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Review

Mitochondrial dysfunction in Parkinson's disease: molecular mechanisms and pathophysiological consequences

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Neurons are critically dependent on mitochondrial integrity based on specific morphological, biochemical, and physiological features. They are characterized by high rates of metabolic activity and need to respond promptly to activity-dependent fluctuations in bioenergetic demand. The dimensions and polarity of neurons require efficient transport of mitochondria to hot spots of energy consumption, such as presynaptic and postsynaptic sites. Moreover, the postmitotic state of neurons in combination with their exposure to intrinsic and extrinsic neuronal stress factors call for a high fidelity of mitochondrial quality control systems. Consequently, it is not surprising that mitochondrial alterations can promote neuronal dysfunction and degeneration. In particular, mitochondrial dysfunction has long been implicated in the etiopathogenesis of Parkinson's disease (PD), based on the observation that mitochondrial toxins can cause parkinsonism in humans and animal models. Substantial progress towards understanding the role of mitochondria in the disease process has been made by the identification and characterization of genes causing familial variants of PD. Studies on the function and dysfunction of these genes revealed that various aspects of mitochondrial biology appear to be affected in PD, comprising mitochondrial biogenesis, bioenergetics, dynamics, transport, and quality control.

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Introduction

Parkinson's disease (PD) is a heterogeneous neurodegenerative disease entity typically diagnosed by its cardinal motor symptoms, including bradykinesia, hypokinesia, rigidity, resting tremor, and postural instability, which are subsumed under the syndrome of parkinsonism. The motor manifestations are attributable to the degeneration of dopaminergic (DA) neurons within the substantia nigra pars compacta (SNc), resulting in dopamine depletion and derangements of neuronal circuits in the basal ganglia target regions of these neurons. Another pathological hallmark of PD is the presence of α-synuclein-containing deposits in neuronal perikarya (Lewy bodies) and processes (Lewy neurites). The role of Lewy bodies in the pathogenic process is discussed controversially. Parkinsonism can occur in the absence of Lewy bodies, for instance in some cases of familial PD or in drug-induced parkinsonism (Davis *et al*, 1979; Langston *et al*, 1999; Nuytemans *et al*, 2010). On the other hand, Lewy body pathology is sometimes found at autopsy in individuals without reported symptoms of parkinsonism (Jellinger, 2009; Adler *et al*, 2010). The manifestation of non-motor symptoms, some of which even precede the motor symptoms, reflect the fact that the neurodegenerative process is not limited to the SNc but has a much wider impact. Non-motor symptoms, such as autonomic dysfunction, sleep abnormalities, depression, and dementia, can contribute considerably to disability, as they usually are not responsive to dopamine replacement therapy.

The etiopathogenesis of sporadic PD, the most common form of parkinsonism, is complex with variable contributions of genetic susceptibility and environmental factors (Figure 1). Ageing is one of the most important risk factors for sporadic PD. Given the demographic trend towards an aged population, the prevalence of PD and thus its socioeconomic burden will increase dramatically in the next decades. Over the last 15 years enormous effort has been taken to unravel the role of genetics in PD pathogenesis. Linkage analyses discovered six genes associated with Mendelian forms of parkinsonism, and genome-wide association studies identified susceptibility genes contributing to the risk for sporadic PD. Strikingly, there is an overlap between Mendelian genes and risk genes in the case of *α-synuclein* and *leucine-rich repeat kinase 2* (*LRRK2*), blurring the traditional boundaries between familial and sporadic PD. The identification of genes associated with parkinsonism has had a major impact on PD research, allowing to dissect molecular pathways implicated in the pathogenesis. From genetic cellular and animal models, it emerged that mitochondrial alterations, oxidative stress, and impaired clearance of misfolded proteins and damaged orga-

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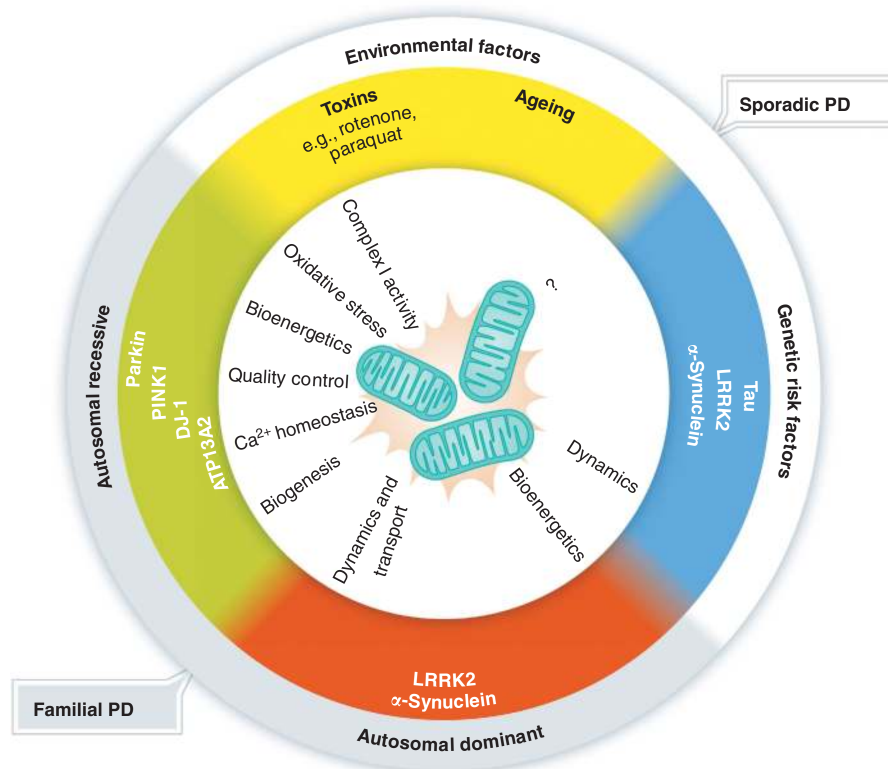

Figure 1 Aetiology of Parkinson's disease (PD) and possible links to mitochondrial integrity. Familial PD is caused by mutations in genes identified by linkage analyses that are inherited in an autosomal recessive or dominant manner. Sporadic PD is considered to be a complex neurodegenerative disease entity with both genetic susceptibility and environmental factors contributing to the etiopathogenesis. Recent genome-wide association studies have identified susceptibility loci, which in two cases (*α-synuclein* and *LRRK2*) overlap with classical PD genes, linking the aetiology of familial parkinsonism with that of sporadic PD. Both genetic and environmental factors influence various mitochondrial aspects, such as bioenergetics, dynamics, transport, and quality control.

nelles by proteasomal and lysosomal degradation pathways contribute to the disease process (reviewed in Dawson *et al*, 2010; Corti *et al*, 2011; Martin *et al*, 2011; and Shulman *et al*, 2011). Moreover, there is increasing evidence that sporadic and familial variants of PD share some common pathways that converge at mitochondria (reviewed in Abou-Sleiman *et al*, 2006; Lin and Beal, 2006; Mandemakers *et al*, 2007; Bogaerts *et al*, 2008; Henchcliffe and Beal, 2008; Schapira, 2008; Vila *et al*, 2008; Van Laar and Berman, 2009; Bueler, 2010; Burbulla *et al*, 2010; Winklhofer and Haass, 2010; and Schon and Przedborski, 2011). In the following, we will review our current knowledge on the role of mitochondria in PD pathogenesis and how these insights have changed our conceptional thinking and may eventually be translated into novel neuroprotective approaches.

Mitochondrial dysfunction in sporadic PD

The role of complex I deficiency and mitochondrial DNA mutations

The first link between parkinsonism and mitochondria became evident in the early 1980s, when it was discovered that a neurotoxin causing a parkinsonian syndrome inhibits mitochondrial respiration. MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), a contaminant of an illicit opioid preparation which was used intravenously by drug addicts, can cross the blood–brain barrier and is taken up by DA neurons via the dopamine transporter after oxidation to MPP^+ (Langston *et al*, 1983; Nicklas *et al*, 1985). Within DA neurons, MPP^+ accumulates in mitochondria and inhibits complex I (NADH ubiquinone oxidoreductase) of the electron transport chain. Although MPTP-induced parkinsonism results from an acute toxic insult and therefore differs from the slow and progressive disease process in sporadic PD, the impact of MPTP has been far reaching. In particular, MPTP and other complex I inhibitors such as rotenone are still being used to model PD in animals and to evaluate therapeutic approaches (reviewed in Hirsch, 2007; Bezard and Przedborski, 2011; and Cannon and Greenamyre, 2011). Interestingly, consumption of fruit and herbal teas from plants of the *Annonceae* family, containing the complex I inhibitor annonacin, has been linked to the high frequency of atypical parkinsonism in Guadeloupe (Caparros-Lefebvre and Elbaz, 1999; Lannuzel *et al*, 2003; Champy *et al*, 2004), further substantiating a causal role of mitochondrial dysfunction in the pathogenesis of at least some parkinsonian syndromes. In support of a direct or indirect role of complex I, the activity of complex I has been reported to be reduced (in the range of 30%) in the SNc and frontal cortex of PD patients at autopsy (Schapira *et al*, 1989; Parker *et al*, 2008). In mitochondrial preparations from PD frontal cortex samples, complex I subunits derived from both mitochondrial and nuclear genomes were found to be oxidatively damaged, reflected by an increase in protein carbonyls (Keeney *et al*, 2006). This study also reported that the levels of an 8-kDa subunit of complex I were

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reduced by 33% in PD frontal cortex, suggesting that oxidative damage may cause misassembly or reduced stability of complex I subunits. Remarkably, in about 25% of PD patients analysed complex I activities were found to be reduced also in platelets (reviewed in Schapira, 2008). This finding may indicate a systemic complex I defect in a subfraction of PD patients due to genetic and/or environmental causes. In a mouse model of mild complex I deficiency induced by the dopamine neuron-specific loss of the Ndufs4 subunit, increased striatal dopamine turnover rates and decreased dopamine release from striatal axon terminals have been observed (Sterky *et al*, 2012). These alterations in striatal dopamine homeostasis may be caused by a reduced vesicular uptake of dopamine due to ATP deficiency followed by enhanced cytosolic dopamine metabolism, suggesting that impaired dopamine release may be an early consequence of mitochondrial impairment (Choi *et al*, 2011; Sterky *et al*, 2012).

Mitochondrial oxidative phosphorylation depends on both mitochondrial and nuclear DNA-encoded proteins. Mitochondrial DNA (mtDNA) encodes 13 proteins that are all subunits of respiratory chain complexes, 22 tRNAs, and 2 rRNAs. Mutations in mtDNA can be either inherited maternally or acquired and typically cause variable phenotypes in cells with high energy demands, such as neurons and muscle cells. Mouse models with defects in genes essential for the maintenance of mtDNA support the notion that alterations in the mitochondrial genome cause respiratory chain deficiencies and phenotypes associated with ageing and age-related diseases (reviewed in Reeve *et al*, 2008; Larsson, 2010; and Park and Larsson, 2011). Transgenic mice expressing a proofreading-deficient version of the mtDNA polymerase γ (POLG) accumulate mtDNA mutations and display features of premature ageing (Trifunovic *et al*, 2004; Kujoth *et al*, 2005). Notably, cosegregation of parkinsonism with mutations in the human *POLG1* gene has been reported in several families (reviewed in Orsucci *et al*, 2011). In support of a causative role of mitochondrial dysfunction in PD, mice with a DA neuron-specific deletion of the mitochondrial transcription factor TFAM, which is essential for mitochondrial transcription and maintenance of mtDNA, develop a parkinsonian phenotype reproducing key features of PD: adult onset, progressive impairment of motor functions responsive to L-DOPA therapy, and loss of midbrain DA neurons (Ekstrand *et al*, 2007). Similarly, expression of mitochondrially targeted *Pst*I endonuclease in DA neurons, which induces double-strand breaks in mtDNA, causes progressive neuronal degeneration and striatal dopamine depletion (Pickrell *et al*, 2011).

An age-dependent increase in mtDNA deletions has been found in individual DA neurons dissected from the SNc of *post mortem* human brain (Bender *et al*, 2006; Kraytsberg *et al*, 2006). Neurons harbouring $>60\%$ of mtDNA molecules with deletions showed a significant decrease in cytochrome c oxidase, three catalytic subunits of which are encoded by mtDNA. Different types of mtDNA deletions were found in the same individual, but each neuron contained only a single mtDNA mutation, indicating that the mutation was acquired and clonally expanded. In comparison with age-matched controls, the amount of mtDNA mutations was slightly higher in DA neurons from PD patients (Bender *et al*, 2006). Moreover, SNc neurons seem to be particularly vulnerable to mtDNA mutations, since hippocampal neurons or pyramidal cortical neurons of aged individuals did not contain high levels of mtDNA mutations.

There is currently no strong evidence that mtDNA mutations are a major primary cause of PD. However, it seems quite plausible that mtDNA mutations accumulate in the course of the disease as a consequence of an increase in cellular stress and mitochondrial replication errors along with a decrease in the fidelity of quality control systems. Once the mtDNA mutations surpass a critical threshold, the resulting respiratory deficiency may contribute to neuronal degeneration and cell death. Of note, complex I is particularly vulnerable to mtDNA damage, since seven of its subunits are encoded by mtDNA.

Mitochondrial dysfunction and oxidative stress

According to a widespread concept, inhibition of complex I decreases mitochondrial ATP production and increases the formation of reactive oxygen species (ROS), which damage mtDNA, components of the respiratory chain and other mitochondrial factors, thereby triggering a vicious circle between mitochondrial impairment and oxidative stress (reviewed in Abou-Sleiman *et al*, 2006; Lin and Beal, 2006; and Henchcliffe and Beal, 2008). This model has been particularly popular to explain the increased vulnerability of SNc DA neurons, since this neuronal population is characterized by a high oxidative burden and a low anti-oxidant capacity. Mitochondria can be both a source and a target of ROS (reviewed in Starkov, 2008 and Murphy, 2009). However, an obligatory link between mitochondrial dysfunction and increased ROS production has been questioned based on weak experimental support for such a scenario *in vivo* (reviewed in Fukui and Moraes, 2008; Gems and Doonan, 2009; and Park and Larsson, 2011). For example, rotenone toxicity has been reported to be caused by spare respiratory capacity rather than oxidative stress. In primary neurons, rotenone does increase the formation of mitochondrial superoxide, however, trapping superoxide fails to reduce rotenone toxicity (Yadava and Nicholls, 2007). In addition, various mouse models with severe respiratory chain deficiency display increased apoptotic cell death but not increased ROS formation or oxidative stress (Wang *et al*, 2001; Kujoth *et al*, 2005; Trifunovic *et al*, 2005; Kruse *et al*, 2008). Moreover, ROS are not always harmful agents, they also act as important signal transducers in a variety of biological processes.

Mitochondrial effects of genes associated with PD

Parkin: a versatile neuroprotective E3 ubiquitin ligase

The *parkin* gene has been identified in 1998 as a causative gene for autosomal recessive parkinsonism (Kitada *et al*, 1998). More than 100 pathogenic *parkin* mutations have been reported, accounting for the majority of autosomal recessive parkinsonism. The *parkin* gene encodes a cytosolic 465 amino-acid protein with a ubiquitin-like (UBL) domain at the N-terminus and an RBR (RING-between-RING) domain close to the C-terminus. The RBR domain is composed of two RING fingers that flank an in-between RING (IBR) domain and coordinates six zinc ions. An additional RING finger domain (RING0) has been

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identified between the UBL and RBR motifs, which contributes to the binding of zinc ions (Hristova *et al*, 2009). Coordination of zinc ions (eight Zn^{2+} in total) is essential for parkin to adopt and maintain its correct three-dimensional structure, consistent with the observation that pathogenic mutations within the zinc-binding motifs are inactivated by misfolding (Cookson *et al*, 2003; Gu *et al*, 2003; Sriram *et al*, 2005; Wang *et al*, 2005b, 2007). Moreover, the cysteine-rich RBR domain renders parkin vulnerable to inactivation by severe oxidative stress (Winklhofer *et al*, 2003; LaVoie *et al*, 2007; Wong *et al*, 2007; Schlehe *et al*, 2008). Oxidatively modified, misfolded parkin has indeed been found in the brains of PD patients, suggesting that inactivation of parkin may also play a role in sporadic PD (Pawlyk *et al*, 2003; Chung *et al*, 2004; Yao *et al*, 2004; LaVoie *et al*, 2005; Wang *et al*, 2005a). In support of this notion, the c-Abl tyrosine kinase has been reported to be activated in DA neurons of sporadic PD patients, leading to the phosphorylation and inactivation of parkin (Ko *et al*, 2010; Imam *et al*, 2011).

The presence of the RBR domain suggested that parkin acts as an E3 ubiquitin ligase, mediating the covalent attachment of ubiquitin moieties to substrate proteins (Shimura *et al*, 2000; Zhang *et al*, 2000). Considerable evidence has been accumulated indicating that parkin can catalyse various modes of ubiquitination, including poly-ubiquitination with different lysine linkages or mono-ubiquitination. The linkage type determines the fate of the ubiquitinated protein; ubiquitin chain linkage via Lys48 typically targets substrates for proteasomal degradation, whereas linkage involving other lysine residues and mono-ubiquitination or multiple mono-ubiquitination are implicated in numerous regulatory processes, such as signal transduction, trafficking, DNA damage response, DNA repair, and autophagy (reviewed in Komander, 2009; Ikeda *et al*, 2010; and Behrends and Harper, 2011). There are two major classes of E3 ubiquitin ligases: HECT ligases transiently accept ubiquitin from an E2 conjugating enzyme at a cysteine residue within the HECT domain to form a thioester, whereas RING-type ligases act as bridging proteins that bring the ubiquitin-charged E2 in close proximity to the substrate, but are not ubiquitinated themselves (reviewed in Deshaies and Joazeiro, 2009). It was recently shown that parkin functions as an RING/HECT hybrid: RING1 binds to a ubiquitin-charged E2 conjugating enzyme, which transfers ubiquitin to a conserved cysteine residue in RING2, thereby forming a thioester between parkin and ubiquitin (Wenzel *et al*, 2011). Ubiquitin is then discharged to a lysine residue of the substrate protein. So far, this mechanism has been demonstrated *in vitro* with recombinant parkin and auto-ubiquitination of parkin as a surrogate substrate, but undoubtedly, these findings have a major impact on our understanding of the substrate specificity of RBR E3 ligases.

To date, about 30 putative parkin substrates have been reported and both degradative and non-degradative ubiquitination were attributed to parkin (reviewed in Dawson and Dawson, 2010). These substrates do not fit into a common pathway that could unravel the function of parkin. However, from a plethora of studies in cellular and animal models it emerged that parkin has a remarkably wide protective capacity. The increased expression of parkin both *in vitro* and *in vivo* protects against cell death in various stress paradigms, such as mitochondrial stress, endoplasmic reticulum (ER) stress, excitotoxicity, and proteotoxic stress (reviewed in Moore, 2006 and Pilsl and Winklhofer, 2012). *Vice versa*, parkin-deficient cells are characterized by an increased vulnerability to stress-induced cell death. Surprisingly, the sensitivity of parkin knockout (KO) mice to neurotoxins, such as MPTP or 6-OHDA seems not to be increased; nigral degeneration in parkin KO mice has only been reported after inflammatory stimulation by lipopolysaccaride (Perez *et al*, 2005; Thomas *et al*, 2007; Frank-Cannon *et al*, 2008). In line with parkin playing a role in the cellular stress response, *parkin* gene expression is considerably upregulated under cellular stress. ATF4 and p53 have been shown to increase parkin expression, whereas c-Jun and N-myc act as transcriptional repressors of *parkin* (West *et al*, 2004; Bouman *et al*, 2011; Zhang *et al*, 2011).

Several viability pathways were reported to be influenced by parkin, including JNK, PI3K, and NF-κB signalling, p53 transcriptional activity, or Bax activation (Cha *et al*, 2005; Yang *et al*, 2005; Fallon *et al*, 2006; Henn *et al*, 2007; Hasegawa *et al*, 2008; da Costa *et al*, 2009; Sha *et al*, 2010; Johnson *et al*, 2012). Parkin has recently been shown to induce the proteasomal degradation of PARIS (parkin-interacting substrate), which acts as a transcriptional repressor of *PGC-1α* (peroxisome proliferator-activated receptor gamma-co-activator 1-alpha) (Shin *et al*, 2011). PGC-1α stimulates mitochondrial biogenesis as a co-activator of various transcription factors, such as NRF (nuclear respiratory factor)-1 and -2 (reviewed in Scarpulla, 2011). Thus, loss of parkin function suppresses mitochondrial biogenesis through an accumulation of PARIS. This study not only provided an important link between the protective activity of parkin and mitochondria but also implicated a transcriptional program in mediating the effects of parkin. Moreover, by generating conditional parkin KO mice Dawson and coworkers could show for the first time that loss of parkin function in adult mice leads to a progressive degeneration of DA neurons which can be suppressed by silencing PARIS expression (Shin *et al*, 2011). This finding supports the notion that developmental compensation accounts for the absence of major phenotypic alterations in germline parkin KO mice.

Parkin at the interface of neurodegeneration and cancer

In an attempt to characterize FRA6E, one of the most active common fragile sites in the human genome located at chromosome 6q25-q27, the *parkin* genomic structure was found to span a large region of FRA6E (Cesari *et al*, 2003; Denison *et al*, 2003a). Common fragile sites are specific loci that are susceptible to chromosomal breaks and rearrangements and seem to play a role in oncogenesis. Studies to detect genomic copy number variations in human ovarian and breast carcinomas identified a common minimal region of loss located within the *parkin* gene. Indeed, decreased or absent parkin expression was observed in various malignancies (Cesari *et al*, 2003; Denison *et al*, 2003a, b; Picchio *et al*, 2004; Wang *et al*, 2004; Agirre *et al*, 2006; Fujiwara *et al*, 2008; Ikeuchi *et al*, 2009; Poulogiannis *et al*, 2010; Veeriah *et al*, 2010; Mehdi *et al*, 2011). Several studies have now provided considerable evidence that *parkin* might be a *bona fide* tumour suppressor gene (TSG). Heterozygous deletion of *parkin* accelerated the development of intestinal adenoma in mice expressing mutant APC, a regulator of Wnt signalling (Poulogiannis *et al*, 2010). Upon γ-irradiation parkin KO mice developed lymphomas in the spleen with a shorter tumour

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latency compared with wild-type mice (Zhang *et al*, 2011). In one line of parkin KO mice lacking exon 3, enhanced hepatocyte proliferation and development of hepatic tumours has been observed (Fujiwara *et al*, 2008). Ectopic expression of parkin in parkin-deficient tumour cells lines (glioma cells or lung cancer cells) resulted in reduced tumour growth after injection of these cells as xenografts into nude mice (Picchio *et al*, 2004; Veeriah *et al*, 2010). Some studies reported that parkin overexpression inhibits cell proliferation, albeit this is not a consistent finding (Picchio *et al*, 2004; Poulogiannis *et al*, 2010; Tay *et al*, 2010; Veeriah *et al*, 2010).

The mechanism underlying the tumour suppressor activity of parkin is not well understood. A previous study identified cyclin E as a substrate of parkin for ubiquitination and proteasomal degradation in neuronal cells (Staropoli *et al*, 2003); therefore, it is tempting to speculate that a decrease in parkin expression results in an accumulation of cyclin E, a cell-cycle regulator required for the transition from G1 to S phase. Increased levels of cyclin E have been observed in some but not all parkin-deficient primary tumours and cancer cell lines (Ikeuchi *et al*, 2009; Tay *et al*, 2010; Veeriah *et al*, 2010; Yeo *et al*, 2012). Based on the effect of parkin on mitochondrial bioenergetics as reviewed further below, it is also conceivable that parkin exerts its tumour suppressor activity via influencing tumour metabolism. Evidence for such a scenario was recently provided by Feng and coworkers (Zhang *et al*, 2011). A hallmark of tumour cells is the switch from mitochondrial energy production to aerobic glycolysis, which is known as the Warburg effect (reviewed in Vander Heiden *et al*, 2009 and Cairns *et al*, 2011). To compensate for the lower efficiency of ATP production by glycolysis compared with mitochondrial respiration, tumour cells increase glucose uptake and utilization. An important role in regulating energy metabolism plays the TSG p53, a transcription factor that promotes mitochondrial respiration and reduces glycolysis via transcription of specific target genes. *Parkin* was recently identified as a p53 target gene, which can mediate effects of p53 on energy metabolism and antioxidant defense (Zhang *et al*, 2011). Parkin deficiency activates glycolysis and reduces mitochondrial respiration in human lung cancer cells and mouse embryonic fibroblasts, thereby contributing to the Warburg effect. Remarkably, parkin can also affect lipid metabolism by regulating fatty acid uptake. In wild-type mice, parkin expression is robustly upregulated upon exposure to a high fat and cholesterol diet (HFD), inducing the stabilization of the fatty acid transporter CD36, whereas parkin KO mice are resistant to weight gain and hepatic insulin resistance under HFD feeding (Kim *et al*, 2011).

Whether germline pathogenic mutations in the *parkin* gene can increase the risk for cancer is difficult to assess given that parkin-linked parkinsonism is rare and a large number of cases would be required for a statistically robust epidemiological study (reviewed in Plun-Favreau *et al*, 2010 and Devine *et al*, 2011).

PINK1: a mitochondrial kinase of complex regulation and processing

The *PINK1* (PTEN-induced putative kinase 1) gene was linked to autosomal recessive early onset PD in 2004 (Valente *et al*, 2004). It encodes a ubiquitously expressed 581 amino-acid protein with an N-terminal mitochondrial targeting sequence (MTS), a transmembrane domain and a highly conserved serine/threonine kinase domain with homology to the Ca^{2+}/calmodulin family. About 30 pathogenic *PINK1* mutations have been identified, among them missense, non-sense, or frameshift mutations, deletions or genomic rearrangements (reviewed in Deas *et al*, 2009; Nuytemans *et al*, 2010; and Corti *et al*, 2011). Most *PINK1* mutations have been described to impair its kinase activity or reduce the stability of the protein, in line with a loss of function mechanism.

The subcellular localization of PINK1 is still debated. PINK1 has been found at the outer and inner mitochondrial membrane and in the cytosol (Silvestri *et al*, 2005; Muqit *et al*, 2006; Haque *et al*, 2008; Lin and Kang, 2008; Weihofen *et al*, 2009; Jin *et al*, 2010; Narendra *et al*, 2010b; Murata *et al*, 2011; Shi *et al*, 2011). From recent research, a complex mechanism of PINK1 targeting and processing has emerged that could provide an explanation for the different observations regarding PINK1 localization. PINK1 seems to be imported via the TOM/TIM23 complexes at the outer/inner mitochondrial membrane in a membrane potential-dependent manner for cleaving off its MTS by the mitochondrial processing protease (Greene *et al*, 2012). PINK1 exposing its kinase domain to the intermembrane space could then be released from the transport pore by lateral diffusion to be further processed by a protease giving rise to a PINK1 fragment, which is subsequently degraded by an MG132-sensitive protease, possibly in the cytoplasm. How this retrotranslocation is mediated, is not known. It has been hypothesized that proteolytic cleavage of a PINK1 import intermediate still associated with the TOM complex leads to a C-terminal PINK1 fragment that reaches the cytoplasm by reverse translocation (Meissner *et al*, 2011). PARL (presenilin-associated rhomboid like protease) has recently been identified as a protease promoting PINK1 cleavage under basal conditions to keep mitochondrial PINK1 levels low (Whitworth *et al*, 2008; Jin *et al*, 2010; Deas *et al*, 2011; Meissner *et al*, 2011; Shi *et al*, 2011). When the mitochondrial membrane potential is dissipated, PINK1 mitochondrial import and processing by PARL is inhibited, leading to the integration of PINK1 into the outer mitochondrial membrane, which is a prerequisite to recruit parkin for the induction of mitophagy (Jin *et al*, 2010; Matsuda *et al*, 2010; Narendra *et al*, 2010b; Meissner *et al*, 2011) (see below). In depolarized mitochondria, endogenous PINK1 is associated with the TOM complex, which may allow rapid reimport of PINK1 after repolarization to switch off the mitophagy pathway (Lazarou *et al*, 2012). Interestingly, PINK1 can be processed in the absence of PARL resulting in a slightly different cleavage pattern, thus, PARL is apparently not the only protease capable of PINK1 processing (Narendra *et al*, 2010b; Shi *et al*, 2011; Greene *et al*, 2012).

PINK1 can increase the resistance to diverse cellular stressors in a kinase-dependent manner (Petit *et al*, 2005; Haque *et al*, 2008; Wood-Kaczmar *et al*, 2008; Gandhi *et al*, 2009; Morais *et al*, 2009; Sandebring *et al*, 2009; Klinkenberg *et al*, 2010; Murata *et al*, 2011; Wang *et al*, 2011b). Of note, PINK1 deficiency in mice, as demonstrated in germline PINK1 KO mice or in shRNA-mediated PINK1 knockdown mice, significantly increases sensitivity of DA neurons to systemic MPTP treatment (Haque *et al*, 2012). Nigrostriatal degeneration in these models can be prevented by virally expressed parkin or DJ-1, suggesting that these genes are

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either downstream or act in parallel pathways to confer stress protection. To get insight into the mechanism underlying the protective activity of PINK1, efforts have been intensified to identify its substrates. Pridgeon *et al* (2007) reported that PINK1 phosphorylates TRAP1 (TNF receptor-associated protein 1), a mitochondrial chaperone of the Hsp90 family also known as Hsp75. Phosphorylation of TRAP1 was shown to be essential for the protective activity of PINK1 against oxidative stress. Plun-Favreau *et al* (2007) identified the mitochondrial serine protease HtrA2/Omi as a PINK1 interactor. A direct interaction between PINK1 and HtrA2/Omi favours the phosphorylation of HtrA2/Omi, which seems to be mediated by the p38 stress kinase pathway. Phosphomimetic HtrA2/Omi mutants show an increased protease activity along with the ability to protect against mitochondrial toxins. In another approach to identify PINK1-interacting proteins by immunopurification, the mitochondrial outer membrane Rho-like GTPase Miro2 and the adaptor protein Milton were found to form a complex with PINK1 (Weihofen *et al*, 2009). By binding to both Miro (two human homologues Miro1 and Miro2) and kinesin heavy chain, Milton links mitochondria to microtubules for axonal transport (Guo *et al*, 2005; Glater *et al*, 2006). The physical interaction of PINK1 and Miro therefore suggested that PINK1 has an impact on mitochondrial transport. In a recent study employing cultured rat hippocampal neurons or *Drosophila* larval neurons, it was indeed observed that the overexpression of either PINK1 or parkin causes an arrest of mitochondrial transport (Wang *et al*, 2011d). Based on the known interaction between PINK1 and Miro, Schwarz and coworkers followed up the idea that PINK1 and parkin may directly modify Miro. Miro1 was shown to be a direct target of PINK1 for phosphorylation, and Miro1 phosphorylated at serine 156 in turn is degraded by the proteasome in a parkin-dependent manner. This observation fits into a model in which degradation of Miro induced by the PINK1/parkin pathway stops mitochondrial movement and helps to sequester damaged mitochondria prior to their elimination by mitophagy (see below). A recent study from Bingwei Lu's laboratory showed that PINK1 and Miro have opposing effects on mitochondrial flux and net velocity in *Drosophila* larval motor neurons (Liu *et al*, 2012). Overexpression of PINK1 or Miro RNAi inhibited axonal mitochondrial transport in both anterograde and retrograde directions, and downregulation of Miro rescued PINK1 mutant phenotypes in *Drosophila* muscles and DA neurons. Liu *et al* also found that overexpression of PINK1 and parkin reduces Miro1 levels in HeLa cells, but phosphorylation of Miro1 at serine 156 was not required for this effect. Intriguingly, whereas Miro protein levels were increased in PINK1 mutant flies, the opposite effect was observed in mammalian models of PINK1 deficiency. In mouse embryonic fibroblasts from PINK1 KO mice or in PINK1 RNAi HeLa cells, Miro protein levels were significantly reduced, possibly reflecting differences between *Drosophila* and mammalian cells to respond to and compensate for PINK1 loss of function. An impact of PINK1 on mitochondrial transport was also observed by Mandelkow and coworkers who found that MARK2 (microtubule affinity-regulating kinase 2) phosphorylates N-terminally truncated PINK1 thereby increasing its kinase activity (Matenia *et al*, 2012). Whereas N-terminally truncated PINK1 overexpressed in chicken retinal ganglion cells promotes anterograde mitochondrial transport and increases the fraction of stationary mitochondria, full-length PINK1 enhances retrograde transport, suggesting that PINK1 may act as a molecular switch between anterograde and retrograde mitochondrial transport depending on the mitochondrial membrane potential.

The mechanisms of protection mediated by PINK1 are not completely understood. It has been suggested that effects on mitochondrial bioenergetics, quality control, and calcium homeostasis are involved. In various PINK1 loss of function models, mitochondrial impairment has been observed, such as alterations in mitochondrial morphology and dynamics, a decrease in mitochondrial membrane potential, and respiration defects (Exner *et al*, 2007; Hoepken *et al*, 2007; Gautier *et al*, 2008; Piccoli *et al*, 2008; Dagda *et al*, 2009, 2011; Gegg *et al*, 2009; Gispert *et al*, 2009; Lutz *et al*, 2009; Sandebring *et al*, 2009; Cui *et al*, 2010; Yuan *et al*, 2010; Amo *et al*, 2011; Heeman *et al*, 2011). Decreased complex I enzymatic activity was found in fly and mouse models lacking PINK1 and suggested to be the primary event leading to synaptic dysfunction under increased energy demand, based on the observation that the mobilization of reserve pool synaptic vesicles during rapid stimulation is impaired at the neuromuscular junction in PINK1-deficient flies and can be rescued by supplementing synapses with ATP (Morais *et al*, 2009). Moreover, downregulation of a complex I component in flies phenocopies several PINK1 mutant phenotypes (Vilain *et al*, 2012). An alternative explanation was provided by Abramov and coworkers who observed an imbalance of calcium homeostasis in PINK1-deficient cells (Gandhi *et al*, 2009). When PINK1 is downregulated by RNAi in neurons derived from human embryonic stem cells, cellular stress results in mitochondrial calcium overload due to the dysfunction of the Na^{+}/Ca^{2+} exchanger. Mitochondrial Ca^{2+} overload in turn stimulates ROS production, inhibits the glucose transporter and reduces respiratory functions.

Parkin and PINK1: team players in the removal of damaged mitochondria

Surprisingly, neither parkin nor PINK1 KO mice show an overt morphological or behavioural phenotype, which may be explained by developmental compensation or functional redundancy. However, both parkin and PINK1-deficient cells are characterized by an increased vulnerability to mitochondrial damage (Casarejos *et al*, 2006; Rosen *et al*, 2006; Henn *et al*, 2007; Paterna *et al*, 2007; Haque *et al*, 2008, 2012; Sandebring *et al*, 2009), indicating that mitochondrial alterations can be compensated under basal but not under stress conditions. Remarkably, parkin or PINK1 mutant flies display a striking and surprisingly similar phenotype, including reduced life span, male sterility, and apoptotic flight muscle degeneration. In flies and cultured cells, parkin can rescue PINK1 deficiency but not *vice versa*, indicating that parkin is acting downstream of PINK1 (Clark *et al*, 2006; Park *et al*, 2006; Yang *et al*, 2006; Exner *et al*, 2007; Lutz *et al*, 2009).

Evidence for a linear PINK1/parkin mitochondrial pathway was first provided by Richard Youle's laboratory. Narendra *et al* (2008) observed that parkin is targeted to damaged mitochondria in a PINK1-dependent manner and induces their degradation by mitophagy, a selective mode of

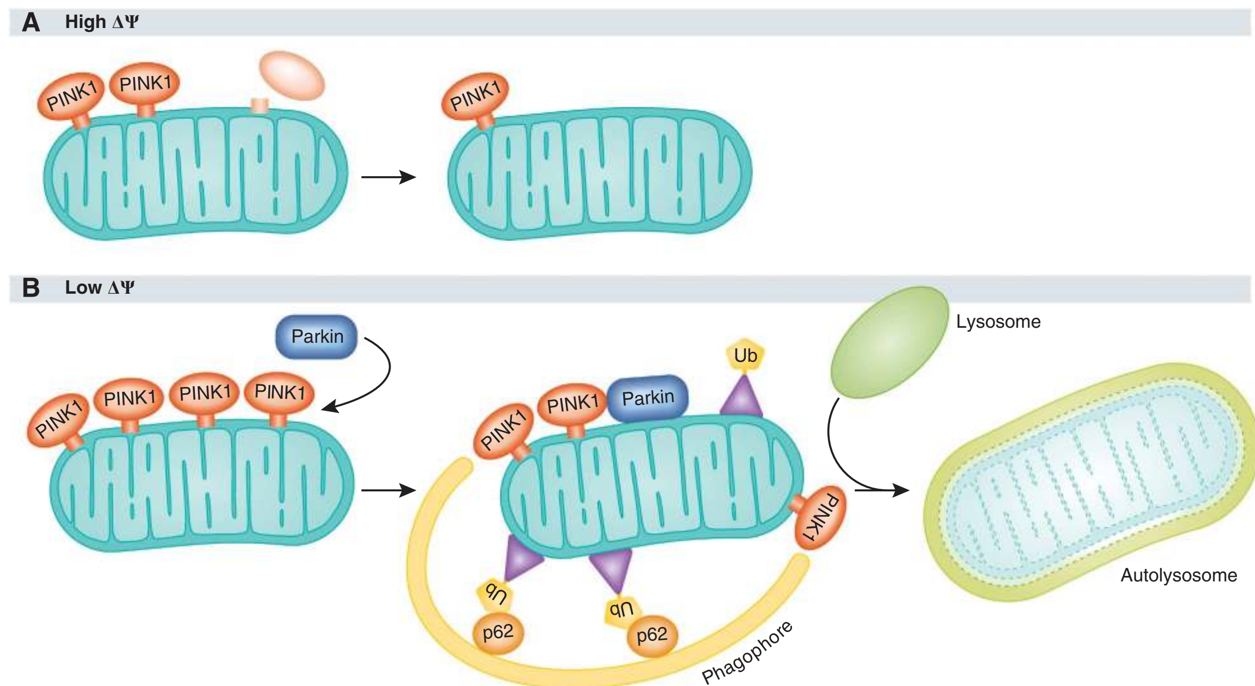

Figure 2 Mechanism of PINK1- and parkin-induced mitophagy. (**A**) When the mitochondrial membrane potential is high, PINK1 is imported into mitochondria, proteolytically processed and rapidly degraded, resulting in mitochondria with low levels of endogenous PINK1 under basal conditions. (**B**) Under conditions of low membrane potential, for example, after uncoupling with CCCP, full-length PINK1 accumulates on the mitochondrial surface, which is essential for the translocation of parkin to mitochondria. Parkin then ubiquitinates mitochondrial proteins at the outer membrane, followed by the recruitment of adaptor proteins, such as p62, which link ubiquitinated cargo to the autophagic machinery. Damaged mitochondria are engulfed by phagophores, which mature into autophagosomes and fuse with lysosomes to autolysosomes, which eventually degrade their content.

autophagy to dispose of dysfunctional mitochondria. When CCCP (carbonyl cyanide 3-chlorophenylhydrazone), an uncoupling agent that dissipates the mitochondrial membrane potential, is added to HeLa cells that do not express endogenous parkin, overexpressed parkin is recruited to mitochondria within 1 h, and mitochondria are cleared from parkin-expressing cells upon prolonged exposure to CCCP. As outlined above, dissipation of the mitochondrial membrane potential by CCCP leads to the accumulation of full-length PINK1 at the outer mitochondrial membrane, which is essential for the mitochondrial translocation of parkin (Figure 2; Geisler *et al*, 2010a; Narendra *et al*, 2010b; Vives-Bauza *et al*, 2010; Seibler *et al*, 2011). Strikingly, the presence of membrane-localized PINK1 is sufficient to recruit parkin, since PINK1 targeted to peroxisomes or lysosomes recruits and activates parkin on the respective organelles (Lazarou *et al*, 2012). In an effort to elucidate the mechanism of parkin-induced mitophagy, several cofactors of this pathway have been identified. Selective autophagy requires specific labelling of the substrates destined for degradation, such as ubiquitination, and subsequent binding of adaptor proteins to recruit the autophagic machinery to the tagged cargo (reviewed in Kirkin *et al*, 2009). The signalling adaptor protein p62 (also termed as SQSTM1, sequestosome-1), which links ubiquitinated cargo to the autophagic machinery by binding to both ubiquitin and LC3, has been described as an essential factor for parkin-induced mitophagy (Ding *et al*, 2010; Geisler *et al*, 2010a; Lee *et al*, 2010b). However, two studies reported that only perinuclear clustering of mitochondria, but not mitophagy was impaired in fibroblasts from p62 KO mice (Narendra *et al*, 2010c; Okatsu *et al*, 2010). The ubiquitin-binding deacetylase HDAC6 has also been implicated in parkin-mediated mitophagy by promoting autophagosome–lysosome fusion (Lee *et al*, 2010b). Ambra1 (activating molecule in Beclin-regulated autophagy) was recently identified as a parkin-interacting protein that promotes autophagic clearance of mitochondria by activating class III PI3K, which is essential for the formation of phagophores (Van Humbeeck *et al*, 2011).

Is parkin-mediated ubiquitination required for mitophagy and which target(s) and mode(s) of ubiquitination are implicated in this process? An increase in ubiquitinated proteins has indeed been detected at mitochondria upon CCCP treatment in parkin-expressing cells but not in cells lacking functional parkin (Geisler *et al*, 2010a; Lee *et al*, 2010b; Matsuda *et al*, 2010; Okatsu *et al*, 2010; Chan *et al*, 2011). A quantitative proteomic approach revealed a significant increase in both Lys48- and Lys63-linked poly-ubiquitination at mitochondria of CCCP-treated HeLa cells overexpressing parkin (Chan *et al*, 2011). Mitochondrial proteins most severely reduced in these cells included the fusion proteins Mfn1 and Mfn2, the mitochondrial transport proteins Miro1 and Miro2, and the import receptor subunit TOM70 (Chan *et al*, 2011). Removal of Mfn1/2 may facilitate the sequestration and clearance of mitochondria by increasing mitochondrial fission, which precedes mitophagy (Twig *et al*, 2008; Tanaka *et al*, 2010). Degradation of Miro1 and Miro2 blocks mitochondrial transport and thus favours clustering of damaged mitochondria (Wang *et al*, 2011d; Liu *et al*, 2012). Mfn2 has been shown to be directly involved in mitochondrial transport by interacting with Miro1/2 and

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Milton (Misko *et al*, 2010), thus by decreasing Mfn1/2 and Miro1/2 protein levels parkin can affect both mitochondrial dynamics and transport. Notably, proteasomal degradation of outer mitochondrial membrane proteins seems to be a prerequisite of parkin-dependent mitophagy, as inhibition of the proteasome abrogates mitophagy (Tanaka *et al*, 2010; Chan *et al*, 2011; Yoshii *et al*, 2011). However, degradative ubiquitination of Mfn1 and Mfn2 (Tanaka *et al*, 2010) or non-degradative ubiquitination of VDAC1 (Geisler *et al*, 2010a) is not essential for parkin-induced mitophagy, since this pathway is not impaired in fibroblasts from Mfn1/2 or VDAC1/3 KO mice, indicating that either the essential substrate(s) for parkin-mediated ubiquitination has yet to be identified, or rather widespread ubiquitination of mitochondrial proteins facilitates mitophagy (Narendra *et al*, 2010a; Tanaka *et al*, 2010; Chan *et al*, 2011). In conclusion, remodelling of the outer mitochondrial membrane by ubiquitin in response to CCCP treatment serves at least two functions: proteasomal degradation of mitochondrial proteins and attracting ubiquitin-binding proteins that recruit the autophagic machinery. Whether PINK1 and parkin interact directly, and if PINK1 phosphorylates parkin and/or parkin ubiquitinates PINK1 is a controversial issue. Some studies provided evidence for such a scenario, others not (Moore, 2006; Kim *et al*, 2008; Shiba *et al*, 2009; Um *et al*, 2009; Sha *et al*, 2010; Vives-Bauza *et al*, 2010; Lazarou *et al*, 2012).

The pathophysiological relevance of mitophagy in PD

In favour of a relevant role of mitophagy in the pathogenesis of PD, pathogenic mutations in both parkin and PINK1 compromise distinct steps in the mitophagic pathway (Geisler *et al*, 2010a, b; Kawajiri *et al*, 2010; Lee *et al*, 2010b; Matsuda *et al*, 2010; Narendra *et al*, 2010b, c; Okatsu *et al*, 2010; Vives-Bauza *et al*, 2010; Chan *et al*, 2011; Seibler *et al*, 2011). However, most of the studies on PINK1/parkin-induced mitophagy used the protonophore CCCP or the ionophore valinomycin to induce mitophagy in PINK1/parkin-overexpressing tumour cells lines or mouse embryonic fibroblasts. Thus, the major critical issues are whether this phenomenon occurs under pathophysiological stress conditions with endogenous expression levels of parkin and PINK1 and whether an impairment of mitophagy contributes to neuronal dysfunction and cell death in PD. So far, studies addressing these important questions did not provide conclusive and coherent answers. It has been reported that parkin is recruited to mitochondria in cultured cells overexpressing a catalytically inactive form of the mitochondrial helicase Twinkle, suggesting that parkin can target mitochondria with mtDNA deletions (Suen *et al*, 2010). In the same study, it was observed that long-term overexpression of parkin in heteroplasmic cybrid cells induces selective elimination of mitochondria with mutations in the *cytochrome c oxidase subunit I* gene (Suen *et al*, 2010). To test for parkin-induced mitophagy *in vivo*, Larsson and coworkers made use of a mouse model of progressive parkinsonism caused by DA neuron-specific loss of the mitochondrial transcription factor TFAM, which is essential for mtDNA maintenance and transcription initiation at mtDNA promoters (Ekstrand *et al*, 2007). These mice display severe respiratory chain deficiency and accumulation of mitochondrial aggregates, however, no evidence was found for mitochondrial translocation of parkin or parkin-induced mitophagy (Sterky *et al*, 2011).

Another crucial aspect concerning the mitophagy pathway seem to be the bioenergetic status of the cells analysed. Berman and coworkers observed that after CCCP treatment parkin efficiently translocates to uncoupled mitochondria in tumour cells, but not in rat primary cortical or striatal/midbrain neurons (Van Laar *et al*, 2011). Typically, tumour-derived cultured cells, such as HeLa cells, do not depend on oxidative phosphorylation, as they generate ATP preferentially by aerobic glycolysis, which can provide a biosynthetic advantage during cell proliferation (reviewed in Vander Heiden *et al*, 2009 and Cairns *et al*, 2011). Remarkably, when HeLa cells are forced into dependence on oxidative phosphorylation by culturing in glucose-free galactose cell culture medium, parkin no longer translocates to uncoupled mitochondria (Van Laar *et al*, 2011). In contrast to the latter study, accumulation of overexpressed YFP-parkin at axonal mitochondria was recently observed in rat hippocampal neurons treated with the complex III inhibitor antimycin A (Wang *et al*, 2011d). As already mentioned above, Schwarz and coworkers could demonstrate that the mitochondrial recruitment of parkin decreases mitochondrial motility as a consequence of parkin-dependent proteasomal degradation of Miro. This mitochondrial arrest may represent an early step of mitophagy, however, whether arrested mitochondria in this model are indeed eliminated by the mitophagy pathway has not been demonstrated. As a conclusion, in neurons that depend on mitochondrial respiration for energy production induction of mitophagy may be more restrictive and possibly regulated in a more complex manner in comparison with tumour cells.

Parkin, PINK1, and mitochondrial dynamics: an impact on fusion or fission?

Although mitochondria appear as solitary, static, bean-shaped double-membrane units in electron micrographs, life-cell imaging revealed that mitochondria are highly dynamic organelles which can change their shape, size, and subcellular localization. These dynamic processes are regulated by mitochondrial fusion, fission, and transport along cytoskeletal tracks. Depending on whether fusion or fission predominates, mitochondria form highly interconnected tubular networks or fragmented, rod-like/spherical structures. The last decade has witnessed remarkable progress in the identification and characterization of the basic mechanisms that govern mitochondrial fusion and fission. The core machinery mediating mitochondrial fusion and fission consists of dynamin-like GTPases, which are tightly regulated at various levels (reviewed in Detmer and Chan, 2007; Westermann, 2010) (Soubannier and McBride, 2009; Campello and Scorrano, 2010; and Oettinghaus *et al*, 2012). Mitofusins (Mfn1 and Mfn2) induce outer mitochondrial membrane fusion, whereas OPA1 mediates fusion of the inner membrane. Mitochondrial fission is dependent on the cytosolic GTPase Drp1, which is recruited to prospective fission sites upon activation, and involves additional fission factors, such as Mff, Mief1, and Fis1. In addition to characterizing the fusion and fission machinery at a molecular level, the impact of mitochondrial dynamics on key cellular processes, such as bioenergetics, apoptosis, autophagy, quality control and stress response pathways is

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still being explored. Beyond any doubt, dysregulation of mitochondrial dynamics can cause or at least contribute to the pathogenesis of various neurological disorders (reviewed in Chan, 2006; Knott *et al*, 2008; Oettinghaus *et al*, 2012; and Pilsl and Winklhofer, 2012). Notably, germline mutations in the *Mfn2* gene are responsible for Charcot-Marie-Tooth type 2A, a peripheral neuropathy affecting both sensory and motor neurons (Zuchner *et al*, 2004), whereas mutations in the *OPA1* gene cause autosomal dominant optic atrophy (Alexander *et al*, 2000; Delettre *et al*, 2000).

Alterations in mitochondrial dynamics have been documented in several genetic PD models. Specifically, in cultured mammalian cells and primary neurons the acute loss of parkin or PINK1 function causes Drp1-dependent mitochondrial fragmentation along with a decrease in the mitochondrial membrane potential and ATP production (Exner *et al*, 2007; Dagda *et al*, 2009; Lutz *et al*, 2009; Sandebring *et al*, 2009; Weihofen *et al*, 2009; Cui *et al*, 2010; Wang *et al*, 2011a). This phenotype can be rescued by increasing mitochondrial fusion (enhanced expression of Mfn2 or OPA1) or decreasing fission (enhanced expression of dominant negative Drp1) (Dagda *et al*, 2009; Lutz *et al*, 2009; Sandebring *et al*, 2009; Cui *et al*, 2010). In contrast to mammalian models, the phenotype of parkin- or PINK1-deficient flies can be reverted by increasing mitochondrial fission or decreasing fusion (Deng *et al*, 2008a; Poole *et al*, 2008; Yang *et al*, 2008). From the observations in the fly model, it was concluded that parkin and PINK1 promote mitochondrial fission. However, in mammalian cells the increased expression of parkin or PINK1 efficiently protects the mitochondrial network from fragmentation induced by cellular stress or Drp1 overexpression (Lutz *et al*, 2009; Sandebring *et al*, 2009; Bouman *et al*, 2011). A possible explanation for the obvious discrepancies are differences between mammals and *Drosophila* to cope with the loss of parkin or PINK1 function. Of note, mitochondrial fragmentation is an immediate response to parkin or PINK1 silencing and is more pronounced after a transient knockdown. Thus, it appears that compensatory strategies can be induced to prevent irreversible cellular damage in parkin- or PINK1-deficient cells. In line with this scenario, we observed that mitochondrial fragmentation occurs in cultured *Drosophila* S2 cells early upon parkin or PINK1 silencing, but is rapidly followed by hyperfusion (Lutz *et al*, 2009). Mitochondrial fusion may be activated in an attempt to dilute dysfunctional mitochondria and to achieve complementation with functional mitochondria. However, this mode of compensation does not promote elimination of damaged mitochondria, explaining why postmitotic cells in tissues with high energy demands are affected in parkin- or PINK1-deficient flies. We did not observe a hyperfusion phenotype upon knocking down parkin or PINK1 expression in mammalian cells; thus, the compensatory mechanisms seem to be different to those in fly cells. In this context, it is important to note that a stable loss of parkin or PINK1 in mammalian cells can obviously be compensated under basal conditions, consistent with the fact that parkin- or PINK1-deficient mice do not show major alterations in mitochondrial morphology. However, parkin- or PINK1-deficient cells including patients' fibroblasts are much more vulnerable to stress-induced mitochondrial fragmentation, suggesting that the compensatory strategies are not

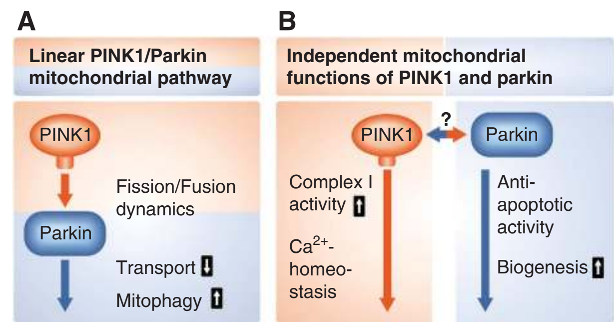

Figure 3 Mitochondrial functions of PINK1 and parkin. (**A**) A linear pathway with PINK1 acting upstream of parkin is implicated in mediating degradation of damaged mitochondria via mitophagy. (**B**) PINK1 and parkin also affect mitochondrial functions via parallel pathways, possibly by acting on mitochondria independently from each other.

sufficient to prevent mitochondrial damage under cellular stress (Hoepken *et al*, 2007; Mortiboys *et al*, 2008; Grunewald *et al*, 2010).

The effects of PINK1 and parkin on mitochondrial dynamics may be secondary to alterations in mitochondrial bioenergetics and/or mitochondrial depolarization (Morais *et al*, 2009; Sandebring *et al*, 2009). In line with this notion, many phenotypes of PINK1-deficient flies, such as male sterility, flight muscle degeneration, and synaptic transmission defects, are rescued by Ndi1p, a yeast NADH dehydrogenase that can bypass electron transport in complex I in mammalian cells. Complex I deficiency in PINK1 mutant flies is not rescued by increasing mitochondrial fission, indicating that PINK1-associated complex I dysfunction acts at least partly upstream of mitochondrial remodelling (Vilain *et al*, 2012). Interestingly, the loss of parkin function in flies does not compromise complex I activity and Ndi1p has no effect on the phenotype of parkin-deficient flies, although parkin can rescue the PINK1 phenotype (Vilain *et al*, 2012). These findings add evidence to the idea that in addition to a simple linear PINK1/parkin pathway, there may exist parallel pathways and independent functions of parkin and PINK1 (Figure 3). Indeed, parkin can prevent cytochrome c release induced by pro-apoptotic BH3 domains, whereas PINK1 does not have this activity (Berger *et al*, 2009).

Parkin has been reported to directly influence the mitochondrial fusion and fission machinery, which makes it even more difficult to decipher the net effect of parkin on mitochondrial dynamics. In the context of parkin-induced mitophagy, it has been observed that parkin mediates ubiquitination and proteasomal degradation of Mfn1 and Mfn2 in response to mitochondrial uncoupling (Poole *et al*, 2008; Gegg *et al*, 2010; Tanaka *et al*, 2010; Ziviani *et al*, 2010; Chan *et al*, 2011; Glauser *et al*, 2011). In contrast to these studies, parkin has been found to induce the proteasomal degradation of the fission-promoting proteins Drp1 or Fis1 (Cui *et al*, 2010; Wang *et al*, 2011a). How can these discrepant observations be explained? Conceptually, parkin may modulate mitochondrial dynamics in a context-specific and possibly cell type-specific manner. An attractive model would be that under moderate stress conditions with only minor mitochondrial damage parkin prevents mitochondrial fragmentation and favours mitochondrial fusion with the remaining healthy mitochondrial population, allowing

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functional complementation. However, when mitochondria are irreversibly damaged in response to severe stress, parkin may promote mitochondrial fission, which facilitates sequestration and elimination of damaged mitochondria via mitophagy.

DJ-1: an ROS-sensitive and ROS-protective protein

Loss-of-function mutations in the *DJ-1* gene are a rare cause of autosomal recessive parkinsonism (Bonifati *et al*, 2003). DJ-1, a ubiquitously expressed protein of 189 amino acids, belongs to an evolutionarily conserved superfamily and shares structural similarities to the stress-inducible *E. coli* chaperone Hsp31 (Lee *et al*, 2003; Lucas and Marin, 2007). DJ-1 forms dimers with extensive contacts covering 35% of the molecular surface of each molecule (Wilson *et al*, 2003). A wide range of obviously unrelated DJ-1 functional activities have been reported, however, there is consensus on the fact that DJ-1 is responsive to and protective against oxidative stress (reviewed in Kahle *et al*, 2009 and Cookson, 2010a). Oxygen species react with the sulphydryl group of a conserved cysteine residue at position 106 to form sulphinic acid, giving rise to a shift in the isoelectric point towards more acidic values (Mitsumoto *et al*, 2001; Canet-Aviles *et al*, 2004). Importantly, this cysteine residue is essential for the stress-protective activity of DJ-1 (Canet-Aviles *et al*, 2004; Taira *et al*, 2004; Meulener *et al*, 2006; Blackinton *et al*, 2009).

DJ-1 KO mice do not display nigrostriatal degeneration, however, they are impaired in scavenging mitochondrial H_2O_2, which has been attributed to the function of DJ-1 as an atypical peroxiredoxin-like peroxidase (Andres-Mateos *et al*, 2007). DJ-1 is mostly found in the cytoplasm, but oxidative stress can induce its translocation to the outer mitochondrial membrane (Canet-Aviles *et al*, 2004; Blackinton *et al*, 2009; Junn *et al*, 2009). Moreover, cell fractionation and immunogold electron microscopy revealed that endogenous Drp1 can be detected in the mitochondrial matrix and intermembrane space (Zhang *et al*, 2005). It has been suggested that parkin, PINK1, and DJ-1 form a complex with E3 ligase activity to degrade misfolded proteins (Xiong *et al*, 2009); however, size exclusion chromatography did not support such a scenario, as parkin, PINK1, and DJ-1 were separated into distinct complexes (Thomas *et al*, 2011). Concerning the maintenance of mitochondrial integrity, DJ-1 shares some features with PINK1 and parkin. Similarly to PINK1 and parkin, DJ-1 increases resistance against mitochondrial toxins and decreases mitochondrial fragmentation in response to mitochondrial damage (Canet-Aviles *et al*, 2004; Kim *et al*, 2005; Zhang *et al*, 2005; Irrcher *et al*, 2010; Krebiehl *et al*, 2010; Thomas *et al*, 2011). DJ-1 deficiency in cultured cells, primary neurons and patients' fibroblasts or lymphoblasts causes mitochondrial fragmentation and depolarization, which can be reverted by parkin or PINK1, but DJ-1 cannot rescue the PINK1- or parkin-deficient phenotype (Exner *et al*, 2007; Irrcher *et al*, 2010; Krebiehl *et al*, 2010; Thomas *et al*, 2011). Mitochondrial alterations induced by DJ-1 loss of function can be prevented by antioxidants, indicating that increased levels of oxidative stress account for the mitochondrial phenotypes (Irrcher *et al*, 2010; Thomas *et al*, 2011). Indeed, brain or skeletal muscle mitochondria from DJ-1 KO mice display increased formation of ROS (Andres-Mateos *et al*, 2007; Irrcher *et al*, 2010).

Notably, DJ-1 maintains its protective activity in the absence of PINK1, thus DJ-1 appears to work rather independently from PINK1 and parkin in a parallel pathway (Thomas *et al*, 2011; Haque *et al*, 2012).

α-Synuclein: a protein of structural plasticity that links sporadic and genetic PD

α-Synuclein is of particular relevance to the etiopathogenesis of PD. The identification of mutations in the gene encoding α-synuclein (*SNCA*) as a cause of familial PD launched the 'molecular era' of PD research (Polymeropoulos *et al*, 1997). Shortly after this discovery, α-synuclein was found to be a major component of Lewy bodies (Spillantini *et al*, 1997). So far, three missense mutations in the *α-synuclein* gene and genomic duplications or triplications have been identified in patients with autosomal dominant parkinsonism (Polymeropoulos *et al*, 1997; Kruger *et al*, 1998; Singleton *et al*, 2003; Chartier-Harlin *et al*, 2004; Farrer *et al*, 2004; Ibanez *et al*, 2004; Zarranz *et al*, 2004). In addition, genome-wide association studies revealed that several single-nucleotide polymorphisms in the *SNCA* gene are strongly associated with PD risk (Satake *et al*, 2009; Simon-Sanchez *et al*, 2009; Edwards *et al*, 2010; Hamza *et al*, 2010; International Parkinson Disease Genomics Consortium *et al*, 2011).

α-Synuclein is a 140 amino-acid protein that is abundantly expressed in the central nervous system of vertebrates. It belongs to the synuclein family, which also includes β- and γ-synuclein. At the N-terminal region it harbours 7 imperfect repeats of 11 amino acids containing a KTKGEV motif which mediate formation of amphipathic α-helices upon membrane binding (Davidson *et al*, 1998; Eliezer *et al*, 2001). The central NAC (non-amyloid β component of Alzheimer's disease amyloid plaques) domain is responsible for the aggregation propensity of α-synuclein (Giasson *et al*, 2001). The acidic C-terminal domain contains several phosphorylation sites and influences the aggregation behaviour of α-synuclein (Okochi *et al*, 2000; Fujiwara *et al*, 2002; Tofaris *et al*, 2006). α-Synuclein is an intrinsically disordered protein in aqueous solution, however, depending on the environment it displays considerable structural flexibility, including membrane-bound α-helical structures, oligomers, protofibrils, and amyloid fibrils, characterized by a cross-β-sheet structure (reviewed in Volles and Lansbury, 2003; Beyer, 2007; and Uversky, 2010). It has recently been reported that endogenous α-synuclein isolated from cultured cells and red blood cells under non-denaturing conditions adopts a helically folded tetrameric structure with enhanced lipid-binding capacity and markedly reduced aggregation propensity in comparison with α-synuclein monomers, suggesting that α-synuclein tetramers are the physiological species (Bartels *et al*, 2011). Formation of stable tetramers with helical secondary structure was also described for recombinantly expressed human α-synuclein in the absence of lipid bilayers or micelles (Wang *et al*, 2011c). However, these findings have been disputed in a joint publication of six different groups (Fauvet *et al*, 2012). Using α-synuclein standards of well-characterized conformational and oligomeric states and applying various methods such as native and denaturing gel electrophoresis techniques, size exclusion chromatography, and an oligomer-specific ELISA, the authors of this study demonstrated that

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native α-synuclein from human, rat, and mouse brains, several mammalian cell lines, and human red blood cells exhibit identical migration patterns and apparent molecular weight as unfolded monomeric recombinant α-synuclein. Thus, the results of the latter study question the existence of physiological α-synuclein tetramers, a critical issue for the development of α-synuclein-specific therapeutic strategies, which mostly aim at preventing α-synuclein oligomerization.

It has now widely been accepted that not the final fibrillar α-synuclein aggregates but rather oligomeric prefibrillar folding intermediates are the major toxic species (reviewed in Lansbury and Lashuel, 2006; Cookson and van der Brug, 2008; and Winklhofer *et al*, 2008). However, the mechanisms of α-synuclein-induced toxicity are still under discussion, hampered by the difficulties in discriminating primary and specific effects from secondary and/or unspecific effects caused by overloading cells with a misfolding-prone protein. Upon overexpression of wild-type or mutant α-synuclein in cellular and animal models alterations in various cellular processes have been reported, including proteasomal and lysosomal degradation, autophagy, and vesicular transport (reviewed in Cookson, 2009; Waxman and Giasson, 2009; Sulzer, 2010; Venda *et al*, 2010; and Vekrellis *et al*, 2011). The physiological function of α-synuclein has also largely remained elusive. Its enrichment in presynaptic terminals and its binding to vesicles suggests a role in vesicle dynamics and synaptic plasticity (reviewed in Auluck *et al*, 2010). A common theme emerging from studies on the function and dysfunction of α-synuclein is that it appears to affect membrane fusion events (Figure 4). Mice lacking α-synuclein were reported to show increased dopamine release upon stimulation, pointing to a role of α-synuclein in negatively regulating dopamine neurotransmission (Abeliovich *et al*, 2000; Yavich *et al*, 2004). Similarly, in α-, β-, and γ-synuclein triple KO mice, an elevated evoked release of dopamine in the nigrostriatal system was observed (Anwar *et al*, 2011). When α-synuclein is overexpressed in PC12 cells evoked catecholamine release is inhibited and docked vesicles accumulate at the plasma membrane (Larsen *et al*, 2006). In primary hippocampal neurons, mild overexpression of α-synuclein in the range of gene duplication or triplication decreases neurotransmitter release, probably by reducing the size of the synaptic vesicle recycling pool (Nemani *et al*, 2010). Similarly, virus-mediated expression of α-synuclein in the SN of adult rats causes a reduction in striatal dopamine release (Lundblad *et al*, 2012). α-Synuclein has been reported to cooperate with presynaptic cysteine-string protein-α (CSPα) to maintain neuronal integrity (Chandra *et al*, 2005). CSPα is a co-chaperone of the Hsp40 family involved in the assembly of SNARE complexes, the core components of a conserved machinery mediating synaptic vesicle membrane fusion during exocytosis (Sharma *et al*, 2011). Strikingly, neurodegeneration observed in CSPα-deficient mice could be rescued by transgenic expression of α-synuclein, whereas deletion of endogenous α-synuclein enhanced the neurodegenerative phenotype (Chandra *et al*, 2005). Concerning the mechanism of this functional interaction it has been demonstrated that α-synuclein directly promotes the assembly of SNARE complexes by simultaneous binding to phospholipids via its N-terminal region and to synaptobrevin-2 via its C-terminal domain (Burre *et al*, 2010; Figure 4).

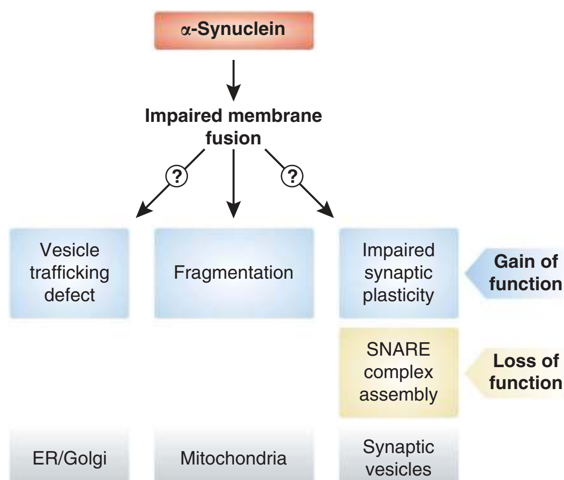

Figure 4 Physiological and pathophysiological functions of α-synuclein on membrane dynamics. Effects of α-synuclein on membrane fusion events can explain why both an increase of α-synuclein expression and a loss of α-synuclein have adverse effects. Overexpression of α-synuclein causes ER/Golgi vesicle trafficking defects and mitochondrial fragmentation, whereas α-synuclein deficiency affects SNARE complex assembly.

In vitro, α-synuclein preferentially binds to small vesicles containing acidic phospholipids and induces ordering of phospholipids at sites of packing defects in the bilayer caused by curvature stress (Davidson *et al*, 1998; Kamp and Beyer, 2006). Local defects in a membrane are required for the formation of a fusion stalk, thus, annealing of α-synuclein to those defects should prevent premature membrane fusion. Indeed, fusion of small unilamellar vesicles is inhibited in the presence of recombinant α-synuclein, and overexpression of α-synuclein in cultured cells and *C. elegans* reduces the fusion rate of mitochondria (Kamp *et al*, 2010). Notably, mitochondrial fragmentation induced by overexpression of α-synuclein can be prevented by PINK1, parkin, or DJ-1 but not by their pathogenic mutants (Kamp *et al*, 2010). Mitochondrial fragmentation upon increased α-synuclein expression was also observed in another study using cultured cells and primary neurons (Nakamura *et al*, 2011). In this study, it was reported that α-synuclein causes an increase in Drp1-independent mitochondrial fission, which is not accompanied by alterations in the morphology of other cellular organelles. Interestingly, α-synuclein-induced mitochondrial fragmentation is not a consequence of mitochondrial depolarization or respiration deficits, it occurs without overt toxicity and is independent of proteins of the mitochondrial fusion and fission machinery (Kamp *et al*, 2010; Nakamura *et al*, 2011). In conclusion, both the increased expression of α-synuclein and the loss of α-synuclein function may impact on membrane fusion, explaining the observed effects of α-synuclein on neurotransmitter release. Similarly, the ER-to-Golgi vesicle trafficking defect observed in several models of α-synuclein overexpression could be attributed to impaired fusion of vesicles at the Golgi membrane (Gitler *et al*, 2008; Figure 4).

There is evidence for a preferential binding of α-synuclein to mitochondria (Nakamura *et al*, 2008), and localization of α-synuclein at mitochondria or even within mitochondria has been reported (Li *et al*, 2007; Cole *et al*, 2008; Devi *et al*, 2008; Parihar *et al*, 2008; Shavali *et al*, 2008; Zhang *et al*, 2008;

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Kamp *et al*, 2010; Nakamura *et al*, 2011). In various models of α-synuclein overexpression structural alterations of mitochondria, increased oxidative stress, and/or bioenergetic defects have been observed (Hsu *et al*, 2000; Martin *et al*, 2006; Stichel *et al*, 2007; Devi *et al*, 2008; Parihar *et al*, 2008; Liu *et al*, 2009; Chinta *et al*, 2010; Loeb *et al*, 2010; Su *et al*, 2010; Choubey *et al*, 2011). α-Synuclein KO mice are resistant or less sensitive to mitochondrial toxins (Dauer *et al*, 2002; Schluter *et al*, 2003; Klivenyi *et al*, 2006) and were reported to have qualitative and quantitative lipid abnormalities with a decrease in cardiolipin content (Ellis *et al*, 2005). *Vice versa*, α-synuclein-overexpressing mice are more vulnerable to mitochondrial toxins (Orth *et al*, 2003; Song *et al*, 2004; Shavali *et al*, 2008), albeit this is not a consistent finding (Rathke-Hartlieb *et al*, 2001; Dong *et al*, 2002). α-Synuclein toxicity in cellular models and in *Drosophila* is mitigated by the mitochondrial chaperone TRAP1, supporting the notion that α-synuclein affects mitochondrial function (Butler *et al*, 2012). α-Synuclein has also been reported to increase autophagy and mitophagy in primary cortical neurons, and depletion of parkin showed beneficial effects on neuronal survival, which led the authors to conclude that inhibition of excess mitophagy is protective in this model (Choubey *et al*, 2011). This observation would be in line with a double transgenic mouse model in which the absence of parkin delayed the neurodegenerative phenotype caused by A30P α-synuclein overexpression (Fournier *et al*, 2009). It should be mentioned that α-synuclein has also been found to impair autophagy (Winslow *et al*, 2010). The effect of parkin on α-synuclein-induced pathology is controversial as well: in various models, a protective effect of parkin on α-synuclein-induced alterations was observed (Petrucelli *et al*, 2002; Yang *et al*, 2003; Lo Bianco *et al*, 2004; Yasuda *et al*, 2007; Khandelwal *et al*, 2010). Furthermore, in another mouse model with a combined transgenic expression of A30P/A53T α-synuclein and a targeted deletion of parkin, mitochondrial pathology was more prominent in comparison with single transgenic mice (Stichel *et al*, 2007).

A striking feature of α-synuclein is its transmissibility to neighbouring cells (reviewed in Brundin *et al*, 2010; Frost and Diamond, 2010; Steiner *et al*, 2011; and Hansen and Li, 2012). The first evidence for this phenomenon came from studies in PD patients that received neural grafts derived from fetal midbrain tissue. At autopsy between 10 and 16 years after transplantation, Lewy body pathology was not only observed in the host striatal neurons but also in the grafted neurons of some PD patients (Kordower *et al*, 2008a, b; Li *et al*, 2008, 2010; Mendez *et al*, 2008). Studies in mice have essentially supported the phenomenon of intercellular α-synuclein transfer. Wild-type neuronal stem cells or embryonic neurons grafted into the brain of α-synuclein-overexpressing transgenic mice can take up host α-synuclein and develop inclusions (Desplats *et al*, 2009; Hansen *et al*, 2011). How can α-synuclein spread from cell to cell? Obviously, α-synuclein is secreted from cultured cells by exocytotic vesicles (Lee *et al*, 2005; Jang *et al*, 2010) or via exosomes, which are endosome-derived vesicles secreted during fusion of multivesicular bodies with the plasma membrane (Emmanouilidou *et al*, 2010; Alvarez-Erviti *et al*, 2011). The mechanism by which α-synuclein is taken up by recipient cells is largely unknown. Passive diffusion, endocytosis, and exosome-mediated uptake have been observed, and it is highly likely that the mode of cell entry depends on the type of α-synuclein species to be taken up (Sung *et al*, 2001; Desplats *et al*, 2009; Park *et al*, 2009; Emmanouilidou *et al*, 2010; Hansen *et al*, 2011). Internalized α-synuclein fibrils generated from recombinant α-synuclein promote aggregation of endogenous α-synuclein in mouse primary hippocampal neurons by a seeding activity and cause synaptic dysfunction and ultimately cell death (Volpicelli-Daley *et al*, 2011). Remarkably, in young asymptomatic A53T α-synuclein transgenic mice, intracerebral injection of brain homogenates from older symptomatic transgenic mice accelerates α-synuclein aggregation and neuronal degeneration, even at regions far beyond injection sites, a phenomenon also observed after injection of synthetic α-synuclein fibrils (Luk *et al*, 2012). The propagation of misfolded protein species by similar mechanisms has also been observed for other proteins associated with neurodegenerative disease, like tau, Aβ peptide and huntingtin, and thus may reflect a unifying mechanism of disease progression.

LRRK2: a GTPase-regulated kinase or a kinase-regulated GTPase?

Mutations in the gene encoding LRRK2 cause autosomal dominant parkinsonism and are the most common cause of familial PD (Paisan-Ruiz *et al*, 2004; Zimprich *et al*, 2004). Moreover, genome-wide association studies identified genetic variants in the *LRRK2* gene as risk factors for sporadic PD (Satake *et al*, 2009; Simon-Sanchez *et al*, 2009; Hamza *et al*, 2011; International Parkinson Disease Genomics Consortium *et al*, 2011). LRRK2 is a 2257 amino-acid protein that belongs to the ROCO protein family, characterized by an Roc (Ras of complex proteins) domain with GTPase activity and a COR (C-terminal of Roc) domain. A kinase domain with sequence similarities to RIPKs (receptor-interacting serine/threonine protein kinases) and MLKs (mixed lineage kinases), a subclass of the MAPKKK (mitogen-activated protein kinase kinase kinases) family, is located C-terminal to the COR domain. In addition, LRRK2 harbours several protein–protein interaction domains, such as a WD40 domain, leucine-rich repeats (LRRs), and an ankyrin domain. To date, six missense mutations have been demonstrated to segregate with familial PD, which cluster to the Roc, COR, and kinase domain.

The modular structure of LRRK2 suggests a role as a signalling protein, which can act as a kinase, GTPase, and/or scaffolding protein. LRRK2 forms dimers, and dimer formation appears to be essential for full catalytic activity (Deng *et al*, 2008b; Gotthardt *et al*, 2008; Greggio *et al*, 2008; Sen *et al*, 2009). LRRK2 shows a weak kinase activity *in vitro* mediating autophosphorylation and phosphorylation of generic substrates (i.e., myelin basic protein) or putative substrates, such as members of the ERM protein family (ezrin/radixin/moesin) that anchor the actin cytoskeleton to the plasma membrane, kinases of the MAP and Ste20 serine/threonine kinase families, 4E-BP, the eukaryotic translation initiation factor 4E-binding protein, or the forkhead box transcription factor FoxO1 (West *et al*, 2005; Gloeckner *et al*, 2006, 2009; Jaleel *et al*, 2007; Imai *et al*, 2008; Parisiadou *et al*, 2009; Kanao *et al*, 2010; Zach *et al*, 2010). It has been reported that the kinase activity is regulated by the GTPase activity, however, the Roc domain can be phosphorylated by the kinase domain, suggesting the

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possibility that not the kinase but the GTPase activity is the main output of LRRK2 and/or that both activities are regulated in a reciprocal manner (Greggio *et al*, 2008, 2009; Kamikawaji *et al*, 2009; Gloeckner *et al*, 2010; Liu *et al*, 2010; Webber *et al*, 2011). This hypothesis is in line with the recent identification of a GTPase-activating protein, ArfGAP1, which enhances the GTPase activity of LRRK2 but is also phosphorylated by LRRK2 (Stafa *et al*, 2012; Xiong *et al*, 2012).

The physiological and pathological functions of LRRK2 are still incompletely understood. It has been reported that pathogenic LRRK2 mutations increase kinase activity and/or decrease GTPase activity. Overexpression of pathogenic LRRK2 mutants in cultured cells, primary neurons or rodents causes cellular toxicity, which is dependent on the kinase activity (Greggio *et al*, 2006; Smith *et al*, 2006; West *et al*, 2007; Ho *et al*, 2009; Dusonchet *et al*, 2011). Indeed, inhibitors of LRRK2 kinase are protective in *in-vitro* and *in-vivo* models of LRRK2-induced neurodegeneration (Lee *et al*, 2010a; Liu *et al*, 2011a). From diverse cellular and animal models of LRRK2 loss and gain of function it emerged that LRRK2 can influence neurite outgrowth, cytoskeleton dynamics, vesicle trafficking, translational control, endocytosis, autophagy, mitochondrial function, MAPK and Wnt signalling, and extrinsic (TNFα/FasL) or intrinsic apoptosis pathways (reviewed in Cookson, 2010b; Berwick and Harvey, 2011; Daniels *et al*, 2011; and Tsika and Moore, 2012). Which of these pathways are relevant to the pathogenesis of PD requires further investigation. Notably, flies expressing the pathogenic G2019S LRRK2 mutant exhibit late-onset loss of DA neurons in selected clusters accompanied by locomotion deficits (Liu *et al*, 2008; Ng *et al*, 2009). In this fly LRRK2 model, coexpression of human parkin protects against DA neurodegeneration induced by ageing or rotenone treatment (Ng *et al*, 2009).

An interesting immunoregulatory function of LRRK2 was recently reported based on genome-wide association studies identifying *LRRK2* as a major susceptibility gene for inflammatory bowel disease (Barrett *et al*, 2008; Franke *et al*, 2010). LRRK2-deficient mice display an increased susceptibility to experimentally induced colitis (Liu *et al*, 2011b). LRRK2 which is expressed in macrophages, dendritic cells, and B lymphocytes can inhibit nuclear translocation of NFAT, a transcription factor regulating immune responses, by increasing its interaction with a cytoplasmic NFAT repressor (Gardet *et al*, 2010; Liu *et al*, 2011b). Notably, this activity of LRRK2 does not involve phosphorylation of NFAT.

So far, little is known about a possible impact of LRRK2 on mitochondria. Upon overexpression in cultured cells, about 10% of LRRK2 was found at the outer mitochondrial membrane (West *et al*, 2005). Mitochondrial localization was also shown for endogenous LRRK2 in mammalian brain tissue by confocal imaging, subcellular fractionation, and electron microscopy (Biskup *et al*, 2006). Mitochondrial pathology has been observed in aged G2019S LRRK2 transgenic mice (Ramonet *et al*, 2011), and the expression of this mutant aggravates mitochondrial alterations in inducible A53T α-synuclein transgenic mice (Lin *et al*, 2009). However, a similar approach using double transgenic mice did not provide support for a pathophysiological interaction of LRRK2 and α-synuclein (Daher *et al*, 2012). Skin fibroblasts derived from five patients carrying the G2019S mutation show a decrease in mitochondrial membrane potential and total ATP production and a trend towards increased mitochondrial interconnectivity (Mortiboys *et al*, 2010). A recent study reported that overexpression of wild-type LRRK2 and pathogenic LRRK2 mutants in cultured cells and primary cortical neurons cause mitochondrial fragmentation by increasing mitochondrial recruitment of Drp1 (Wang *et al*, 2012). Notably, LRRK2-induced toxicity could be blocked by decreasing mitochondrial fission or increasing fusion. Further studies are needed to determine whether these mitochondrial effects of LRRK2 are direct or indirect and what the underlying mechanism might be.

Controversies and unanswered questions

Why are SNc DA neurons particularly vulnerable?

A key challenge in the neurodegeneration field has been to understand the selective vulnerability of neuronal subpopulations. SNc DA neurons are considered special in terms of oxidative stress management and calcium homoeostasis. Sources of ROS in DA neurons are the enzymatic and non-enzymatic metabolism of cytoplasmic dopamine. Vesicular dopamine is protected from oxidation, thus, an increase in oxidation-prone cytoplasmic dopamine is toxic to neurons. VMAT2 is the vesicular monoamine transporter responsible for dopamine uptake into presynaptic vesicles, and dysfunction of VMAT, for example as a consequence of decreased mitochondrial ATP production, can induce nigrostriatal degeneration (Caudle *et al*, 2007). Moreover, the amount of iron, which can trigger ROS formation via the Fenton reaction, has been reported to be high in SNc DA neurons, whereas antioxidative components, such as reduced glutathione, are expressed at low levels (Kish *et al*, 1985; Chinta and Andersen, 2008). Oxidative stress may also arise from excitotoxicity and mitochondrial dysfunction, which can trigger a vicious circle. Excitotoxicity results from overstimulation of glutamate receptors and elicits a cascade of harmful events including calcium influx that can challenge the storage capacity of the ER and mitochondria. SNc DA neurons are vulnerable to excitotoxicity based on rich glutamatergic input from the subthalamic nucleus. A decrease in ATP production, for example as a consequence of mitochondrial dysfunction, can compromise the ATP-dependent regulation of glutamate receptors favouring their overstimulation.

Calcium toxicity has been suggested as another determinant of selective vulnerability. Adult ventrolateral SNc DA neurons engage L-type $Ca_V1.3$ calcium channels for rhythmic pacemaking, in contrast to less vulnerable DA neurons of the ventral tegmental area which use sodium channels for pakemacing activity (Chan *et al*, 2007). Reliance on these L-type calcium channels increases with age and leads to sustained elevated cytoplasmic calcium levels, which may stimulate mitochondrial respiration and ROS production. An imbalance in calcium homeostasis can be enhanced by mitochondrial and ER stressors or genetic mutations in stress-protective genes, resulting in mitochondrial calcium overload, which impairs ATP synthesis and consequently, ATP-dependent cellular processes.

Clearly, none of the current hypotheses can sufficiently explain the selective vulnerability of SNc DA neurons, rather, a combination of the phenomena described above as well as

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some yet unknown factors are responsible for the preferential susceptibility of this neuronal population.

Is mitochondrial complex I inhibition a causal factor for PD?

Generic complex I inhibitors, such as MPTP, rotenone, or annonacin, can induce parkinsonian syndromes in humans and animal models, however, it is not clear whether their mode of action is restricted to complex I inhibition. Compatible with the complex I inhibition hypothesis, ectopic expression of Ndi1p, a yeast single-subunit NADH dehydrogenase that is insensitive to MPTP and rotenone, has been shown to protect DA neurons from MPTP and rotenone toxicity *in vitro* and *in vivo* (Sherer *et al*, 2003; Seo *et al*, 2006; Richardson *et al*, 2007; Marella *et al*, 2008). The role of complex I inhibition in PD has been challenged by a study using mice that lack functional *Ndufs4*, a gene encoding a subunit implicated in assembly and function of complex I. Ndufs4 KO mice show neuronal degeneration in the cerebellum, olfactory bulb, and vestibular nuclei, whereas DA neurons are not affected (Kruse *et al*, 2008; Quintana *et al*, 2010; Choi *et al*, 2011). Moreover, midbrain primary cultures derived from Ndufs4 KO mice are not protected against MPP^+ or rotenone, suggesting that toxicity of these drugs is not caused by complex I inhibition (Choi *et al*, 2008). Ndufs4-deficient DA neurons are even more sensitive to rotenone, and complex I-independent mechanisms have been reported to contribute to the toxicity of rotenone, such as microtubule depolymerization (Ren *et al*, 2005; Choi *et al*, 2011). However, it was recently shown that complex I activity is only partially decreased but not abolished in Ndufs4 KO mice due to the formation of respiratory supercomplexes with a stabilizing effect of complex III (Calvaruso *et al*, 2012; Sterky *et al*, 2012). This finding can explain why Ndufs4-deficient neurons are still sensitive to MPTP and rotenone toxicity. DA neuron-specific conditional Ndufs4 KO mice do not show overt nigrostriatal degeneration, only a mild decrease (7.5%) in tyrosine hydroxylase-positive neurons at 24 months of age (Sterky *et al*, 2012). Nevertheless, striatal dopamine turnover is increased and dopamine release is decreased in Ndufs4-deficient mice, which may reflect an early consequence of mitochondrial dysfunction. These findings support the notion that complex I deficiency can contribute to the pathogenesis of PD.

The most convincing link between PD genes and complex I activity was provided for PINK1. In fact, there is strong experimental evidence for a central role of complex I in PINK1-associated mitochondrial pathology. Complex I enzymatic activities are reduced in PINK1-deficient mice and flies (Gautier *et al*, 2008; Morais *et al*, 2009). In flies, expression of the yeast complex I equivalent Ndi1p rescues several PINK1-deficient phenotypes, and downregulation of complex I subunits phenocopies some PINK1-associated alterations (Vilain *et al*, 2012). A recent screen for genetic modifiers in PINK1-deficient flies identified Heix, a gene encoding a prenyltransferase involved in vitamin K2 biosynthesis (Vos *et al*, 2012). In the fly model, vitamin K2 was able to rescue mitochondrial morphological defects and to maintain ATP production, which has been attributed to its ability to serve as a mitochondrial electron carrier. The molecular mechanism of these interactions and its significance in mammalian models requires further investigation.

How can discrepant findings in Drosophila and mammalian PD models be explained?

The *Drosophila* model has contributed valuable insights into PD pathogenesis. Importantly, it has been helpful to characterize the function of PD-associated genes and to identify genetic modifiers, thereby focusing research activities on key biochemical pathways. The strikingly similar phenotype of PINK1 and parkin mutant flies has revealed that parkin acts downstream of PINK1 to maintain mitochondrial integrity. Various effects of PINK1 and parkin on mitochondria have been described in different models, including mitochondrial morphology, dynamics, bioenergetics, transport, and quality control. Rather than a single linear pathway integrating all these effects described, diverse pathways account for these manifold activities that converge on mitochondria. Consistent with this notion, there are different activities of PINK1 and parkin that are either dependent or independent on each other.

What is still causing confusion in the field is the fact that opposing effects are reported from genetic fly and mammalian models. For example, the PINK1 or parkin mutant phenotypes in *Drosophila* can be rescued by decreasing mitochondrial fusion and increasing fission, whereas the acute loss of PINK1 or parkin in cellular mammalian models is reverted by increasing mitochondrial fusion or decreasing fission. A similar phenomenon has been reported for affecting mitochondrial transport. In PINK1 mutant flies, Miro levels are increased and Miro RNAi can rescue PINK1 mutant phenotypes (Liu *et al*, 2012). However, in human cells Miro levels are decreased in the absence of PINK1 and Miro overexpression can revert PINK1 deficiency (Weihofen *et al*, 2009; Liu *et al*, 2012). One plausible explanation for these seemingly discrepant findings is that mammalian cells have elaborate compensatory strategies that may be absent or different in flies. In fact, compensation can at least partly explain why genetic PD mouse models lack a striking phenotype and why patients with mutations in PD genes develop symptoms only after some decades when coping mechanisms may be less efficient due to ageing.

Does dysfunctional mitophagy cause PD?

A role of parkin and PINK1 in mitochondrial quality control is appealing and can explain beneficial mitochondrial effects of these PD genes in different models. Beyond any doubt, parkin-induced autophagic removal of mitochondria is a robust phenomenon in cultured cells treated with mitochondrial uncouplers, such as CCCP. Parkin-induced mitophagy is strictly dependent on PINK1 expression, since parkin cannot translocate to uncoupled mitochondria in the absence of PINK1. There is an ongoing discussion as to whether PINK1/parkin-mediated mitophagy is a physiologically and pathophysiologically relevant pathway *in vivo*. The main criticism is that CCCP is a drastic unphysiological stressor that causes uncoupling of all mitochondria within seconds. Parkin-induced mitophagy has mostly been studied in established tumour cell lines or mouse embryonic fibroblasts, which preferentially produce ATP by glycolysis. In contrast, neurons depend on oxidative phosphorylation for ATP production and therefore may not easily discard their mitochondria. In parkin- or PINK1-deficient flies, enlarged aggregated mitochondria have been observed, however, this is not necessarily a consequence of defective mitochondrial

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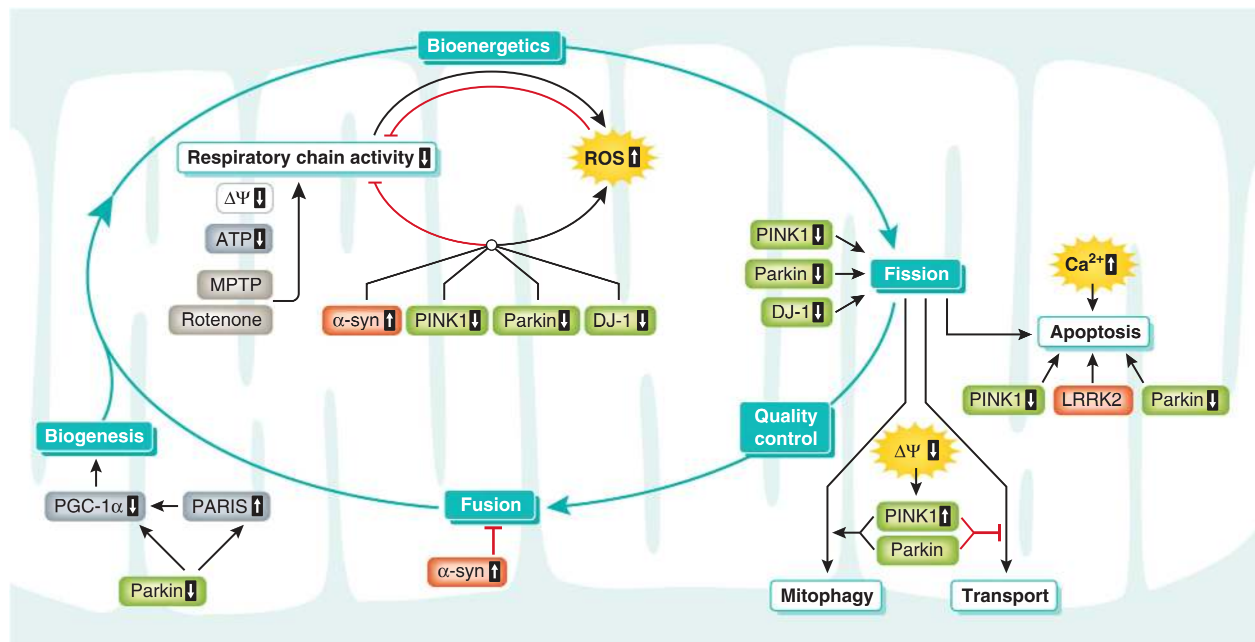

Figure 5 PD genes are implicated in various aspects of mitochondrial biology. Products of autosomal recessive PD genes (shown in green) or autosomal dominant PD genes (shown in red) directly or indirectly affect a wide spectrum of mitochondrial processes, including their life cycle, bioenergetic capacity, quality control, dynamic changes of morphology and connectivity (fusion, fission), subcellular distribution (transport), and the regulation of cell death pathways.

clearance. In fact, it has not been demonstrated conclusively that the phenotypic alterations in parkin or PINK1 mutant flies are caused by an impairment of mitophagy. Moreover, parkin can rescue PINK1 mutant flies, which argues against defective mitophagy being responsible for the PINK1-deficient phenotype, given that parkin cannot promote mitophagy in the absence of PINK1.

Perspective and future directions

Research into the function and dysfunction of PD-associated genes revealed that at least some of these genes interface with pathways regulating various aspects of mitochondrial biology (Figure 5). In fact, there is compelling evidence that mitochondrial dysfunction is a common denominator of sporadic and familial PD. Genome-wide association studies indicated that genetic variants of the classical PD genes *α-synuclein* and *LRRK2* increase the risk of developing sporadic PD, adding to the notion that sporadic and familial disease entities share common pathways. Emerging evidence suggests that bioenergetic deficits and dysregulation of the mitochondrial quality control rather than oxidative stress alone are relevant players in the pathogenesis of PD. Hence, a plethora of challenging issues remains to be addressed. First, it will be important to disentangle direct from indirect mitochondrial effects. Second, results from *in-vitro* studies should be validated in suitable *in-vivo* models to prove the physiological or pathophysiological relevance of the observed effects. Third, we need to increase our understanding of the interaction of disease or susceptibility genes with environmental factors in triggering and/or promoting neurodegeneration. Most importantly, the ultimate aim would be to translate basic research into clinical practice by exploiting mitochondrial pathways as a rationale to intervene early in the pathogenic process. To this aim, it would be helpful to have reliable biomarkers or imaging techniques to identify patients, which are likely to benefit from mitochondria-based strategies.

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Conflict of interest

The authors declare that they have no conflict of interest.

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