SENP5-Tg (SENP5 (a SUMO isopeptidase) overexpressing) murine hearts, a decrease in SUMO attachment to Drp1 has been detected, leading to an enlargement of mitochondria at the early developmental stage. This was also accompanied by decreased cardiomyocyte proliferation and elevated apoptosis [167]. Excess mitochondrial fragmentation due to an upregulation of Drp1 contributes to impaired mitophagy and elevated mitochondrial ROS. Parkin is not abundant and has been found to be dispensable for constitutive mitophagy in normal hearts [168]. Removal of Drp1 by tamoxifen-inducible knockout in the adult mouse hearts, however, not only prevented mitochondrial fission, but also markedly upregulated Parkin, thus provoking mitophagic mitochondrial depletion that contributed to a lethal cardiomyopathy [168]. Concomitant conditional Parkin deletion with Drp1 ablation in adult mouse hearts prevented the upregulation of Parkin, while increasing 6-week survival, with an improved ventricular ejection fraction, mitigating adverse cardiac remodeling, and decreasing cardiac myocyte necrosis and replacement fibrosis [168]. Short-term observation of heart failure in mice subjected to transverse aortic constriction (TAC), however, showed that Drp1-associated mitochondrial autophagy is transiently activated and then downregulated in the mouse heart in response to pressure overload [169]. Downregulation of mitochondrial autophagy in this scenario plays an important role in mediating the development of mitochondrial dysfunction and heart failure, which can be reversed by injection of Tat-Becn1, a potent inducer of autophagy [169]. It has also been reported in murine hearts subjected to pressure overload that there is an aberrant activation of MMP-9 causing degradation of the gap junction protein connexin-43 (Cx-43) in the ventricular myocardium [170, 171]. The reduction in levels of Cx-43 was associated with increased fibrosis and ventricular dysfunction in heart failure [170]. Usage of the small molecule inhibitor of Drp1 – *mdivi-1*, normalized the decreased ratio of MFN2 to Drp1 [172] as well as the expression levels of MMP-9 and Cx-43, thus showing an improved cardiac function [171, 173, 174]. The intricate link between mitochondrial biogenesis and dynamics and mitophagy can also be seen from the use of Brg1 (Brahma-related gene 1) /Brm (Brahma) double mutant mice (cardiomyocyte-specific and inducible deletion of the SWI/SNF (SWI/SNF/Sucrose Non-Fermentable) ATP-dependent chromatin remodeling complexes BRG1 and BRM in adult mice) [175]. These double-mutant mice exhibited increased mitochondrial biogenesis, increases in ‘mitophagy’, and late stage decrease in

mitochondrial-shaping proteins that led to small, fragmented mitochondria following which a severe cardiomyopathy and death occur [175].

Mitochondrial Fusion Proteins

Cardiac ablation of both Mfn1 and Mfn2 causes a mild cardiomyopathy [98, 99]. Cardiac ablation of Mfn2 alone in the adult murine heart only manifests as increased sensitivity to acute IRI and development of late-onset LV dysfunction at 17 months of age. The underlying mechanisms associated with this include impaired autophagy, defective lipid metabolism, and decreased mitochondrial respiration (primarily at complex III) [10]. Silencing of MARF (mitochondrial assembly regulatory factor – orthologue of mammalian, Mitofusin) and OPA1 in the *Drosophila* fly heart tube leads to dilated cardiomyopathy, a phenomenon which could be rescued by over-expressing either of the human Mitofusins (MFN1 or MFN2) or superoxide dismutase 1, implicating impaired mitochondrial fusion and oxidative stress in the pathogenesis of the heart failure [176]. A further study conducted by the same group refined the details by demonstrating that scavenging ROS by overexpressing superoxide dismutase (SOD) or suppressing ROMO1 prevented mitochondrial and heart tube dysfunction provoked by OPA1 RNAi, but not by mitofusin/MARF RNAi. In contrast, enhancing the ability of endoplasmic/sarcoplasmic reticulum to handle stress by expressing Xbp1, an ER stress-responsive transcription factor, rescued the cardiomyopathy of mitofusin/MARF insufficiency without improving that caused by OPA1 deficiency [166]. This again reiterates, at least in *Drosophila* heart tubes, the fact that mitochondrial fission per se does not always lead to cardiac dysfunction. Rather, it is the co-occurrence of mitochondrial fission with enhanced ROS and ER stress that induces cardiomyopathy. Similarly, rat hearts followed for twelve to eighteen weeks after myocardial infarction exhibited a decrease in MFN2, an increase in Fis1, and no change in OPA1 expression [177]. The lack of Mfn2 also impairs Parkin-mediated mitophagy leading to an accumulation of damaged ROS-producing organelles and progressive heart failure [9, 178]. To circumvent this, suppressing mitochondrial ROS provides benefits in preventing depolarization of the mitochondria, respiratory impairment and structural degeneration. Nevertheless, it should be noted that suppression of mitochondrial ROS in these settings should be controlled as overly suppressing mitochondrial ROS was associated with impaired secondary autophagic pathways, suggesting the importance of mitochondrial ROS alongside mitochondrial dynamics in mediating mitophagy and minimize cardiac failure [179]. The lack of Parkin and ensuing disruption of mitophagy causes an accumulation of enlarged, hollow “donut” mitochondria with dilated cardiomyopathy [180]. Induction of mitochondrial fission prevented the cardiomyopathy and corrected mitochondrial

dysfunction demonstrating the link among improper mitochondrial fusion, defective mitophagy and organ dysfunction [180].

Levels of OPA1 were also found to be decreased in a post-MI rat heart failure model and human dilated and ischemic cardiomyopathy tissue samples [181]. Heterozygous levels of OPA1 also reduced mitochondrial DNA copy number and decreased expression of nuclear antioxidant genes at 3–4 months [182]. Nevertheless, baseline cardiac function was found to be normal in OPA1-deficient mice, although cardiomyopathy associated with mitochondrial fragmentation and impaired mitochondrial function developed at 12 months of age [182]. The reason for the decline of OPA1 levels in heart failure requires further investigation. Similarly during initial compensatory cardiac hypertrophy, an increase in OPA1 and decrease in Drp1 occur in concert with decreased Parkin and Sirt1/AMPK-PGC-1 α signalling, signifying a compromised mitochondrial remodelling system [183]. A recent study by Wai et al. demonstrated that dilated cardiomyopathy and heart failure was induced when cardiac-specific ablation of Yme11 in mice activated OMA1 and hastened OPA1 proteolysis, leading to mitochondrial fragmentation and altered cardiac metabolism [184]. This deleterious scenario was rescued by OMA1 deletion, which prevented OPA1 cleavage [184].

Acute Ischemia-Reperfusion Injury and Cardioprotection

Mitochondrial Fission Proteins

Mitochondrial dysfunction induced by acute ischemia/reperfusion injury (IRI) is a critical determinant of cardiomyocyte death following acute myocardial infarction. In 2006, Brady et al. first made the observation that mitochondria undergo fission when HL-1 cardiac cells were subjected to simulated ischemia, generating fragmented mitochondria, a change in mitochondrial morphology which persisted into simulated reperfusion, and was reversed in the presence of pharmacological inhibition of p38 MAPK [185]. Two years later, Plotnikov et al. [186] noted that mitochondria in rat primary renal tubular epithelium and fibroblasts also underwent fragmentation during simulated IRI, and this effect was prevented by pre-treating the cells with either SkQ1 (a mitochondrial anti-oxidant), Li⁺ (a non-specific GSK-3 β inhibitor) or insulin. Whether mitochondrial fission induced by acute IRI was merely an epiphenomenon or a determinant of cell death in this setting, was investigated by our group, when we found that genetic inhibition of Drp1 in HL-1 cardiac cells (using a dominant-negative mutant of Drp1) [130], not only inhibited the mitochondrial fission induced by acute IRI but also reduced cell death in this setting, highlighting mitochondrial fission as a potential therapeutic target for cardioprotection. Our group went on to demonstrate that mitochondria in adult murine hearts also undergo fission in response to acute IRI, and that preventing this using *mdivi-1*, a

small molecule inhibitor of Drp1, attenuated the death of adult cardiomyocytes subjected to simulated IRI and reduced in vivo MI size in the adult murine heart following acute IRI [130]. Subsequent studies have confirmed that adult cardiac mitochondria undergo fission in response to acute IRI, and that targeting Drp1 with dominant negative mutants [187], siRNA [188], *mdivi-1* [173, 189], P110 (a peptide inhibitor of the interaction between hFis and Drp1) [190], or Dynasore (a non-specific dynamin inhibitor) [191] reduced MI size in small animal models of acute IRI. Pharmacological inhibition of mitochondrial fission at the onset of reperfusion using *mdivi-1* [173], P110 [190] and most recently nanoparticle-mediated drug delivery of Mdivi1 (which was shown to be more effective than administering *mdivi-1* alone [192], has been shown in experimental studies to reduce MI size in small animal *ex* and in vivo models of acute IRI, demonstrating a clinically-relevant time-point for cardioprotection in AMI patients. However, a recent study in HL-1 cardiac cells failed to demonstrate any benefit with *mdivi-1* administered at the onset of reoxygenation, and actually showed an increase in cell death – the reason for which is unclear [193]. Whether therapeutic inhibition of mitochondrial fission can reduce MI size in a clinically-relevant large animal model remains to be determined. Given the potential non-specific effects of *mdivi-1* [194], novel, more -specific inhibitors of mitochondrial fission are needed to translate this therapeutic approach into the clinical setting. In this regard, the discovery of other components of the mitochondrial fission machinery such as ER-mediated mitochondrial pre-constriction and the Drp1 docking proteins in the OMM, Mff and MiD49/51, may provide novel therapeutic targets for inhibiting mitochondrial fission. It is important to appreciate that although acute inhibition of mitochondrial fission induced by acute IRI is cardioprotective, the chronic inhibition of Drp1 may be detrimental to the heart as the process of mitochondrial fission is necessary for the removal of damaged mitochondria by mitophagy [112, 196]. This was nicely illustrated by Ikeda et al. [83] who demonstrated that conditional cardiac-specific ablation of Drp1 induced mitochondrial elongation, suppressed mitophagy, increased MPTP opening susceptibility, resulting in a cardiomyopathy and increased MI size following acute IRI.

There are several unanswered questions concerning mitochondrial fission as a mediator of cell death in acute IRI, and as a target for cardioprotection. Firstly, the mechanism through which acute IRI induces mitochondrial fission is not clear - potential explanations include:

- (1) mitochondrial ROS [186, 189] which is generated during acute IRI is known to induce mitochondrial fission. Plotnikov et al. showed that pre-treatment of SkQ1 (a mitochondrial ROS scavenger) prevented the mitochondrial fission induced by simulated IRI [186], and Zaja et al. demonstrated that Trolox, a ROS scavenger, decreased pSer616 Drp1 levels and mitochondrial fission in HL-1 cardiac cells following simulated IRI [189].
 - (2) cytosolic calcium overload which occurs during acute IRI may activate calcineurin thereby dephosphorylating and activating Drp1 [61, 195]. Hom et al. [196] demonstrated in both neonatal and adult cardiomyocyte that increasing cytosolic calcium concentration induced Drp1-dependent mitochondrial fission.
- Secondly, the mechanism through which inhibiting mitochondrial fission induced by acute IRI protects the heart is not clear. We have shown that inhibiting mitochondrial fission may protect the heart against acute IRI by decreasing MPTP opening susceptibility [130], although the mechanisms linking mitochondrial fission to MPTP opening remain unclear and need to be further investigated.
- A number of signaling pathways and/or cardioprotective interventions have been shown to inhibit Drp1-dependant mitochondrial fission induced by acute IRI:
1. Wang et al. [195] demonstrated that the p53-microRNA499-calcineurin-Drp1 axis operates in the heart to mediate mitochondrial fission and cardiomyocyte death in the setting of acute IRI in the heart, and therapeutic inhibition of this pathway was found to reduce MI size in murine hearts.
 2. Using H9C2 myoblasts, Kim et al. [197] found that the mitochondrial scaffolding protein A-kinase anchoring protein 121 (AKAP121) inhibits mitochondrial fission following simulated IRI AKAP121 through PKA-dependent inhibitory phosphorylation of Drp1 and PKA-independent inhibition of Drp1-Fis1 interaction.
 3. Pride et al. [198] have shown that nitrate-induce preconditioning protect H9C2 myoblasts against simulated IRI by activating PKA, phosphorylating Drp1 and inhibiting mitochondrial fission induced by acute IRI.
 4. Kuzumic et al. [199] demonstrated that trimetazidine protected rat neonatal cardiomyocytes from palmitate-induced mitochondrial fission and dysfunction.
 5. Zaja et al. [189] found that cyclin dependent kinase 1 (Cdk1) and protein kinase C isoform delta (PKC- δ) bind to and increased Drp1 activity, in the setting of simulated IRI in HL-1 cardiac cells.
 6. Using an experimental model of isoproterenol (β -adrenergic receptor agonist)-induced ischemic injury in the rat heart, Xue et al. [200] demonstrated that vagal nerve stimulation attenuated the expression of the mitochondrial fission proteins (Drp1, hFis1), prevented mitochondrial fragmentation and inhibited MPTP opening, and decreased MI size, via the muscarinic-3 receptor/Ca²⁺/calmodulin-dependent kinase kinase β /AMPK signaling pathway.
 7. DJ-1, also known as Park7 (Parkinson's Disease autosomal recessive, early onset 7), is an evolutionarily

conserved 189-amino acid protein, that has been shown to protect the brain and heart against acute IRI through its protective effects on mitochondrial function [201, 202]. Shimizu et al. [203] have demonstrated that DJ-1 reduces MI size by modifying the SUMOylation (a post-translational modification process in which small ubiquitin-like modifier proteins are covalently and reversibly conjugated to target proteins) of Drp1 and inhibiting mitochondrial fission in the murine heart [203].

Mitochondrial Fusion Proteins

The roles of the mitochondrial fusion proteins Mfn1, Mfn2 and OPA1 in susceptibility to acute IRI have been recently investigated in HL-1 cardiac cells, neonatal rodent cardiomyocytes, and adult rodent cardiomyocytes with both interesting and sometimes surprising results. The over-expression of Mfn2 in HL-1 cardiac cells was shown to induce mitochondrial elongation, lower MPTP opening susceptibility and reduce cell death following simulated acute IRI [130], and Mfn2 ablation in neonatal cardiomyocytes was shown to increase cell death following simulated IRI [99], findings which are consistent with the notion that mitochondrial fission induced by acute IRI is detrimental. However, in the adult heart, the genetic ablation of either cardiac-specific Mfn1 or Mfn2 was found to have opposing and unexpected effects with pleomorphic mitochondria, less MPTP opening and reduced cell death [107, 148, 204]. Similarly, although cardiac-specific ablation of both Mfn1 and Mfn2 (DKO) resulted in mitochondrial fragmentation, decreased respiratory function, and impaired myocardial contractile function [205], it resulted in less MPTP opening and reduced MI size following acute IRI. The reason for the unexpected results of ablating Mfn1 and/or Mfn2 in the heart are not known but may relate to pleiotropic effects of Mfn2. The absence of Mfn2 in the DKO mice increased the distance between SR and mitochondria and resulted in less mitochondrial calcium overload during acute IRI which may have, in part, contributed to the protected phenotype observed in the DKO mice [205]. These findings suggest that the acute inhibition of Mfn2 during acute IRI may provide a novel cardioprotective strategy and that the mechanism underlying this beneficial effect appears to be independent of its effects on mitochondrial morphology.

Partial ablation of OPA1 promotes formation of clusters of fused mitochondria and altered cristae with increased mitochondrial size. Sensitivity to MPTP opening however, was decreased [206]. OMA1 is activated in a BAX- and BAK-dependent fashion [207]. Activated OMA1 cleaved OPA1, an event that is critical for remodeling of mitochondrial cristae [207]. OMA1 knockdown in neuronal cells or renal proximal tubular cells prevented OPA1 proteolysis, fragmentation of mitochondria, cytochrome c release and cell death during ATP-depleted ischemia [208, 209]. The pro-survival kinase, Akt as well as its

pharmacological activator, EPO has also been postulated to confer cardioprotection by means of elongating the mitochondria via modulation of Mfn1 [210], a finding similarly recapitulated by the pro-survival extracellular-signal-regulated kinase (ERK) [211]. Recent studies in the liver demonstrated a downregulation of SIRT1 following hepatic IRI, which was partially attributable to activation of calpains [212], while genetic overexpression or pharmacological activation of SIRT1 using resveratrol or SRT1720 markedly suppressed defective autophagy, onset of the mitochondrial permeability transition, and hepatocyte death after I/R [213]. The studies also identified Mfn2 as a novel target of SIRT1 in which SIRT1 deacetylates Mfn2 to mediate autophagy induction [212, 213]. Male adult rats exposed to four 5-min cycles of limb ischemia interspersed by 5 min of limb reperfusion, immediately prior to myocardial ischemia and 120 min of reperfusion (MI + RIPC group) experienced a smaller infarct size (−28%), increased mitochondrial fusion protein OPA1, and preserved mitochondrial morphology [214]. A mild overexpression of OPA1 also protected against cardiac ischemic injury measured in the form of reduced LDH release in 5 months old mice subjected to a Langendorff model of 40 min of ischemia followed by 15 min of reperfusion [215]. Whether this modest expression of the long form of OPA1 (~10% in OPA1^{tg} hearts versus ~0.6% of total OPA1 in WT) is able to reduce infarct size in an in vivo model of myocardial infarction remains to be investigated. The role of the mitochondrial-shaping proteins in susceptibility of the adult heart to acute IRI is complex and not easily determined, further complicated by the lack of an OPA1 or Mfn-specific activator.

Left Ventricular Hypertrophy

Mitochondrial Fission Proteins

The development of left ventricular hypertrophy (LVH) has been linked to fragmentation of the mitochondria and enhanced mitophagy, due to an upregulation of Drp1 in a cell model of phenylephrine-induced cardiomyocyte hypertrophy [174, 177]. In order to prevent the development of LVH, Givvimani et al. employed *mdivi-1* to maintain the mitochondrial network and prompt the release of pro-angiogenic factors (CD31 and VEGF) while reducing collagen deposition [174], effects that were mirrored a year later in a quantitative phosphoproteomics study using myocardial samples at different time points following transverse aortic banding (TAB) [216]. The hypertrophic response and oxygen consumption were reduced in response to treatment with *mdivi-1* while the absence of *mdivi-1* saw phosphorylation of DRP1 S622 and subsequent mitochondrial translocation in TAB-treated mouse hearts and phenylephrine-treated rat neonatal cardiomyocytes [216]. These findings suggest a potential therapeutic strategy in acute inhibition of mitochondrial fission proteins to salvage LV hypertrophy.

Mitochondrial Fusion Proteins

MEK1/2–Erk1/2 expression is upregulated in LVH leading to a proliferation of VSMC, a phenomenon inhibited by Mfn2 (formerly known as hyperplasia suppressor gene or HSG) [217]. The same group used different experimental models of LVH (phenylephrine induced LVH in neonatal rat cardiomyocytes, spontaneously hypertensive rats, β 2-adrenergic transgenic mice, and pressure overload LVH by transverse aortic constriction) to determine the downregulation of Mfn2 and upregulation of Erk1/2 [217]. Angiotensin-II treatment in neonatal rat myocytes similarly decreased expression of Mfn2 while elevating Akt levels. Over-expressing MFN2 reversed the angiotensin-ii-induced LVH in both neonatal cardiomyocytes and the intact rat heart [218]. In mice with cardiac-specific Mfn2 KO, the downregulation of Mfn2 impairs tethering of mitochondria to ER and subsequent Ca²⁺ signalling [9, 99]. Using the model of transverse aortic constriction (TAC), Piquereau et al. demonstrated that a partial deficiency in OPA1 also increases the susceptibility to LVH and cardiac dysfunction, albeit whether resuming the levels of OPA1 could rescue this phenotype is unknown [206].

Anthracycline Cardiomyopathy

The aetiology of myocardial contractile dysfunction induced by anthracycline cardiotoxicity is unclear, although mitochondrial dysfunction and the production of ROS have been implicated. Marechal et al. [219] demonstrated the presence of mitochondrial fragmentation in hearts subjected to doxorubicin, and this change in mitochondrial morphology could be prevented by the MPTP inhibitor, CsA. The importance of mitochondrial fission to the pathophysiology of doxorubicin cardiomyopathy was investigated by Gharanei et al. [172], who found that treatment with the Drp1 inhibitor, *mdivi-1*, attenuated mitochondrial fragmentation, prevented the cardiac dysfunction and increase in MI size induced by doxorubicin in the isolated rat heart, indicating the therapeutic potential for inhibiting mitochondrial fission as a strategy for preventing doxorubicin cardiomyopathy. Crucially, *mdivi-1* was found to have no effect on survival of a cancer cell line.

Vascular Effects of Mitochondrial Fusion and Fission Proteins

Mitochondrial Fission Proteins

In native non-proliferative vascular smooth muscle cells (VSMC), the mitochondria are static and mainly ovoid in shape whereas during proliferation, the mitochondria become more mobile with varying shapes [220]. Angiotensin II stimulation of the cells activates Drp1 which will then interact with PKC- δ and subsequent MEK1/2–ERK1/2 signalling cascade and

MMP2 [221]. Hyperproliferation of VSMCs also constitutes a pathogenic mechanism in diabetic vascular complications, associated with increased ROS leading to excessive Drp1-mediated mitochondrial fission [222]. Inhibiting Drp1 via *mdivi-1* impaired the proliferative response [221, 222]. Similarly, the presence of a positive feedback of ROS/mROS-DRP1 contributes to the suppression of pulmonary arterial smooth muscle cells (PASMC) apoptosis in hypoxic pulmonary vascular remodeling, a phenomenon circumvented by the use of ROS inhibitors such as N-acetylcysteine and mitochondrial-derived ROS inhibitor TEMPO [223]. The mechanism underlying pulmonary arterial hypertension (PAH) lies in the hyperproliferation of the PASMC, during which mitochondria divide to ensure equal redistribution. Fragmentation of the mitochondria during hyperproliferation is due to an upregulation of Drp1 and downregulation of Mfn2, coupled with Cyclin B1/CDK1 phosphorylation of Drp1 at Ser-616. Reversal of PAH was achieved using the small molecule inhibitor of Drp1 – *mdivi-1* [60].

Mitochondrial Fusion Proteins

Originally known as a hyperplasia suppressor gene (HSG), Mfn2 functions to inhibit VSMC proliferation in different vasculo-proliferative conditions due to PKA-induced phosphorylation of Mfn2 at Ser442 [224, 225]. Over-expression of Mfn2 in an experimental animal model of angioplasty balloon-induced neointimal injury was found to inhibit VSMC proliferation, oxidized LDL and subsequent atheroma formation and carotid artery restenosis. Down-regulating Mfn2 conversely, enhanced VSMC proliferation, coupled with an increase in fatty acid oxidation and decrease in glucose oxidation. Conflicting data where down-regulation of Mfn2 in HeLa cells or T/G HA-VSMC cells suppressed proliferation has also been shown where interrupted autophagosome-lysosome fusion and impaired bioenergetics are the underlying factors [226].

During hypoxia-induced pulmonary hypertension in rats, downregulation of Mfn2 activates the PI3K/Akt pathway, thereby causing more cells to enter the S + G2/M phase of the cell cycle and inhibiting the mitochondrial apoptosis pathway [227]. These observations suggest that modifications in mitochondrial morphology and bioenergetics may underlie the hyperproliferative features of the VSMC, although the optimum balance of Mfn2 and associated mechanisms determine the endpoint of whether activation or suppression of proliferation occurs. In the settings of PAH, both Mfn2 and PGC-1 α have been found to be down-regulated in PASMCs. Over-expression of Mfn2 reversed the phenotype of PAH. Similarly, both Mfn2 and PGC-1 α were down-regulated in PASMC in two different experimental models of PAH, and in patients with PAH while Mfn2 reversed this phenotype. This seems to implicate Mfn2 as a novel therapeutic target for PAH [60], although whether preventing mitochondrial fission via the use of *mdivi-1* may be beneficial remain to be determined.

Other Cardiac Conditions

Intriguingly, a recent experimental study by Sharp et al. [228] has demonstrated that pharmacological inhibition of Drp1 using *mdivi-1* improved time to return of spontaneous circulation and hemodynamics, thereby resulting in improved survival and neurological outcomes, in a murine model of cardiac arrest. Another interesting example is the work by Sumida et al. [229] where they showed that mouse kidney subjected to acute IRI induced mitochondrial fragmentation in the heart, resulting in apoptosis and cardiac dysfunction, the effects of which could be reverse by pharmacological inhibition of Drp1 using *mdivi-1*.

Diabetes

In obesity and type 2 diabetes (DM), the loss of mitochondrial function in white adipose tissue is associated with a reduction in whole body insulin sensitivity. It has been demonstrated in the kidneys of streptozotocin-induced diabetic mice that there is an upregulation of translocase of inner mitochondrial membrane 44 (Timm44) [230]. Timm44 anchors mitochondrial heat-shock protein 70 to the translocase of inner mitochondrial membrane 23 complex and facilitates the import of mitochondria-targeted pre-proteins into the mitochondrial matrix. Over-expression of Timm44 in mice fed high-fat/high-sucrose chow protects from type 2 diabetes and obesity. Adipocyte size and pro-fission proteins were reduced while the pro-fusion proteins were induced when the mice were fed standard chow [230]. The discrepancy behind these varying levels of mitochondrial-shaping proteins in these mice when fed different diets remains unexplained although mitochondria ultimately became elongated.

Mitochondrial biogenesis remains unperturbed in the diabetic myocardium, in accordance with the absence of a difference in citrate synthase activities and PGC1- α protein expression [231]. Electron microscopy imaging of right atrial tissue sections also showed no difference in interfibrillar mitochondrial density between diabetic and nondiabetic patients [231]. However, a shift toward smaller cardiac mitochondria was observed in DM with a significantly lower mean mitochondrial length (1.14 μm) in diabetic versus (1.41 μm) in nondiabetic patients, due to a lower expression level of Mfn1 [231]. Interestingly, the content of myocardial MFN1 protein correlated negatively with HbA1C concentrations. Autophagy levels in the diabetic myocardium remained normal as well, albeit Atg5 was significantly decreased [231]. These observations established the association of mitochondrial dysfunction (mitochondrial fragmentation with decreased Mfn1) with decreased contractile performance in cardiac muscle of diabetic patients before the onset of clinical cardiomyopathy. Obesity, conversely, was not associated with any major perturbation of mitochondrial function [231], although a study conducted in

the same year using high-fat diet -induced obese mice showed the opposite [232]. A recent study also showcased the Drp1 monitoring of the mitochondrial network which is important for glucose-stimulated insulin secretion in pancreatic beta cells [233]. The optimum balance between fusion and fission may, thus, govern the proper function of the pancreatic beta cells and susceptibility to diabetes.

The presence of diabetes has been reported to render the heart resistant to cardioprotective strategies such as ischemic preconditioning and postconditioning. Yu et al. [234] found that hyperglycemia blocked the cardioprotective effect elicited of sevoflurane postconditioning by inducing mitochondrial fission in neonatal rat cardiomyocytes, an effect which was reversed using the Drp1 inhibitor, *mdivi-1*.

Therapeutic Targeting of Mitochondrial Fusion and Fission Proteins

A number of strategies have been investigated to modulate mitochondrial morphology including: genetic approaches such as micro-RNAs [235]; upregulation of heme oxygenase-1 (HO-1) to mediate mitochondrial quality control [236]; restoration of Connexin43 networks [237]; and even exercise to upregulate mitochondrial biogenesis factors [238]. Remote ischemic preconditioning (RIPC) has been shown to induce upregulation of OPA1 [214], and sevoflurane postconditioning has been reported to suppress the decline of OPA1 and increase in f Drp1 and Parkin induced by IRI [234].

Pharmacological interventions to target mitochondrial morphology have included the use of melatonin [239, 240], *Tribulus terrestris L.* fruit methanol extract [241], mitochondria-targeted molecules MitoQ and SS31 that have been shown to upregulate the pro-fusion proteins while down-regulating the pro-fission proteins as well as inducing the mitochondrial biogenesis genes PGC1 α , PGC1 β , Nrf1, Nrf2 and TFAM [242], coenzyme Q10 to rescue loss of Mfn2-associated respiratory impairment [243], resveratrol or SRT1720 to induce

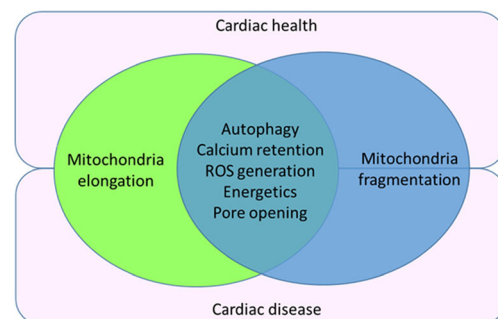


Fig. 3 Image showing the co-dependency of both mitochondrial fusion and fission in cardiac health and disease. Changes in mitochondrial morphology in the form of fusion and fission can affect autophagy, calcium signaling, ROS generation, mitochondrial energetics and MPTP opening, all of which can impact on cardiac health and disease

SIRT1 [213] or even metformin to inhibit ROS-associated mitochondrial fission by upregulating Drp1 phosphorylation (Ser 637) in an AMPK-dependent manner, and then suppressing ER stress [244]. The GLP-1 peptide-mimetic exenatide which has been used for the treatment of type 2 diabetes prevents neointimal layer formation in response to endothelial damage and atherosclerotic lesion formation in aortic tissue. Torres et al. have shown that GLP-1 induced a Ser-637 phosphorylation in the mitochondrial fission protein Drp1, and decreased Drp1 mitochondrial localization, thereby inhibiting VSMC migration and proliferation [245].

Drugs that have been documented to protect the heart by inhibiting the MPTP such as cyclosporine-A may well serve to modulate mitochondrial morphology, although this needs to be investigated. Similarly, although well-known mitochondrial morphology modulators such as *mdivi-1* have been shown to protect the heart against acute IRI, their potential off-target effects and efficacy in a large animal model and humans remains to be tested. The optimum time to modulate mitochondrial morphology during the course of cardiac dysfunction progression to achieve a beneficial effect is not easy to determine. The drug delivery vehicle which may affect the potency of the drug is another crucial factor to be taken into account. In addition, one should realize that the hope of conferring cardioprotection by simply modulating mitochondrial morphology towards a defined phenotype may not be beneficial in the long-term given that it will interfere with mitochondrial homeostasis by disturbing the balance of mitochondrial fusion and fission (see Fig. 3) [246].

Conclusions

Emerging studies suggest that changes in mitochondrial morphology occur in different cardiac disease and pharmacological targeting of the mitochondrial fusion and fission proteins may provide a novel therapeutic strategy for treating cardiac diseases such as acute myocardial infarction, LVH, PAH, and heart failure. However, it is important to take into consideration the pleiotropic roles of the mitochondrial shaping proteins especially the fusion proteins, Mfn2 and OPA1, when targeting the mitochondrial shaping proteins as a treatment for cardiac disease. Furthermore, although acute inhibition or activation of the shaping proteins may be beneficial in certain cardiac diseases, the chronic inhibition or activation of either mitochondrial fusion or fission proteins may have detrimental off-target effects.

Compliance with Ethical Standards

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Conflict of Interest All authors declare no conflict of interest.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

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