

Integrated pathways of parkin control over mitochondrial maintenance – relevance to Parkinson's disease pathogenesis

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Mitochondrial dysfunction and oxidative stress are considered among the main molecular mechanisms implicated in Parkinson's disease (PD) pathogenesis. Here, we focus on the deficiency of *PARK2* and its product parkin, which is relevant to both familial and sporadic PD pathogenesis. Parkin emerges as an important regulator of processes that maintain mitochondrial quality. We focus on the parkin-dependent aspects of mitochondrial biogenesis, including mtDNA replication, transcription, mitophagy, mitochondrial fusion, fission, and transport. We discuss possible underlying molecular mechanisms, exerted by parkin in cooperation with other mitochondrial maintenance factors such as TFAM, PGC-1alpha, mortalin, HSP70/HSC70 and LRPPRC, all of them implicated in PD pathogenesis. We review numerous models of lipopolysaccharide toxicity that demonstrate how mitochondrial biogenesis and mitophagy are induced simultaneously to cope with mitochondrial dysfunction. The spatial and temporal interdependence of mitochondrial quality pathways underscores the importance of an integrative approach for future studies.

Key words: Parkinson's disease, *PARK2*, parkin, mitochondrial biogenesis, mitochondrial quality control, TFAM, PGC-1alpha

INTRODUCTION

Mitochondrial dysfunction is consistently observed at various stages of both sporadic and genetic Parkinson's disease (Abou-Sleiman et al. 2006). Over the recent 10 years evidence has been mounting that parkin integrates mitochondrial maintenance and quality control. *PARK2* gene mutations cause juvenile and early onset PD. Parkin deficiency in PD patients, and also in numerous cellular and animal PD models leads to mitochondrial dysfunction. Here, we provide the state of the art regarding the parkin involvement in mtDNA damage recognition and repair, mitophagy, mitochondrial fusion, fission, transport and biogenesis. We focus on the interdependence among these processes and aim to provide their integrated picture. We concentrate and hypothesize on the functional versatility of parkin, which exerts control over mitochondrial physiology not only as a multifaceted

ubiquitin E3 ligase but, possibly, as a *bona-fide* transcription factor. We also discuss the possible involvement of parkin deficiency in sporadic PD pathogenesis.

ROLE OF *PARK2* AND PARKIN IN PARKINSON'S DISEASE

PARK2 mutations in PD

PARK2 is a large gene, spanning 1.3 Mb. More than 200 *PARK2* mutations are known, scattered over all twelve exons (The Parkinson Disease Mutation Database, accessed May 29, 2013). They comprise point mutations, small insertions/deletions and exon rearrangements (Mata et al. 2004). Homozygous and compound heterozygous *PARK2* mutations are the most frequent cause of autosomal recessive early onset Parkinson's disease (EOPD) and juvenile PD (Hattori et al. 1998, Kitada et al. 1998, Klein and Lohmann 2009). They are also found in about 1% of LOPD (late onset PD) patients (age at onset – AAO>50 years) (Klein et al. 2007). A debate is ongoing whether the *PARK2* mutations-associated PD and sporadic PD

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overlap pathophysiologically (van Eimeren et al. 2010). Single heterozygous *PARK2* mutations occur with similar frequency in PD patients and in healthy individuals and their role in PD pathogenesis is not well understood (Klein et al. 2007, Bruggemann et al. 2009). Furthermore, since common *PARK2* polymorphisms could potentially influence the protein activity, numerous association studies have addressed their role in PD risk, often with contradictory results (The Parkinson Disease Mutation Database, accessed March 12, 2013).

Role of alternatively spliced isoforms of *PARK2* transcripts

The understanding of the role of *PARK2* mutations in PD pathogenesis is further complicated by the finding that alternatively spliced isoforms devoid of exon 5 or exon 4 are expressed in the brain (Kitada et al. 1998, Sunada et al. 1998, Tan et al. 2005). The exon 4 splice variant (E4SV) results in a truncated protein lacking two RING domains, present both in the substantia nigra (SN) and leukocytes. The E4SV/wild type parkin ratio is higher in sporadic PD patients compared to healthy controls (Tan et al. 2005). Differential expression of alternatively spliced transcripts in different tissues provides another level of parkin activity regulation. For instance, in peripheral leukocytes an isoform lacking exons 3–5 is the most abundant *PARK2* transcript (Sunada et al. 1998).

Landscape of clinical phenotypes related to *PARK2* mutations

PARK2 mutations cause, in most cases, a PD form indistinguishable from idiopathic PD, characterized by a good response to L-DOPA and slow progression, sometimes accompanied by less typical manifestations like dystonia, motor fluctuations, early dyskinesias, and symmetrical onset (Abbas et al. 1999, Hayashi et al. 2000, Lucking et al. 2000, Gouider-Khouja et al. 2003, Lohmann et al. 2003, Varrone et al. 2004, Khan et al. 2005a, Ribeiro et al. 2009, Semenova et al. 2012). Substantia nigra neurodegeneration and gliosis have been also observed (Mori et al. 1998, Hayashi et al. 2000, Sasaki et al. 2004, Pramstaller et al. 2005). Although initially it seemed that Lewy body-pathology was basically absent in brains of PD patients with *PARK2* mutations (Ishikawa and Takahashi 1998,

Mori et al. 1998, Hayashi et al. 2000, van de Warrenburg et al. 2001), a few subsequent brain *post-mortem* studies have reported the presence of alpha-synuclein-positive inclusions (Farrer et al. 2001, Pramstaller et al. 2005, Ruffmann et al. 2012, Sasaki et al. 2004), reviewed in (Poulopoulos et al. 2012). Interestingly, age at onset of PD decreased with increasing number of mutated *PARK2* alleles (Sun et al. 2006, Klein et al. 2007). Also substantia nigra hyperechogenicity increased with the number of mutated parkin alleles (Walter et al. 2004, Hagenah et al. 2007).

Asymptomatic single *PARK2* mutation carriers show a reduction of striatal F-DOPA uptake compared to control subjects in positron emission tomography (PET) (Hilker et al. 2001, 2002, Khan et al. 2002). In another PET study, 69% of asymptomatic single *PARK2* mutation carriers demonstrated subclinical loss of putamen dopaminergic function (Khan et al. 2005b). They manifested no signs of parkinsonism at neurological examination after 5-year follow-up and had very slow rates of nigrostriatal dysfunction progression, in comparison to idiopathic PD patients (a mean of 0.56% vs. 9–12% annual reduction in putamen F-DOPA uptake) (Pavese et al. 2009). These results suggest that either few or none of the subjects with a single *PARK2* mutation will develop clinical parkinsonism (Pavese et al. 2009). Interestingly, functional magnetic resonance imaging (fMRI) in another group of asymptomatic single *PARK2* mutation carriers showed additional recruitment of motor cortical areas during simple motor tasks, indicative of compensatory response (van Nuenen et al. 2009).

Does parkin matter in sporadic PD?

Parkin activity has been found compromised in post-mortem brains from sporadic PD (sPD) patients with Lewy bodies (LBs), in cellular and animal PD models, by oxidative (Wang et al. 2005, Wong et al. 2007, Meng et al. 2011), nitrosative (Chung et al. 2004, Yao et al. 2004) or dopamine-related stress (LaVoie et al. 2005). Regarding the latter, dopamine has been shown to modify parkin covalently inducing its aggregation and inactivating its ubiquitin E3 ligase function. Catechol-modified parkin has been detected in substantia nigra, but not in other regions of healthy human brain, and, indeed, its activity is decreased in brains of sPD patients (LaVoie et al. 2005). These results underscore the selective vulnerability of affected brain regions in PD. Interestingly, the parkin

levels in blood of PD patients was also decreased (Vinish et al. 2010). Recently, parkin sulphydration has been found to enhance its catalytic activity (Vandiver et al. 2013). This physiological modification is markedly depleted in the brains of PD patients (Vandiver et al. 2013).

Collectively, the cited results suggest that various, possibly overlapping, pathways lead to a progressive loss of parkin function in dopaminergic neurons during physiological aging, as well as, in sporadic Parkinson disease. Some of the mechanisms will be discussed in details below.

THE PARKIN PROTEIN IS A MULTIFUNCTIONAL E3 UBIQUITIN LIGASE

Structure and activities

PARK2 gene codes for a protein of 465 amino acids, parkin. The C-terminus of the protein comprises a motif characteristic for ubiquitin ligases: RBR (RING between RING), containing RINGI and RINGII domains, separated by the IBR (In-Between-RING) domain (Marin et al. 2004). The N-terminus contains UBL domain (UBiquitin-Like) and a UPD domain (Unique Parkin Domain) comprising a recently identified RING0 domain (Hristova et al. 2009). Parkin functions as a RBR ubiquitin E3 ligase catalyzing the transfer of ubiquitin from E2 to a plethora of substrates and acting as a scaffolding protein (Shimura et al. 2000). However, HECT-like E3 ligase activity of parkin has been identified recently in a cell-free assay (Lazarou et al. 2013). Parkin usually forms poly-ubiquitin chains through lysine 48 (K48) residue of ubiquitin, targeting substrates for proteasomal degradation [reviewed in (Dawson and Dawson 2010, Rankin et al. 2011)]. However, parkin is also capable of mediating monoubiquitination (Fallon et al. 2006, Hampe et al. 2006, Matsuda et al. 2006, Joch et al. 2007, Moore et al. 2008, Chen et al. 2010, Ramanathan and Ye 2012) and K63-linked polyubiquitination (Doss-Pepe et al. 2005, Lim et al. 2005). The latter modifications are considered as proteasome-independent and can signal other cellular processes, such as protein sorting and transport, transcription regulation, and DNA repair (reviewed in Dawson and Dawson 2010, Chew et al. 2011, Ramanathan et al. 2012). Moreover, parkin auto-regulates its E3 ligase activity through autoubiquitination (Chaugule et al. 2011)

Various molecular mechanisms have been proposed to underlie the pathogenic effects of *PARK2* mutations (Dawson and Dawson 2010). The primary hypotheses have focused on the ubiquitin-proteasome dysfunction due to impairment of the ubiquitin E3 ligase activity of parkin (Shimura et al. 2000) which in turn would lead to the accumulation of parkin substrates in the cell. However, some reports have suggested that only RINGII domain point mutations (affecting the catalytic core of the enzyme) impair the E3 ligase activity (Hampe et al. 2006, Matsuda et al. 2006). Many other pathogenic *PARK2* mutations change the general physical characteristics of the protein such as its stability and/or solubility, promoting aggregate formation (Sriram et al. 2005, Hampe et al. 2006). Moreover, pathogenic *PARK2* mutations do not seem to impair the binding of substrates, e.g., p38, CDCrel-1, α -tubulin, α 4 proteasome subunit (Hampe et al. 2006), or influence their levels in PD animal models (Goldberg et al. 2003). Recently, more and more observations from PD animal models, and later from PD patient-derived tissues, and PD cellular models have pointed to a key role of parkin in the regulation of mitochondrial maintenance. Some of the mitochondria-mediated effects of parkin require its presence within this cellular compartment, which is discussed below.

Subcellular localization

In subcellular fractionation studies endogenous and ectopically expressed parkin is mainly cytosolic, though it has been also detected in the outer mitochondrial membrane, Golgi complex, synaptic vesicles, and postsynaptic densities (Shimura et al. 1999, Kubo et al. 2001, Fallon et al. 2002, Darios et al. 2003). In ultrastructural analyses of adult mouse brain, overexpressed parkin associated with cytoplasmic vesicles, endoplasmic reticulum, the outer nuclear membrane, the nuclear matrix, and the outer mitochondrial membrane (OMM) (Stichel et al. 2000).

Fractionation experiments, immunofluorescent and immunoelectron microscopy in various cultured cell lines (including a dopaminergic neuroblastoma, SH-SY5Y, a rhabdomyosarcoma, RD, a cerebellar medulloblastoma, TE671, a monkey kidney, COS-1 and a rat myocyte L6 cell line) corroborated the presence of endogenous parkin within the mitochondrial compartment, possibly associated with inner mitochondrial membrane (IMM), and in mitochondrial

protein extracts (Kuroda et al. 2006, Davison et al. 2009, Rothfuss et al. 2009). In contrast, other researchers found that parkin (both endogenous and exogenous) translocates from the cytosol to mitochondrial surface only upon chemically-induced mitochondrial depolarization (Narendra et al. 2008, Matsuda et al. 2010, Rakovic et al. 2010, Yang et al. 2011). However, the latter studies only demonstrate a substantial difference between the quantities of parkin present in mitochondrial and cytoplasmic fractions, rather than confirm the lack of the protein in mitochondria.

Fractionation experiments, especially for an overexpressed protein, should be interpreted with caution since they are prone to artifacts. For example, parkin overexpression increases its proportion in the mitochondrial fraction (Matsuda et al. 2010). Thus, further *in vivo* studies on endogenous parkin are needed to confirm unambiguously its presence inside mitochondria.

LOSS OF *PARK2* FUNCTION LEADS TO MITOCHONDRIAL DYSFUNCTION

Lessons from animal models

Early evidence of parkin involvement in mitochondrial maintenance comes from animal PD models based on *PARK2* deficiency. They exhibited mitochondrial dysfunction along with increased oxidative stress, although the majority recapitulated hardly any PD-like features, such as dopaminergic (DA) neuron degeneration.

For instance, *Caenorhabditis elegans* with a deleted parkin gene was more susceptible to apoptosis induced by rotenone, a known complex I inhibitor, in comparison with control animals (Ved et al. 2005). *Drosophila* parkin null mutants were characterized by a reduced lifespan, male sterility and locomotor defects due to flight muscle cells degeneration, accompanied by a loss of a subset of dopaminergic neurons, inflammation and oxidative stress markers upregulation (Greene et al. 2003, 2005). In that model, mitochondrial dysfunction preceded the pathological changes occurring in muscles and spermatids (Greene et al. 2003). Recently, it was shown that DA neurons from *Drosophila* parkin mutants accumulate enlarged, depolarized mitochondria to a greater extent than do cholinergic neurons, which underscores the higher and selective vulnerability of the DA neurons to mitochondrial dysfunction (Burman et al. 2012).

In mice, *PARK2* exon 3 deletion led merely to a nigrostriatum dysfunction, without a loss of dopaminergic neurons, accompanied by lowered expression of mitochondrial complex I and IV subunits. The mice had also an impaired respiratory efficiency in striatum-isolated mitochondria, and increased oxidative stress (Palacino et al. 2004). In other parkin deficiency models, mice showed only minimal deficits in behavior, and dopamine neurotransmission, with a preserved number of SN DA neurons (Goldberg et al. 2003, Itier et al. 2003, Von Coelln et al. 2004, Perez and Palmiter 2005). It has been proposed that the lack of PD-like phenotypes and DA neurons degeneration in various mice models of germline parkin deletion could have resulted from developmental compensatory mechanisms (for review see Dawson et al. 2010). Indeed, young parkin-null-mice showed a compensatory elevation of the antioxidant GSH and stress induced proteins (glutathione reductase (GR), glutathione peroxidase (GPx), chaperones CHIP and HSP70) which could protect dopamine neurons from death (Itier et al. 2003, Rodriguez-Navarro et al. 2007). In contrast, aged parkin-null mice lack these compensatory mechanisms, have a shorter lifespan, a PD-like motor dysfunction, reduced number of DA neurons and high levels of tau (Rodriguez-Navarro et al. 2007).

The aforementioned counter-balancing responses can be avoided by creating conditional parkin knockout in adult mice, which indeed leads to a progressive DA neurons degeneration (Shin et al. 2011). The compensatory mechanisms fail also in mice with parkin germline knockout exposed additionally to chronic systemic lipopolysaccharide (LPS), where degeneration of SN neurons is observed (Frank-Cannon et al. 2008). LPS causes inflammatory response, but it also triggers oxidative stress and mitochondrial dysfunction, followed by either apoptosis or autophagy and mitochondrial biogenesis, the latter two processes being cytoprotective, as shown in various cellular and *in vivo* models (discussed below). Macrophages from parkin-null mice have elevated TNF, IL-1 β and iNOS mRNAs compared to controls, indicating that parkin may also downregulate the expression of inflammatory mediators (Tran et al. 2011).

On the other hand, overexpression of a human mutant parkin in *Drosophila* and mice resulted in progressive motor impairment and degeneration of DA neurons, indicative of a dominant negative effect exerted by the mutant parkin (Sang et al. 2007, Lu et

al. 2009). Prolonged stress activates oxidative processes in mitochondria and inhibits activity of the antioxidant system, causing development of energy deficiency in brain cells (Kuchukashvili et al. 2012).

Taken together, the animal models lacking functional parkin highlight the crucial role of the protein in mitochondrial maintenance and oxidative stress response. However, the causal relation between the parkin deficiency and PD pathogenesis is difficult to observe in germline parkin deletion models due to the developmental compensatory mechanisms. Typical PD hallmarks become only evident in conditional parkin knockouts, in aged animals or upon additional administration of a stressogenous factor, such as LPS.

More evidence from PD patient-derived tissues and *in vitro* models

Another line of evidence showing the parkin's crucial role in mitochondrial physiology comes from *in vitro* studies involving PD patient-derived tissues and PD cellular models.

It has been shown, that leukocytes and fibroblasts from EOPD patients, carrying *PARK2* mutations, have a reduced activity of complex I, as compared to respective controls (Muftuoglu et al. 2004, Mortiboys et al. 2008). Moreover, EOPD fibroblasts, with homozygous and heterozygous compound *PARK2* mutations, showed a decline in mitochondrial membrane potential (MMP) and ATP levels, accompanied by enhanced mitochondrial branching (Mortiboys et al. 2008). The MMP collapse was further aggravated by a switch to a galactose medium, forcing the cells to use mostly oxidative phosphorylation as an energy source. Indeed, it has been recently shown that parkin deficiency activates glycolysis and reduces mitochondrial respiration in various cell lines, leading to the Warburg effect – a shift towards anaerobic respiration (Zhang et al. 2011). Likewise, parkin ectopic expression reduces the rate of glycolysis and lactate production in the cytosol, promoting aerobic respiration (Zhang et al. 2011).

An almost complete parkin deficiency, obtained by silencing with small interfering RNAs (siRNA), was sufficient to trigger mitochondrial dysfunction in wild-type fibroblasts (Mortiboys et al. 2008). However, 50% parkin silencing, which models haploinsufficiency, did not change any of the aforementioned mitochondrial parameters, indicating the importance of the dose (Mortiboys et al. 2008). Treatment of fibroblasts

carrying *PARK2* mutations with L-2-oxothiazolidine-4-carboxylic acid (OTCA) – a known antioxidant and glutathione precursor – normalized MMP and restored ATP levels, likely through a compensatory upregulation of complex II activity (Mortiboys et al. 2008). In two other models, fibroblasts with *PARK2* compound heterozygous mutations (c. del exon 2–3/del exon 3, c.101delAG/del exon 3–4, respectively), displayed ultrastructural mitochondrial and peroxisomal abnormalities, impaired energy metabolism, lipid peroxidation due to upregulated ROS production (Pacelli et al. 2011), a 22% decrease in mtDNA level and less sufficient repair mechanisms in comparison to control fibroblasts (Rothfuss et al. 2009).

To sum up, evidence on parkin's role in mitochondrial maintenance from numerous parkin deficiency models (PD patient-derived tissues, cellular and previously mentioned animal models) is consistent. Lack of parkin leads to abnormal mitochondrial morphology and results in a decrease of various mitochondrial physiology parameters, such as the activity of electron transport chain complexes, ATP levels, mitochondrial membrane potential, and mtDNA level. However, the underlying molecular mechanisms by which parkin controls mitochondria, remain obscure.

PARKIN REGULATES MITOCHONDRIAL TURNOVER

Possible mechanisms of mitochondrial biogenesis regulation by parkin

Although parkin seems to be involved in a plethora of highly interconnected processes, such as mitophagy, fusion, fission and mitochondrial transport, first we would like to focus on mitochondrial biogenesis that is the least known area of parkin activity.

Parkin binds mtDNA and TFAM

In 2006 Kuroda and colleagues provided the first evidence for parkin regulating mitochondrial biogenesis and their results were subsequently confirmed by Rothfuss and coworkers (2009). *In vitro* overexpression of wild-type (wt) parkin increased mtDNA transcription and replication, mtDNA-encoded respiratory chain proteins level and mitochondrial mass, which was abrogated by parkin mutations, localized in all functional parkin domains (Kuroda et al. 2006, Rothfuss et al. 2009) (see

also Fig. 1). These results suggest that the induction of mitochondrial biogenesis by parkin is not specifically related to its RINGII domain, which is thought to determine the ubiquitin E3 ligase activity (Hampe et al. 2006, Matsuda et al. 2006). Parkin dose-dependently stimulated the *in vitro* mitochondrial transcription from both LSP and HSP mtDNA promoters in the presence of partially purified TFAM fraction (Kuroda et al. 2006), which could potentially contain other mitochondrial biogenesis factors. Compatible with the results of parkin

overexpression, endogenous parkin silencing by siRNA significantly decreased the levels of mitochondria-encoded mRNAs (Kuroda et al. 2006). Moreover, it has been shown that overexpressed parkin and TFAM interact with each other, as demonstrated by co-immunoprecipitation and electromobility shift assay (Kuroda et al. 2006). Importantly, both overexpressed and endogenous parkin binds mtDNA, most likely *via* TFAM, in cell culture, and *in vivo* – in murine and human brain (Kuroda et al. 2006, Rothfuss et al. 2009).

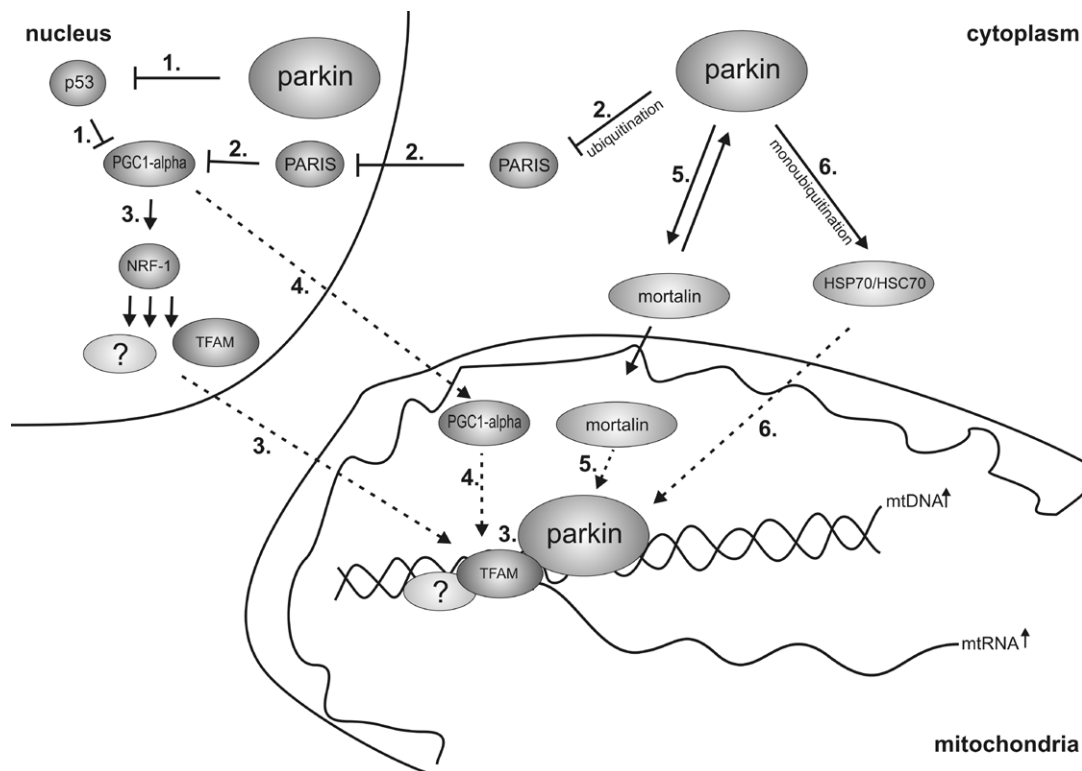


Fig. 1. Potential mechanisms of mitochondrial biogenesis regulation by parkin. Although parkin is predominantly a cytosolic protein, it has been also detected in mitochondria and in the nucleus. The presence of parkin in these compartments is further corroborated by some of the following functional observations: (1) Parkin may induce PGC-1alpha-dependent mitochondrial biogenesis through ubiquitin E3 ligase – independent repression of p53 transcription; (2) Parkin may enhance mitochondrial biogenesis by targeting to proteasome a negative transcriptional regulator of PGC-1alpha – PARIS; (3) Parkin complexed with TFAM binds mtDNA *in vivo* while its overexpression *in vitro* enhances both mtDNA transcription and replication. This effect may be direct or PGC-1alpha/NRF-1 – dependent. However, it is not known what downstream targets of PGC-1alpha/NRF-1 could mediate upregulation of mtDNA and mtRNA. Although TFAM seems to be a good candidate, in parkin deficiency models a decrease in TFAM expression levels was not observed. Parkin could modulate directly TFAM activity rather than its level, or influence other transcriptional/replication machinery proteins (designated with the interrogation mark), e.g., TFB2M, TWINKLE, etc. (4) PGC-1alpha could be speculated to induce mitochondrial transcription and replication directly since it has been found in mitochondrial nucleoids. (5) Overexpressed parkin interacts with mortalin, but the molecular mechanism of the reciprocal regulation of these two proteins remains to be established. Mortalin knockdown leads to decreased mtDNA copy number, reversible by parkin overexpression, which indicates that mortalin could enhance mtDNA replication and that parkin and mortalin could operate within the same molecular pathway. (6) Parkin multiple monoubiquitinates HSP70/HSC70. HSP70/HSC70 has been detected in mtDNA nucleoids and thus could potentially regulate mtDNA replication. Dashed lines indicate pathways that require further experimental verification.

TFAM is the principal mtDNA maintenance factor that organizes it into nucleoid-like structures, displaying both promoter-specific and non-specific mtDNA binding (Larsson et al. 1998, Alam et al. 2003, Campbell et al. 2012). TFAM, along with TFB1M, TFB2M (mitochondrial transcription factor B1M and B2M) and mitochondrial RNA polymerase (POLRMT) forms the basal mtDNA transcription apparatus (Shutt and Shadel 2010). Apart from controlling transcription it regulates mtDNA replication through RNA priming for the first strand synthesis. Both TFAM and parkin have been implicated in the recognition of nuclear and mitochondrial DNA damage and repair (Yoshida et al. 2002, Noack et al. 2006, Hayashi et al. 2008, Kao 2009, Rothfuss et al. 2009). TFAM also interacts with p53, possibly mediating mtDNA damage-induced apoptosis (Yoshida et al. 2003).

Collectively, the above observations suggest several scenarios by which parkin could enhance mitochondrial transcription and replication. A direct stimulation of mitochondrial biogenesis by parkin could be envisioned, but it needs to be verified in functional studies. Alternatively, the effect could be indirect, through the control of the level and/or activity of proteins responsible for mitochondrial biogenesis. In this case, TFAM, known to interact with parkin, could be a good candidate. Still, other mitochondrial biogenesis factors, such as TFB2M, NRFs (nuclear respiratory factors), PGC-1alpha (peroxisome proliferator-activated receptor-gamma co-activator alpha), mortalin, etc. cannot be excluded as mediators (Fig. 1). As a third possibility, transcriptional control of nuclear genes involved in mitochondrial physiology by parkin can be considered. We discuss the suggested scenarios below.

Parkin increases PGC-1alpha levels through PARIS downregulation

Another mechanism linking the E3 ligase function of parkin with mitochondrial biogenesis regulation has been recently proposed by Shin and colleagues (2011). They have found that parkin controls the levels of PARIS (parkin interacting substrate, or ZNF746) – a negative transcriptional regulator of PGC-1alpha – through proteasome-targeting ubiquitination (Shin et al. 2011). Upon parkin inactivation (either in carriers of *PARK2* mutations or in sporadic PD patients through oxidative/nitrosative stress) PARIS is not degraded, which inhibits expression of PGC-1alpha and downstream acting nuclear respiratory factor 1 (NRF-1) (Shin et al. 2011) (see also Fig. 1).

PGC-1alpha adjusts the metabolic activity to changing external stimuli, co-ordinating mitogenesis, gluconeogenesis, myogenesis and adipogenesis (Goffart et al. 2009, Gleyzer and Scarpulla 2011). For instance, PGC-1alpha co-activates nuclear respiratory factors (NRF1, NRF2) – the key players in the control of mitochondrial biogenesis and antioxidant defense. The NRFs, in turn, regulate the expression of the majority of nuclear genes encoding subunits of the respiratory complexes. They also act on genes whose products take part in mtDNA transcription and replication (TFAM, TFB1M, TFB2M, POLRMT, POLG, and RMRP – the RNA component of mitochondrial endoribonuclease); genes encoding mitochondrial translation apparatus (ribosomal proteins, aminoacyl-tRNA synthetases), mitochondrial protein import and assembly machinery (TOM20, TOM70, COX17), and heme biosynthetic enzymes (Scarpulla 2008a,b).

PARIS accumulates in the SN and the striatum of sporadic PD brains and in conditional parkin knockout (KO) mice. PARIS up-regulation and subsequent downregulation of the PGC-1alpha/NRF-1 pathway expression (at the mRNA and protein levels) precedes the loss of DA neurons in mice with conditional parkin inactivation (Shin et al. 2011). In line with the above findings, a meta-analysis of genome-wide expression studies, has demonstrated that PGC-1alpha and its target genes involved in cellular bioenergetics are downregulated in sporadic PD patients in comparison with control groups (Zheng et al. 2010, Taccioli et al. 2011). In particular, downregulated were mRNAs coding for electron transport chain (ETC) elements, mitochondrial protein import, folding, and translation machinery (Zheng et al. 2010).

In contrast to the Shin and coauthors (2011) results, Pacelli and others (2011) have found that PGC-1alpha mRNA and protein is up-regulated in EOPD patients with *PARK2* compound heterozygous mutation devoid of the full-length protein. This apparent inconsistency could reflect a blocked compensatory response to rescue mitochondrial function through PGC-1alpha up-regulation in PD patients, that is absent in conditional parkin knockout mice (Shin et al. 2011).

It could be speculated that the compromised parkin activity in brains of PD patients (sporadic or with *PARK2* mutations) leads to PARIS accumulation, downregulation of the PGC-1alpha-NRF1 pathway (Shin et al. 2011) and downstream mitochondrial biogenesis factors such as TFAM or

TFB2M, and decreased mtDNA transcription and replication. Nonetheless, those observations were made in different models. Whether they overlap in time and space in PD patient's brains (both sporadic or due to *PARK2* mutations), remains to be established. The downstream effectors of the parkin-regulated PARIS-PGC-1alpha-NRF1 pathway remain unknown.

Interestingly, the TFAM mRNA level decreased in sporadic PD brains (Zhang et al. 2005), but no changes were observed, in PD patients with *PARK2* mutations or parkin KO mice, as it could be expected (Pacelli et al. 2011, Shin et al. 2011). Likewise, we did not observe TFAM protein level changes upon transient overexpression of wild-type or mutated parkin variants, in western blot analysis (Gaweda-Walerych and Zekanowski 2013). These results suggest that mtDNA/RNA changes observed upon parkin overexpression and in PD patients with *PARK2* mutations do not result from TFAM expression changes. Though it cannot be excluded that the changes are very subtle or that parkin could modulate directly or indirectly TFAM activity. A recent discovery of PGC-1alpha in mitochondrial nucleoids, where it interacts with TFAM, raises the possibility of a direct regulation of mitochondrial transcription and/or replication with the omission of NRFs and their target genes (Aquilano et al. 2010). Notably, numerous nuclear transcription factors have been detected in mitochondria, along with their putative responsive elements within mtDNA, which implicates them in direct regulation of mitochondrial DNA transcription and replication (Psarra and Sekeris 2008).

Could parkin control PGC-1alpha levels *via* TP53 transcriptional repression?

Parkin could possibly enhance PGC-1alpha expression also through p53 downregulation (see Fig. 1). Parkin has been recently found to act as a TP53 transcriptional repressor *in vivo* and *in vitro* (da Costa et al. 2009). Upon parkin overexpression, reduced p53 expression, transcriptional activity, and promoter transactivation is observed (da Costa et al. 2009). Concordantly, parkin depletion upregulates p53 mRNA and protein levels in fibroblasts and mouse brains (da Costa et al. 2009). The effect is independent of the parkin E3 ubiquitin ligase function, and is abolished by pathogenic *PARK2* mutations. An enhanced p53 expression was found in human brains from patients

with sporadic PD and those with AR-JP (autosomal recessive juvenile PD) caused by parkin missense and deletion mutations (da Costa et al. 2009, Mogi et al. 2007).

It has been proposed that a loss of telomerase activity during aging induces p53 activation, which in turn decreases expression of PGC-1alpha/beta and their downstream key targets regulating mitochondrial biogenesis (Fig. 1) (Sahin et al. 2011). In the context of telomere dysfunction, germ-line TP53 deletion or PGC-1alpha overexpression counteract the accelerated aging phenotype and restore normal mitochondrial function (Sahin et al. 2011). These results underscore a direct link between telomere-associated aging and mitochondrial function.

On the other hand, human and mouse *PARK2* genes contain p53-responsive elements (RE) (Zhang et al. 2011). p53 interacts *in vivo* with the p53 RE in human parkin intron 1 (Zhang et al. 2011). It has been also shown that p53 increases parkin transcription more than 20 fold *in vitro* (Zhang et al. 2011). Nonetheless, in human neuroblastoma SH-SH5Y cells, where parkin represses p53 expression, p53 does not regulate parkin expression. Hence, the positive p53 control over parkin transcription seems to be cell type/tissue-specific (Zhang et al. 2011).

Taken together, parkin has been suggested to act upstream of p53 (da Costa et al. 2009) and PGC-1alpha (Shin et al. 2011), while p53 upstream of PGC-1alpha (Sahin et al. 2011), in independent studies. Therefore, further experiments are required to elucidate whether parkin upregulates PGC-1alpha level through downregulation of p53. For example, it would be useful to analyze simultaneously the correlations among parkin, PGC-1alpha and p53 levels in PD patient brains vs. controls.

Parkin and mortalin

Proteomic analyses have revealed that overexpressed parkin interacts with mitochondrial proteins with chaperone functions: mortalin, HSP60, and LRPPRC (Davison et al. 2009, Rakovic et al. 2011b, Yang et al. 2011). HEK293 cells overexpressing parkin demonstrated a decrease in mitochondrial, and an increase in cytoplasmic levels of mortalin and HSP60, as compared to controls (Davison et al. 2009).

Mortalin is a mitochondrial matrix chaperone of the heat shock protein 70 family, present also in the endo-

plasmic reticulum, cytoplasmic vesicles, and the cytosol. Mortalin, like other chaperones interacts with a vast array of proteins, which confers its functional versatility. For example, apart from parkin, mortalin interacts with other PD related proteins, such as DJ-1 and PINK1 (Davison et al. 2009, Rakovic et al. 2011b, Yang et al. 2011). Mortalin is an essential mediator of mitochondrial biogenesis and maintenance due to its role in the import of newly synthesized nuclear-encoded polypeptides from the cytoplasm, through the TOM and TIM complexes (translocases of the outer and inner membranes, respectively) (Deocaris et al. 2006). In the mitochondrial matrix it facilitates proper folding and assembly of both imported polypeptides and those synthesized on mitochondrial ribosomes, acting in concert with mitochondrial HSP60 (Cheng et al. 1989, Koll et al. 1992). In mitochondria mortalin antagonizes oxidative stress injury and repairs misfolded domains imposed by various stressors (Deocaris et al. 2006). In yeast and *Caenorhabditis elegans* a loss of function of mortalin homologues results in abnormal mitochondrial morphology, aggregate formation, reduced levels of ATP and mitochondrial HSP60 (Kawai et al. 2001, Kimura et al. 2007). Conversely, overexpression of mortalin resulted in an extended lifespan of the nematode and of human cells (Yokoyama et al. 2002, Kaul et al. 2003).

Several lines of evidence have implicated mortalin in PD pathogenesis. First, proteomic analyses revealed decreased mortalin levels in PD brains, and in animal and cellular PD models (Jin et al. 2005, Jin et al. 2006, Chiasserini et al. 2011, Shi et al. 2008). Moreover, reduced mortalin levels in the frontal cortex of PD patients correlated with disease progression (Shi et al. 2008). Furthermore, four potentially pathogenic variants in the gene encoding mortalin were identified in PD patients from Spain (two missense R126W, P509S, and one intron 8 insertion of 17 bp, predicted to affect RNA splicing) and from Germany (A476T). The aforementioned variants were absent in control groups (De Mena et al. 2009, Burbulla et al. 2010). Chemical inhibition of mortalin deregulated electrophysiological activity in corticostriatal brain slices from 6-OHDA rat PD model and sham-operated animals, mimicking the alterations observed in a rotenone PD model (Costa et al. 2008, Chiasserini et al. 2011).

Overexpression of mutated mortalin variants as well as siRNA-mediated knockdown of endogenous mortalin by 50% led to increased reactive oxygen species

(ROS) level and reduced the mitochondrial membrane potential, further exacerbated under proteolytic stress, which could be rescued by wt mortalin (Burbulla et al. 2010). Fibroblasts from a PD patient carrying the A476T mortalin mutation displayed fragmented mitochondria (Burbulla et al. 2010). Stable overexpression of wt mortalin *in vitro*, but not its mutated variants, increased mitochondrial branching, connectivity and elongation (Burbulla et al. 2010). Lentivirus-mediated knockdown of mortalin in HeLa cells (which do not express parkin) resulted in a decreased mtDNA copy number, which was reversible upon parkin ectopic expression (Yang et al. 2011). In that study, the mortalin-deficient cells had an increased susceptibility to oxidative insult, exhibiting a collapse of mitochondrial membrane potential, ROS accumulation, fragmented mitochondrial network and increased apoptosis, but only when additionally stressed with H₂O₂ (Yang et al. 2011). All those changes were rescued upon overexpression of wild-type, but not mutant, parkin. In HeLa cells expressing parkin and treated with carbonyl cyanide 3-chlorophenylhydrazone (CCCP), endogenous mortalin and wild-type parkin could be co-immunoprecipitated (Yang et al. 2011). The parkin RING1 domain seems to be crucial for mortalin binding, since its deletion impaired the ability to interact with mortalin (Yang et al. 2011). However, parkin was recruited to mitochondria upon CCCP treatment both in mortalin-deficient cells and controls, indicating that mortalin is not necessary for this stage of mitophagy (Yang et al. 2011, Rakovic et al. 2013).

Collectively, the above results indicate that not only overexpressed, but also endogenous parkin could interact with mortalin and modulate the level of various mitochondrial chaperones. The ability of parkin to rescue the mitochondrial mortalin knockdown phenotype (characterized by a decreased mtDNA amount) in a cellular model suggests that the two PD proteins may operate in the same molecular pathway. The putative mechanism of reciprocal regulation between parkin and mortalin, remains to be established.

Parkin and HSP70/HSC70

The mitochondrial nucleoid comprises numerous proteins, the majority of which are involved in mtDNA replication and transcription and nucleoid structure formation. As it has turned out recently, mtDNA nucleoids also bind numerous chaperons, such as HSP70,

HSP60, LRPPRC (LRP130), prohibitin and AAA ATPase (Wang and Bogenhagen 2006). Chaperones regulate the dynamic interaction between replication forks and associated proteins in bacterial and eukaryotic systems (Wang and Bogenhagen 2006). HSP60 binds mtDNA in yeast (Kaufman et al. 2003), while HSP70 is an eukaryotic homolog of the dnaK protein of *E. coli*, taking part in replication of bacterial and phage DNA (Alfano and McMacken 1989). Similarly to parkin, HSP70 is mainly cytoplasmic, but it is also present in mitochondrial nucleoids (Wang and Bogenhagen 2006). Parkin and HSP70/HSC70 pathways seem interconnected. First, parkin has been shown to multiple mono-ubiquitinate HSP70/HSC70 (Moore et al. 2008). Moreover, HSP70/HSC70 levels are up-regulated in brains of sporadic PD cases and parkin null mice but not in brains of patients with juvenile PD (JPD) (Rodriguez-Navarro et al. 2007, Moore et al. 2008). Third, an elevated HSC70 mRNA level was identified among five optimal predictors of a blood molecular signature for sporadic PD (Molochnikov et al. 2012).

Interestingly, some known ubiquitin E3 ligases such as MDM2 or the co-chaperone CHIP (carboxyl terminus of HSP70-interacting protein) – have also chaperone activity, and both, similarly to parkin, also regulate p53 transcription activity (Tripathi et al. 2007, Wawrzynow et al. 2007). Distinct protein domains are proposed to regulate the chaperone and ubiquitin E3 ligase activities (Wawrzynow et al. 2007). Thus, it can be speculated that parkin itself could also act as a passive chaperone, for example, for TFAM or other mitochondrial biogenesis factors.

To sum up, HSP70/HSC70 seem to play a parkin-dependent role in PD pathogenesis. Whether it could be related to mtDNA expression remains to be investigated.

Parkin *versus* LRPPRC and HNRNPK

Recent proteomic analyses have indicated that parkin may interact with and modulate levels of proteins directly involved in mitochondrial gene expression (Davison et al. 2009). For instance, in HEK293 cells overexpressed parkin interacts with LRPPRC and decreases HNRNPK level in a proteasome – dependent manner (Davison et al. 2009).

The LRPPRC protein (leucine rich PPR-motif containing protein, also known as LRP130) is a member of

the pentatricopeptide repeat (PPR) family comprising seven proteins localizing to mitochondria (Shutt and Shadel 2010). LRPPRC (in complex with HNRNPK) binds poly(A) mRNA in mitochondria and nucleus. It plays a role in post-transcriptional regulation of all mtDNA-encoded mRNAs (but not rRNAs), increasing their stability (Mili and Pinol-Rama 2003, Gohil et al. 2010). Also HNRNPK alone regulates translation of some nucleus-encoded mRNAs for mitochondrial proteins (Davison et al. 2009). Thus, parkin could potentially regulate levels of mtDNA-encoded mRNAs through a direct influence on LRPPRC and/or HNRNPK. However, the study by Davison and coauthors (2009) was done in whole cell lysates. More research is needed to pinpoint the character of HNRNPK level regulation by parkin and the interaction between parkin and LRPPRC.

Rakovic and colleagues (2011b) sequenced the LRPPRC gene in 46 atypical EOPD patients and found over 20 variants, including four novel ones. Three non-coding variants had higher frequencies than reported in the SNP NCBI database (<http://www.ncbi.nlm.nih.gov/>) (Rakovic et al. 2011b).

It remains to be established whether the identified variants influence *LRPPRC* expression or LRPPRC structure and function. That preliminary results could be an interesting starting point for combined association and functional studies of *LRPPRC* gene mutations.

Perspectives for mitochondrial therapy:
PGC-1alpha and TFAM as therapeutic targets in PD?

Many PD therapeutic approaches targeting mitochondrial dysfunction have been previously proposed (Yokoyama et al. 2011), including upregulation of PGC-1alpha and TFAM. To date, PGC-1alpha has been shown to rescue the dopaminergic neuron loss in various cellular PD models (based on rotenone administration, or A53T- α -synuclein neurotoxicity), while its deficiency aggravates MPTP-induced damage in mice (St-Pierre et al. 2006, Zheng et al. 2010). In the case of PGC-1alpha there are some reservations that should be taken into account. First, there is a substantial functional redundancy with two other members belonging to the family of PGC-1 co-activators, PGC1beta and PGC-1-related coactivator (PRC). They are all able to co-activate nuclear respiratory factors (NRF1, NRF2) and trans-activate

the NRFs-responsive genes involved in mitochondrial biogenesis (Scarpulla 2011). Thus, depending on the design, in some functional studies it would be difficult to discern the effects exerted by particular members of the PGC-1 family. Second, as a sustained overexpression of PGC-1 α in the rat nigrostriatal system leads to metabolic alterations followed by DA neuron degeneration (Ciron et al. 2012), future therapeutic approaches should maintain physiological levels of the protein.

Also TFAM has been demonstrated to protect against oxidative stress and/or enhance mitochondrial respiratory functions in cellular and animal models (Noack et al. 2006, Hayashi et al. 2008). Gene therapy based on transfection of recombinant TFAM alone or mtDNA-complexed TFAM leads to a marked improvement of various mitochondrial functions deregulated in cybrids from PD patients (decreased mtDNA and mtRNA levels, accompanied by impaired respiratory capacity) (Keeney et al. 2009). Eleven weeks after a single recombinant TFAM-mtDNA complex application, mtDNA and mtRNA levels were restored and PGC-1 α mRNA, TFAM and ETC protein levels increased, indicative of an induction of mitochondrial biogenesis and also of a feedback loop between TFAM and/or mtDNA levels and PGC-1 α . Control cybrids or those exhibiting limited mitochondrial dysfunction, did not respond to the therapy, which suggests selectivity of the treatment without an interference with the normal mitochondrial function.

It is worth mentioning that no PD-causative mutations have been found so far within PGC-1 α , NRFs, TFAM, and TFBM genes, etc. However, a few association studies, but none of PD GWAS reports, indicate that the common variants of these genes may modulate the PD risk (Alvarez et al. 2008, Sánchez-Ferrero et al. 2009, Gaweda-Walerych et al. 2010, Clark et al. 2011).

Although rescuing mitochondrial function through up-regulation of mitochondrial biogenesis factors seem a promising therapeutic approach, further experiments in different animal and cellular PD models are required to confirm its feasibility and limitations.

PARKIN COOPERATES WITH PINK1 TO MEDIATE MITOPHAGY

In 2008 Narendra and coworkers discovered a new role of parkin – in directing dysfunctional mitochondria for degradation by autophagy (mitophagy)

(Narendra et al. 2008). They showed, that upon exposure to the protonophore CCCP, parkin translocates to mitochondria with a collapsed membrane potential, which results in their fragmentation and autophagy (Narendra et al. 2008). The discovery was subsequently confirmed by other groups (reviewed by Tanaka 2010, Mizushima and Komatsu 2011, Pilsl and Winklhofer 2012). Shortly after that, it was found that PINK1 is another important key player that recruits parkin to damaged and depolarized mitochondria (Geisler et al. 2010a, Matsuda et al. 2010, Narendra et al. 2010, Vives-Bauza et al. 2010). Mutations in the *PARK6* gene, coding for PINK1, a mitochondrial serine-threonine kinase, were discovered in 2004 in autosomal recessive EOPD cases (Valente et al. 2004). It has turned out that parkin and PINK1 display similar functional characteristics. PINK1 germline knockouts in flies and mice result in similar phenotypes as do parkin: a decline in complex I activity, ATP level, MMP, an increase in ROS; without overt DA neuron degeneration (Clark et al. 2006, Park et al. 2006, Yang et al. 2006, Kitada et al. 2007, 2009, Zhou et al. 2007, Gautier et al. 2008, Gispert et al. 2009, Morais et al. 2009). Moreover, similar compensatory mechanisms (upregulation of antioxidative pathways) are observed in PINK1 and parkin deficiency animal models (Hoepken et al. 2007, Wood-Kaczmar et al. 2008, Dagda et al. 2009, Grunewald et al. 2009). Both *PARK6* and *PARK2* mutations, associated with PD, impair autophagy (Geisler et al. 2010b, Mizushima and Komatsu 2011, Pilsl and Winklhofer 2012).

It would be interesting to determine whether PINK1, similarly to parkin, also plays a role in mitochondrial biogenesis, interacts with mtDNA, enhances mtDNA transcription and replication, etc. So far, PINK1 and parkin were shown to share some interacting partners involved in mitochondrial biogenesis, e.g., mortalin, HSP60, and LRPPRC. PINK1 also interacts with mitochondrial translation initiation factor 3 (MTIF3) (Rakovic et al. 2011b).

Despite the established link between defective mitophagy and monogenetic PD, its role in sporadic PD still needs to be characterized, using various approaches and models (patient-derived cell cultures, postmortem brains, and different genetic/toxin based PD animal models).

Despite the recent substantial progress in understanding mitophagy, many conceptual gaps remain unanswered. For instance, PINK1/parkin dependent ubiquit-

ination of various mitochondrial outer membrane proteins, such as mitofusin1/2, Tom20, VDAC, has convincingly been shown to act as an autophagy trigger, but the exact mechanism of mitochondria recognition by the autophagosome remains to be established (Gegg et al. 2010, Geisler et al. 2010a, Mizushima and Komatsu 2011). Additionally, the mitophagy phenomenon awaits confirmation in physiological conditions, i.e. *in vivo* with endogenous parkin and other important key-players, and in different metabolic contexts. To date, the results regarding mitophagy in cells that rely on oxidative phosphorylation are contradictory (Van Laar and Berman 2009, Van Laar et al. 2011, Cai et al. 2012a,b), reviewed in (Pils and Winklhofer 2012). In contrast to mitochondria utilizing mostly glycolytic metabolism, in neurons, or HeLa cells forced into aerobic respiration, CCCP treatment did not recruit parkin to mitochondria (Van Laar et al. 2011). Others report that parkin-dependent neuronal mitophagy does occur in neurons, after MMP dissipation (Seibler et al. 2011), but it is much slower and compartmentally restricted, as compared to nonneuronal cells (Cai et al. 2012a,b). Moreover, it has been shown that endogenous levels of parkin are not sufficient to initiate mitophagy upon loss of the mitochondrial membrane potential in human primary fibroblasts and induced pluripotent stem derived neurons (Rakovic et al. 2013). Experiments in MitoPark mice (characterized by selective inactivation of both *TFAM* alleles in substantia nigra resulting in mtDNA depletion and respiratory chain deficiency) showed that ectopically expressed parkin (endogenous was undetectable with immunocytochemistry) localizes exclusively to the cytoplasm in DA neurons and is not recruited by dysfunctional mitochondria (Sterky et al. 2011). Morphologically, MitoPark neurons display accumulation of large mitochondria in the perikaryon and in proximal dendrites but never in DA target areas that co-occur with small fragmented mitochondria. The neurodegeneration phenotype of the MitoPark mice did not change upon additional parkin ablation. Those authors suggested that parkin-mediated autophagy could be switched on, only when the membrane potential decreases below a certain threshold. In the MitoPark mice, despite the mtDNA depletion, MMP was high enough to allow for the protein import into mitochondria (Sterky et al. 2011). Indeed, as previously shown, subtle MMP differences between cybrids carrying different mtDNA mutations (COXI, cytochrome c oxidase I, vs. cytochrome b) decide about parkin recruitment (Suen et al. 2010). Moreover, even rho0 cells, lacking mtDNA

altogether, preserve some mitochondrial membrane potential due to the activity of the F1-ATPase subcomplex (Buchet and Godinot 1998, Appleby et al. 1999, de Vries et al. 2012). It should be also taken into account that TFAM plays a role in the spatial organization of mtDNA and interacts *in vivo* with mtDNA and parkin. Hence, it is tempting to speculate that TFAM is a necessary element in the parkin-mediated mitophagy and its lack could have interfered with the process in the MitoPark mice.

In contrast to the MitoPark mice, in a model of mtDNA lesions, overexpression of mutated TWINKLE (a mitochondrial DNA helicase) increased parkin translocation to mitochondria in HeLa cells. TWINKLE gene mutations disrupt mtDNA replication, lead to multiple mtDNA deletions and cause autosomal dominant progressive external ophthalmoplegia (adPEO) that can feature parkinsonian symptoms (Spelbrink et al. 2001, Baloh et al. 2007). Likewise, overexpression of parkin in heteroplasmic cybrid lines carrying a mixture of wt- and COXI- mutated mtDNAs led to parkin and LC3 (microtubule-associated protein 1 light chain 3, an autophagosome marker) co-localization on mitochondria, followed by elimination of the mutated mtDNAs, increase in the fraction of wild-type mtDNA and restoration of cytochrome c oxidase activity (Suen et al. 2010). The proportion of parkin translocated to mitochondria further increases in COXI cybrids upon inhibition of: the F1-ATPase subcomplex (which further decreases MMP) or fusion, the latter process enabling functional complementation among mitochondria (Suen et al. 2010).

Taken together, both genetically (mtDNA mutations/damage) and chemically induced loss of MMP causes parkin recruitment to mitochondria. Interestingly, mitophagy is crucially dependent on a certain minimal level of endogenous parkin (Gilkerson et al. 2012). Both above-mentioned issues gain importance in the light of the parkin deficiency (discussed in the chapter entitled “Does parkin matter in sporadic PD?”) and the very high level of somatic mtDNA mutations in substantia nigra of sporadic PD patients (Mawrin et al. 2004, Bender et al. 2006, Kraytsberg et al. 2006).

Since both parkin and TFAM bind mtDNA and are implicated in mtDNA repair, it could be speculated that parkin directly or *via* TFAM recognizes mtDNA mutations/damage. Parkin could induce mitochondrial biogenesis to replace defective mtDNA and damaged organelles. Such a scenario would require a presence of parkin inside mitochondria. *In vivo* studies on

endogenous parkin are needed to confirm unambiguously its presence inside mitochondria and its involvement in mtDNA dynamics.

Does mitochondrial biogenesis accompany mitophagy?

Mitochondrial biogenesis can be stimulated simultaneously with mitophagy to cope with mitochondrial dysfunction. As a result, removal of damaged mitochondria is accompanied by their replacement with new ones, as evidenced by various cellular models of lipopolysaccharide (LPS) toxicity (Suliman et al. 2003, Hickson-Bick et al. 2008, Yuan et al. 2009). Below, we discuss the sequential dynamics of the cellular response to LPS-induced mitochondrial dysfunction, oxidative stress and inflammation. These processes are observed also in the course of PD pathogenesis (Suliman et al. 2003). Early events following LPS exposure include mtDNA oxidation, mitochondrial GSH depletion, and a decrease of the mtDNA copy number and mitochondrial RNAs (mtRNAs). As a result mRNAs of mitochondrial biogenesis factors (NRF-1/2, TFAM, POLG, mtSSB – mitochondrial single strand binding protein) are upregulated, followed by restoration of mtDNA copy number and the level of mitochondrial transcripts, within 48h (Suliman et al. 2003). Concordant results were obtained *in vivo*, in the rat heart, i.e., LPS treatment led to GSH depletion, lipid and protein oxidation, increase in a 3.8 kb mtDNA deletion, decrease in mtDNA copy number, and diminished expression of mitochondrially (especially complex I and IV) and nucleus-encoded ETC proteins (Suliman et al. 2004). The impaired mitochondrial parameters were immediately responded to by up-regulation of TFAM, POLG, PGC-1alpha, NRF-1, NRF-2, UCP-2, and UCP-3 mRNAs, indicative of mitochondrial biogenesis induction (Suliman et al. 2004). Similarly, in neonatal rat cardiomyocytes, administration of sub-lethal doses of LPS, rather than triggering apoptosis up-regulated mitochondrial biogenesis along with autophagy (Hickson-Bick et al. 2008). The authors observed higher levels of TFAM and PGC-1alpha mRNAs, NRF-1 accumulation in nucleus, and an increased amount of microtubule-associated light chain 3 (LC3) protein, the element of autophagosomes (Hickson-Bick et al. 2008). Further studies confirmed increased ROS generation and glutathione depletion after LPS treatment (Yuan et al. 2009). Induction of autophagy was

also observed, *in vitro* and *in vivo* in transgenic rats upon administration of LPS or TNFalpha, the latter one being also activated by LPS (Yuan et al. 2009). The authors showed that autophagy could be triggered (independently) by exposure to LPS, NO, TNFalpha, or H₂O₂. On the other hand, autophagy could be suppressed by inhibition of nitric oxide synthase, TNFalpha, p38MAPK, or administration of an antioxidant (N-acetyl-cysteine) (Yuan et al. 2009). Induction of autophagy by rapamycin administration, prior to LPS exposure protected cells against LPS toxicity by attenuating ROS production, indicating a cytoprotective role of autophagy, possibly by elimination of dysfunctional mitochondria and/or supply of amino acids for *de novo* glutathione synthesis (Yuan et al. 2009).

Interestingly, chronic LPS administration can trigger degeneration of DA neurons (that are otherwise spared) in parkin germline knockout mice, accompanied by subtle motor symptoms (Frank-Cannon et al. 2008). It is not known whether parkin is recruited to mitochondria in LPS-induced mitochondrial dysfunction. It has only been reported that in microglia, macrophages and neurons, treatment both with LPS and TNFalpha caused a transient and dose-dependent decrease in parkin expression, which was shown to be NFkappaB dependent (Tran et al. 2011).

The observations from cited experiments on LPS suggest that mitophagy is very likely accompanied by mitochondrial biogenesis, inviting an integrative experimental approach in future studies. In particular, it remains to be established whether parkin stimulates mitochondrial biogenesis in various mitophagy models.

PARKIN REGULATES MITOCHONDRIAL DYNAMICS

Mitochondria form a dynamic, interconnected network within a cell, undergoing continuous fusion and fission. Both processes seem equally important in maintaining healthy mitochondria. They fine-tune the mitochondrial network in response to changing physiological conditions (Detmer and Chan 2007). They are important for cell growth (segregation of mitochondria during cell division) and death, mitochondrial membrane potential, respiration, the distribution of mitochondria within the cell, formation and function of synapses and dendritic spines, mitochondrial quality

control, especially in post-mitotic cells such as neurons (for review, see Detmer and Chan 2007, Van Laar and Berman 2009, Perier and Vila 2012). Three GTPases are involved in the fusion: the mitofusins MFN1 and MFN2 located in the OMM, and the dynamin-related protein OPA1 located in the IMM. The basal fission machinery comprises an OMM protein FIS1 that mediates recruitment of the dynamin-related GTPase DRP1 to the mitochondrial surface.

Mitochondrial fusion promotes functional complementation through dilution of damaged (e.g., oxidatively modified) proteins, lipids and mtDNA (Detmer and Chan 2007). Fission aids mitochondrial transport to nerve terminals, mitophagy and apoptosis (Perier and Vila 2012). DRP1 is crucial for proper subcellular distribution of mitochondria and its mutations result in elongated mitochondria absent from synapses and unable to support normal neurotransmission (Verstreken et al. 2005).

It is known that some ubiquitin E3 ligases control mitochondrial dynamics (Karbowski et al. 2007). Indeed, parkin may regulate the fusion and fission through proteasome-targeting ubiquitination of MFN1/2 (Gegg et al. 2010, Poole et al. 2010, Tanaka et al. 2010, Ziviani et al. 2010, Glauser et al. 2011, Rakovic et al. 2011a), and DRP-1 (Wang H et al. 2011). Both PINK1 and parkin mutations impair ubiquitination of mitofusins in human fibroblasts (Rakovic et al. 2011a). It has been proposed that downregulation of mitofusins by parkin aids mitophagy, through prevention or delay in the refusion of mitochondria (Tanaka et al. 2010, Ziviani and Whitworth 2010).

Mammalian models with parkin or PINK1 deficiency have featured DRP-1-dependent mitochondrial fragmentation that could be rescued by enhanced fusion (for review, see Pils and Winklhofer 2012). Apparently contrary results have been obtained in *Drosophila* models lacking parkin or PINK1, where an excessive fusion that could be reversed by enhanced fission has been observed (Deng et al. 2008, Poole et al. 2008, Yang et al. 2008, Park et al. 2009). However, these discrepancies could have arisen from differences in the timing of the observations, as *Drosophila* cells with RNAi-silenced parkin or PINK1 do display mitochondrial fragmentation, but as an early and transient phenotype, followed by a rapid compensatory up-regulation of fusion (Lutz et al. 2009). Compatible with the results of parkin deficiency, overexpression of parkin or PINK1 reverses mitochondrial fragmentation induced by DRP-1, alpha-synuclein overexpression, or

rotenone treatment (Kamp et al. 2010, Thomas et al. 2011, Pils and Winklhofer 2012).

On the other hand, inhibition of mitochondrial fission favors mutant over wild-type mitochondrial DNA, probably by impairing mitophagy that eliminates dysfunctional mitochondria (Malena et al. 2009). In accordance with the latter observation, parkin overexpression selects for wt mtDNA in heteroplasmic cybrids carrying COXI mutation (Suen et al. 2010). Whether it happens through enhancing mitophagy (preceded by increased fragmentation) or rather the induction of mitochondrial biogenesis remains to be clarified. Collectively, it could be stated that the parkin along with PINK1 modulate the mitochondrial network dynamics, creating an additional link between PD and mitochondrial dysfunction.

PARKIN REGULATES MITOCHONDRIAL TRANSPORT

Mitochondria in neurons are transported along microtubules with the aid of the motor proteins, kinesins and dyneins, responsible for the anterograde and retrograde movement, respectively (Hirokawa 1998). A mitochondrial outer membrane GTPase Miro (Fransson et al. 2003) and a cytosolic protein Milton form a motor/adaptor complex that anchors mitochondria to the kinesin motor and the microtubule cytoskeleton (Stowers et al. 2002, Guo et al. 2005). Both parkin and PINK1 interact with and regulate the mitochondrial transport machinery. Parkin binds tubulin/microtubules independently of its E3 ligase activity, which may not only stabilize, but also dock parkin (and possibly mitochondria or misfolded proteins?) on microtubules (Yang et al. 2005). Overexpressed parkin has been shown to rescue the dysfunction of the microtubule system observed in fibroblasts derived from PD patients with *PARK2* mutations (Cartelli et al. 2012). PINK1 interacts with the Miro and Milton proteins that regulate the mitochondrial transport along microtubules (Weihofen et al. 2009). It has been proposed that PINK1-mediated phosphorylation of Miro activates its proteasomal degradation in a parkin-dependent manner leading to a detachment of Miro from kinesin and subsequent block of mitochondrial transport (Wang X et al. 2011). In line with these findings Liu and coworkers (2012) reported that in *Drosophila* and HeLa cells PINK1 or parkin overexpression decreased the Miro level by enhancing its ubiquitination and inhibited the anterograde and retrograde transport of axonal mito-

chondria, while a loss of Miro led to perinuclear clustering of damaged mitochondria.

These findings reveal yet another level of mitochondrial quality control exerted by PINK1 and parkin that, through downregulation of mitochondrial motility, impedes spreading of damaged mitochondria throughout the cell and facilitates their autophagy.

CONCLUSIONS

In this review we discussed possible roles of parkin in genetic and sporadic PD, predominantly in the context of mitochondrial dysfunction.

Maintenance of a healthy mitochondrial network within the cell requires a concerted regulation of mitochondrial biogenesis, fusion, fission, transport and mitophagy. As discussed above, these processes are highly interconnected in time and space. Numerous pieces of evidence indicate that parkin plays multiple roles in the regulation of mitochondrial functioning (Fig. 2). It seems to be involved in the responses to mtDNA damage and mitochondrial membrane potential collapse, and to regulate mitophagy, mitochondrial fusion, fission, transport, and biogenesis. Is parkin indeed such a versatile guardian of healthy mitochondria? Since we have taken into consideration various observations from many different models: animal, cellular, mitochondrial toxin-based and cybrid ones, there is a concern that some of the reported phenomena may be artifactual due to methodological limitations, while others authentic, but causally unrelated.

Hence, here we propose the most important questions that should be addressed.

Does parkin coordinate mitochondrial maintenance merely by controlling levels of mitochondria-related proteins through ubiquitin E3 ligase activity? The best documented examples of this mode of parkin action include ubiquitination of PARIS (controlling PGC-1 α -NRF-1 pathway), MFN1/2, DRP-1, and Miro, unifying mitochondrial biogenesis, mitophagy, mitochondrial dynamics and transport (Fig. 2). What other activities of parkin could be involved? More studies are required to shed light on the ability of parkin to modulate transcriptional activity of other proteins, directly or through UPS-unrelated ubiquitination.

Parkin seems to regulate at least some aspects of mitochondrial biogenesis. Although only exogenous parkin has been shown to enhance mtDNA replication/transcription (Kuroda et al. 2006, Rothfuss et al.

2009), parkin deficiency resulted in decreased mtDNA and mtRNA levels, thereby corroborating the overexpression findings (Kuroda et al. 2006, Rothfuss et al. 2009). In addition, parkin was also able to rescue the decreased mtDNA copy number induced by mortalin knockdown in a cellular model (Burbulla et al. 2010). The next important step would be to elucidate the role of endogenous parkin in mtDNA transcription and replication. We have proposed here a few possible pathways that could be involved (Fig. 1). Whether it is exerted by a direct interaction with mtDNA or an indirect control over mitochondrial biogenesis factors, requires experimental verification.

Overexpressed parkin interacts with a plethora of mitochondrial proteins: TFAM, mortalin, HSP70/HSC70, LRPPRC and HNRNPK. Are these proteins true mediators of parkin effects on mitochondrial biogenesis? The next step would be to corroborate these interactions for endogenous parkin and to identify the underlying molecular mechanisms.

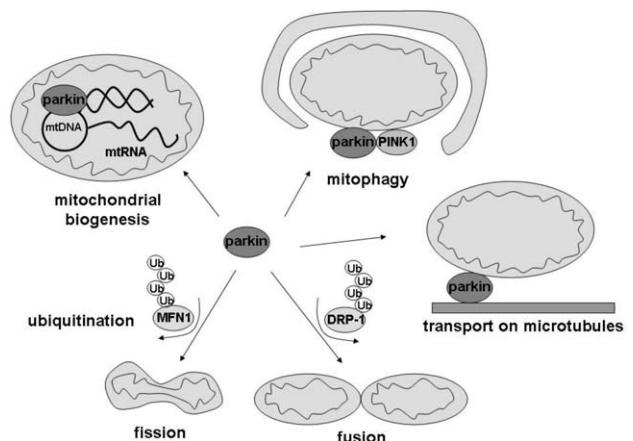


Fig. 2. Parkin controls many aspects of mitochondrial physiology. Parkin seem to be involved in: (1) mitochondrial biogenesis; (2) mitophagy; (3) mitochondrial transport; (4) mitochondrial fusion; (5) mitochondrial fission. The representation is schematic, in reality all the processes are highly interconnected and may occur concurrently. One possible mechanism by which parkin can control fission and fusion is through proteasome-targeting ubiquitination of MFN1/2 and DRP-1 proteins. For example, downregulation of mitofusins by parkin is thought to aid mitophagy, transiently maintaining “pro-fission” state of mitochondria. Parkin is recruited by PINK1 to damaged and depolarized mitochondria to switch on mitophagy. Parkin binds microtubules and is able to rescue the dysfunction of the microtubule system observed in fibroblasts derived from PD patients with *PARK2* mutations.

To sum up, an integrative approach is required that would analyze simultaneously the role of parkin in all the above-mentioned highly interconnected mitochondrial physiology pathways.

It seems very likely that depending on the type, and the extent of mitochondrial dysfunction, different pathways of mitochondrial quality control are engaged. Some of the cited reports indicate that fusion and mitochondrial biogenesis could be the first line of defense triggered by moderate mitochondrial dysfunction. Parkin could induce fusion, for example through ubiquitination of DRP-1. When these mechanisms would turn out to be insufficient to overcome the damage, parkin could increase mitochondrial fission by ubiquitination of MNF1/2, which would aid in mitophagy. The latter process could be accompanied by mitochondrial biogenesis that would enable reconstruction of the mitochondrial network (Suen et al. 2010, Arnold et al. 2011, Pilsel and Winklhofer 2012). In addition, parkin control over mitochondrial transport would enable to impede spreading of damaged mitochondria and facilitate autophagy. On the other hand, it could facilitate the distribution of newly synthesised mitochondria within the cell.

Some of the aforementioned compensatory processes (over-activated and often sequentially blocked) may lead to mitochondrial network deregulation, which blurs the interpretation of the observed phenomena in terms of their cause and effect. For example, morphology examination does not always enable to discern between mitochondrial biogenesis vs. accumulation of dysfunctional mitochondria, which gains on significance especially in the light of the compartmentalized regulation of mitochondrial dynamics in neurons (Arnold et al. 2011). To untangle the complicated relationships among the biochemical and genetic players of PD pathological process, high throughput methods should be used to analyze simultaneously genome, transcriptome, and proteome accompanied by imaging techniques.

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