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Mitochondrial dynamics and mitophagy in Parkinson's disease: A fly point of view

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ABSTRACT

Mitochondria are double membrane-bounded organelles residing in the cytoplasm of almost all eukaryotic cells, which convert energy from the disposal of organic substrates into an electrochemical gradient that is in turn converted into ATP. However, the ion gradient that is generated through the oxidation of nutrients, may lead to the production of reactive oxygen species (ROS), which can generate free radicals, damaging cells and contributing to disease. Originally described as static structures, to date they are considered extremely plastic and dynamic organelles. In this respect, mitochondrial dynamics is crucial to prevent potential damage that is generated by ROS. For instance, mitochondria elongate to dilute oxidized proteins into the mitochondrial network, and they fragment to allow selective elimination of dysfunctional mitochondria via mitophagy. Accordingly, mitochondrial dynamics perturbation may compromise the selective elimination of damaged proteins and dysfunctional organelles and lead to the development of different diseases including neurodegenerative diseases.
In recent years the fruit fly *Drosophila melanogaster* has proved to be a valuable model system to evaluate the consequences of mitochondria quality control dysfunction *in vivo*, particularly with respect to PINK1/Parkin dependent dysregulation of mitophagy in the onset of Parkinson's Disease (PD). The current challenge is to be able to use fly based genetic strategies to gain further insights into molecular mechanisms underlying disease in order to develop new therapeutic strategies.
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1. Mitochondria: from structure to function.

The term Mitochondria comes from the composed Greek word *mitos* (filament) and *chondrion* (granule) and was coined in 1898 by German doctor Karl Benda to describe filamentous-type organelles, which were first observed in the 1850s by Swiss physiologist Albert Von Kölliker. Between 1850 and 1880, several scientists independently observed in different cell types the presence of these organelles, which vary in number, size and subcellular localization (Ernster and Schatz, 1981).

With the advent of advanced biochemistry-based techniques, light was shed on their physiological function. Several researchers independently hypothesized the presence of mitochondria resident enzymatic complexes that were responsible for processing oxygen. In the second half of the twentieth century, Serrano et al. reported the purification and properties of a proton-translocating adenosine triphosphatase complex, which was isolated from mitochondria of bovine heart (Serrano et al., 1976). Two years later, the so-called theory of chemiosmosis was proposed, according to which the flow of hydrogen ions through an enzyme complex present in the mitochondria, would provide the potential energy that is required for ATP synthesis. The transformation of potential energy into metabolic energy in the form of ATP involved the oxidation of oxygen (Boyer et al., 1977; Mitchell, 1977). This theory earned the British scientist Peter Mitchell the Nobel Prize for chemistry in 1978. About twenty years later, scientists Paul Boyer, John Walker and Jens Skou independently showed that the passage of protons through the ATP synthase, which acts as a mechanical force, causes the rotation of a part of this protein, catalyzing the formation of ATP via phosphorylation of a molecule of ADP (Groth and Walker, 1996). This discovery earned them the Nobel Prize for Chemistry in 1997.

Thanks to the development of the electron microscope in 1931, it was possible to analyze the microscopic structure of the mitochondrion and characterize the intimate structure of the mitochondrion at a resolution of several orders of magnitude higher than that of the optical microscope. This organelle consists of a double layer of lipid membrane, which allows distinguishing five distinct compartments: the outer mitochondrial membrane (OMM), intermembrane space (IMS) (between

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the outer membrane and the inner one), the inner mitochondrial membrane (IMM), the cristae (compartments that are formed by invagination of the inner mitochondrial membrane) and the matrix (the space surrounded by the IMM). The OMM contains channel proteins, called Porins, which allow the free diffusion of small metabolites. In this respect, the outer membrane envelopes the organelle, separating the IMS from the cytoplasm, yet its content is metabolically similar to the cytoplasm. Molecules that are larger that 5 kDa contain a specific mitochondrial targeting signal and they are actively transported across the OMM into the IMS by a subset of proteins called translocases that, upon ATP hydrolysis, actively import specific metabolites intended to be part of the IMS (or the matrix).

The OMM has originally been considered a mere containment enclosure of the mitochondrion. However, recent works attributed to the OMM characteristics of physiological and signaling importance. For instance, points of close contact were observed between the OMM and the nearby endoplasmic reticulum (ER) called MAMs (mitochondria-associated ER-membranes) (Naon and Scorrano, 2014), which have proved to play strategically in the propagation of cellular signals, including those that control lipid metabolism, calcium homeostasis and cell death (Ernster and Schatz, 1981; Rizzuto et al., 2000; McBride et al., 2006). In particular, mitochondria largely contribute to calcium (Ca^{2+}) homeostasis at the MAMs. Although they require Ca^{2+} for the operation of mitochondrial resident enzymatic complexes, mitochondria are relatively inefficient Ca^{2+} up-taker. Nevertheless, they can uptake Ca^{2+} via the mitochondrial Ca^{2+} uniporter (MCU), a newly identified Ca^{2+} transporter (De Stefani et al., 2011) that, despite its low Ca^{2+} affinity, imports Ca^{2+} at the MAMs, where high content Ca^{2+} microdomains are forming. Of note, aberrations in ER-mitochondria juxtaposition have been described in cellular models of different neurodegenerative diseases, including Alzheimer's, Huntington's and Parkinson's disease (Area-Gomez et al., 2012; Cali et al., 2013a; Cali et al., 2011; Cali et al., 2013b; Costa and Scorrano, 2012). Although the exact cause for neuronal loss is not clear, it is plausible that the neurodegeneration observed might be caused by malfunction of the synaptic nerve transmission, which depends on proper communication between mitochondria and ER at the MAMs.

The IMM contains the enzymatic complexes that are responsible for the transformation of energy that comes from the processing of the organic substrates into electrochemical gradient, which is then converted into metabolic energy (ATP), through the ATP synthase. The electrochemical gradient is generated through the oxidation of nutrients, via the operation of a chain of enzymes, which resides within the IMM and the IMS. The energy released by the passage of the electrons through the protein complexes of the enzymatic chain, is used to actively pump protons out of the mitochondrial matrix, into the intermembrane space, creating a proton gradient. The energy stored in form of the proton gradient (potential energy) is then used to produce ATP (metabolic energy), thanks to the exergonic passage of the protons through the ATP synthase. The IMM has a structure similar to the plasma membrane of bacteria and, unlike the OMM, does not contain Porin channels. It is therefore completely impermeable to any molecule present outside. One particular molecule must then be actively transported into the mitochondrial matrix via protein translocases. The IMM forms numerous invaginations, which folds back into pockets, called cristae (Frey and Mannella, 2000; Mannella et al., 2001). The main function of such invaginations is to extend the surface for the respiratory complexes so that mitochondria respiratory capacity can be greatly amplified. Cristae size and shape can change depending on intracellular signaling. For example, cristae remodeling occurs upon activation of programmed cell death: cristae junctions become wider to release cytochrome c (Frezza et al., 2006), which in turn activates cysteine proteases Caspases, the main executers of programmed cell death. In addition, respiratory chain complexes assemble into quaternary structure, called supercomplexes, which formation and stability depend on cristae shape (Cogliati et al., 2013). Accordingly, the efficiency of mitochondrial respiration in response to changes in cell metabolism or upon stress, depends on cristae shape. Interestingly, the proteins that control cristae architecture at the IMM and ER-mitochondria juxtaposition at the MAMs do cooperate to respond to changes in metabolism (Sood et al., 2014), which suggests a previously uncharacterized inter organelle coordinated process.

All these evidences clearly suggest how the dynamic orchestration of intra and inter compartments interaction is an absolute requirement for the modulation of mitochondrial activity.

2. The master regulators of mitochondrial dynamics

Originally described as static structures, mitochondria are now widely considered extremely plastic and dynamic organelles. Indeed, eukaryotic cells maintain the overall shape of their mitochondria by balancing the opposing processes of mitochondrial fission and fusion. Mitochondria shape and dynamic is not random and tightly correlates to mitochondria functions, which include, beyond energy conversion, the biosynthesis of amino acids and steroids, the beta-oxidation of fatty acids, modulation of Ca^{2+} signaling and amplification of apoptosis (Ernster and Schatz, 1981; Rizzuto et al., 2000; McBride et al., 2006). Mitochondrial shape is regulated by a set of proteins that respond to cellular cues such as phosphorylation or ubiquitination. Mitochondria shaping proteins have pleiotropic functions, participating in apoptosis, tethering of mitochondria to other organelles, calcium signaling and regulation of autophagy. The players in mitochondrial network remodeling are dynamin-related proteins, large GTPases that participate in fusion, fission and tubulation of membranes (McNiven et al., 2000). The dynamin-related GTPases Optic Athropy 1 (OPA1) and Mitofusins (MFNs) have been identified as the main regulator of mitochondrial fusion, while the Dynamin Like Protein (DRP1) and FIS1 are responsible for mitochondrial fission. MFNs are responsible for the fusion of the OMM. In mammals there are two MFNs, MFN 1 and 2, displaying a similar structure with a terminal GTPase domain, two hydrophobic heptad repeats (HR) and two transmembrane domains that insert them on the OMM. Despite their high homology, they exhibit distinct functions (Eura et al., 2003; Koshiba et al., 2004). They both form homo- and heterodimers, and force OMM to fuse upon conformational changes led by GTP hydrolysis (Chen et al., 2003). Both MFN1 and MFN2 are required for mitochondrial fusion (Koshiba et al., 2004). However, while the main role of MFN1 is to control mitochondria tethering *in trans* and, to promote fusion in cooperation with OPA1(Cipolat et al., 2004; de Brito and Scorrano, 2008), the role of MFN2 is more elusive. MFN2 levels correlate with oxidative metabolism of skeletal muscle (Bach et al., 2003) and the proliferative ability of vascular smooth muscle cells by sequestering the protooncogene Ras (Chen et al., 2004). Moreover, MFN2 forms complexes that enable the tether between mitochondria and ER at the MAMs, impinging on lipid transfer and synthesis, mitochondria energy metabolism, Ca^{2+} transfer between the two organelles and Ca^{2+} dependent cell death (de Brito and Scorrano, 2008). The direct role of MFN2 in the formation of ER-mitochondria molecular bridges originally described by De Brito et al.(de Brito and Scorrano, 2008) has been recently challenged by an electron microscopy study that indicate increased ER-mitochondria interaction in MFN2 deficient cells (Cosson et al., 2012). However, in this study the authors arbitrary defined sites of tethering as those regions of ER-mitochondria distance of 10 or less nm. Remarkably, a parallel study that also used electron microscopy to measure the contacts and did not introduce any arbitrary tie, produced the opposite result that MFN2 removal results in decreased sarcoplasmic reticulum-mitochondria juxtaposition (Chen et al., 2012). Another recent study, which took advantage of both electron and confocal based microscopy techniques agreed with Cosson et al. conclusions, that MFN2 ablation increases ER-mitochondria tethering (Filadi et al., 2015). However in this case the analysis of the confocal images was based on selection of an arbitrary plane section and not on a three dimensions volume rendering reconstruction of Z stack acquired images.

Since ER-mitochondria tethers develop on three dimensions, this analysis is prone to misinterpretation as it misses the interactions that do not develop in the analyzed section. Of note, several independent works showed that Ca^{2+} (Chen et al., 2012; Sugiura et al., 2013) and lipid transfer (Area-Gomez et al., 2012; Hailey et al., 2010; Hamasaki et al., 2013; Wasilewski et al., 2012), both functional counterparts of ER-mitochondria physical interaction, is diminished in cells lacking MFN2. Certainly both schools acknowledge the involvement of MFN2 in the regulation of ER-mitochondrial interaction and controversial results might depend on lack of definitive definition of ER-mitochondrial functional tethering distance.

The other protein involved in mitochondrial fusion, OPA1, is anchored on the IMM and most of the protein is exposed to the IMS (Olichon et al., 2002). In humans there are 8 splice variants of OPA1, while in mice there are only four (Akepati et al., 2008). Its activity is regulated by proteolitic cleavage (Ehses et al., 2009) and both long and short forms are needed for fusion (Song et al., 2007). OPA1 is not only involved in mitochondria IMM fusion in a MFN1-dependent manner, but it also plays a role in controlling cell death by regulating the size of mitochondria cristae junctions. Heterocomplexes between proteolytic processed or unprocessed OPA1 regulate the width of the cristae junctions, thus affecting the release of cytochrome c (Frezza et al., 2006; Ishihara et al., 2006).

On the other side, DRP1, MFF (Mitochondrial Fission Factor), FIS1, MiD49 and MiD51 regulate mitochondrial fission. The large GTPase DRP1 is a dynamin-related protein which has a role in both mitochondria and peroxisomes fission (Schrader, 2006). DRP1 has mainly a cytosolic localization and it translocates to mitochondria in response to Ca^{2+}-dependent cellular signals. Cytosolic Ca^{2+} rise, associated with mitochondrial depolarization, leads to Calcineurin activation and dephosphorylation of DRP1 on Ser637 and concomitant translocation of DRP1 to mitochondria (Cereghetti et al., 2008), where it is stabilized by sumoylation (Harder et al., 2004). Once on mitochondria, DRP1 oligomerizes and interacts with its putative interactors on the OMM (Fis1, MFF, MiD49 and MiD51)(Loson et al., 2013), forming a ring-shaped structure, which constricts around the mitochondrial tubular structure, inducing mitochondrial fission (Loson et al., 2013; Mears et al., 2011). Protein Kinase A (PKA)-dependent phosphorylation of DRP1 on Ser637 prevents DRP1 translocation thus allowing unopposed fusion (Cribbs and Strack, 2007; Chang and Blackstone, 2007). PKA activity is dependent on cellular levels of cyclic AMP (cAMP), thus cAMP seems to have an important role in mitochondrial shape remodeling, although the relationship between mitochondrial morphology and bioenergetics is much more complex. Furthermore, DRP1 can be activated by a phosphorylation at Ser600 by calmodulin-dependent kinase 1(Han et al., 2008) (CaMKIα) or at Ser616 by Cyclin-Dependent Kinase 1 (CDK1) (Taguchi et al., 2007), meaning that the regulation of this protein and, consequently, of mitochondrial morphology is tightly regulated by many different Ca^{2+}-dependent proteins.

FIS1 is a membrane protein homogenously distributed in the OMM via a transmembrane domain located at the C-terminal region, and a small portion of region facing the IMS. The cytoplasmic region contains six alpha helices, four of which (a2–a5) form two tetratricopeptide repeat (TPR)-like domains that allow protein–protein interaction (Suzuki et al., 2003). FIS1 overexpression results in mitochondrial fission, but since it does not possess enzymatic activity, its role is probably restricted to anchoring effector proteins to mitochondria. Accordingly, mitochondrial fragmentation by FIS1 overexpression can be blocked by expression of dominant negative mutants of DRP1 (James et al., 2003). Evidences suggest that FIS1 acts as an interactor for DRP1 in the OMM (Yoon et al., 2003); however, FIS1 does not seem to be absolutely required for binding DRP1 to mitochondria, since downregulation of FIS1 only partially blocks DRP1 recruitment to the organelles (Lee et al., 2004). MFF is an integral protein of the OMM that has been reported to participate in mitochondrial fission, by recruiting DRP1 to mitochondria in a FIS1- independent manner, acting as a putative adaptor. Although Fis1 was the first proposed DRP1 receptor to be identified on the OMM, MFF appears to have a more important role in recruiting DRP1 and promoting mitochondrial fission (Otera et al., 2010). Recently, two novel OMM resident proteins, MiD49 and MiD51, have been found to be able to promote fission in the absence of FIS1 and MFF, thus operating as *bona fide* DRP1 receptors (Loson et al., 2013). Indeed, FIS1, MFF, MiD49 and MiD51 can each recruit DRP1 and promote mitochondrial fission independently pointing to a potential activation of each of them depending on the cell type or specific physiological conditions. Recent works, also suggest that mitochondrial fission events predominantly occur at the contact sites between mitochondria and ER. Interestingly, DRP1 and MFF have been found to localize at these contact sites (Friedman et al., 2011), suggesting an important role for the ER in the regulation of mitochondrial dynamics.

3. Est modus in rebus: the mitochondria quality control

Although oxidative phosphorylation is a vital part of metabolism, it produces ROS such as superoxide and hydrogen peroxide, which lead to propagation of free radicals, that may oxidize mitochondrial own lipids, proteins and DNA (Scherz-Shouval and Elazar, 2011), damaging cells and contributing to disease and senescence. Therefore, mitochondria are set at a central point of the equilibrium between health and disease. In this respect, the adaptation of energy supply to energy demand is central to cellular vital bioenergetic homeostasis and is critically regulated by dynamics and turnover of the mitochondrial population. The balance between biogenesis and degradation of mitochondria is tightly controlled by two major catabolic processes in the cytosol. The ubiquitin–proteasome system is able to proteolytically degrade mitochondrial outer membrane proteins, whereas the autophagy–lysosome pathway can eliminate mitochondria as whole organelles in a process termed mitophagy. Importantly, mitophagy can be employed by cells to selectively degrade dysfunctional mitochondria in order to maintain a healthy mitochondrial network and to control mitochondrial components, products and by-products, a mechanism called mitochondrial quality control (QC). To serve as QC, mitophagy needs specialized molecules that sense dysfunctional mitochondria and mark them for autophagic degradation. Several studies have lined out the importance of Parkin, an E3 ubiquitin ligase and a PD-related gene, and the serine/threonine protein kinase PINK1, also a PD-related gene, as key players in this process (Ziviani et al., 2010; Narendra and Youle, 2011). In healthy cells Parkin resides in the cytosol whereas the precursor of PINK1 is continuously imported into the intermembrane space of mitochondria *via* the translocase of outer mitochondrial membrane (*TOM*) complex. Inside mitochondria the full length form of PINK1 is processed by the mitochondrial proteases mitochondrial processing peptidase (MPP), AFG3-like AAA ATPase 2 (AFG3L2) and presenilin-associated rhomboid-like protein (PARL). The short form of PINK1 is then released into the cytosol and subsequently degraded by the proteasome (Deas et al., 2011; Jin et al., 2010; Yamano and Youle, 2013) (Fig. 1, left panel). When cellular stress conditions lead to loss of mitochondrial membrane potential, which in experimental models of cancer cell lines, *Drosophila* cells, mouse embryonic fibroblasts and primary neurons is artificially induced by treatment with the uncoupler CCCP (Ziviani et al., 2010; Narendra et al., 2012; Youle and Narendra, 2011; Narendra et al., 2008), PINK1 cleavage fails and it accumulates on the outer mitochondrial membrane (OMM). Once stabilized on the OMM, PINK1 first autophosphorylates (Okatsu et al., 2012) and subsequently phosphorylates ubiquitin (Koyano et al., 2014), MFN (Chen and Dorn, 2013) and Parkin (Sha et al., 2010), thus inducing Parkin recruitment to mitochondria and activation of its ubiquitin E3 ligase activity (Fig. 1, right panel). In this scenario phosphorylated MFN was shown to function as a tag to induce Parkin translocation from the cytosol to the mitochondria (Chen and Dorn, 2013). The Parkin-dependent K48-mediated polyubiquitination of several target proteins on the OMM, such as MFN, the voltage-dependent anion channel VDAC, the kinesin anchor protein

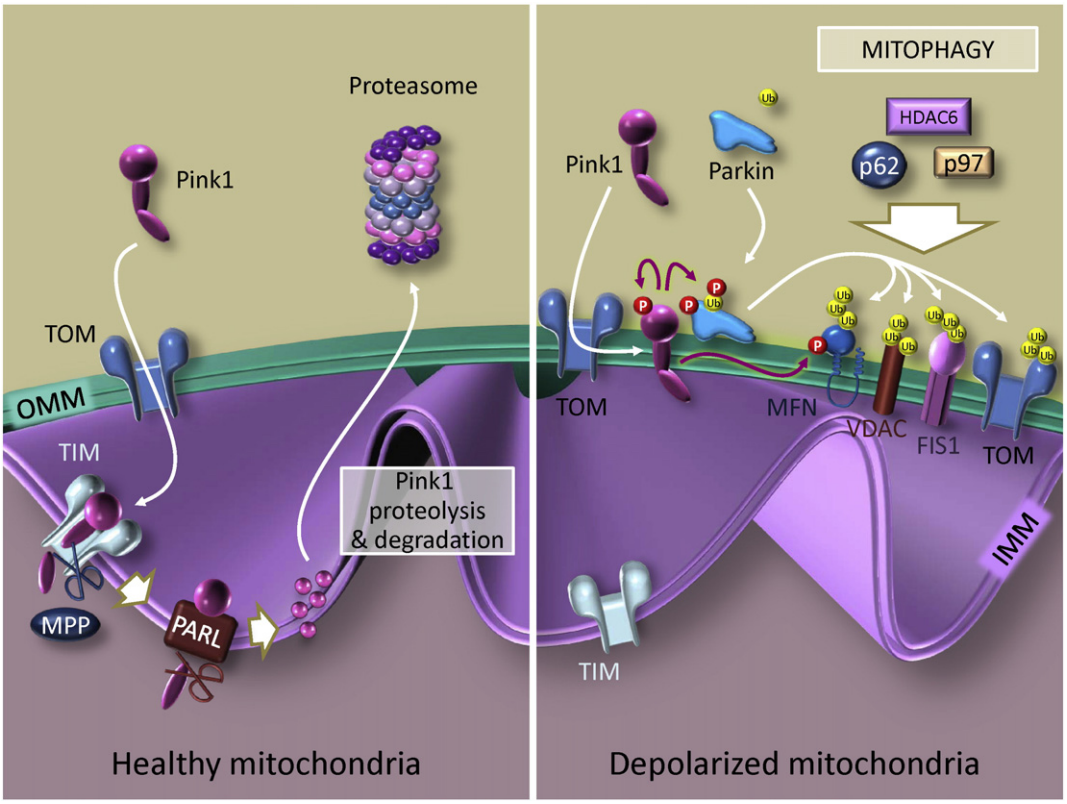

Fig. 1. The Pink/Parkin pathway in mitophagy. In healthy mitochondria (left panel), PINK1 is targeted to the outer mitochondrial membrane (OMM) owing to its mitochondrial target sequencing (MTS). It is then imported into the inner mitochondrial membrane (IMM) through the TOM/TIM complex and cleaved by the TIM-associated mitochondrial processing peptidase (MPP). MPP-cleaved PINK1 is thereafter further processed by the presenilin associated rhomboid-like protease (PARL), and it rapidly undergoes proteasome-dependent degradation. In depolarized mitochondria (right panel), TIM-mediated import of mitochondria is impaired, and PINK1 accumulates on the OMM. The OMM-accumulation of PINK1 will lead to the selective recruitment of Parkin, via PINK1-dependent phosphorylation of ubiquitin and Parkin. PINK1-dependent phosphorylation of both Parkin and ubiquitin is sufficient to fully activate Parkin E3 ubiquitin activity, which results into ubiquitination of Parkin targets on the OMM (among them MFN, TOM20, VDAC and Fis1). Ultimately, ubiquitinated proteins serve to recruit essential adaptors such as p62, HDAC6 or p97, which will tether the phagophore membrane and induce mitophagy.

Miro, and the autophagy adaptor p62(Ziviani et al., 2010; Sarraf et al., 2013) induces their proteasomal degradation and recruitment of the autophagic machinery, resulting in mitophagy (Ziviani et al., 2010; Geisler et al., 2010; Kim et al., 2008; Matsuda et al., 2010; Narendra et al., 2010a). Several studies have shown that Parkin also catalyzes other forms of ubiquitination that regulate subcellular localization and protein-protein interactions, rather than proteasomal degradation (Mukhopadhyay and Riezman, 2007). It was hypothesized that the prevention of mitochondrial fusion through degradation of MFN on the one hand and the arrest of mitochondrial motility via degradation of the GTPase Miro linking mitochondria to the cytoskeleton for kinesin-mediated transport on the other hand help to "quarantine" unhealthy mitochondria, thus facilitating their autophagic engulfment (Wang et al., 2011).

Another way for mitochondria to get rid of damaged and oxidized proteins, is the Drp1-independent budding of mitochondria-derived vesicles (MDVs) which can be targeted either to lysosomes or peroxisomes (Neuspiel et al., 2008; Soubannier et al., 2012a; McLelland et al., 2014). This process is cargo-selective and can be induced by oxidative stress, mitochondrial damage and specific nutrients. The vesicle fate is primarily determined by its cargo. MDVs containing the outer membrane mitochondria-anchored protein ligase MAPL were shown to be targeted to peroxisomes (Neuspiel et al., 2008) whereas MDVs carrying TOM20 or pyruvate dehydrogenase fuse with lysosomes (Soubannier et al., 2012a). Delivery to the lysosomes is independent of ATG5 and LC3 and mitochondrial depolarization, indicating that vesicle delivery is a complementary process to mitophagy. Since the formation of MDVs occurs in the presence of actively respiring mitochondria it was hypothesized that this pathway is an early response to oxidative stress, whereas mitophagy is rather induced by late-stage mitochondrial damage. Interestingly, a specific sub-type of MDVs targeted to the lysosomes is regulated by PINK1 and Parkin. Ectopic expression of wildtype Parkin but not PD-associated mutant Parkin in Hela cells promotes the biogenesis of MDVs (McLelland et al., 2014). Parkin was shown to colocalize with MDVs in a PINK1-dependent manner, and to stimulate their formation in response to antimycin A, an inhibitor of respiratory chain complex III potently increasing ROS levels. These findings implicate that PINK1 and Parkin have a duplicate function in mitochondrial QC and operate even at early stages in order to salvage mitochondria by selectively extracting damaged components. Only when this first step of QC fails, mitochondria are targeted for mitophagy.

Recently, AMBRA1, an upstream autophagy regulator and Parkin interactor was identified as another central player in mitophagy. AMBRA1 was shown to enhance Parkin-mediated mitophagy through binding of the autophagosome adaptor LC3. In normal conditions AMBRA1 is present at the mitochondria where it binds to and is inhibited by the pro-apoptotic protein Bcl-2 (Strappazzon et al., 2011). Upon induction of mitophagy, AMBRA1 binds to LC3 through a LIR (LC3 interacting region) motif, thereby regulating both Parkin-dependent and -independent mitochondrial clearance. Mitochondrial AMBRA1 was shown to control arrangement of the mitochondrial network around the nucleus and to cause mitochondrial depolarization. Authors hypothesized that AMBRA1 might facilitate mitochondrial

clearance by bringing damaged mitochondria onto autophagosomes via its interaction with LC3.

4. Faber est suae quisque fortunae: consequences of impaired mitochondrial clearance.

Dysregulation of the QC pathway leads to the accumulation of damaged mitochondria, resulting in increased oxidative stress, decreased mitochondrial Ca^{2+} buffering capacity and loss of ATP, all factors particularly harmful in postmitotic cells such as neurons. Several studies indeed have shown that mutations in the PINK1 and Parkin genes *Park6* and *Park2* are linked to hereditary forms of early-onset familial Parkinson's disease (PD), suggesting that PINK1/Parkin- mediated mitophagy is critical for the maintenance of normal mitochondrial function in cells (Youle and Narendra, 2011).

PD is one of the most common neurodegenerative disorders, characterized by the gradual degeneration of multiple neuron types including dopaminergic neurons in the *substantia nigra* of the mid brain. This causes several motor impairments such as muscle rigidity, resting tremor, bradykinesia and postural instability as well as non-motor symptoms including dementia, and psychiatric problems, such as depression and anxiety. A pathologic hallmark of the disease is the formation of Lewy bodies, protein aggregates composed of α-synuclein, ubiquitin and other proteins. Most cases of PD are sporadic with no known cause. However, a small percentage of genetically-linked PD cases caused by mutations in genes including α-synuclein (Polymeropoulos et al., 1997), Parkin (Kitada et al., 1998), PINK1(Valente et al., 2004), LRRK2(Paisan-Ruiz et al., 2004) and UCHL (Ragland et al., 2009) have been identified and these manifest indistinguishable dopaminergic neuron loss and similar clinical symptoms compared to sporadic cases. Therefore the knowledge gained from studies of inherited PD will likely elucidate disease mechanisms for sporadic PD as well. At the moment there is no cure that can stop disease progression and most treatment approaches are based on dopamine replacement. Nevertheless, this can only ameliorate some motor symptoms but not the non-motor symptoms and does often cause unwanted side effects. Thus, there is an urgent need for developing therapies that target the disease from its origin in the underlying alterations of cellular pathways.

Several studies demonstrated a clear link between mitochondrial dysfunction and the onset of PD. Indeed, exposure to mitochondrial toxins, such as rotenone, paraquat and MPTP causing oxidative stress and dysfunctional mitochondria results in loss of dopaminergic neurons and PD-like symptoms (Langston et al., 1983; Bove et al., 2005). Furthermore, most of the proteins related to PD are directly or indirectly linked to mitochondria and contribute to the QC pathway.

5. The fruit fly as a valuable tool to model human diseases

Studying PD in human subjects is constrained by technical and ethical issues. Furthermore, the work with human cells can only partially be related to the tissue, organ or whole-body level. Thus, it is essential to develop suitable animal models for studying new therapeutic strategies targeting the actual pathogenic mechanisms. These models open up the possibility to address cellular processes in the context of functional neuronal circuits and can be used to confirm data on molecular pathways obtained in cell lines. Although the mouse is a highly valid model organism due to easy genetic manipulation and a genome that is very similar to humans, in some cases it fails to reproduce human disease pathology (Dawson et al., 2010). Embryonic knockout mice both for PINK1 and Parkin do neither display loss of dopaminergic neurons nor have any behavioral alterations and are thus considered a poor PD model, which at the very best can be employed to model only the early impairments caused by pathogenic mutations. As a consequence these KO mice cannot be used to develop neuroprotective strategies and to test promising drugs since there is no neurodegenerative phenotype to recover from. However, recently adult conditional parkin KO mice model were analyzed and showed a progressive loss of dopaminergic neurons (Shin et al., 2011), demonstrating that this PD-like phenotype is probably lost in embryonic KO mice through compensatory effects during development.

Among various model organisms, the fruit fly *Drosophila melanogaster* has emerged as an especially effective model to study PD pathology. As soon as it became obvious that most of the genes implicated in human diseases have at least one fly homolog (Reiter et al., 2001), *Drosophila* became a powerful tool to elucidate the molecular and cellular mechanisms that underlie these disorders. Compared to higher organisms *Drosophila* offers some attractive features; these are especially suited for studying complex biological processes. *Drosophila* is ideally tractable at the genetic, biochemical, molecular and physiological levels. First of all the flies can be easily maintained in large numbers in stocks and populations without specialized instrumentation. *Drosophila* has a short life-cycle resulting in the production of a large number of progeny over a short, 10-day generation period (St Johnston, 2002). For the purpose of genetic screens, *Drosophila* provides two benefits in that its genome is comprised of only 4 pairs of chromosomes, as opposed to 16 in the yeast strain *Saccharomyces cerevisiae*, or 23 in humans, thus simplifying genetic inheritance. The second advantage is that mutants can be created quite easily by molecular techniques using P-element transposons for loss-of-function studies (Adams and Sekelsky, 2002; Rubin and Spradling, 1982), tissue-specific downregulation or overexpression of proteins by the bipartite transcription activation system UAS-GAL4 (Brand and Perrimon, 1993) or site-specific gene integration via specific donor plasmids (Venken et al., 2011). Furthermore, the use of X-rays and other mutagenic agents makes it possible to generate large collections of mutant stocks (St Johnston, 2002). Another possibility is the screen of chemical compounds in the already established disease model in order to pick out those that ameliorate the phenotype. This approach was successfully used in fly models of adult-onset, age-related neurodegeneration and led to the complete rescue of disease-related phenotypes (Chang et al., 2008). Several key features of *Drosophila*, such as the compound eye, provide unique methods for studying mutational effects by simple visual observation of the resulting phenotype (St Johnston, 2002).

Thus, *Drosophila* provides an excellent model organism through the compromise between simple cultivation, genetics and phenotypic scoring, while key cellular processes are evolutionary conserved.

6. What Drosophila taught us about PD

A suitable model organism to study PD should have homologs to the disease-related genes and should possess neurobiological cellular processes (such as synapse formation and neuronal communication) and neurobiological bases of behavior (such as sensory perception, aspects of learning and memory formation) that are similar to those found in humans. All of these criteria are fulfilled by *Drosophila*. The fly genome encodes homologs of PINK1 and Parkin, and its adult brain shows clusters of dopaminergic neurons, which degenerate upon treatment with rotenone (Nassel and Elekes, 1992), as shown in mammals. Indeed, the first in vivo results, showing that PINK1 and Parkin operate within the same pathway came from studies in mutant flies. Parkin loss of function flies display reduced lifespan, male sterility and severe defects in flight and climbing abilities. Importantly, they show dramatic mitochondrial alterations (Greene et al., 2003) and indirect flight muscle degeneration (Whitworth et al., 2005). Aged Parkin mutant flies have decreased levels of tyrosine hydroxylase, a marker of dopaminergic neurons, and further investigation showed loss of a subset of the latter. *Drosophila* PINK1 mutants exhibit male sterility, slower climbing speed and defects in flight ability (Clark et al., 2006; Park et al., 2006). Similar to flies lacking Parkin, they display striking mitochondrial abnormalities such as disrupted cristae resulting in reduced ATP levels and mtDNA subsequently leading to apoptosis in flight muscles. The number of

dopaminergic neurons is slightly but significantly reduced. As listed here, PINK1 and Parkin *Drosophila* mutants share marked phenotypic similarities. Transgenic expression of Parkin suppresses PINK1 loss of function phenotypes, whereas transgenic expression of PINK1 cannot compensate for Parkin loss (Clark et al., 2006; Park et al., 2006). Furthermore, double mutants for both genes display identical phenotypes to either single mutant. Thus, work in *Drosophila* provided first *in vivo* evidences that PINK1 and Parkin function in a common pathway with PINK1 acting upstream of Parkin (Deng et al., 2008; Narendra et al., 2010b; Poole et al., 2008; Vives-Bauza et al., 2010). Further *Drosophila in vivo* studies have identified upstream and downstream regulators of the PINK1/Parkin pathway. Assays including ectopic expression in the *Drosophila* eye, genetic interaction using double mutants and epistasis experiments revealed that the mitochondrial protease HtrA2/Omi acts downstream of PINK1, independently of Parkin (Whitworth et al., 2008; Tain et al., 2009a). *HtrA2* mutant flies are viable but exhibit mild mitochondrial defects, loss of flight and climbing ability, male infertility, and sensitivity to oxidative stress and mitochondrial toxins, a phenotype similar to other PD *Drosophila* models. *PINK1*:*HtrA2* double mutants display an identical phenotype to *PINK1* mutants alone, suggesting they act in a common pathway, whereas *Parkin*:*HtrA2* double mutants display a stronger phenotype than either mutant alone, suggesting HtrA2 acts in a parallel pathway to Parkin (Tain et al., 2009a). Another mitochondrial protease rhomboid 7 was shown to act upstream of PINK1 and Parkin and to be required for cleaving the precursor forms of PINK1 and Omi (Whitworth et al., 2008). Rhomboid 7 is the *Drosophila* homolog of PARL, which promotes cleavage of vertebrate Omi (Chao et al., 2008).

Besides mapping components of the PINK1/Parkin pathway, *Drosophila* served also to identify genetic modifiers of *PINK1* and *Parkin*. Overexpression of the translation inhibitor *Thor*, the *Drosophila* homolog of mammalian *EIF4E-BP1*, was shown to suppress PD-related impairments such as dopaminergic neuron loss, locomotor deficits and muscle degeneration *in vivo* (Tain et al., 2009b). Furthermore, *PINK1* and *Parkin Drosophila* mutant phenotypes could be pharmacologically rescued by the treatment with the TOR inhibitor rapamycin that activates 4E-BP *in vivo*. Mitochondrial alterations could be ameliorated by rapamycin in *PARK2*-deficient human cells as well (Tain et al., 2009b). Thus, pharmacologic modulation of 4E-BP activity may represent a therapeutic approach for PD.

Importantly, the expression of human PINK1 or Parkin in *Drosophila* abolishes phenotypical alterations of PINK1 or Parkin loss of function flies, underlining functional conservation of the PINK1/Parkin-pathway between both species. This is supported by the fact that PD patient fibroblasts also show alterations in mitochondrial morphology and mitochondrial respiration with lowered complex I activity and ATP production (Mortiboys et al., 2008) as well as by the finding that neurons derived from pluripotent stem cells of PD patients display impaired Parkin translocation (Seibler et al., 2011).

Drosophila has also been a key player in demonstrating that PINK1 and Parkin promote mitophagy *in vivo* under normal physiological conditions. This has long been unclear, since all insights in the PINK1/Parkin mitophagy pathway have been gained based on toxin-treated cell models and PINK1 or Parkin overexpression conditions that are far from physiological. A proteomic *in vivo* approach in *Drosophila* was used to compare the rates of mitochondrial protein turnover in wildtype compared to *Parkin* or *PINK1* mutant flies (Vincow et al., 2013). *Parkin* null mutants showed a significantly decreased mitochondrial protein turnover, similar to but less severe than in autophagy-deficient *Atg7* mutants. This finding demonstrated that the PINK1/Parkin pathway induces mitophagy *in vivo*. Surprisingly, the nonmitophagic turnover of several mitochondrial respiratory chain (RC) subunits showed greater impairment in *Parkin* and *PINK1* mutant flies than in *Atg7* mutants, thus describing an additional role of the PINK1/Parkinpathway in regulating RC proteins. Loss of PINK1 and/or Parkin activity has already been shown to cause RC impairments, particularly in complex I (Mortiboys et al., 2008; Morais et al., 2009; Amo et al., 2011) and this was associated to pathogenesis of PD (Zhu and Chu, 2010). Thus, impairment of RC turnover and with this accumulation of damaged proteins, as previously shown in *PINK1* and *Parkin* mutant flies (Pimenta de Castro et al., 2012) could account for the respiratory deficits found in both familial and sporadic PD patients. PINK1 was shown to regulate complex I activity by phosphorylating its NDUFA10/ND42 subunit. An RNAi based screen in *Drosophila* cells for genes that regulate the PINK1/Parkin pathway identified the complex I subunit ND42(Pogson et al., 2014). *PINK1* mutant flies display lowered complex I activity (Morais et al., 2009), as observed in PD patient fibroblasts (Mortiboys et al., 2008). Overexpression of ND42 in *PINK1* mutant flies restores complex I activity and is able to partially rescue flight and climbing ability. The same could not be observed in *Parkin* mutant flies. These results indicate that the *in vivo* rescue is due to restoring complex I activity rather than promoting mitophagy and support the hypothesis that PINK1 modulates complex I independently of its role with Parkin in mitophagy.

Interestingly, defects in mitochondrial morphology, cell death, muscle degeneration and locomotor deficits in PINK1 and Parkin loss of function *Drosophila* models can be suppressed by simultaneous overexpression of DRP1 or downregulation of Marf, fly homolog of mammalian mitofusins (Deng et al., 2008; Poole et al., 2008). This is consistent with results obtained in MFN1/MFN2 KO mouse embryonic fibroblasts, where increased Parkin translocation to depolarization-induced fragmented mitochondria could be observed (Narendra et al., 2008). On the other hand phosphorylated MFN2 was shown to be a molecular tag for Parkin translocation (Chen and Dorn, 2013), although Parkin translocation is not completely abolished in MFN RNAi knockdown cells (Ziviani et al., 2010). These findings might seem contradictory at first sight but can be explained by the fact that MFNs have pleiotropic functions ranging from the regulation of mitochondrial fusion (Chan, 2006), oxidative metabolism (Bach et al., 2003) and cell proliferation (Chen et al., 2004) to mitochondria-ER tethering, impinging on lipid transfer and Ca^{2+} homeostasis (de Brito and Scorrano, 2008). Thus, alterations of MFNs transcript levels or post-translational modifications do probably not affect only one of these functions and the physiological outcome might depend on the complex interplay of all of them and possibly correlates to a specific cell type/model organism or cellular circumstances. In this respect, lack of good mammalian models might be limiting our understanding of pathophysiology of potential MFN-dependent degeneration in the context of PD. For instance, it is possible that functional abnormalities induced by MFN2 ablation or mutations in mammals are compensated by MFN1, therefore limiting insights into the functional role of MFN2 *in vivo* at the physiological level. In this respect, the fruit fly is an ideal *in vivo* model system to address this question as *D. melanogaster* only possesses an ubiquitous MFN-christened Mitochondrial assembly regulatory factor called Marf.

In addition, not all of the PINK1 deficiency-related phenotypes can be rescued by the increase of fission or the decrease of fusion and might not be the result of impaired mitophagy but depend on a more general role of PINK1 in controlling mitochondrial fitness and health as e.g. by phosphorylation of complex I. Indeed, genetic or pharmacological interventions that improve mitochondrial respiratory chain electron transport (Vos et al., 2012) or restore proton motive force (Vilain et al., 2012), or enhance mitochondria biogenesis (Tufi et al., 2014), or provide mitochondrial substrates downstream Complex I (Gandhi et al., 2009), proved to efficiently rescue PINK1 related dysfunctions and PINK1 mutant phenotype both *in vitro* and *in vivo*.

Furthermore, alterations in assembly of the electron transport chain complexes can be rescued as well by increasing Drp1 gene dosage (Liu et al., 2011) and heterozygosity of Drp1 in a PINK1 or Parkin mutant background is lethal. In cultured *Drosophila* cells, Parkin was shown to induce MFN ubiquitination and proteasomal degradation (Ziviani et al., 2010), whereas loss of either PINK1 or Parkin resulted in MFN accumulation. These data all support the hypothesis that the PINK1/

Parkin pathway promotes mitochondrial fission or inhibits mitochondrial fusion providing a novel therapeutic strategy through gene dosage-dependent manipulation of mitochondrial dynamics.

7. *Drosophila* in the validation of new therapeutic targets — deubiquitinating enzymes as Parkin antagonists.

One recently emerging approach is focused on the search for Parkin-antagonizing deubiquitinating enzymes (DUBs), catalyzing the removal of ubiquitin from substrates. Alteration of expression level or activity of these DUBs could lead to an attractive new therapeutic strategy for PD. This becomes particularly important with regard to the finding that ubiquitin, besides tagging proteins for proteasomal degradation, can function as a signaling molecule modulating the activity of its target and modifying its subcellular localization or ability to interact with other proteins. The human proteome contains five subclasses of DUBs among which the largest group is named ubiquitin-specific protease family (USP). Recent works showed that three members of this family USP8, USP15 and USP30, which were identified by RNAi screen in U2OS cells (Durcan et al., 2014), tandem affinity purification and mass spectrometry (Cornelissen et al., 2014) or a human cDNA library screen (Bingol et al., 2014), modulate auto-ubiquitination of Parkin and Parkin-mediated mitophagy (Fig. 2). Also in this field *Drosophila* proved to be a perfectly suitable *in vivo* tool to validate data on molecular pathways obtained in cell lines. The *Drosophila* genome encodes around 40 DUBs. Among these, CG8334 displays the highest sequence homology to human USP15. Knockdown of CG8334 in a Parkin RNAi background rescued Parkin-related mutant phenotypes such as the accumulation of mitochondrial clumps in indirect flight muscles, vacuolization of flight muscle cells, alterations of mitochondrial cristae, decreased mitochondrial membrane potential and climbing ability (Cornelissen et al., 2014). These were the first *in vivo* results demonstrating that indeed Parkin and USP15 have antagonizing effects on mitochondrial morphology and mitophagy, confirming data previously obtained in cell models. More in detail, USP15 was shown to inhibit CCCP-induced mitophagy in Parkin-transfected Hela cells depending on its DUB activity and RNAi-mediated silencing of USP15 enhanced Parkin-mediated mitophagy in the same model as well as in human dopaminergic neuronal SH-SY5Y cells and primary fibroblasts from healthy human subjects (Cornelissen et al., 2014). Furthermore, USP15 KD was able to rescue the mitophagy defect of Parkin and PINK1 mutant PD patient fibroblasts. Interestingly, authors were able to demonstrate that the Parkin-opposing effect of USP15 indeed was due to its direct role in deubiquitinating Parkin targets on the outer mitochondrial membrane and that USP15 KD lead to the accumulation of ubiquitinated Parkin substrates such as MFN2 after depolarization (Fig. 2).

Another study demonstrated that USP30 has a similar function in antagonizing Parkin-induced mitophagy via deubiquination. USP30 was identified in a human cDNA library screening as the only candidate that robustly blocked mitophagy and at the same time is localized on the OMM (Bingol et al., 2014). Overexpression of USP30 in dopaminergic SH-SY5Y cells reduced CCCP-induced recruitment of autophagic markers and mitochondrial ubiquitination. A mass spectrometry approach identified 41 proteins that are oppositely regulated by Parkin and USP30, among these the mitochondrial protein TOM20 whose ubiquitination was shown to be a mitophagy-promoting signal. Strikingly, downregulation of the fly USP30 (CG3016) in *Drosophila* Parkin or PINK1 mutant backgrounds could rescue mitochondrial abnormalities and ameliorate climbing ability as well as dopamine depletion in the brain. As a model of PD, flies were treated with the mitochondrial toxin paraquat inducing dopamine depletion and resulting in reduced climbing performance. RNAi-mediated knockdown of USP30 specifically in dopaminergic neurons via the dopamine decarboxylase driver completely rescued the paraquat-induced behavioral deficit and prevented dopamine depletion in fly heads. These results demonstrate that the beneficial effect of USP30 silencing after mitochondrial damage is occurring in dopaminergic neurons and provide further *in vivo* evidence that the regulation of DUBs is a promising therapeutic strategy for PD (Fig. 2).

8. Conclusions

By converting the energy that is trapped in the electrochemical gradient, mitochondria are undoubtedly considered the cell power plant and indispensable to the life of all eukaryotic cells. Nevertheless, they also actively participate in the pathways leading to cell death. In this respect, mitochondria are at the intriguing, yet not fully characterized, intersection point between life and death and a better understanding of their functions and malfunctions would be instrumental to gain insights in human pathologies. Not surprisingly, mutations in genes that affect mitochondrial functions have been linked to the onset of multifactorial human pathologies like cancer, Alzheimer's and Parkinson's diseases and diabetes. With respect to neurodegenerative diseases, and particularly to PD, ROS formation and oxidative stress resulting from oxidative phosphorylation-dependent redox reactions, has been clearly linked to

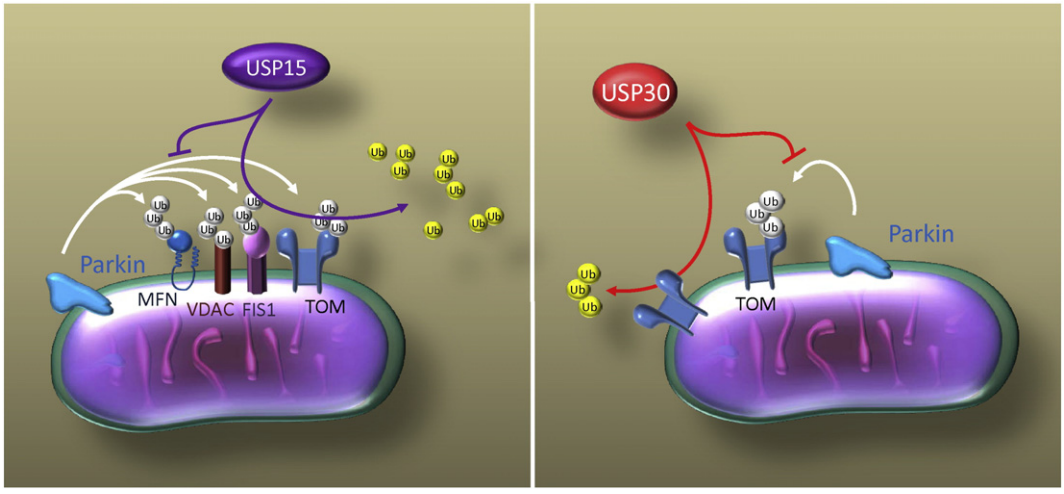

Fig. 2. DUBs role in mitochondria quality control. By impacting on the ubiquitination levels of Parkin targets, USP15 and USP30 affect Parkin translocation and Parkin-dependent mitophagy. USP15 deubiquitinates different Parkin targets on mitochondria, and knockdown of USP15 fly homolog CG8334, in a Parkin RNAi background rescues Parkin-related mutant phenotypes. USP30 targets TOM20, another Parkin putative substrate. USP30 downregulation promotes mitophagy via its effect on TOM20 ubiquitinated levels. Accordingly, downregulation of the fly USP30 (CG3016) in *Drosophila* Parkin or PINK1 mutant backgrounds rescue PINK1 and Parkin mutants abnormalities.

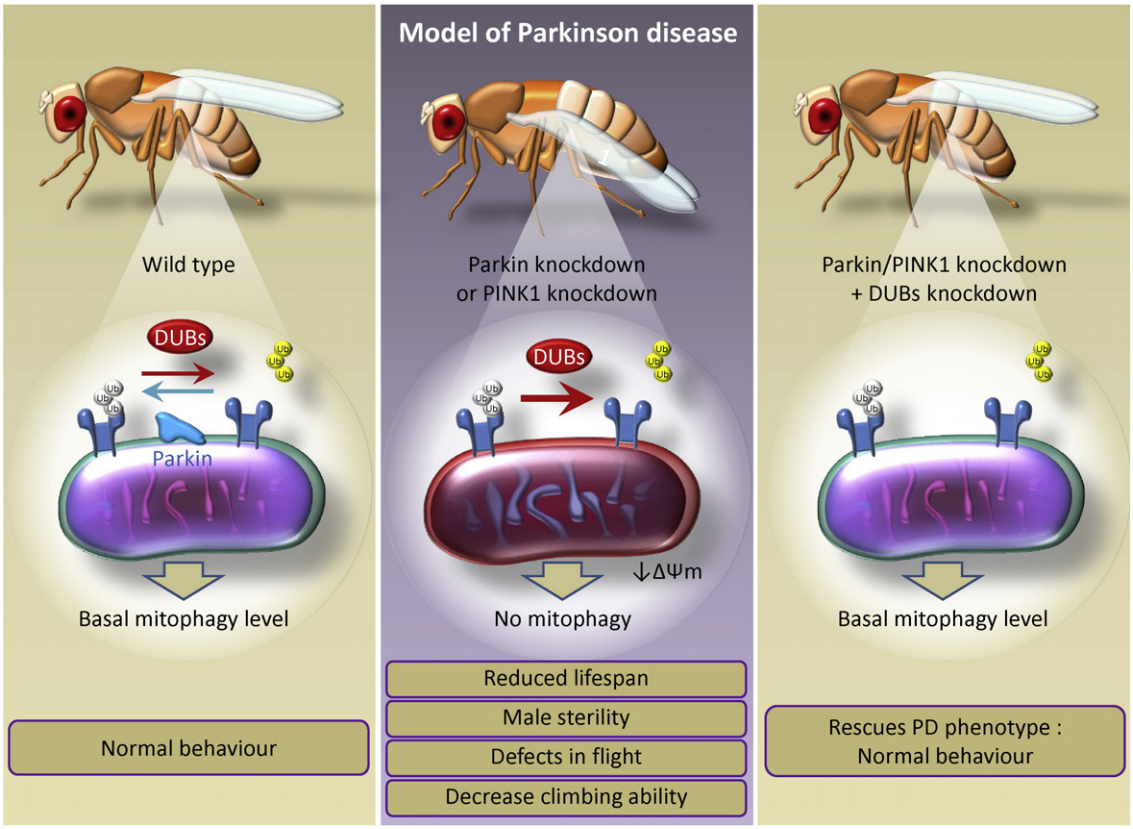

Fig. 3. Fly-based *in vivo* screening. The identification of specific DUB/DUBs that counteract Parkin activity in the ubiquitination of mitophagy substrates is emerging as one of the most promising approaches to promote mitophagy in PINK1/Parkin deficient system. In this respect, the fruit fly has proved to be a valuable model system to dissect functional defects underlying PD pathogenesis *in vivo* and screen for the effect of both genetic or chemical inhibition of specific Parkin-opposing DUBs *in vivo*, which might ameliorate PINK1/Parkin mutant abnormalities.

PD onset (Youdim and Lavie, 1994; Yoshikawa, 1993). ROS may oxidize mitochondrial lipids and proteins and induce DNA damage: cells need to promptly respond in order to avoid cell demise. One possibility to efficiently handle damaged components is via mitochondrial complementation, where damaged components are diluted into the mitochondria network upon mitochondrial fusion and subsequently degraded (Ono et al., 2001; Nakada et al., 2001). Degradation of damaged mitochondrial components can occur upon formation of mitochondria-derived vesicles that engulf and shuttle selected cargoes to the lysosome in a LC3/ATG-independent manner (Soubannier et al., 2012a; Soubannier et al., 2012b). However, when damage accumulates above a certain threshold, it is safer for the cell to eliminate the entire organelle *via* mitophagy (McLelland et al., 2014; Parone et al., 2008; Twig et al., 2008). In this respect, mitochondrial asymmetric division is a pre-requisite to segregate debris and promote mitophagy of selected dysfunctional mitochondria via PINK1/Parkin (Youle and Narendra, 2011; McLelland et al., 2014).

The fruit fly *Drosophila* has provided key insights in revealing alteration of the PINK1/Parkin mitophagy pathway and it has proved to be a valuable tool to dissect functional defects underlying PD pathogenesis *in vivo*. In contrast to embryonic mice KO models, *Drosophila* PINK1 and parkin mutants display key PD-related phenotypes such as dopaminergic neuron loss and motor impairments and at the same time reproduce molecular pathways characterized in patient fibroblasts, such as impairment in mitochondrial bioenergetics. Also, the fly relative low cost of maintenance, its rapid life cycle and the small size, makes it the perfect model system for *in vivo* high-throughput screening of chemical libraries like those of small compounds that might impact mitophagy and be beneficial in ameliorating PINK1/Parkin mutant phenotype (Fig. 3).

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