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REVIEW

Mitochondrial fusion and fission proteins: novel therapeutic targets for combating cardiovascular disease

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Mitochondria are no longer considered to be solely the static powerhouses of the cell. While they are undoubtedly essential to sustaining life and meeting the energy requirements of the cell through oxidative phosphorylation, they are now regarded as highly dynamic organelles with multiple functions, playing key roles in cell survival and death. In this review, we discuss the emerging role of mitochondrial fusion and fission proteins, as novel therapeutic targets for treating a wide range of cardiovascular diseases.

LINKED ARTICLES
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Abbreviations
Cdk1, cyclin-dependent kinase 1; Drp1, dynamin-related peptide; ER, endoplasmic reticulum; ESC, embryonic stem cell; Fis1, human fission protein 1; IMM, inner mitochondrial membrane; IRI, ischaemic reperfusion injury; L-OPA1, long form of optic atrophy factor 1; LV, left ventricular; LVH, left ventricular hypertrophy; MAM, mitochondria-associated membranes; MFF, mitochondrial fission factor; MFN1, mitofusin 1; MFN2, mitofusin 2; MI, myocardial infarction; MiD49/MiD51, mitochondrial dynamics proteins of 49 kDa and 51 kDa; MOMP, mitochondrial outer membrane permeabilization; MPTP, mitochondrial permeability pore; OMM, outer mitochondrial membrane; OPA1, optic atrophy factor 1; PAH, pulmonary arterial hypertension; PARL, presenilins-associated rhomboid-like protein; PGC-1α, peroxisome proliferator-activated receptor γ coactivator 1-α; PINK1, PTEN-induced putative kinase 1; RIP, receptor-interacting serine-threonine kinases; S-OPA1, short/cleaved form of optic atrophy factor 1; VSMC, vascular smooth muscle cells

Introduction

Mitochondria are essential for eukaryotic life, meeting the cells' energetic requirements through oxidative phosphorylation. This is especially true in the heart, where the mitochondria occupy around 30% of the total cell volume and produce a staggering 30 kg of ATP per day, in order to sustain normal contractile function of the heart. While their primary role is to generate ATP, mitochondria also play a number of important roles which affect cell survival and death (Pellegrini and Scorrano, 2007).

These important secondary roles mean that mitochondrial biology is a rejuvenated field, with mitochondrial dysfunction at the root of numerous diseases. In particular, the field of mitochondrial dynamics, in which mitochondria are dynamic organelles, and are able to change their morphology by undergoing fusion and fission, has recently been investigated and shown to be relevant to the cardiovascular system

(Ong *et al.*, 2013). This review investigates the role of the mitochondrial fusion and fission proteins as novel therapeutic targets for combating cardiovascular disease.

Changing mitochondrial morphology by fusion and fission

Proteins involved in shaping mitochondria are grouped as either mitochondrial fusion proteins (joining individual mitochondria together to become one), mitochondrial fission proteins (dividing one mitochondrion into two mitochondria) or mitochondrial distribution proteins (those which influence mitochondrial morphology through movement of mitochondria around the cell such as Miro and Milton) (see Figure 1 for summary).

Mitochondrial fusion

The fusion proteins are the outer mitochondrial membrane (OMM) proteins, mitofusin 1 (MFN1) and mitofusin 2 (MFN2), and the inner mitochondrial membrane (IMM) protein, optic atrophy factor 1 (OPA1) (as shown in Figure 1). These proteins hydrolyze GTP to fuse two neighbouring mitochondria together to allow sharing of mitochondrial DNA, proteins and metabolites. MFN1 and MFN2 mediate OMM fusion through the formation of either heterodimers [i.e. MFN1 binding to MFN2, and the most efficient method of fusion (Hoppins *et al.*, 2011)] or homodimers (MFN2 binding to MFN2) (Chen *et al.*, 2003; Koshiba *et al.*, 2004). Fusion of IMM is mediated by OPA1 (Malka *et al.*, 2005; Song *et al.*, 2009), which itself is regulated by nuclease cleavage at the mRNA level, and proteolytic cleavage at the protein level (Song *et al.*, 2007). In the absence of OPA1, MFN1 and MFN2

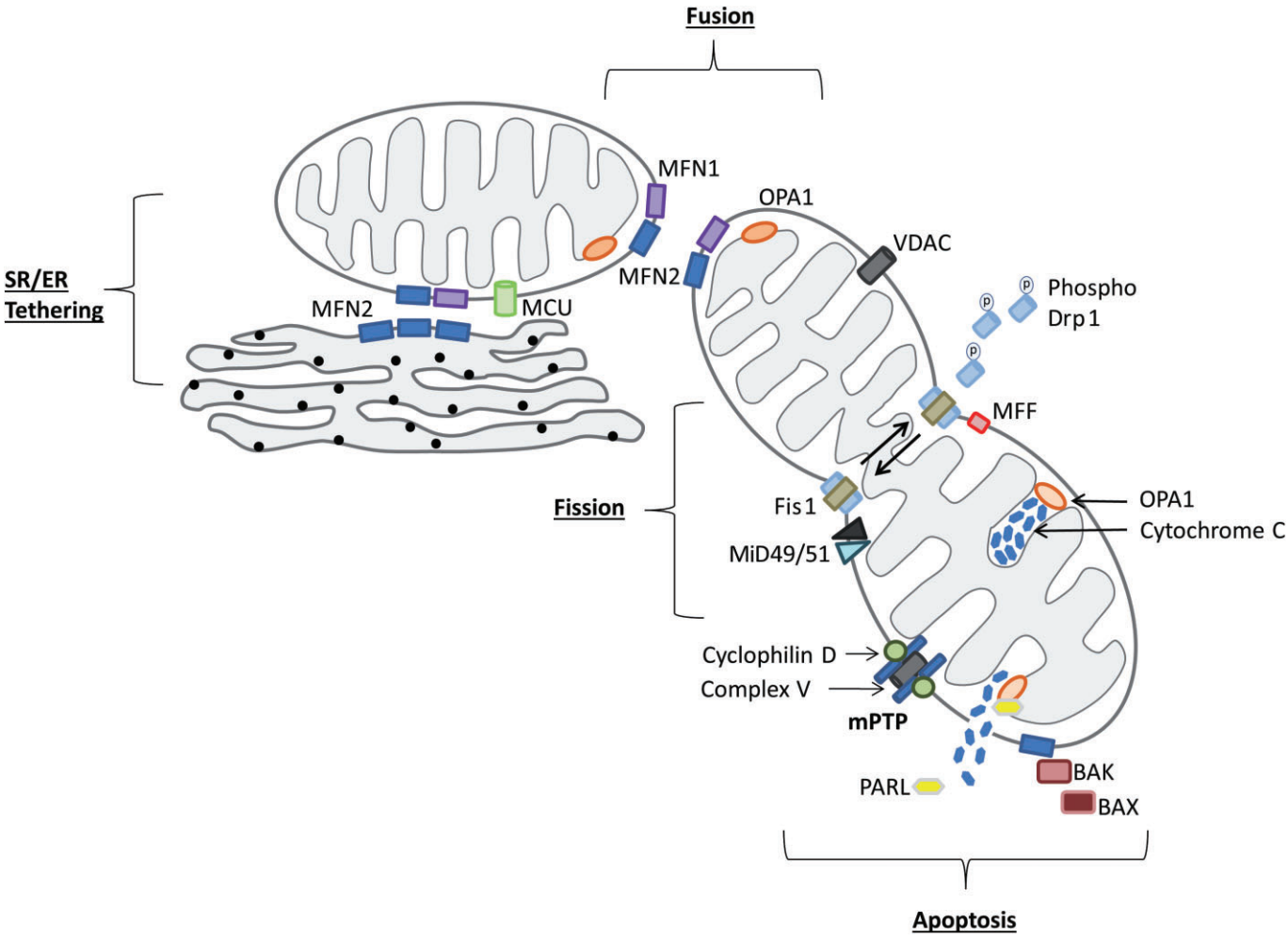

Figure 1

Mitochondrial fusion and fission proteins and their roles in changing mitochondrial morphology. MFN1, MFN2 and OPA1 are all key to mediating mitochondrial fusion, while Drp1 interacts with Fis1, MFF and MiD49/51 to mediate mitochondrial fission. Secondary to these mitochondrial-shaping roles, MFN2 tethers the SR/ER to the mitochondria allowing efficient Ca^{2+} transfer between the organelles. The mitochondria are also key to apoptosis with BAK and BAX binding to MFN2 stabilizing Drp1 binding. Drp1 binding leads to mitochondrial fragmentation and the release of cytochrome *c*. Cytochrome *c* is normally sequestered by OPA1 in the cristae, although the OPA1 oligomers maintaining cristae shape are disrupted by BAK and BAX binding.

mediate only superficial mitochondrial fusion with the IMMs remaining unfused. This lack of IMM fusion and matrix content mixing result in loss of mitochondrial heterogeneity and the occurrence of metabolic disturbances, confirming that a key role of mitochondrial fusion is the mixing of matrix contents (mitochondrial DNA, metabolites and proteins) (Chen *et al.*, 2005; Song *et al.*, 2009). Whole body genetic ablation of MFN1 and MFN2 results in embryonic lethality due to a major placental defect (Chen *et al.*, 2003). Mutations in human MFN2 are responsible for the autosomal dominant neurodegenerative disease Charcot-Marie-Tooth type 2A, a peripheral sensorimotor neuropathy (Zuchner *et al.*, 2004). Mutations in the OPA1 gene were first identified in 2000 and found to be associated with the human neurogenerative condition, autosomal dominant optic atrophy (Delettre *et al.*, 2000). How a deficiency in the mitochondrial fusion protein MFN2 or OPA1 results in such a specific neurological condition is currently unknown.

Mitochondrial fission

Mitochondrial fission describes the fragmentation or division of one mitochondrion into two or more mitochondria. Its main functions include: (i) increasing mitochondrial numbers to ensure mitochondria are passed on to daughter cells during mitosis (Taguchi *et al.*, 2007); (ii) enabling shuttling of mitochondria to other regions of the cell; (iii) signalling to the cell that it is damaged and needs to be removed by mitophagy thereby maintaining a healthy mitochondrial network (Lee *et al.*, 2011); and (iv) the initiation of apoptotic cell death (Lee *et al.*, 2004).

The process of mitochondrial fission occurs through two coupled mechanisms. The first is the inhibition of the mitochondrial fusion proteins, and the second is the recruitment of the mitochondrial fission protein (see Figure 1), dynamin-related protein 1 (Drp1), from the cytosol (where it is primarily located) to the OMM where it mediates mitochondrial scission, through its interaction with other mitochondrial fission proteins: human fission factor-1 (Fis1), mitochondrial fission factor (MFF), and mitochondrial dynamics proteins of 49 and 51 kDa (MiD49 and MiD51, respectively) (Yoon *et al.*, 2003; Gandre-Babbe and van der Bliek, 2008; Otera *et al.*, 2010; Palmer *et al.*, 2011; Loson *et al.*, 2013). The pro-apoptotic protein PUMA (a BH3 member of the Bcl-2 protein family) has been reported to be critical for the recruitment of Drp1 to the mitochondria (Wang *et al.*, 2009; Din *et al.*, 2013). PUMA is reported to either displace BAX and Bad from their cytosolic sequestering proteins (Ren *et al.*, 2010) which in turn causes Drp1 to translocate to the mitochondria (Din *et al.*, 2013), or actually be present at the mitochondria where it aids Drp1 binding and assembly to mediate the scission events (Wang *et al.*, 2009). Once Drp1 has bound to the OMM on one of its numerous receptors, it oligomerizes into a helical structure encircling the mitochondrion, and upon GTP hydrolysis, it constricts the mitochondrial membrane, until the lipid bilayers are sufficiently destabilized to break apart from each other (Mears *et al.*, 2011). Drp1 then reverts back to its monomeric structure and moves back to the cytosol, leaving two mitochondria behind.

The dominance and specific roles of MFF, MiD49/51 and Fis1 in Drp1-mediated fragmentation are controversial (for recent reviews see Elgass *et al.*, 2013; Otera *et al.*, 2013). It is reported that MFF can recruit Drp1 independently of Fis1; while MiD49 and MiD51 can act independently of both MFF and Fis1 (Loson *et al.*, 2013). The MiD proteins are reported to be Drp1 receptors on the mitochondrial membrane (Palmer *et al.*, 2011), while MFF is reported to be a key protein in the recruitment of other proteins (potentially more Drp or other, as yet unidentified proteins) (Gandre-Babbe and van der Bliek, 2008) or a *bone fide* Drp1 receptor (Otera *et al.*, 2010). With the oligermization of Drp1 less efficient without Fis1, it has a potential role as an assembly chaperone protein (Otera *et al.*, 2010; Loson *et al.*, 2013), although this role is distinct from MFF (Gandre-Babbe and van der Bliek, 2008). Clarification of the roles of MiD49/51, MFF and Fis1, the pathways (mitosis or apoptosis) in which Drp1 binds specifically to each protein could yield potential therapeutic targets.

Recently, it has been reported that the interaction of mitochondria with the endoplasmic reticulum (ER) (Friedman *et al.*, 2011), inverted formin 2 (a formin that accelerates both actin polymerization and depolymerization) and the actin component of the cytoskeleton (De Vos *et al.*, 2005; Korobova *et al.*, 2013), may mark out sites for Drp1-mediated mitochondrial fission. The proposed paradigm suggests that the ER encircles mitochondria at sites of fission, and ER-associated inverted formin 2 then stimulates actin polymerization, providing the force required for partial constriction of the mitochondria, thereby facilitating the translocation of Drp1 to these pre-constriction contact sites in the OMM. The actual mechanism through which Drp1 localizes to these pre-constricted ER-contact sites on the OMM, and the roles MFF and MiD49/51 play in this process remains to be determined.

Regulation of the mitochondrial fission and fusion proteins

Recent studies have begun to describe the mechanisms that regulate the function of the mitochondrial fission and fusion proteins, providing insight into how mitochondrial dynamics may integrate into cell death and survival pathways.

Regulation of Drp1. Drp1 recruitment from the cytosol to the OMM is the major factor governing mitochondrial fission. As such, mitochondrial fission is regulated by the post-translational modification of Drp1, which alters both its localization and affinity for oligomerization. A number of post-translational modifications have been reported to occur on Drp1 (see Table 1) including sumoylation (Figueroa-Romero *et al.*, 2009), phosphorylation (see below), ubiquitination (Nakamura *et al.*, 2006), S-nitrosylation (Cho *et al.*, 2009), O-GlcNAcylation (Gawlowski *et al.*, 2012) and so on (reviewed in Otera *et al.*, 2013). The most extensively reported Drp1 post-translational modification is phosphorylation, which occurs at Ser^{616} and Ser^{637} (in reference to the sequence of the human Drp1). Phosphorylation on Ser^{616} by cyclin-dependent kinase 1 (Cdk1)/cyclin B, a key mitotic kinase, induces Drp1 translocation to the mitochondria, where mitochondrial fragmentation ensures an equal division of the mitochondrial network between the daughter cells (Taguchi *et al.*, 2007; Marsboom *et al.*, 2012). Due to the slow mitotic turnover of adult cardiac myocytes, this form of Drp1 regulation may occur on an infrequent basis in the

Table 1

Post-translational modification of Drp1 by phosphorylation

Reference	Cell-type	Regulator	Drp1 phosphorylation site	Effect
Taguchi *et al.*, 2007	HeLa	Cyclin-dependent kinase (Cdk1)	Ser^{585} with no effect of GTPase activity (Ser^{585} is the rat Drp1 splice variant 1 of Ser^{616})	Cdk1 phosphorylates Drp1 and activates it during cell division
Chang and Blackstone, 2007	HeLa	PKA	Ser^{637} in the GTPase effector domain	PKA phosphorylates Drp1 and inhibits GTPase activity
Cribbs and Strack, 2007	PC12	PKA Calcineurin Calcium	Ser^{656} with no effect of GTPase activity (Ser^{656} is the rat Drp1 splice variant 1 of Ser^{637})	PKA phosphorylates Drp1 and inhibits GTPase activity preventing apoptosis Calcineurin and calcium dephosphorylates Drp1 and activates it enhancing apoptosis
Cereghetti *et al.*, 2008		Calcineurin Calcium	Ser^{637}	Calcineurin and calcium dephosphorylates Drp1 and activates it enhancing apoptosis
Han *et al.*, 2008	HeLa neurones	Ca^{2+}/calmodulin-dependent PKI 1 (CaMKIα)	Ser^{600} (Ser^{600} is the Drp1 splice variant of Ser^{637})	CaMKIα phosphorylates Drp1 and activates it
Marsboom *et al.*, 2012	Pulmonary arterial smooth muscle cells	Cdk1	Ser^{616}	Cdk1 phosphorylates Drp1 and activates it during cell division
Din *et al.*, 2013	Neonatal cardiomyocytes	Pim-1	Ser^{637}	Pim-1 phosphorylates Drp1 and inhibits fission

adult heart. Phosphorylation at the other site, Ser^{637}, is mediated by PKA, Cam kinase and Pim1. Phosphorylation at Ser^{637} inhibits Drp1 oligomerization, suppresses mitochondrial fission and prevents cell death (Table 1). Indeed, phosphorylation by Pim1 is reported to play a key role in the sequestering of Drp1 in the cytosol (Din *et al.*, 2013).

Conversely, dephosphorylation by calcineurin induces mitochondrial fragmentation through Drp1 recruitment to the mitochondria (Cribbs and Strack, 2007; Chang and Blackstone, 2007). Dephosphorylation of this site is reported to initiate the transition towards apoptosis, with high Ca^{2+} concentrations, ATP depletion and the loss of mitochondrial membrane potential, all initiators of the calcineurin-dependent mitochondrial fission pathway (Cribbs and Strack, 2007; Estaquier and Arnoult, 2007; Cereghetti *et al.*, 2008; Sandebring *et al.*, 2009; Cho *et al.*, 2010). In neonatal cardiomyocytes, it has recently been demonstrated that high glucose and the presence of diabetes induces mitochondrial fragmentation through the O-GlcNAcylation of OPA1 (Makino *et al.*, 2011) and Drp1 (Gawlowski *et al.*, 2012), resulting in the dephosphorylation of Ser^{637} and its translocation to the OMM.

Regulation of mitofusins. A prerequisite for mitochondrial fusion is a mitochondrial membrane potential (Legros *et al.*, 2002), ensuring the mixing of healthy mitochondrial proteins and mtDNA. MFN1 has been reported to have higher GTPase activity than MFN2 (Ishihara *et al.*, 2004), and this activity appears to be regulated by guanine nucleotide binding protein-β subunit 2 (Zhang *et al.*, 2010). The binding of guanine nucleotide binding protein-β subunit 2 to MFN1 appears to decrease its mobility, allowing clustering of MFN1 to occur in specific foci and increasing mitochondrial fusion (Zhang *et al.*, 2010).

MFN2 regulation has been reported to be mainly dependent on protein expression, rather than any protein modifications that alter GTPase activity or recruit MFN2 to the mitochondria. Under conditions of enhanced metabolic demand, peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α) and PGC-1β have been shown to increase MFN2 expression (Liesa *et al.*, 2008; Zorzano, 2009), linking mitochondrial fusion to changes in mitochondrial biogenesis.

Conversely, both MFN1 and MFN2 are reported to be regulated by ubiquitination, which immediately inhibits their activity, as well as providing the signal for these proteins to be degraded (Gegg *et al.*, 2010). This modification inhibits mitochondrial fusion and is key to the process of removing damaged mitochondria by mitophagy (Gegg *et al.*, 2010; Tanaka *et al.*, 2010). The loss of the mitochondrial membrane potential, which indicates a loss of mitochondrial function, recruits the kinases PTEN-induced putative kinase 1 (PINK1) or JNK to the OMM (Leboucher *et al.*, 2012). The phosphorylation of MFN2 on Thr^{111} and Ser^{442} by PINK1 then stabilizes the binding of Parkin, which ubiquitinates MFN2 (Chen and Dorn, 2013). The effect of the ubiquitination is twofold; a direct and immediate inhibition of MFN2 activity, thus preventing the damaged mitochondria fusing with the healthy mitochondrial population, and the recruitment of p62 (sequestosome 1) to mediate mitochondrial autophagy (Gegg

et al., 2010; Tanaka *et al.*, 2010; Glauser *et al.*, 2011; Chen and Dorn, 2013). This modification and inhibition is not just limited to MFN2, as Parkin-mediated ubiquitination of MFN1 has also been reported (Gegg *et al.*, 2010; Glauser *et al.*, 2011). Thus the MFN proteins are regulated by their presence on the OMM, allowing mitochondrial fusion to occur when in contact with other mitochondria. As such, mitochondrial fusion must also be regulated through the proteins that manoeuvre the mitochondria around the cell, such as Miro and Milton (Koutsopoulos *et al.*, 2010). Interestingly, the activity of both Miro and Milton has been reported to be regulated through their binding to MFN2 (Misko *et al.*, 2010), demonstrating that mitochondrial fusion can also be controlled by the presence of these proteins in the OMM.

Regulation of OPA1. The regulation of OPA1 is complex and is dependent upon cleavage at both the mRNA transcript and protein levels (Delettre *et al.*, 2001; Ishihara *et al.*, 2006; Griparic *et al.*, 2007; Ehses *et al.*, 2009; Song *et al.*, 2009). There are reported to be eight transcript variants of the OPA1 gene generated through the differential splicing of exon 4 (Delettre *et al.*, 2001). Furthermore, each OPA1 isoform generated from these transcripts has two cleavage sites; S1 which is located on the N-terminus and cleaved by the mitochondrial processing peptidase to generate long form of optic atrophy factor 1 (L-OPA1), and S2 which is targeted by Yme1, presenilins-associated rhomboid-like protein (PARL), OMA1 and paraplegin to generate S-OPA1 (short OPA1) (Cipolat *et al.*, 2006; Griparic *et al.*, 2007; Song *et al.*, 2007; Quiros *et al.*, 2012). The sum of these processes results in five distinct OPA1 bands on electrophesis gels ranging from 100 to 85 kDa (Griparic *et al.*, 2007; Song *et al.*, 2007).

Yme1, PARL and paraplegin are all proteases reported to cleave OPA1 at S2 to create OPA1 short forms (Cipolat *et al.*, 2006; Ishihara *et al.*, 2006; Song *et al.*, 2007). The cleavage of OPA1 is classified as either constitutive or inducible. Constitutive cleavage refers to OPA1 cleavage to form a range of fusion competent OPA1 isoforms, and is mediated by PARL and OMA1 (Cipolat *et al.*, 2006; Frezza *et al.*, 2006; Sanjuan Szklarz and Scorrano, 2012). PARL is reported to be sensitive to the phosphorylation status of OPA1, implicating another layer of regulation (Pellegrini and Scorrano, 2007), while Yme1 is only able to cleave certain splice variants of OPA1, with some being totally insensitive to Yme1 cleavage (Griparic *et al.*, 2007). Inducible cleavage occurs in response to apoptotic stimuli to generate S-OPA1 isoforms, which have no fusion abilities, and is reported to also be mediated by OMA1 (Head *et al.*, 2009). Genetic knockout of OMA1 results in mice that are unable to adapt their metabolism to utilize the appropriate substrates, revealing the importance of OMA1-mediated cleavage (Quiros *et al.*, 2012). Formation of L-OPA1 is totally reliant upon *de novo* synthesis, with S-OPA1 unable to convert back to L-OPA1 (Griparic *et al.*, 2007).

The reason for such regulation of OPA1 splice lengths is that each OPA1 variant has a different solubility, which governs their localization within the inner mitochondrial membrane and their ability to mediate mitochondrial fusion of the IMMs. Controversy exists as to whether just the long forms of OPA1 are fusion competent (Ishihara *et al.*, 2006), or whether a mixture of isoforms varying in length is required (Song *et al.*, 2007). The long forms of OPA1 are reported to be stabilized by Higd1-a, with knockdown of Higd1-a resulting in excessive processing of OPA1 into the short form and collapse of cristae, fragmentation of the mitochondrial network, the loss of mitochondrial DNA stability and a retardation of cellular growth (An *et al.*, 2013). In response to the loss of mitochondrial membrane potential, OPA1 is rapidly cleaved at the protein level into shorter forms (by a protease other than Yme1) and is classified as inducible OPA1 cleavage (Griparic *et al.*, 2007). The loss of L-OPA1 reduces the ability of the mitochondrion to fuse, essentially signalling the mitochondria for autophagy (Duvezin-Caubet *et al.*, 2006).

Such debate as to the processing of OPA1 arises because of the large number of splice variants (at both mRNA and protein level), cleavage being mediated by several different proteases, and cleavage occurring in response to both pro-fusion and pro-apoptotic pathways. Indeed, the L-OPA1, are likely to be required to mediate IMM fusion; while OPA1 processing (by paraplegin or OMA1) to create shorter, less active forms of OPA also occurs to inhibit fusion (Duvezin-Caubet *et al.*, 2006; Ishihara *et al.*, 2006; Ehses *et al.*, 2009). The inhibition of OPA1 immediately following the loss of a mitochondrial membrane potential, serves as a block of mitochondrial fusion, preventing the damage spreading to healthy mitochondria. As such, the regulation of OPA1 activity is the key to a mitochondrion's ability to fuse and remain part of the healthy network.

Mitochondrial fusion and fission proteins and cell death

The mitochondrial fusion and fission proteins have been shown to play critical roles in a number of different cell death pathways, with the mitochondria as a point of convergence for many of them (see Figure 1). However, their interplay with components of the cell death pathway can be quite complex and continues to be elucidated.

Apoptosis. Mitochondria play a major role in the initiation of the intrinsic pathway of apoptotic cell death and, in this respect, are controlled by a subclass of the Bcl-2 protein family. This subclass is further categorized into anti-apoptotic and pro-apoptotic proteins, such as BAX and BAK. In healthy cells, BAX is predominantly cytosolic, until pro-apoptotic stimuli cause BAX translocation to the mitochondria to mediate mitochondrial outer membrane permeabilization (MOMP), releasing cytochrome *c* along with other pro-apoptotic factors into the cytosol. Mitochondria are known to undergo fragmentation on induction of apoptosis, which has been suggested to facilitate MOMP. However, the interplay between the mitochondrial fusion and fission proteins, the members of the Bcl-2 protein family and MOMP is quite complex and is incompletely understood (Youle and Strasser, 2008).

Interestingly, both BAX and BAK are reported to colocalize with MFN2 at the outer mitochondrial membrane (Karbowski *et al.*, 2002; Neuspiel *et al.*, 2005). Indeed, BAX-mitochondrial binding, apoptosis and cytochrome *c* release are all inhibited by the expression of constitutively active MFN2 (Neuspiel *et al.*, 2005). Therefore, during apoptosis, BAX could bind and inhibit MFN2, preventing any further mitochondrial fusion and prepare the mitochondria for

fragmentation. If one proposes MFN2 as the BAX receptor, this may also explain why MFN2 knockouts are more resistant to apoptosis. With the BAX and BAK binding sites no longer present, binding of these pro-apoptotic proteins at the mitochondria is likely to be reduced.

MFN2 has also been proposed to decrease mitochondrial membrane stability, encouraging pores to form in the OMM and facilitate the opening of the mitochondrial permeability pore (MPTP) (Papanicolaou *et al.*, 2012c). Potentially, the presence of MFN2 may reduce the stability of the mitochondrial membrane, making it easier for Drp1 to mediate mitochondrial fragmentation and stimulate cytochrome *c* release. Finally, the role of non-oligomerized or monomeric BAX and BAK in mitochondrial fusion has recently been evaluated in the context of MPTP opening and necrotic cell death (Whelan *et al.*, 2012). This study confirmed that BAX/BAK double-knockout cells contain fragmented mitochondria and the double-knockout cells were resistant to MPTP opening and necrotic cell death. MFN2-deficient cells, tested in the same study (Whelan *et al.*, 2012), also exhibited resistance against MPTP opening, supporting the observation that MFN2 promotes this process. Furthermore, monomeric BAX was demonstrated to be responsible for the observed sensitivity to MPTP. This may be in agreement with the notion that the localization of BAX to the OMM lowers the threshold for forming hemifusion-related holes, and if there is sufficient stress overload in the matrix/IMM, this hole can be employed in the precipitous exchange of ions during MPTP opening.

Drp1. Drp1 is also reported to colocalize with BAX at the OMM and mediate mitochondrial fragmentation in response to apoptotic stimuli. While Drp1 mutants failed to prevent BAX-mitochondrial binding, there were no subsequent changes in mitochondrial morphology, indicating that BAX recruits Drp1 rather than *vice versa* (Karbowski *et al.*, 2002). The recruitment of Drp1 to the mitochondria has been reported to be independent of Fis1, with Drp1 binding to the mitochondria stimulated by BAX and stabilized by ubquitination by the small ubiquitin-like modifier (Wasiak *et al.*, 2007). Once at the OMM, Drp1 mediates mitochondrial fragmentation, the loss of the membrane potential and facilitates the release of cytochrome *c*. These studies implicate Drp1 as a key player in apoptosis, with Drp1 inhibition reported to slow, rather than fully inhibit apoptosis (Frank *et al.*, 2001; Estaquier and Arnoult, 2007). This may be due to the release of other pro-apoptotic proteins (other than cytochrome *c*) from the mitochondria when Drp1 is inhibited. However, there is growing evidence that apoptosis is able to proceed independently of Drp1 (Parone *et al.*, 2006; Wakabayashi *et al.*, 2009) despite the fact that mitochondrial fission mediated by Drp1 is considered to be a key hallmark of apoptosis (Martinou and Youle, 2006). As such, whether therapeutic inhibition of Drp1 would be effective in preventing apoptosis is still controversial.

OPA1. OPA1 is also regarded as an anti-apoptotic protein, with its overexpression reducing cell death and its knockdown increasing cell susceptibility to apoptosis (Olichon *et al.*, 2003; Lee *et al.*, 2004; Estaquier and Arnoult, 2007; Song *et al.*, 2007). Localized in the IMM, OPA1 maintains IMM integrity and is able to sequester cytochrome *c* within the cristae (Frezza *et al.*, 2006). While both long insoluble and short soluble forms of OPA1 are found at the cristae junctions, it is the short form that is reported to be anti-apoptotic. Cleavage by PARL increases the amount of shorter soluble OPA1, which is able to form oligomers and mediate cristae remodelling (Cipolat *et al.*, 2006; Frezza *et al.*, 2006; Sanjuan Szklarz and Scorrano, 2012). Such remodelling is reported to keep the cristae junctions tight, sequestering the cytochrome *c* within the cristae thereby inhibiting apoptosis. To mediate cytochrome *c* release, OPA1 oligomerization is disrupted by Bid, which opens up the cristae releasing cytochrome *c* into the intermembranous space (Frezza *et al.*, 2006). In this regard, BAX- and BAK-mediated fission can also release cytochrome *c* into the cytoplasm leading to caspase activation (Scorrano *et al.*, 2002). Furthermore, OPA1 cleavage is responsible for the protective response to heat shock, with PARL knockout mice being more susceptible to apoptosis. As such, this pathway is physiologically relevant (Cipolat *et al.*, 2006).

Mitophagy. Mitophagy is required to remove damaged mitochondria and maintain a healthy mitochondrial network for normal cell function. The role of MFN2 in cellular autophagy has recently been explored in the adult murine heart by Zhao *et al.* (2012). These authors found that the cardiac-specific deletion of MFN2 in the adult heart resulted in the accumulation of autophagosomes, which was mostly shown to be due to impaired fusion of autophagosomes with lysosomes (Zhao *et al.*, 2012). In this particular study, the role of MFN2 in mediating mitophagy was not clear, although starvation was found to increase its association with Ras-related protein, an autophagosome maturation-related protein (Zhao *et al.*, 2012).

The current paradigm suggests that mitochondrial membrane depolarization of damaged mitochondria signals PINK1 translocation to the OMM where it mediates Parkin recruitment to the damaged mitochondria and induces their mitophagic removal by ubiquitination (Karbowski *et al.*, 2002). Chen and Dorn (2013) have provided experimental data suggesting that in damaged mitochondria, PINK1 phosphorylates and activates MFN2 so the it can act as a receptor for Parkin on the OMM and mediate autophagy. These findings were shown to be consistent with the observation that hearts deficient in MFN2 displayed impaired mitochondrial respiration and went on to develop a dilated cardiomyopathy (Chen and Dorn, 2013).

In summary, these two experimental studies suggest another non-fusion pleiotropic role for MFN2 as a critical mediator of cellular autophagy and mitophagy. Given the importance of these processes in the healthy and diseased cardiovascular system, the therapeutic potential for manipulating these processes may be mediated via MFN2.

MPTP. The opening of the MPTP is a critical determinant of cell death. Therefore, preventing or delaying its opening increases cell survival. This is of particular importance in the setting of acute ischaemic reperfusion injury (IRI), where MPTP opening at the point of reperfusion mediates cardiomyocyte death, and inhibiting MPTP opening at this time prevents cell death (Hausenloy *et al.*, 2003; Hausenloy and Yellon, 2003). The mitochondrial fusion and fission proteins have been reported to influence MPTP opening susceptibility

and, therefore, affect cell viability. In the HL-1 cardiac cell line, Ong *et al*. (2010) found that inhibiting mitochondrial fission during acute IRI could prevent MPTP opening. Similarly, MFN2 ablation in neonatal cardiomyocytes was also found to increase MPTP opening susceptibility (Papanicolaou *et al*., 2011). However, the results of manipulating the mitochondrial fusion proteins in the adult heart have had opposing effects on MPTP opening susceptibility. Genetic ablation of MFN1, MFN2 or OPA1 has been reported in the adult heart to reduce MPTP opening susceptibility, the mechanism for which is unclear. It has been suggested that the mitofusins may act as indirect mediators of MPTP formation (Papanicolaou *et al*., 2012c). During the process of mitochondrial fusion, membrane destabilization occurs. As well as leading to a leak of solutes from the mitochondria, the destabilization of the membrane may make it easier for MPTP formation to occur. Conversely, in the MFN1 and MFN2 knockout mice, the lack of mitochondrial fusion stabilizes the mitochondrial membranes, thus reducing the ability of the pore proteins to break the membrane, and form the MPTP (Papanicolaou *et al*., 2012c). OPA1 down-regulation may inhibit pore formation through a similar stabilization of the inner mitochondrial membrane, or the disturbances in cristae formation may alter the way Ca^{2+} is handled within the mitochondria (Piquereau *et al*., 2012). Whether the mitofusin proteins or OPA1 play a direct role in MPTP formation remains to be determined, although how these proteins would interact with the ATP synthase, a recently proposed component of the MPTP is unclear (Giorgio *et al*., 2013).

Programmed cell necrosis. The current paradigm has suggested that cell death by necrosis is an unregulated process. However, a pathway of programmed cell necrosis has recently been described, in which the cytokine TNF-α activates the receptor-interacting serine-threonine kinases (RIP), RIP1 and RIP3, and their interaction with the mixed lineage kinase domain like protein resulting in ROS generation, calcium overload and the opening of the MPTP (Vanlangenakker *et al*., 2008). RIP3 forms a complex mitochondrial protein phosphatase PGAM5, which recruits Drp1 to the OMM by dephosphorylating its Ser^{637} site causing mitochondrial fragmentation (Wang *et al*., 2012). These findings implicate a role for Drp1 in mediating programmed cell death necrosis. Importantly, and relevant to the heart, pharmacological inhibition of this pathway, using Necrostatin-1 to target RIP1, has been reported to limit myocardial infarction (MI) size and prevent post-left ventricular (LV) remodelling in animal models of acute IRI (Lim *et al*., 2007; Oerlemans *et al*., 2012).

Tethering to ER or sarcoplasmic reticulum. Tethering between the ER and mitochondria was initially observed in the 1970s using electron microscopy (Morre *et al*., 1971). Subsequent studies have gone on to show that this interaction is physiologically important, and they have identified some of the key proteins which mediate this interaction including the voltage-dependant anion channel (Szabadkai *et al*., 2006), Tespa1 (Matsuzaki *et al*., 2013), Trichoplein/mitostatin (Cerqua *et al*., 2010) and, most recently MFN2 (de Brito and Scorrano, 2008), although the latter has not been universally reported (Cosson *et al*., 2012). The contact region between the ER and mitochondria is known as the mitochondrial associated membrane (MAM) and contains proteins and phospholipids which facilitate inter-organelle signalling, energy regulation and apoptosis all of which occur at the ER-mitochondria interface (Vance, 1990).

Scorrano's group (de Brito and Scorrano, 2008) first proposed the key role for MFN2 in tethering these organelles together. Present at both the mitochondrial and ER membranes, MFN2 is enriched within the MAM region (see Figure 1). As such, MFN2 present on the ER membrane is able to interact with either MFN1 or MFN2 on the mitochondrial membrane, and tether the organelles together. Loss of MFN2 diminishes this interaction between the mitochondria and ER, increasing the distance between the two organelles. The interaction mediated by MFN2 is physiologically important for IP3 signalling, as well as preventing ER stress. Indeed, Ngoh *et al*. (2012) showed that MFN2 expression increases under ER stress, and without this adaptive tethering, apoptosis follows.

MFN2-mediated ER-mitochondrial tethering is regulated by MITOL (ubiquitin ligase) (Sugiura *et al*., 2013). Interestingly, MITOL only ubiquitinates mitochondrial MFN2 on lysine residues 63 and 192, while ER MFN2 remains unmodified. Rather than signalling MFN2 for degradation, ubquitination increases the affinity of MFN2 for GTP, stimulating MFN2 activity. Through this mechanism, MFN2 undergoes GTP-dependent oligomerization and facilitates the formation of mitochondrial ER tethering domains (Sugiura *et al*., 2013).

The dynamic nature of cardiac contractility is mirrored by alterations in energy demand, which must be met by mitochondrial respiration. Key to this supply-demand coupling is the interaction and close proximity of the sarcoplasmic reticulum (SR) and mitochondria. With Ca^{2+} stimulating both contraction and mitochondrial respiration (specifically the Kreb's cycle dehydrogenases), the transfer of Ca^{2+} from the SR to the mitochondria enables a tight coupling of energy demand and supply (Chen *et al*., 2012b). Central to this pathway is the uptake of Ca^{2+} into the mitochondria through the mitochondrial Ca^{2+} uniporter, whose molecular identity was recently discovered (de Brito and Scorrano, 2008; Baughman *et al*., 2011; De *et al*., 2011). Despite the mitochondrial Ca^{2+} uniporter possessing a low affinity for Ca^{2+} (10 μM) (Kirichok *et al*., 2004), mitochondrial Ca^{2+} uptake is rapid, suggesting that high concentrations of Ca^{2+} exist (around 25 μM) (Giacomello *et al*., 2010) within a very localized area to the SR (Rizzuto *et al*., 1998). Mitochondrial tethering to the SR through MFN2 enables the Ca^{2+} present in these subcellular microdomains to stimulate mitochondrial respiration on a beat-to-beat basis (Csordas *et al*., 2010; Giacomello *et al*., 2010; Chen *et al*., 2012b).

The effect of disturbing the interaction between the SR and mitochondria by ablating MFN2 or by a pharmacological strategy is not clear, but it may in part contribute to the development of a cardiomyopathy through its effect on Ca^{2+} signalling.

Mitochondrial fusion and fission proteins and the adult heart

The majority of experimental studies investigating the phenomenon of mitochondrial dynamics have been restricted to

Table 2

Effect of ablating the mitochondrial fusion proteins in the adult heart

Study	Fusion protein	Murine model	Mitochondrial morphology (TEM)	MPTP susceptibility	IRI sensitivity	Other effect
Papanicolaou *et al.*, 2012a	MFN1	Cardiac knockout (KO)	Smaller	Reduced	Reduced	No phenotype
Papanicolaou *et al.*, 2011	MFN2	Cardiac KO	Pleomorphic, larger	Reduced	Reduced	Modest LV hypertrophy, mild LV impairment
Wang *et al.*, 2012	MFN2	Cardiac KO	Pleomorphic, larger	Not tested	Increased	Impaired mito function and autophagy
Chen *et al.*, 2011	MFN1/MFN2	Cardiac KO	Smaller	No change	Not known	Impaired mito function
Piquereau *et al.*, 2012	OPA1	Whole body OPA1 +/–	Pleomorphic larger	Reduced	Not known	

immortal cell lines because their mitochondrial networks are highly mobile, unconstrained by sarcomeres, easy to image and the cells themselves are compliant to genetic manipulation. More recently, mitochondrial fusion and fission proteins have been investigated in the adult heart, in which mitochondria have a distinct arrangement and in which mitochondrial movement is constrained by the cellular architecture.

The mitofusins and the adult heart

In terms of the cardiovascular system, a number of experimental studies have investigated the mitofusins in cardiac and vascular cell lines and neonatal cardiomyocytes in which mitochondrial movement is unrestricted. In the adult heart, however, the role of the mitofusins in the adult heart have been somewhat varied and in some cases unexpected (see Table 2). This difference may be due in part to the pleiotropic non-fusion effects of mitofusins, in particular MFN2 (de Brito and Scorrano, 2008). Of particular interest and importance is the recent observation suggesting that mitochondrial dynamics may be relevant to the adult heart, with the description of 'nanotunneling' and 'kiss-and-tell' events in isolated adult cardiomyocytes (Huang *et al.*, 2013; Kasahara *et al.*, 2013). 'Kiss-and-tell' events were observed between adjacent mitochondria and are hypothesized to mediate local mixing of mitochondrial material. 'Nanotunneling' was also observed as a means of communication between mitochondria that are more remote. The highest rate of fusion events was observed in the perinuclear region where the mitochondria are not restricted by the sarcomeres, as with the interfibrillar mitochondria. Interestingly, these fusion events occurred relatively infrequently (compared to mitochondrial mobility in HeLa and MEF cell lines) with mitochondrial material taking up to a day to transverse the cell (Huang *et al.*, 2013). These observations add substantial evidence to the idea that adult cardiac mitochondria are dynamic, and form a large interconnected network as observed in other tissues. An early study by Chen *et al.* (2003) was the first to report that mice deficient in both MFN1 and MFN2 die *in utero* during the mid-gestation period, highlighting the critical role the mitofusins play in embryonic development. Cardiac-specific ablation of both MFN1 and MFN2 in the embryo was shown to be lethal at day 9.5–10.5, demonstrating that these mitochondrial fusion proteins also play a critical role in cardiac development (Chen *et al.*, 2011).

Papanicolaou *et al.* (Papanicolaou *et al.*, 2011; Chen *et al.*, 2012b) first investigated the effects of conditional ablation of cardiomyocyte-specific MFN2 (α-MHC-*Cre*) in the adult murine heart. Interestingly, they found that subsarcolemmal mitochondria in this heart were pleomorphic and slightly enlarged, and that the hearts themselves displayed modest LV hypertrophy and a mild deterioration in LV systolic function. MFN2 ablation was found to be associated with a decreased sensitivity to MPTP opening and reduced susceptibility to acute IRI, despite there being no major effect on mitochondrial function other than a mild mitochondrial membrane depolarization (Papanicolaou *et al.*, 2011; Chen *et al.*, 2012b). In contrast, the effect of genetic ablation of MFN2 using small interfering RNA in neonatal cardiomyocytes had opposing effects with mitochondrial fragmentation, increased susceptibility to MPTP opening and less cell death following oxidative stress. These studies demonstrate the cell-specific effects of MFN2 ablation. Zhao *et al.* (2012) have also investigated the effects of cardiac-specific MFN2 ablation (Mlc2v-*Cre*) in the adult murine heart. At 4 months of age, there was no obvious cardiac phenotype in MFN2 depleted hearts (Zhao *et al.*, 2012). Although they were able to confirm the appearance of pleomorphic large mitochondria in these hearts, the MFN2 knockout hearts also displayed increased sensitivity to acute IRI and developed late-onset LV dysfunction at 17 months of age (Zhao *et al.*, 2012). These findings were associated with a major metabolic disturbance with impaired autophagy, defective lipid metabolism and decreased mitochondrial respiration (primarily at complex III) (Zhao *et al.*, 2012). The explanation for the observed differences in effects of MFN2 ablation in the adult heart is not clear. Whether the difference in response to MFN2 ablation can be attributed to the use of different cardiac-specific promoters is not known.

Papanicolaou *et al.* (2012b) were also the first to investigate the effects of deleting cardiomyocyte-specific MFN1 (Myh6-*Cre*) in the adult murine heart from embryonic day 9.5. They found evidence of mitochondrial fragmentation (smaller spherical mitochondria), preserved mitochondrial function, resistance to oxidative stress in terms of mitochon-

drial membrane depolarization and cell viability, and reduced sensitivity to MPTP opening. The explanation for the differing effects on MFN1 versus MFN2 ablation on mitochondrial morphology is not known but may be attributed to the high profusion affinity of MFN1.

The effect of genetic ablation of both MFN1 and MFN2 in the adult murine heart (8 weeks of age) has also been investigated. It resulted in mitochondrial fragmentation, impairment in mitochondrial respiration, and a severe lethal cardiomyopathy after 7–8 weeks, suggesting that the mitofusins are essential for normal cardiac mitochondrial morphology and respiratory function (Chen *et al.*, 2011; Papanicolaou *et al.*, 2012a).

OPA1 and the adult heart

Mice completely deficient in OPA1 die *in utero*, confirming the importance of this protein in embryonic development and maintenance of mitochondrial integrity (Davies *et al.*, 2007). As an alternative approach, mice partially deficient in OPA1 (heterozygous OPA1 +/–) have been used to investigate the role of OPA1 in the adult heart (Piquereau *et al.*, 2012; Chen *et al.*, 2012a). In this transgenic mouse, there was a 50% reduction in myocardial OPA expression, enlarged mitochondria with disturbed cristae and altered mitochondrial organization, with a mild cardiac phenotype at 3 and 6 months, but severe deterioration and heart failure occurring at 12 months. Indeed, at 12 months, the mutation of OPA1 led to cardiac dysfunction with a reduced cardiac output, blunted inotropic reserve and reduced pressure-volume loops (Chen *et al.*, 2012a). This reduction in cardiac reserve was associated with mitochondrial dysfunction with a general reduction in complex activity and a blunted respiratory response when stimulated with either ADP or FCCP. In addition, the 10-week-old mouse was more susceptible to pressure-load LV hypertrophy when challenged with the transverse aortic constriction model (Piquereau *et al.*, 2012). Coupled with a reduction in mitochondrial DNA, these results suggest that maintenance of mitochondrial integrity, at either the DNA or protein level, is a key physiological requirement for mitochondrial fusion in the adult heart. The requirement for OPA1 in the adult heart is also the same for skeletal muscle, in which OPA1 plays a pivotal role (Chen *et al.*, 2010).

Drp1 and the adult heart

The Drp1-knockout mouse is embryonically lethal at embryonic day 12.5 and surprisingly, there are no phenotypical differences between the wild type and heterozygote Drp1-knockout mouse (Manczak *et al.*, 2012). These studies demonstrate a vital role for Drp1 during development (Ishihara *et al.*, 2009; Wakabayashi *et al.*, 2009), and that Drp1 localization, rather than its quantity may regulate its action. Despite the lack of a Drp1-knockout mouse, many of its roles in mitochondrial fission (apoptosis, mitophagy and increasing the mitochondrial network) are likely to hold true in the heart, since these functions of Drp1 have been reported in numerous other tissues and appear fundamental to mammalian life. The only effect of Drp1 that is unlikely to occur in the heart, or occur on a very infrequent basis, is its effect in mitosis as cardiomyocytes rarely undergoing replication.

While there are few studies directly investigating the role and molecular mechanisms of Drp1 in cardiac tissue (due to the aforementioned difficulties of manipulation and imaging in the heart), those studies conducted on cardiac tissue, have focused mainly on the involvement of Drp1 in apoptosis. Indeed, several studies have all reported that inhibition of Drp1 reduces cardiac cell death post-IRI (Ong *et al.*, 2010; Din *et al.*, 2013; Gao *et al.*, 2013) or prevents cardiac dysfunction following aortic banding (Givvimani *et al.*, 2012). These studies are discussed in greater detail later on, but they all demonstrate that Drp1 and mitochondrial fission play a key role in the regulation of apoptosis in cardiac tissue. As mentioned previously, not all mitochondrial fission should be considered detrimental. Drp1 recruitment to the mitochondria mediated by Bnip3 serves to mediate mitophagy in cardiomyocytes (Lee *et al.*, 2011), and, because of the high amount of oxidative phosphorylation that occurs in the heart, this pathway is especially important to remove damaged mitochondria and maintain the quality of mitochondria.

Mitochondrial fusion/fission proteins as therapeutic targets

The postnatal period

In the first few days of postnatal cardiac growth there are drastic changes in cardiac metabolism and intracellular architecture as the heart switches from the placental circulation and anaerobic metabolism to the pulmonary circulation and oxidative phosphorylation. The role of the mitochondrial fusion and fission proteins in mediating this adaptive response has recently been investigated (Papanicolaou *et al.*, 2012a). It was found that over the first 7 days of postnatal cardiac growth, mitochondrial morphology changes from fragmented and randomly orientated to more elongated mitochondria aligned to myofibrils; this change is associated with a significant increase in the expression of MFN1 and MFN2 (Papanicolaou *et al.*, 2012a). Crucially, transgenic mice (loxP/Myh6-Cre) deficient in cardiac-specific MFN1 and MFN2 from the late embryonic period display severe mitochondrial dysfunction at 7 days (abnormal mitochondrial structure, down-regulated mitochondrial biogenesis genes, reduced mitochondrial DNA), develop cardiomyopathy, and all die before 14 days old, implicating an important role for the mitofusins in cardiac growth over the initial postnatal period (Papanicolaou *et al.*, 2012a).

Another critical change, which occurs at the time of birth, is closure of the ductus arteriosus (DA) in response to the increase in oxygen thereby diverting blood from the right ventricle into the pulmonary circulation. Failure of the DA to close can result in pulmonary congestion and failure to thrive. Hong *et al.* (2013) have investigated the role of Drp1-mediated mitochondrial fission in oxygen-induced DA closure. They provided evidence that oxygen-induced phosphorylation of Drp1 at Ser^{616} induces mitochondrial fission in DA smooth muscle cells, which then generates mitochondrial-signalling ROS required to activate DA closure through a complex mechanism involving inhibition of oxygen-sensitive potassium channels, membrane depolarization of DA smooth muscle cells, L-type channel activation, increased intracellular calcium and subsequent

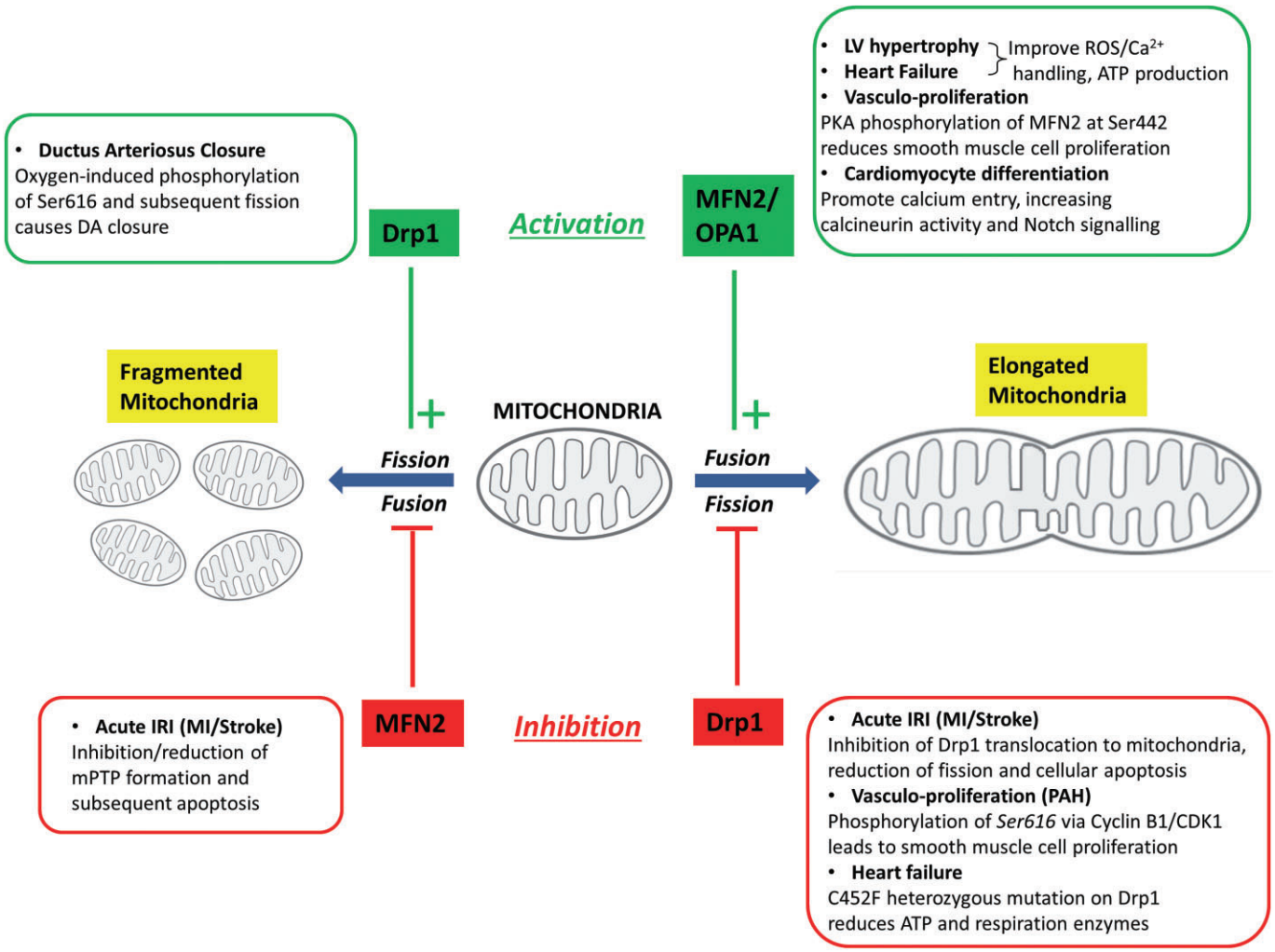

Figure 2

This scheme depicts the mitochondrial fission and fusion proteins as novel therapeutic targets for treating cardiovascular disease. It is important to bear in mind that chronic therapeutic targeting of these shaping proteins may have detrimental effects and that there may be off-target effects as these mitochondrial-shaping proteins may have non-fusion or non-fission pleiotropic actions within the cell.

vasoconstriction of the DA (Hong *et al.*, 2013). These findings implicate Drp1 as a potential therapeutic target for mediating DA closure (see Figure 2).

Vascular smooth muscle cell (VSMC) proliferation

VSMC proliferation and hyperplasia is a key feature of a variety of cardiovascular diseases including atherosclerosis, hypertension, pulmonary arterial hypertension (PAH), and its existence contributes to the failure of coronary artery bypass vein grafts and to the restenosis following percutaneous coronary intervention. As such, novel therapeutic targets are required to inhibit VSMC proliferation in these settings. In this regard, the mitochondrial fusion and fission proteins may provide novel targets for preventing this pathological process.

MFN2 and proliferation of VSMCs. Chen *et al.* were the first to implicate MFN2, as a novel hyperplasia suppressor gene (HSG), capable of inhibiting VSMC proliferation in a variety of vasculo-proliferative conditions (Chen *et al.*, 2004). These authors found that the expression of HSG (MFN2) in VSMCs was reduced in several experimental models of vasculo-proliferation, and HSG (MFN2) overexpression inhibited VSMC proliferation in angioplasty balloon-induced neointimal injury, oxidized LDL and subsequent atheroma formation and restenosis in rat carotid arteries. These vasculo-proliferative effects appeared to be mediated by inducing apoptotic death of VSMCs through the suppression of the Ras-Raf-MAPK-Erk1/2 and PI3K-Akt signalling pathways (Chen *et al.*, 2004; Guo *et al.*, 2007). The same researchers found that PKA-induced phosphorylation of MFN2 at Ser^{442} is central to the anti-proliferative effect of MFN2 on VSMCs (see Figure 2) (Zhou *et al.*, 2010).

Drp1 and proliferation of VSMCs. Chalmers *et al.* (2012) have investigated the role of the mitochondrial fission protein, Drp1, in VSMCs in relation to their proliferative response.

They found that in native non-proliferative quiescent VSMCs mitochondria were fairly static, mainly ovoid in shape whereas during proliferation, the mitochondria became more mobile and displayed various shapes. Interestingly, treatment of VSMCs with the Drp1 inhibitor, Mdivi-1, was able to inhibit the proliferative response, suggesting that mitochondrial fission may be required for the proliferation of VSMCs. The requirement for mitochondrial fission in VSMC proliferation has been recently explored in the setting of PAH (see Figure 2).

PAH. PAH, in which the pulmonary arteries become obstructed, results in right ventricular hypertrophy and heart failure. Despite new vasodilator therapy, the morbidity and mortality remain high (15% death at 1 year) (Thenappan *et al.*, 2010). Therefore, novel therapeutic targets for preventing the progression of PAH are desirable. In this regard, recent experimental data have implicated the mitochondrial fusion and fission proteins as potential novel therapeutic targets for treating PAH.

The ability of pulmonary arterial smooth muscle cells (PASMC) to hyperproliferate is an essential part of the pathophysiology underlying PAH. It has been demonstrated that this pathological process is dependent on the ability of the mitochondria to undergo division as this allows the equal redistribution of mitochondria during cell proliferation. Marsboom *et al.* (2012) noted that in PAH, the mitochondria in PASMCs are fragmented, findings which were associated with the up-regulation of Drp1 and the down-regulation of MFN2 (see Figure 2). They demonstrated that mitochondrial fission, mediated by cyclin B1/Cdk1 phosphorylation of Drp1 at Ser^{616}, is required for the hyperproliferation of PASMCs. Interestingly, treatment with the small molecule Drp1 inhibitor, Mdivi-1, was shown to prevent the progression of PAH in three different experimental models of PAH, suggesting Drp1-mediated mitochondrial fission as a novel therapeutic target for PAH (Marsboom *et al.*, 2012). However, preventing PAH by using this therapeutic approach would require prolonged and chronic inhibition of mitochondrial fission, the result of which may be detrimental over the long term, given the essential role mitochondrial fission plays in maintaining a healthy mitochondrial network. The same research group have gone on to implicate the mitochondrial fusion protein, MFN2, as a potential therapeutic target for treating PAH. They found that in two different experimental models of PAH, and in patients with PAH, both MFN2 and PGC-1α were down-regulated in PASMCs. Importantly, they demonstrated that genetic ablation of MFN2 and PGC-1α induced mitochondrial fragmentation and worsened PAH, whereas in contrast, over-expressing MFN2 was able to prevent the progression of PAH.

Heart failure

A critical role for the mitochondrial fusion and fission proteins in normal development and function of the heart has been indicated by the finding that deficiencies in either the mitochondrial fusion or fission proteins result in the development of a cardiomyopathy.

OPA1 and heart failure. The changes in mitochondrial morphology that occur with ischaemia-induced heart failure have been investigated by Chen *et al.* (2009), using a post-MI rat heart failure model and human dilated and ischaemic cardiomyopathy tissue samples. These authors observed mitochondrial fragmentation and decreased myocardial levels of OPA1 (Chen *et al.*, 2009). A subsequent experimental study by the same authors has recently demonstrated that heterozygous mice deficient in OPA1 had reduced mitochondrial DNA copy number and decreased expression of nuclear antioxidant genes at 3 to 4 months of age (Chen *et al.*, 2012a). However, baseline cardiac function was normal in these OPA1-deficient mice, although at 12 months of age, the mice developed a cardiomyopathy associated with mitochondrial fragmentation and impaired mitochondrial function (Chen *et al.*, 2012a). The reason for the decline in OPA1 levels in heart failure requires further investigation.

Mitofusins and heart failure. Silencing of mitochondrial assembly regulatory factor orthologue of mammalian mitofusin and OPA1 in the *Drosophila* fly heart tube resulted in a dilated cardiomyopathy, which could be rescued by overexpressing either of the human mitofusins (MFN1 or MFN2) or superoxide dismutase 1, implicating impaired mitochondrial fusion and oxidative stress in the pathogenesis of heart failure (Dorn *et al.*, 2011).

Conditional cardiac-specific ablation of MFN1 and MFN2 in the adult murine heart has been reported to result in a severe lethal dilated cardiomyopathy after 6–8 weeks, a finding which was associated with mitochondrial fragmentation and impaired mitochondrial respiration, implicating a role for the mitofusins in maintaining normal mitochondrial function in the adult heart (Chen *et al.*, 2011).

Drp1 and heart failure. Ashrafian *et al.* (2010) have described a novel mutation in the Drp1 gene (C452F) which gives rise to an autosomal dominant form of dilated cardiomyopathy in the python mouse. Although the homozygous mutation is embryonically lethal, the heterozygous form survives until adulthood and develops a severe dilated cardiomyopathy after 11 weeks, a finding which was associated with reduced content of mitochondrial respiratory enzymes and ATP (Ashrafian *et al.*, 2010).

Acute IRI

Ischaemic heart disease is the leading cause of death and disability worldwide. Its clinical manifestations are due to the detrimental effects of acute IRI on the myocardium. The susceptibility of the heart to acute IRI and its recovery is critically dependant on the function of its mitochondria. Mitochondrial dysfunction in response to acute IRI and subsequent opening of the MPTP at reperfusion are critical determinants of cell death. Therefore, the preservation of mitochondrial viability and the prevention of MPTP opening during acute IRI are important therapeutic strategies for cardioprotection. Recent experimental data suggest that manipulating the mitochondrial fission and fusion proteins in the heart may affect the susceptibility to acute IRI, providing novel therapeutic targets for cardioprotection.

Mitochondrial fission proteins as targets for cardioprotection. In response to acute IRI, the mitochondrial fission protein, Drp1, has been demonstrated to translocate to the OMM and induce

mitochondrial fission (Ong *et al*., 2010; Din *et al*., 2013). In the HL-1 cardiac cell line, Ong *et al*. (2010) showed that genetic or pharmacological (using Mdivi-1) inhibition of Drp1 during simulated IRI prevented the opening of the MPTP and reduced cell death. In the murine heart, Mdivi-1-induced inhibition of Drp1 reduced cell death in isolated cardiomyocytes subjected to simulated IRI and reduced MI size in the adult murine heart subjected to *in vivo* acute IRI. A number of experimental studies have subsequently confirmed Drp1 to be a therapeutic target for cardioprotection in the adult heart. Din *et al*. (2013) found that using Mdivi-1 to inhibit Drp1 translocation to the OMM during acute IRI protected neonatal murine cardiomyocytes and the adult murine heart. Interestingly, the same authors were able to reduce Drp1 translocation to the mitochondria in simulated IRI through the overexpression of Pim1, implicating this kinase as another mechanism through which cardioprotection through the modulation of Drp1 may be achieved. Using the non-specific dynamin inhibitor, Dynasore, to inhibit Drp1 during acute IRI, Gao *et al*. (2013) were able to protect the isolated adult murine heart. Inhibiting mitochondrial fission has also been reported to protect the kidney and the brain against acute IRI, suggesting that therapeutic targeting of mitochondrial fission may be beneficial in other organs (Zhang *et al*., 2013). Most recently, a specific peptide inhibitor of Drp1, named P110, has been used to demonstrate that inhibiting mitochondrial fission at reperfusion can reduce myocardial infarct size and prevent adverse LV remodelling post-MI in the adult rat heart (Disatnik *et al*., 2013). The discovery of other components of the mitochondrial fission machinery, such as MFF and MiD49/51, raises the possibility of inhibiting these other proteins to mediate cardioprotection. However, this therapeutic strategy will only be useful in protecting the heart against acute episodes of IRI, as this can be achieved by transient pharmacological inhibition of mitochondrial fission. Chronic inhibition of mitochondrial fission would be detrimental to the heart and other organs as this process is critical to maintaining a health mitochondrial network.

Mitochondrial fusion proteins as targets for cardioprotection. The role of the mitochondrial fusion proteins (MFN1, MFN2 and OPA1) as targets for cardioprotection has produced some unexpected findings. This may relate to their well-established non-fusion effects, which may interfere with the myocardial response to acute IRI. In the HL-1 cardiac cell line, Ong *et al*. (2010) found that overexpressing MFN1 or MFN2 prevented the opening of the MPTP and reduced cell death following simulated IRI. These findings were consistent with those of Papanicalou *et al*. (Papanicolaou *et al*., 2011; Chen *et al*., 2012b), who demonstrated that small interfering RNA knockdown of MFN2 delayed MPTP opening and rendered neonatal cardiomyocytes more susceptible to oxidative stress.

The genetic manipulation of the mitochondrial fusion proteins in the adult heart has had contrasting effects in terms of susceptibility to MPTP opening and sensitivity to acute IRI (see Table 2). The deletion of MFN1, MFN2 or OPA1 appears to render the heart less resistant to MPTP opening, and in the case of MFN1 and MFN2, the hearts have been reported to be more resistant to acute IRI. The explanation for this is not clear, but could possibly be related to the non-fusion effects of the mitofusins described earlier. Although partial genetic ablation of OPA1 has been shown to delay MPTP opening, its effect on acute IRI susceptibility has not been investigated (Piquereau *et al*., 2012). Similar to the mitofusins, OPA1 has non-fusion pleiotropic effects such as inhibiting apoptosis by preventing cytochrome *c* release, and therefore, the effect of manipulating OPA1 on the susceptibility to acute IRI may be less straightforward to predict.

Experimental studies thus far have investigated the effects of genetically ablating the mitochondrial fusion proteins. However, the effect of overexpressing the mitochondrial fusion proteins in the adult heart on sensitivity to acute IRI is currently unknown. Whether this would worsen the response to acute IRI is unclear.

In summary, the role of the mitochondrial fusion proteins in the adult heart in terms of susceptibility to acute IRI is quite complex. However, the development of small molecule inhibitors of MFN1 and MFN2 may provide a novel therapeutic strategy for cardioprotection.

LV hypertrophy (LVH)

Hypertrophy of the left ventricle can be both physiological (in response to exercise) and pathological (congenital or acquired – most often a detrimental response to an increase load) and can lead to an increased risk of arrhythmias, regions of ischaemia and heart failure (Frey and Olson, 2003; Frey *et al*., 2004). As such, novel therapeutic agents are required to prevent the progression of LVH and reduce the onset of heart failure.

Mitochondrial fusion proteins and LVH. A number of experimental studies have implicated the mitochondrial fusion proteins MFN2 and OPA1 as potential therapeutic targets for treating LVH. Previous work by Fang *et al*. demonstrated that MFN2 (formerly known as hyperplasia suppressor gene or HSG) can inhibit VSMC proliferation through the suppression of MEK1/2-Erk1/2 (Fang *et al*., 2007), a signalling pathway that is up-regulated in LVH. As such, they investigated whether MFN2 can also suppress pathological LVH. They found that MFN2 expression was down-regulated and Erk1/2 up-regulated in four different experimental models of LVH (phenylephrine induced LVH in neonatal rat cardiomyocytes, spontaneously hypertensive rats, β_2-adrenoceptor transgenic mice and pressure overload LVH by transverse aortic constriction) (Fang *et al*., 2007). Yu *et al*. (2011) went on to demonstrate that MFN2 is down-regulated and Akt up-regulated in neonatal rat cardiomyocytes treated with angiotensin-II, and that genetic overexpression of MFN2 could prevent angiotensin-II-induced LVH in both neonatal cardiomyocytes and the intact rat heart. While these studies all show a reduction in total MFN2 protein, there is also the possibility that these observations are merely due to the reduction in mitochondrial mass *per se*. It is, therefore, important to quantify the amount of mitochondrial dynamic protein to total mitochondrial mass in order to obtain the correct conclusion, especially in pathologies that are associated with dynamic changes in mitochondrial number. Consistent with a role for MFN2 in hypertrophy are the observations of cardiac hypertrophy in mice with cardiac-specific MFN2 knockout (Papanicolaou *et al*., 2011; Chen and Dorn, 2013). These implicated MFN2 as a potential cause, and

therefore target for treating LVH. MFN2 and the mitochondria are presumably implicated in such a condition through the generation of ROS, and an energy supply–demand mismatch. One can hypothesize that by increasing the activity of MFN2 in the early stages of hypertrophy, the mitochondrial network is maintained, ensuring healthy mitochondria, which produce less ROS. Furthermore, given the ability of MFN2 to tether mitochondria to the SR, this pathway is not only important in the maintenance of Ca^{2+} homeostasis, but also in energy supply–demand coupling. Through these mechanisms, MFN2 could reduce the need for the heart to hypertrophy pathologically.

Another mitochondrial fusion protein, OPA1, may also be a potential therapeutic target for preventing LVH. Mice that are partially deficient in OPA1 are more susceptible to LVH and cardiac dysfunction induced by total aortic constriction (Piquereau *et al.*, 2012). While the anti-hypertrophic effects of OPA1 overexpression in the adult heart are yet been investigated, it is supposed that beneficial effects would be observed. The benefits could operate on two levels, the maintenance of a healthy mitochondrial network (less ROS, better ATP coupling etc.) and a reduction in apoptosis. With OPA1 a strongly anti-apoptotic protein (as previously discussed), OPA1 manipulation may reduce the apoptosis of cardiomyocytes, reducing subsequent collagen deposition and the associated decline in cardiac function. If the next lines of investigation prove MFN2 and OPA1 to be beneficial in the treatment of hypertrophy, the next challenges are to discover drugs that will specifically activate these fusion proteins, and pinpoint the exact time-point of disease progression where activation of these proteins would prove most beneficial.

Mitochondrial fission proteins and LVH. In a recent study it was reported that Drp1 is up-regulated, and MFN2 and OPA1 are down-regulated in a cell model of phenylephrine-induced cardiomyocyte hypertrophy, suggesting a change in the balance of mitochondrial morphology to fragmentation may be associated with the development of LVH (Javadov *et al.*, 2011). Drp1 has recently been investigated as a potential mediator of LVH given its role in mediating mitophagy, a process that may contribute to the pathogenesis of LVH and heart failure (Givvimani *et al.*, 2012). Treatment with the Drp1 inhibitor Mdivi-1 prevented the progression of LVH and development to heart failure induced by pressure overload transaortic constriction. Treatment with Mdivi-1 was associated with the maintenance of the mitochondrial population, a release of pro-angiogenic factors (CD31 and VEGF) and a reduced collagen deposition. Assuming that Mdivi-1 is acting upon the mitochondria through Drp1 inhibition, this study implicates changes in mitochondrial dynamics as a key stage in the development of pathological hypertrophy (Givvimani *et al.*, 2012).

Differentiation of stem cells into cardiomyocytes

In the cardiomyocyte differentiation of stem cells, changes in mitochondrial function and architecture are required to cater for the increased metabolic demands of the differentiated beating cardiomyocyte (reviewed in Rehman, 2010). For the embryonic stem cell (ESC) to differentiate into a cardiomyocyte, there needs to be a metabolic switch from anaerobic glycolysis to oxidative phosphorylation (Chung *et al.*, 2007). Crucially, this change in mitochondrial metabolism has been reported to require a change in mitochondrial morphology from fragmented rounded mitochondria (lacking cristae) present in the ESC, to an elongated interconnected mitochondria (with well-developed cristae) closely aligned with the myofibrils of the differentiated contractile cardiomyocyte (Chung *et al.*, 2007). This change in mitochondrial morphology was associated with alterations in the expression of Drp1 and MFN2 as well as expected changes in the metabolic transcriptome (Chung *et al.*, 2007). A recent experimental study has shown that the presence of OPA1 and MFN2 are required in the development of the heart, with developmental arrest occurring at e13.5 (Kasahara *et al.*, 2013). Similarly, differentiation from ESC to cardiac myocytes was associated with an increased expression of MFN2 and OPA1. While knockout of these genes failed to affect mitochondrial biogenesis, the mitochondrial network failed to elongate and the cells were no longer able to differentiate into beating cardiomyocytes. Through a mechanism that was unrelated to ATP production, the interruption of mitochondrial fusion reduced signalling calcium entry, which suppressed calcineurin activity and Notch signalling (Kasahara *et al.*, 2013). These findings suggest that therapeutic targeting of mitochondrial fusion and fission protein may allow one to facilitate the cardiac differentiation of stem cells in future regenerative therapy.

Limitations and future therapeutic potential

Despite the therapeutic potential of pharmacologically targeting mitochondrial fusion and fission proteins to treat cardiovascular disease, several hurdles still need to be overcome to make this a clinical reality. These include the ability to target the therapy in terms of treatment duration and its organ-specificity. Given the important physiological roles the mitochondrial fusion and fission proteins play in normal cellular physiology, therapeutic activation or inhibition of either the mitochondrial fission or fusion proteins is likely to have detrimental effects as both processes are required to maintain a healthy mitochondrial network for normal cell function. As such, the application of this therapeutic approach may be limited to temporary modulation in acute rather than chronic cardiovascular conditions. Another important issue to consider will be the ability to target the drug to a specific organ and avoid off-target side effects. One potential approach could be the intracoronary delivery of the drug or coating coronary stents with the drug, thereby enabling the local delivery of the drug to the myocardium or the coronary endothelium.

Summary

Mitochondria are now regarded as highly dynamic organelles with multiple functions. Emerging data suggest that the mitochondrial fusion and fission proteins may provide novel therapeutic targets for treating a variety of cardiovascular diseases including acute IRI, heart failure, LVH, pulmonary arterial

hypertension and diabetes mellitus (see Figure 2). There is also the potential to target mitochondrial fusion and fission proteins to promote DA closure and facilitate cardiomyocyte differentiation from stem cells for regenerative therapy.

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Conflict of interest

None.

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