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The role of PTEN-induced kinase 1 in mitochondrial dysfunction and dynamics

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Abstract

Mutations in *parkin*, *PTEN-induced kinase 1 (PINK1)* and *DJ-1* can all cause autosomal recessive forms of Parkinson disease. Recent data suggest that these recessive parkinsonism-associated genes converge within a single pathogenic pathway whose dysfunction leads to the loss of *substantia nigra pars compacta* neurons. The major common functional effects of all three genes relate to mitochondrial and oxidative damage, with a possible additional involvement of the ubiquitin proteasome system. This review highlights the role of the mitochondrial kinase, PINK1, in protection against mitochondrial dysfunction and how this might relate to loss of substantia nigra neurons in recessive parkinsonism.

Keywords

parkinsonism; parkin; Drp1; fission; fusion; oxidative stress

Introduction

Defects in mitochondrial metabolism are implicated in many common diseases of aging. Of these, Parkinson disease (PD) is a common neurodegenerative disorder that is characterized in part by the loss of dopaminergic neurons in the *substantia nigra pars compacta* (SNpc). Although mitochondrial defects have been proposed to be part of the pathophysiology of many age-related neurodegenerative disorders, there are several distinct and complementary lines of evidence suggesting links between mitochondria and PD. These ideas have been re-emphasized in the last few years by the discovery of mutations in genes that are directly associated with mitochondrial function where the phenotype includes loss of SNpc neurons, as seen in PD.

One piece of supportive data for the hypothesis that mitochondria may be important in PD is that the mitochondrial complex I inhibitors, rotenone and MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), lead to death of dopaminergic cells in the SNpc (Betarbet et al., 2000, Langston et al., 1983, Langston et al., 1984, Liou et al., 1996, Ricaurte et al., 1986). Therefore, damage to mitochondria, specifically to complex I is sufficient to produce a phenotype that mimics the cell loss in PD.

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One specific link between PD and mitochondria is that complex I activity of the electron transport chain is diminished in the *substantia nigra* of PD patients (Schapira et al., 1989). Inhibition of electron transport produces damaging reactive oxygen species (ROS) and limits the capacity of mitochondria to generate ATP, which has profound effects on the cell. This type of continuous oxidative insult induces cell death, which in many model systems includes cytochrome c release via the mitochondrial permeability transition pore and features typical of apoptosis (Tsujimoto and Shimizu, 2007).

Oxidative damage secondary to ROS generation has been proposed to be associated with the pathogenesis of PD (Dexter et al., 1989, Shimura-Miura et al., 1999, Sofic et al., 1992, Yoritaka et al., 1996, Youdim et al., 1989). ROS are capable of damaging many macromomolecules including nuclear and, especially, mitochondrial DNA (mtDNA), which is not histone bound. Mammalian mitochondrial DNA is a circular molecule of 16.6 kb, present in several copies per mitochondria which encode 13 subunits of respiratory chain complexes I, III–V, which are essential components of oxidative phosphorylation (OXPHOS). MtDNA also encodes the 12S and 16S rRNA genes and the 22 tRNA genes required for mitochondrial protein synthesis. The remaining mitochondrial OXPHOS proteins, the metabolic enzymes, the DNA and RNA polymerases, the ribosomal proteins and the mtDNA regulatory factors are all encoded by nuclear genes, synthesized in the cytosol and then imported into the mitochondria (Wallace, 1997). A supportive piece of evidence for a mitochondrial pathophysiology of PD is the observation that mtDNA abnormalities are found in patients with PD (Rana et al., 2000, Swerdlow et al., 1998, Swerdlow et al., 1996). Furthermore, although mtDNA deletions are rarely found in nigral neurons in young subjects, they are found increasingly in the same neuronal groups in older subjects (Bender et al., 2006, Kraytsberg et al., 2006).

However, decreased complex I activity in PD patients is also seen in peripheral tissues of PD patients (Krige et al., 1992, Parker et al., 1989, Wallace et al., 1992, Blake et al., 1997), and it is therefore unclear why neurons in general, and SNpc neurons in particular, would be susceptible to loss of complex I activity. Furthermore, consistent mtDNA mutations have not been identified in either familial or sporadic forms of parkinsonism (Tan et al., 2000). One possible interpretation of this data is that mtDNA mutations are secondary to the pathological process of PD and are not causal for the disease. The age-related loss of mtDNA integrity and biochemical mitochondrial function may be a precipitating event for neuronal damage that is fundamentally driven by other events that are yet to be described.

These several lines of evidence are largely derived from studies of sporadic PD, which accounts for the majority of all cases and is not familial in nature. However, there are rare inherited diseases that partially overlap with the clinical syndrome of PD. These have been given designations of PARK1-14 in OMIM (http://www.ncbi.nlm.nih.gov/omim/) and are discussed elsewhere. This review will focus on autosomal recessively inherited early onset disease where parkinsonism is a major clinical feature.

It is important at this point to distinguish parkinsonism from PD. Parkinsonism is a clinically defined syndrome that describes predominantly motor problems (tremor, slowness of movement, rigidity and instable upright posture) deriving from loss of SNpc neurons. Parkinson disease, at least in the way the term is currently used, is a clinico-pathological diagnosis that requires the presence of protein deposition as Lewy bodies superimposed on a picture of neuronal loss. Importantly, PD is not ‘simply’ a nigral disease and includes both non-dopaminergic and non-motor symptoms. Recessive parkinsonism tends to be earlier onset, have a milder course, respond better to symptomatic treatment and have fewer non-motor complications such as dementia compared to sporadic PD. This distinction between PD and parkinsonism is partly an issue of terminology but it is important here because if these are

different diseases (ie having a different underlying cause), we have to be careful about extrapolating from one to the other in relation to mitochondrial mechanisms.

There are three genes for autosomal recessive parkinsonism: *parkin*, *DJ-1* and *PINK1*. Several recent lines of data suggest that recessive parkinsonism-associated genes converge within a single pathogenic pathway whose dysfunction leads to the loss of SNpc neurons. The major common functional effects of all three genes relate to mitochondria and oxidative damage, with a possible additional involvement of the ubiquitin proteasome system (UPS) (for a recent review on the UPS and neuronal dysfunction, see Tai and Schuman, 2008) (Tai and Schuman, 2008). This review will focus specifically on the role of the mitochondrial kinase, PINK1, in protection against mitochondrial dysfunction and how this might relate to loss of SNpc neurons in recessive parkinsonism.

PINK1 and mitochondrial dysfunction

The *PINK1 (phosphatase and tensin homolog (PTEN)-induced kinase 1)* gene consists of eight exons, encoding a 581-amino acid protein with a predicted molecular mass of 62.8 kilodaltons. PINK1 mRNA is expressed ubiquitously, but the highest expression levels are found in the heart, skeletal muscle, and testes and intermediate levels of expression are found in the liver, kidney pancreas and brain (Unoki and Nakamura, 2001, Taymans et al., 2006). In the brain, expression is primarily neuronal in the hippocampus, *substantia nigra* and cerebellar Purkinje cells (Blackinton et al., 2007).

The PINK1 protein has a central domain with homology to serine/threonine kinases (Valente et al., 2004a) and exhibits autophosphorylation activity *in vitro* (Nakajima et al., 2003, Beilina et al., 2005, Silvestri et al., 2005). An N-terminal mitochondrial-targeting signal (MTS) is sufficient for mitochondrial import of PINK1 (Muqit et al., 2006, Silvestri et al., 2005) and the protein can be found the outer mitochondrial membrane (OMM) (Gandhi et al., 2006, Weihofen et al., 2009), the mitochondrial intermembrane space (IMS) (Plun-Favreau et al., 2007, Pridgeon et al., 2007, Silvestri et al., 2005) and the inner mitochondrial membrane (IMM) (Gandhi et al., 2006, Lin and Kang, 2008, Muqit et al., 2006, Pridgeon et al., 2007, Silvestri et al., 2005). However, PINK1 also localizes to the cytosol (Beilina et al., 2005, Haque et al., 2008, Lin and Kang, 2008, Takatori et al., 2008, Weihofen et al., 2008, Weihofen et al., 2009, Zhou et al., 2008). Recent work suggests that the topology of PINK1, including the endogenous protein, relies on the presence of a transmembrane domain located after the MTS to insert the N-terminal tail into the outer mitochondrial membrane with the kinase domain facing the cytoplasm (Zhou et al., 2008). This suggested orientation would imply that physiological PINK1 substrates are localized in the outer mitochondrial membrane or possibly in the cytosol at the mitochondrial surface. The cytoplasmic pool of PINK1 found in overexpression models may represent excess protein that is shed from the mitochondrial surface; it is of interest that this pool is rapidly degraded by the proteasome (Lin and Kang, 2008, Takatori et al., 2008).

The first reported *PINK1* mutations, G309D and the truncation mutant W437X, were identified in patients of Spanish and Italian origin, respectively (Valente et al., 2004a). Over 20 pathological mutations have now been identified in *PINK1* varying from point mutations, truncations and whole gene heterozygous deletions (Klein et al., 2006, Marongiu et al., 2007, Zadikoff et al., 2006), which between them account for between 1–9% of cases with early onset parkinsonism (Healy et al., 2004, Li et al., 2005, Rohe et al., 2004, Tan et al., 2006, Tan et al., 2005, Valente et al., 2004b).

Because they are recessively inherited, pathogenic mutations are predicted to inactivate the protein, leading to a loss of function. Some mutations cause loss of function in very simple way. For example, L347P is less stable than wild type PINK1 (Beilina et al., 2005, Moriwaki

et al., 2008), probably due to increased protein turnover by the proteasome (Moriwaki et al., 2008). Other pathogenic mutations such as G309D are stable have decreased *in vitro* kinase activity (Beilina et al., 2005), measured using autophosphorylation as a convenient assay.

However, it is difficult to say whether mutations outside the kinase domain affect kinase activity or whether they work in some other fashion. *PINK1* C-terminal truncations identified produce clinical phenotypes to missense *PINK1* mutations (Rohe et al., 2004). The C-terminal regions of many protein kinases contain functional motifs that control catalytic activity of the kinase domain and/or bind regulatory proteins and substrates (Jeffrey et al., 1995, Niefind et al., 1998, Nolen et al., 2001). Deletion of the C-terminal region of PINK1 results in decreased kinase activity when expressed in baculovirus-infected insect cells (Sim et al., 2006) but enhanced kinase activity when recombinant PINK1 was expressed in *E. coli* (Silvestri et al., 2005). Again, these studies measured autophosphorylation activity and so an open question is whether these types of assays capture the true effects on mutations under more physiological conditions.

Two putative mitochondrial substrates for PINK1, tumor necrosis factor type 1 receptor associated protein 1 (TRAP1) (Pridgeon et al., 2007) and the serine protease Omi/HtrA2 (high temperature requirement protein A2) (Plun-Favreau et al., 2007), have been identified. TRAP1 has been reported to be a direct substrate for PINK1, based on in vitro assays using phospho-serine specific antibodies but the site of modification is not known. TRAP1 has been shown to localize primarily in the mitochondrial matrix, but it has also been found in the IMS (Pridgeon et al., 2007) and at extramitochondrial sites (Cechetto et al., 2000). Omi/HtrA2 is phosphorylated at Ser142 by p38γ and this is modified by the presence or absence of PINK1. Whether Omi/HtrA2 is therefore a direct PINK1 substrate is unclear and it is possible that differences in cell viability resulting from PINK1 inactivation (see below) might indirectly affect Omi/HtrA2 through other kinases, including p38. Omi/HtrA2 is released from the intermembrane space of the mitochondria during apoptosis to the cytosol where it interacts with the inhibitor of apoptosis protein (IAP) (Strauss et al., 2005). If the model of PINK1 topology proposed by Zhou et al. is correct, then neither TRAP1 nor Omi/HtrA2 would be substrates of PINK1 under physiological conditions, as such substrates would need to be present on the cytoplasmic edge of the mitochondria. However, it is not clear how much endogenous PINK1 is present in different mitochondrial locations under basal conditions and under conditions of cellular toxicity, where PINK1 has been shown to have a functional role.

Such a neuroprotective effect has been demonstrated in cell culture models under various forms of cellular stress. PINK1 has been shown to protect against cell death induced by proteasome inhibition (Muqit et al., 2006, Valente et al., 2004a, Wang et al., 2007) and oxidative damage with rotenone (Deng et al., 2005) and MPP^+, the active metabolite of the complex I inhibitor MPTP (Deng et al., 2005, Haque et al., 2008, Tang et al., 2006). PINK1 has also been shown to protect against MPTP toxicity *in vivo* in a kinase-dependent fashion (Haque et al., 2008). Interestingly, the MTS is not required for the protective function of overexpressed PINK1 *in vivo* against complex I inhibition (Haque et al., 2008), which supports the idea that the functional part of the molecule is exposed to the cytoplasmic surface (Zhou et al., 2008). Presumably, high levels of cytoplasmic PINK1 can substitute for endogenous protein in this context by phosphorylating substrates at the mitochondrial surface or in the cytoplasm in the vicinity of mitochondria. Loss of function mutations (Hoepken et al., 2008, Hoepken et al., 2007, Pridgeon et al., 2007, Valente et al., 2004a) or PINK1 silencing (Clark et al., 2006, Gautier et al., 2008, Wang et al., 2006, Wood-Kaczmar et al., 2008) enhances susceptibility to cell death mediated by oxidative damage.

Prolonged ROS exposure can cause mitochondrial dysfunction, in part because proteins with iron sulfur clusters that are involved in oxidative phosphorylation and the electron transport

chain are sensitive to oxidative stress (Gardner and Fridovich, 1991). PINK1 silencing also results in mitochondrial respiratory dysfunction. PINK1 knockout mice exhibit impaired striatal mitochondrial respiration and decreased activity of aconitase (Gautier et al., 2008), an iron sulfur cluster-containing enzyme involved in oxidative phosphorylation. This loss of mitochondrial aconitase activity is an intracellular indicator of superoxide generation and oxidative damage. In addition, the impaired mitochondrial respiration in these mice can be exacerbated by exposure of isolated mitochondria to heat shock or H_20_2, or by letting the animals age (Gautier et al., 2008). Similarly, analysis of fibroblasts obtained from a patient carrying the homozygous W437X PINK1 nonsense mutation showed a lower mitochondrial respiratory activity (Piccoli et al., 2008). ATPase activity was lower in W437X patient fibroblasts when cultured with galactose, which causes cells to utilize oxidative phosphorylation instead of relying on glycolytic ATP, which suggests a basal oxidative phosphorylation deficit in these cells (Piccoli et al., 2008). Complex I respiratory defects were also found in primary fibroblasts isolated from patients homozygous for G309D PINK1 (Piccoli et al., 2008).

In addition to oxidative stress, loss of mitochondrial membrane potential ($\Delta\psi_m$) can cause mitochondrial dysfunction. Mitochondrial membrane potential results from the charge imbalance across the inner mitochondrial membrane as the respiratory chain builds up the proton gradient that is required for oxidative phosphorylation. One mechanism resulting in the decrease of $\Delta\psi_m$ results from the permeabilization of the inner mitochondrial membrane by oxidative stress or Ca^{2+} overload. Inner mitochondrial membrane permeabilization results in increased mitochondrial matrix volume, reduced matrix electron density and the disorganization of the cristae—the internal compartments formed by the inner membrane of a mitochondrion (Nieminen et al., 1997, Petronilli et al., 1999). Mitochondrial membrane potential is maintained with wild type PINK1 overexpression and decreased by the presence of the G309D mutation after treatment with the proteasome inhibitor MG-132 (Valente et al., 2004a), heterozygous PINK1 mutations (Abou-Sleiman et al., 2006) or PINK1 silencing (Exner et al., 2007). Parkin, another recessive parkinsonism gene can also affect $\Delta\psi_m$ (Mortiboys et al., 2008) and decrease mitochondrial swelling once parkin has translocated to the outer mitochondrial membrane (Darios et al., 2003). Recently it has been shown that when parkin is overexpressed it is selectively recruited to mitochondria with low membrane potential (Narendra et al., 2008). Therefore, whether by direct or indirect mechanisms, PINK1 and parkin influence the ability of mitochondria to maintain $\Delta\psi_{m,}$ particularly under conditions such as oxidative stress or a shift to galactose as a respiratory substrate to stress the cell.

In combination, the results above show that PINK1 is a mitochondrial kinase that promotes cell survival, particularly under conditions of oxidative/metabolic stress. Although the precise physiological substrate(s) of PINK1 are not resolved, two have been proposed and it is clear that the kinase activity is important. Given a likely functional pool of PINK1 at the cytoplasmic face of the organelle, the protein is positioned to potentially play a role in many aspects of mitochondrial function. The remainder of this review will focus on one facet of mitochondria, namely the regulation of morphology, which is a regulated and dynamic process in many cells and tissues.

PINK1 and mitochondrial dynamics

The combinatorial consequences of mitochondrial dysfunction and oxidative stress, both of which are modified by PINK1, are known to influence mitochondrial morphology. Free radical generation by the respiratory chain (Jezek and Hlavata, 2005) and reactive oxygen species (Pletjushkina et al., 2006) modify mitochondrial morphology (Benard and Rossignol, 2008), leading to rapid mitochondrial fragmentation (Yu et al., 2006).

Three groups concurrently reported abnormal mitochondrial phenotypes, and a genetic interaction between *PINK1* and *parkin*, in *Drosophila* models (Clark et al., 2006, Park et al., 2006, Yang et al., 2006). The phenotype associated with *PINK1* knockout in *Drosophila* was found to be similar to that of *parkin* knockout, with altered mitochondrial morphology consisting of fragmentation and disintegration with loss of cristae, flight defects attributable to the degeneration of flight-wing muscle. Such flies also have an increased sensitivity to oxidative stress (Greene et al., 2003, Pesah et al., 2004) and male sterility due to ineffective spermatid maturation (Clark et al., 2006, Park et al., 2006). Transgenic expression of parkin could rescue both the *PINK1*-null phenotypes of male sterility and mitochondrial abnormalities (Clark et al., 2006, Park et al., 2006). However, *PINK1* transgenic flies could not ameliorate the *parkin*-null phenotype, suggesting that *PINK1* lies upstream of *parkin* in a common genetic pathway. Also, the phenotype of double *parkin/PINK1* null flies resembled that of single mutants, suggesting that *PINK1* and *parkin* do not act in parallel (Clark et al., 2006, Park et al., 2006).

A similar PINK1/parkin relationship may hold in mammalian cells where fragmented and truncated mitochondrial phenotypes associated with the loss of PINK1 could be rescued by overexpression of parkin (Exner et al., 2007). Additionally, altered cristae were observed in PINK1 deficient cells, suggesting one similarity with the *Drosophila* models (Exner et al., 2007). In contrast, no gross mitochondrial morphological defects were found in *PINK1* knockout mice (Kitada et al., 2007), even though functional effects were seen (Gautier et al., 2008). This suggests that mitochondrial morphology changes can result from loss of PINK1 function but may possibly be secondary to other changes. It would therefore seem important to understand the mechanism(s) involved in morphological changes in mitochondria in PINK1 deficient cells or tissues to understand the precise role of PINK1 in this process.

Mitochondria are dynamic organelles that continually undergo the opposing processes of fusion and fission to maintain a distinct morphology. The balance between fission and antagonizing fusion events regulate mitochondrial morphology, which includes controlling the shape, number and length of mitochondria. The shape of the mitochondria corresponds to the metabolic status (Rossignol et al., 2004) and the health of the cell (Youle and Karbowski, 2005). The process of fusion is necessary for maintaining mitochondrial function, including mitochondrial inner membrane potential, respiration, and genomic content (Chen et al., 2003). Fission events mediate apoptosis by regulating the release of pro-apoptotic factors from the intermembrane space to the cytosol. Proteins involved in mitochondrial dynamics originally identified in yeast are highly conserved in mammals. The fission mediators include Drp1 (Dnm1 in yeast) and Fis1, while the mediators of fusion are Mitofusins 1 and 2 (Mfn) (Fzo1 in yeast) and Opa1 (Mgm1 in yeast) (Westermann, 2008). However, additional proteins have been found in mammals that do not appear in yeast, such as Bax (Karbowski et al., 2006) and Endophilin B1 (Karbowski et al., 2004), which also influence mitochondrial dynamics. Yeast also have mitochondrial dynamics players that have not been identified in mammals (Coonrod et al., 2007). Therefore, although many aspects of mitochondrial dynamics are highly conserved from single cell organisms to humans, there are species differences, especially in some of the modulatory machinery for dynamics. Figure 1 illustrates the localization of mammalian proteins involved in mitochondrial dynamics.

In mammalian cells, the large GTPase, dynamin-related protein 1 (Drp1) mediates division of the mitochondrial network (Hoppins et al., 2007, Okamoto and Shaw, 2005). Drp1 belongs to the conserved dynamin large GTPase superfamily that controls membrane tubulation and fission (Praefcke and McMahon, 2004). Dynamin, a Drp1 homolog, is assembled in the cytosol and forms spirals around endosomes to mediate fission from the plasma membrane. Helices of dynamin mediate lipid tubule scission by constricting and twisting the anchored tubules upon GTP cleavage (Danino et al., 2004, Roux et al., 2006). Presumably acting like dynamin,

Drp1 oligomerizes into ring-like structures at division sites on the outer mitochondrial membrane to initiate fission in a GTP-dependent manner (Ingerman et al., 2005). However, unlike dynamin, Drp1 contains a carboxy-terminal GTPase effector domain (GED), which facilitates intra- and intermolecular interactions that regulate GTPase activity (Chang and Blackstone, 2007, Zhu et al., 2004). Dominant negative mutations that block Drp1 GTPase activity result in an elongated mitochondrial phenotype through the inhibition of GTP-mediated fission (Otsuga et al., 1998, Smirnova et al., 2001).

Soluble Drp1 is mostly cytosolic, but can be recruited to the mitochondria to form punctate foci at sites of mitochondrial fission (Smirnova et al., 2001, Wasiak et al., 2007). Both actin filaments and microtubules are integral in the recruitment of Drp1 to mitochondria (De Vos et al., 2005, Varadi et al., 2004). Drp1 recruitment to the outer mitochondrial membrane occurs via a transient interaction with Fis1 (Yoon et al., 2003, Yu et al., 2005), a tetratricopeptide domain protein that contains a C-terminal transmembrane domain localized to the outer mitochondrial membrane and an N-terminus, which protrudes into the cytosol (Yoon et al., 2003, Dohm et al., 2004, Suzuki et al., 2003). It has been suggested that Fis1 acts as a limiting factor in mitochondrial fission, supporting the notion that Fis1 acts as a receptor for Drp1 on the outer mitochondrial membrane (Yoon et al., 2003). However, Drp1 can assemble on mitochondria in the absence of Fis1 (Lee et al., 2004, Stojanovski et al., 2004), leaving the mechanism of Drp1 localization to mitochondria unclear.

Three large GTPases from the dynamin family, Mitofusin 1 (Mfn1), Mitofusin 2 (Mfn2) and Optic atrophy protein 1 (Opa1), are conserved from yeast to mammals and mediate mitochondrial fusion. It has been demonstrated that Mfn1 and Mfn2 regulate fusion of the outer mitochondrial membrane while Opa1 is responsible for fusion of the inner mitochondrial membrane (Meeusen et al., 2006, Meeusen et al., 2004). Although both Opa1 and Mfn1/2 are necessary for mitochondrial fusion, no direct interactions between the two proteins have been observed in mammalian cells. However, Mfn1 was reported to be required for Opa1-dependent inner mitochondrial membrane fusion (Cipolat et al., 2004).

Mitofusins 1 and 2 localize to the outer mitochondrial membrane through two transmembrane domains (Santel and Fuller, 2001) orienting their N-terminal GTPase domains and C-terminal coiled-coil regions towards the cytosol (Rojo et al., 2002) and facilitate the docking of adjacent mitochondria through a tethering mechanism (Koshiba et al., 2004). Trans-mitochondrial tethering occurs when the C-terminal coiled-coil regions of Mfn1 and Mfn2 interact (Chen et al., 2003, Ishihara et al., 2004, Koshiba et al., 2004, Meeusen et al., 2004). The GTPase activity of the mitofusins is necessary for fusion activity in addition to the maintenance of mitochondrial membrane potential (Hales and Fuller, 1997, Hermann et al., 1998, Santel and Fuller, 2001). Some experiments suggest that Mfn1 and Mfn2 proteins are functionally redundant (Chen et al., 2003); however, Mfn1 and Mfn2 seem to have specialized functions despite their similarities in protein structure (Cipolat et al., 2006, Eura et al., 2003). It has been shown that increased GTPase activity of Mfn1 mediates more efficient GTP-dependent mitochondrial tethering than Mfn2 (Ishihara et al., 2004). Using co-immunoprecipitation, anti-apoptotic Bcl-2 and Bcl-xL have been shown to specifically interact with Mfn2 to enhance mitochondrial fusion (Delivani et al., 2006). Mitofusin interactors also include the pro-apoptotic Bcl-2 family members Bax and Bak, which have been shown to interact with both Mfn1 and Mfn2 (Brooks et al., 2007). It has been proposed that mitofusins and Bcl-2 family proteins interact to regulate mitochondrial fusion in healthy cells (Karbowski et al., 2006).

Opa1 is located in the inner mitochondrial membrane, facing the intermembrane space (Olichon et al., 2002) and is required for fusion but not mitochondrial docking (Chen et al., 2005, Cipolat et al., 2004, Olichon et al., 2003). A single gene encodes mammalian Opa1 with 8 transcript variants resulting from alternative splicing (Delettre et al., 2001). Opa1 splice

variants are differentially proteolyzed and yield various long and short isoforms that influence membrane association (Satoh et al., 2003). Longer isoforms, which retain the N-terminal transmembrane segment and the MTS, are more tightly connected to the inner mitochondrial membrane when compared to the shorter isoforms lacking residues required for membrane insertion (Ishihara et al., 2004). Numerous proteases have been identified in Opa1 processing that include paraplegin (Ishihara et al., 2004), PARL (presenilin-associated rhomboid-like) (Cipolat et al., 2006, McQuibban et al., 2003) and Yme1 (Griparic et al., 2007, Song et al., 2007). The identification of multiple splice variants of Opa1 in mammalian cells suggests that many versions of Opa1 may be necessary to differentially regulate mitochondrial morphology with respect to damaging cellular triggers. Cleavage of Opa1 is enhanced under conditions of decreased inner mitochondrial membrane potential suggesting its activity is coordinated with the energetic state of the mitochondria, supporting the notion that high membrane potential is required for fusion (Duvezin-Caubet et al., 2006, Griparic et al., 2004, Ishihara et al., 2004, Meeusen et al., 2006).

Recently it has been proposed that the parkinsonism-associated genes *PINK1* and *parkin* regulate mitochondrial dynamics through interaction with the fission/fusion machinery. Specifically, it is thought that PINK1 and parkin promote mitochondrial fission in *Drosophila.* Introducing an extra copy of *Drp1* rescues wing posture abnormalities caused by degeneration of indirect flight muscle in *PINK1* mutants with both *PINK1* and *parkin* deficient mutants (Deng et al., 2008, Karbowski et al., 2006, Poole et al., 2008, Yang et al., 2008). Fewer swollen and more intact cristae structures were observed in tissue with *Drp1* overexpression when compared to the flight muscle in wild type flies (Poole et al., 2008, Deng et al., 2008). However, *Drp1* overexpression had no effect on mitochondrial morphology in the wild type (ie PINK1 +/+) background (Yang et al., 2008).

As an alternative to the *dPINK1* knockdown model, *dPINK1* overexpression was examined in the compound eye of *Drosophila*, where ectopic *dPINK1* expression causes disruption of ommatidia. Genetic perturbations of *dDrp1* that reduce its activity suppress the *dPINK1* eye overexpression phenotype (Poole et al., 2008). However, although the compound eye is a useful structure to assess genetic relationships, phenotypes associated with *dPINK1* overexpression compared to knockout are functionally different, as *parkin* does not rescue PINK1 overexpression effects (Yang et al., 2008).

Inactivation of *PINK1* in *Drosophila* also leads to loss of dopaminergic neurons (Park et al., 2006, Yang et al., 2006) and overexpression of *Drp1* restores dopamine levels to normal in *dPINK1* null mutant (Yang et al., 2008). GFP labelled mitochondria in dopaminergic neurons in flies are aggregated and tubular in *dPINK1* mutant or *dPINK1* RNAi lines (Yang et al., 2008). Increasing *Drp1* dosage in this mutant *dPINK1* background promotes uniform mitochondrial distribution and eliminates aggregate formation (Yang et al., 2008). *Drp1* overexpression in a wild type background did not influence mitochondrial morphology in this study. Studies in cultured S2R *Drosophila* cells indicate that downregulation of Drp1 results in a singular perinuclear mitochondrial aggregate that resembles the phenotype demonstrated by *dPINK1* silencing (Yang et al., 2008). However, double knockdown of *dPINK1* and *dDrp1* demonstrates extreme fusion of the mitochondrial networking (Yang et al., 2008). Additionally, expression of the Fis1 *Drosophila* homolog in PINK1 silenced S2R cells suppresses the mitochondrial phenotype. Together these data suggest that *Fis1* and *Drp1* are epistatic to *PINK1*.

Some studies characterizing the genetic interactions between *PINK1* and *parkin* in *Drosophila* testes suggest that these parkinsonism associated genes may inhibit mitochondrial fusion (Deng et al., 2008) rather than promote mitochondrial fission. In *Drosophila*, mitochondrial morphology can be examined during spermatogenesis, where germ cells

undergo multiple rounds of mitosis and meiosis with incomplete cytokinesis to yield interconnected early spermatids (Fuller, 1993). During spermatid maturation at the ‘onion stage’, the mitochondria aggregate adjacent to the nucleus (Fuller, 1993) and fuse into two large mitochondrial masses that are arranged by layers of wrapped mitochondrial membrane into a structure called the nebenkern (Lindley, 1980). Dramatic morphological changes occur when the two interlocked subunits of the nebenkern elongate with the growing microtubule-based axenome to promote membrane remodeling and individualization (Fuller, 1998). The *Drosophila* genome encodes two Mfn homologs that regulate mitochondrial fusion, *Fuzzy onion* (*Fzo*) and *Marf* (*Mitochondrial assembly regulatory factor*). *Fzo* expression is limited to the testes and mutations cause mitochondrial fusion deficits and male sterility due to defective nebenkern formation (Hales and Fuller, 1997). *Marf* is expressed in germline and somatic cells (Hwa et al., 2002). During spermatogenesis, *dPINK1* or *dparkin* loss of function results in defects in mitochondria morphology as observed by the vacuolated ‘onion stage’ nebenkern (Clark et al., 2006, Deng et al., 2008, Riparbelli and Callaini, 2007). *Fzo* mutants exhibit onion-staged nebenkern composed of many small mitochondria and, as a consequence, nebenkern borders appear irregular but this is morphologically different from the PINK1 phenotype (vacuolated with regular borders). Double mutants with *dPINK1* and *Fzo* loss of function exhibit a single elongated mitochondrial derivative and irregular nebenkern borders associated with *Fzo* mutants were rescued by *dPINK1* mutation (Deng et al., 2008). These data support the notion that PINK1 plays a role in mitochondrial morphology but suggest that PINK1 is not a core component of the fusion/fission machinery but rather plays a modulatory role.

The *Drosophila* genome also encodes a single homolog of *Opa1* (Yarosh et al., 2008) that functions to regulate mitochondrial fusion of the inner mitochondrial membrane. Loss of function mutations in *Drosophila Opa1* or *Mfn2* homologs suppresses the *dPINK1* and *dparkin* flight and climbing phenotypes (Poole et al., 2008). Moreover using the *dPINK1* overexpression eye phenotype, alterations that decrease dOpa1 and Marf activity enhance the eye phenotype associated with PINK1/parkin pathway activation (Poole et al., 2008). PINK1-dependent inhibition of fusion was further studied in the indirect flight muscle using mitochondrial directed GFP and phalloidin to simultaneously label mitochondria and actin, respectively. In addition to muscle specific downregulation of *Marf* in flies, *dOpa1* RNAi in muscle resulted in mitochondrial fragmentation that is relatively similar to *dDrp1* overexpression in transgenic flies (Deng et al., 2008).

These studies of mitochondrial dynamics in *Drosophila* models have therefore provided a great deal of insight into the relationships of PINK1 and parkin to mitochondrial morphology, especially in flight muscles and in testes. However, PINK1 silencing studies performed in mammalian cells (Exner et al., 2007, Weihofen et al., 2009) or *C. elegans* (Ichishita et al., 2008) have shown very different effects of PINK1/parkin on morphology. The genetic interaction between PINK1 and parkin initially identified in *Drosophila* is conserved in mammalian cells in that parkin can rescue loss of PINK1. However, PINK1 deficient mammalian cells have a fragmented and truncated mitochondrial morphology, which would suggest an imbalance towards fission (Exner et al., 2007, Weihofen et al., 2009). Data from our own studies in mammalian cells also supports a fragmented mitochondrial phenotype after PINK1 silencing (KJ Thomas, unpublished observations) and parkin deficient fibroblasts have decreased mitochondrial connectivity (Mortiboys et al., 2008). Additionally, in a high throughput system for examining modulators of mitochondrial morphology in *C. elegans*, knockdown of the PINK1 homolog in that species also resulted in fission, not fusion. What possible explanations could there be for apparently disparate results in different models?

The dynamic life cycle of a mitochondrion is heavily dependent upon mitochondrial membrane potential (Duvezin-Caubet et al., 2006, Griparic et al., 2004, Ishihara et al., 2004, Meeusen et

al., 2006, Twig et al., 2008). The downregulation of PINK1 causes loss of mitochondrial membrane potential in several mammalian systems (Abou-Sleiman et al., 2006, Exner et al., 2007, Valente et al., 2004b). Therefore, loss of $\Delta\psi_m$ associated with the loss of PINK1 may be a primary event that leads to secondary effects mitochondrial morphology that may result in apparent fission or fusion depending on the tissue/cell type or status. As recent data suggests that fusion and fission are linked (Twig et al., 2008), the apparent occurrence of either fusion or fission may not be instructive as to which is the primary event. Coupled with evidence comparing PINK1 mutants with mutations in the fission/fusion machinery (Deng et al., 2008), the most parsimonious interpretation is that the effects of PINK1 on morphology are indirect.

An additional question regarding the relationship between PINK1 and mitochondrial morphology occurs because of evidence of multiple roles of mitochondria in apoptosis. Mitochondrial membrane permeabilization is often considered the “point of no return” in events leading to cell death as during apoptosis, the permeability of the outer mitochondrial membrane increases allowing soluble, intermembrane space proteins to be released into the cytoplasm (Adrain et al., 2001, Patterson et al., 2000, Susin et al., 1999). PINK1 has been shown to block apoptosis induced by staurosporine, a general kinase inhibitor (Gelmetti et al., 2008, Petit et al., 2005, Wood-Kaczmar et al., 2008) and degeneration of indirect fly muscles in *Drosophila* from knockout animals includes apoptosis. Overexpression of PINK1 and parkin in cultured cells can prevent the release of cytochrome c and caspase activation (Darios et al., 2003, Gelmetti et al., 2008, Petit et al., 2005, Pridgeon et al., 2007). However, apoptosis is associated with mitochondrial fission and there is therefore difficult to understand how a molecule could promote fission but limit apoptosis. It is known that Drp1-mediated mitochondrial fission and fragmentation of the mitochondrial network occurs during programmed cell death in *Drosophila* (Abdelwahid et al., 2007, Goyal et al., 2007). In recent studies on overexpression of parkin, increased expression did not induce fission (as would be predicted from the idea that PINK1/parkin promotes fission or limits fusion) but instead resulted in the formation of extended organelles that were then degraded by autophagy (Narendra et al., 2008). One possibility is, again, that the effects on morphology are secondary to the direct effects of PINK1, which may include protection of the organelle. Clearly, the resolution of these questions will require a more detailed understanding of the mechanism(s) by which PINK1 and its partner, parkin, influences both cellular viability and mitochondrial morphology in different tissues and cells.

Mechanisms involved in the PINK1/parkin pathway and effects on mitochondria

How do PINK1 and parkin influence mitochondria? One current controversy surrounds the localization of PINK1 and parkin. The majority of parkin is cytoplasmic in most cells, including neurons (Cookson et al., 2003, Hase et al., 2002), although a fraction is found at the outer mitochondrial membrane (Darios et al., 2003, Narendra et al., 2008). Kuroda et al. suggested that parkin preferentially localizes to the mitochondrial matrix, and mitochondrial biogenesis is enhanced through an association with mitochondria transcription factor A in dividing cells (Kuroda et al., 2006). As previously described, PINK1 is found in both the mitochondria (Beilina et al., 2005, Gandhi et al., 2006, Plun-Favreau et al., 2007, Lin and Kang, 2008, Muqit et al., 2006, Pridgeon et al., 2007, Silvestri et al., 2005, Weihofen et al., 2009) and the cytoplasm (Beilina et al., 2005, Haque et al., 2008, Lin and Kang, 2008, Takatori et al., 2008, Weihofen et al., 2008, Weihofen et al., 2009, Zhou et al., 2008). Whether PINK1 and parkin proteins physically interact is unclear. It is plausible that they associate in the cytoplasm before PINK1 is transported to the mitochondria or perhaps they interact after mitochondrial import and processing of PINK1.

Alternatively, PINK1 and parkin might modify one another indirectly. For example, they may share substrates, such as the mitochondrial proteins released into the cytoplasm under stress conditions or other proteins mediating cell death and/or survival. Parkin may only ubiquitylate substrates that have been phosphorylated by PINK1. It is also possible that parkin can only function as a ubiquitin ligase after phosphorylation by PINK1 whose kinase activity is upregulated in response to oxidative stress.

Might post-translational regulation of key players in the mitochondrial dynamics process be targets for phosphorylation or ubiquitylation by PINK1/parkin, respectively? Drp1 activity is enhanced by cyclin-dependent kinase 1/cyclin B phosphorylation of Ser618 (human Drp1; Ser585 in rat Drp1) during mitosis (Taguchi et al., 2007) and cAMP-dependent protein kinase A (PKA) inactivates Drp1 GTPase activity by phosphorylation of Ser637 (human Drp1; Ser656 in rat Drp1) which results in mitochondrial fusion (Chang and Blackstone, 2007, Cribbs and Strack, 2007). The Ser637 residue is conserved in *Drosophila*, but according to these reports if Drp1 phosphorylation in flies mimics that which occurs in mammalian cells, PINK1 would limit mitochondrial fission. Whether PINK1 influences mitochondrial morphology through direct phosphorylation of Drp1 remains to be determined.

SUMOylation also regulates Drp1 activity and mitochondrial morphology (Harder et al., 2004) (Zunino et al., 2007). SUMOylation of Drp1 enhances its activity, positively regulating mitochondrial fission. During apoptosis, more Drp1 protein associates with the outer mitochondrial membrane and Drp1 SUMOylation increases (Wasiak et al., 2007), resulting in increased mitochondrial fission. Parkin has been shown to physically interact *in vitro* and *in vivo* with SUMO1 to increase its nuclear transport and self-ubiquitination (Um and Chung, 2006). Downregulation of SUMO1 may inhibit mitochondrial fission caused by excess parkin turnover in the *dPINK1*/*dparkin* pathway and the epistatic *Drp1*.

The mitofusins, which regulate fusion events in yeast (Fzo1) and mammals, may be regulated by ubiquitylation. During the mating of yeast, mitochondria fragment and the levels of Fzo1 decrease. Chemical inhibition (Neutzner and Youle, 2005) or genetic inactivation (Escobar-Henriques et al., 2006) of the proteasome attenuates the loss of Fzo1. Fzo1 is removed from the outer mitochondrial membrane and degraded following lysine 48-dependent ubiquitination to regulate its function in a process similar to ERAD (ER-associated degradation) removal and degradation of membrane spanning proteins. Mammalian mitofusin protein levels increase after exposure to proteasome inhibitors in culture (Karbowski et al., 2007), suggesting the ubiquitin proteasome pathway regulates these fusion proteins.

As an E3 ligase, parkin can promote both degradative and nondegradative forms of ubiquitination (Doss-Pepe et al., 2005, Joch et al., 2007, Lim et al., 2006). For instance, ubiquitin lysine 48-linked polyubiquitin chains target substrates to the proteasome (Imai et al., 2000), whereas ubiquitin lysine 63-linked chains control non-degradative processes including kinase activation, DNA repair, translational regulation and endocytosis of membrane proteins (Doss-Pepe et al., 2005, Joch et al., 2007, Moore et al., 2008, Pickart and Fushman, 2004). It is notable that ubiquitin lysine 63-linked chains promote the degradation of membrane proteins by the lysosome, a putative link supporting the notion that parkin may be a quality control mechanism in *Drosophila* whereby the PINK1/parkin pathway promotes fission to degrade impaired mitochondria by autophagy. In fact, it has been shown that overexpression of parkin in mammalian cells selectively recruits dysfunctional mitochondria with low membrane potential and promotes their autophagy (Narendra et al., 2008). The loss of parkin associated with recessive parkinsonism suggests that parkin-mediated ubiquitination of specific cellular substrates is required for the survival of dopaminergic neurons. At the present it is unclear how parkin deficiencies might precipitate defects in mitochondrial function and whether this relates

to the accumulation of toxic parkin substrates or loss of a non-degradative regulatory role of parkin-mediated ubiquitination.

Morphological changes in mitochondria can be influenced by directed movement or anchoring of these organelles by various components of the cytoskeleton (Rube and van der Bliek, 2004). In cultured cells, disruption of the microtubule network affects the distribution of mitochondria (Heggeness et al., 1978), suggesting that mitochondria require microtubules for normal cellular distribution. Secondary messengers, such as calcium, can influence movement of mitochondria along microtubule tracks. For instance, mitochondrial motility is blocked by increased mitochondrial calcium uptake (Rintoul et al., 2003), suggesting that local calcium buffering limits mitochondrial movement. Moreover, calcium signaling can influence the function of molecular motors (Marston, 1995) that facilitate mitochondrial movement along cytoskeletal elements. Recently, it was shown that PINK1 forms a multi-protein complex with an atypical calcium-dependent GTPase Miro and the adaptor protein Milton (Weihofen et al., 2009), a complex which links the heavy chain of kinesin to mitochondria for anterograde transport of mitochondria along microtubules (Glater et al., 2006, Stowers et al., 2002). Overexpression of Miro and Milton rescued mitochondrial fragmentation associated with the loss of PINK1 and increased mitochondrial localized PINK1 protein, further suggesting a role for PINK1 at the outer mitochondrial membrane and linking PINK1 function to mitochondrial trafficking (Weihofen et al., 2009). These aspects of mitochondrial transport may be especially important in neurons where mitochondria are transported out of synapses. Future studies should address the effects of PINK1/parkin pathway perturbations not only on mitochondrial calcium regulation and buffering but also investigate the effect of the differences between metabolically homogeneous and heterogeneous pools of mitochondria on mitochondrial transport and trafficking.

Other putative links between defects in mitochondrial transport and recessive parkinsonism include the association of parkin with the cytoskeleton or the influence of oxidative stress on cytoskeletal function. Parkin is reported to associate with cytoskeletal elements, such as actin filaments (Huynh et al., 2000) and microtubules (Ren et al., 2003). Studies suggest that the binding of parkin stabilizes microtubules (Yang et al., 2005). Stabilization of the cytoskeletal network may be an important link between parkin and mitochondrial dynamics because disruption of microtubules can cause aberrant mitochondrial distribution (Yaffe et al., 1996). On the other hand, this interaction may simply provide transport of misfolded proteins for degradation (Yang et al., 2005). Additionally, oxidative stress is associated with microtubule disorganization (Annunen-Rasila et al., 2007) and oxidative insult can impair mitochondrial trafficking (Liu et al., 2008). The idea of oxidation being detrimental to mitochondrial plasticity is further supported by the notion that rotenone selectively kills neurons via a microtubule-dependent mechanism (Ren and Feng, 2007). While the shape and connectivity of the mitochondria are likely to influence the ability of the cytoskeleton to move the organelle, it is unknown if altered transport is the result of defects in morphology or vice versa.

Concluding remarks

In addition to summarizing the role of PINK1 in mitochondrial function, this review has attempted to critically analyze the newly proposed relationship between parkinsonism and mitochondrial dynamics. Evolutionarily conserved from yeast to humans, mitochondrial morphogens regulate the genome, membrane integrity, bioenergetic connectivity and turnover of mitochondria. We suggest here that the PINK1/parkin pathway is also conserved from *Drosophila* to mammals but those differences in the output, in terms of mitochondrial morphology, imply that it is likely that the primary effect is on mitochondrial function and any changes in morphology are probably secondary. Figure 2 illustrates the possible direct effects of PINK1/parkin on the life cycle of a mitochondrion, which may influence other aspects of

organellar function, such as transport or degradation (mitophagy), in addition to mitochondrial membrane potential. Future work needs to address in detail the biochemical roles of PINK1/parkin in terms of substrates for both proteins, as well as their relationship to each other.

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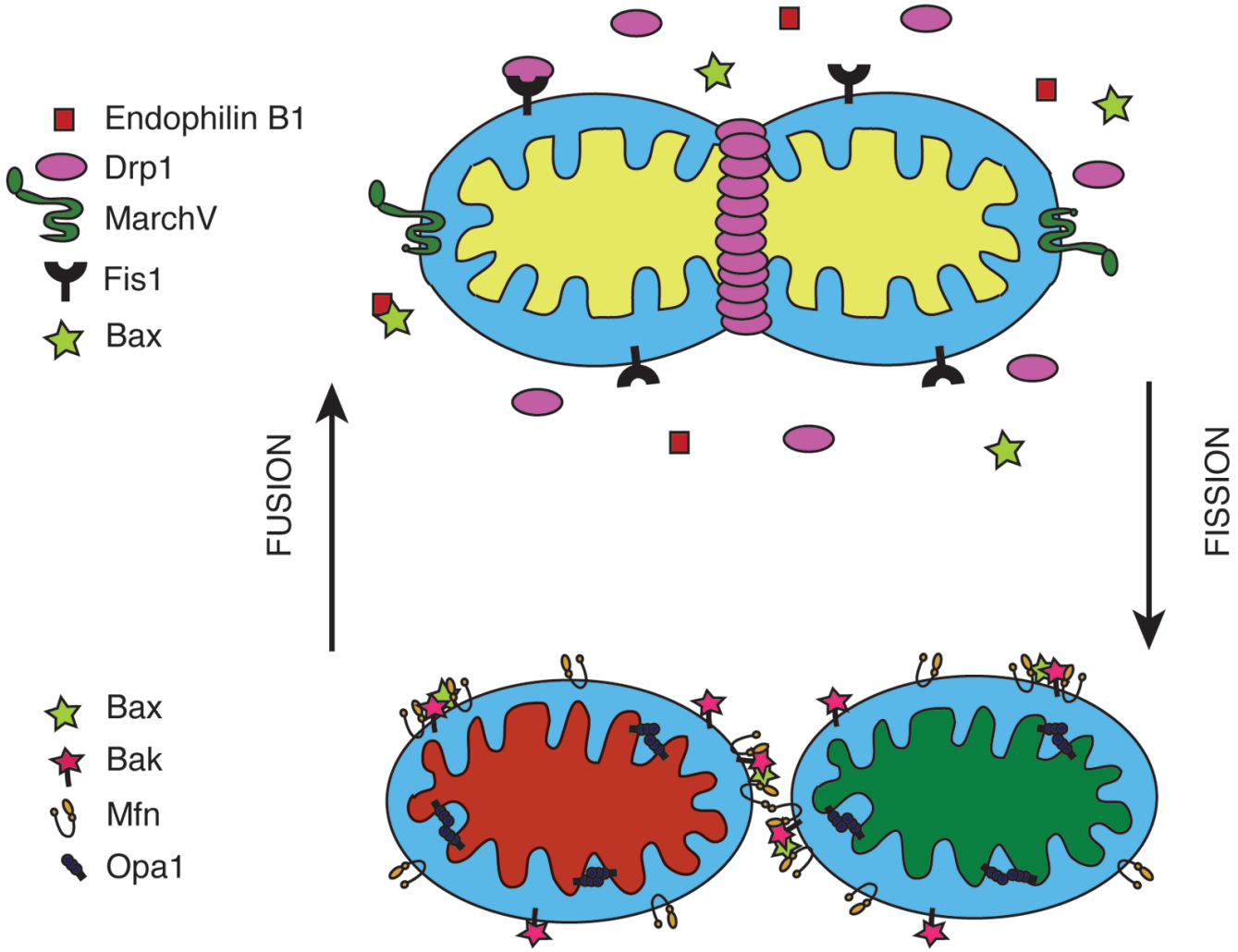

Figure 1. Mitochondrial fission and fusion machinery

A schematic of the localization of the proteins involved in mitochondrial dynamics is illustrated. Mitochondrial fission divides a mitochondrion into two daughter units by the coordinated actions of Drp1, Endophilin B1, Fis1 and MarchV. Tethering of two mitochondria results in mitochondrial fusion of the membranes mediated by Mfn and Opa1. Bcl-2 family proteins, Bax and Bak are involved primarily in the induction of apoptosis, which influences mitochondrial dynamics.

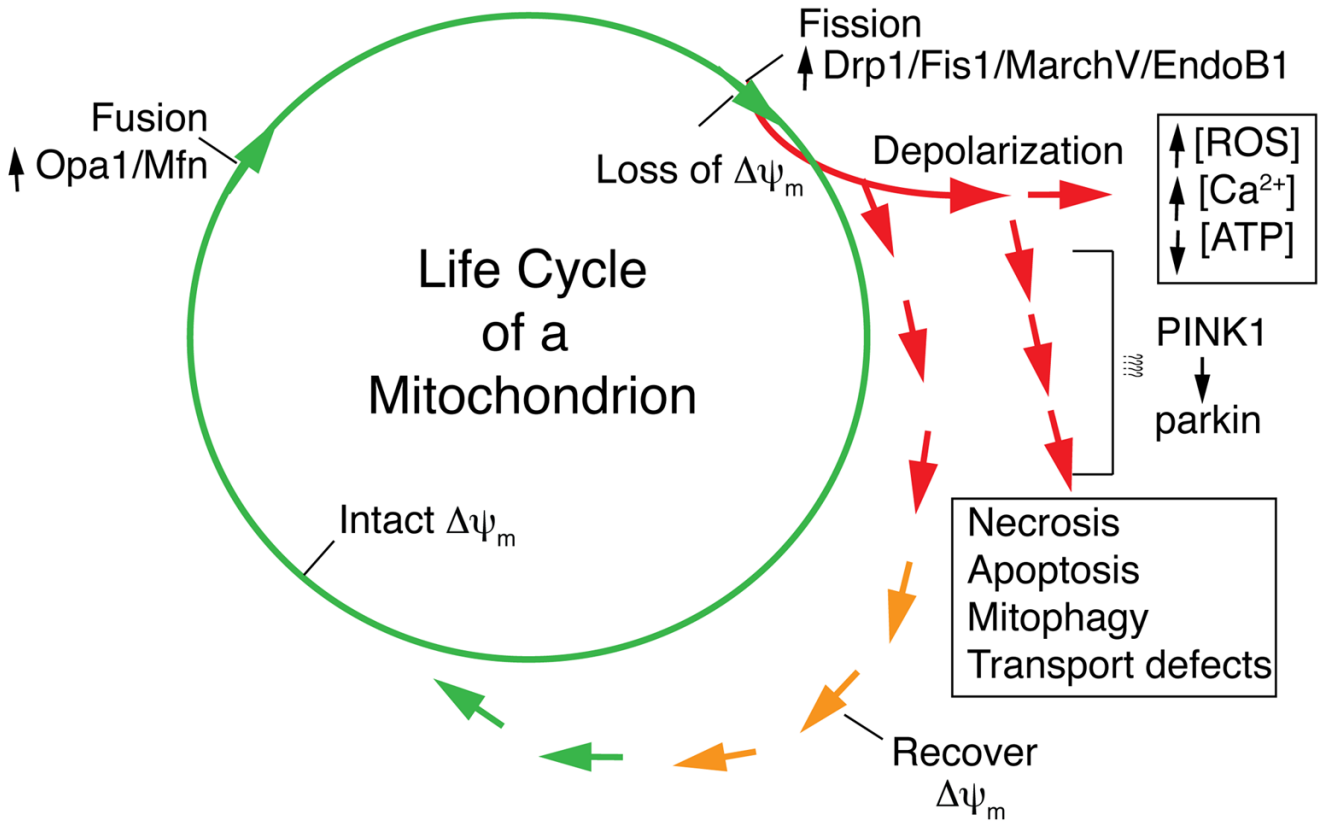

Figure 2. Model for the life cycle of a mitochondrion

This proposed model for the life cycle of a mitochondrion reflects mitochondrial dynamics and turnover (adapted from (Twig et al., 2008)). In a healthy cell, the antagonizing events of mitochondrial fusion and fission are dependent upon mitochondrial membrane potential. Following a mitochondrial fission event, fragmented mitochondria may either maintain an intact membrane potential (green line) or depolarize (red line). Should mitochondrial membrane depolarization take place prior to fission, it is unlikely to proceed to fusion without repolarization. Consequences of depolarization include the production of reactive oxygen species (ROS), increased cytosolic [Ca^{2+}] or loss of ATP with subsequent effects on various types of cell death and/or impaired mitochondrial transport and trafficking. PINK1 lies upstream of parkin in a conserved genetic pathway as indicated. Exactly how this relates to mitochondrial morphology is unclear, but we suggest here that the major effect of PINK1/parkin might be on the turnover of damaged mitochondria and thus may be tangential to the fusion/fission machinery.