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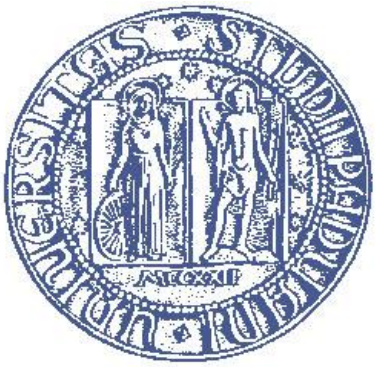

SCUOLA DI DOTTORATO DI RICERCA IN: BIOSCIENZE E BIOTECNOLOGIE
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CICLO XVIII

ANALYSIS OF THE ROLE OF MITOCHONDRIAL MORPHOLOGY IN AUTOPHAGY

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Indice

1. Riassunto dell'attivita' svolta

I mitocondri sono organelli fondamentali nella vita e nella morte cellulare. I mitocondri sono complessi organelli a doppia membrana che formano un network subcellulare molto dinamico. La loro struttura e' fondamentale per la loro funzione e per questo la morfologia di questi organelli continua a cambiare, passando da un network molto interconnesso fino alla divisione in singole unita'. Componenti chiave che regolano la forma dinamica dei mitocondri sono le proteine mitocondriali pro-fissione Drp1 e Fis1 e le proteine mitocondriali pro-fusione Mfn1/2 e Opa1.

Non solo i mitocondri sono la sede della catena respiratoria, in cui avviene la conversione dell'energia, ma sono anche fondamentali per la regolazione di apoptosi e autofagia. Inoltre, sono responsabili della regolazione dell'omeostasi del Calcio e dello stress ossidativo, della differenziazione e crescita cellulare, e dell'amplificazione di cascate di segnale. Recentemente, disfunzioni nel funzionamento dei mitocondri sono state legate all'insorgenza di varie malattie; in particolare, in malattie neurodegenerative, come il morbo di Parkinson, e' stato riscontrato l'accumulo di mitocondri non funzionali.

Il morbo di Parkinson e' la seconda malattia neurodegenerativa piu' comune, dopo il morbo di Alzheimer. Questa malattia e' caratterizzata dalla degenerazione selettiva dei neuroni dopaminergici della Substantia Nigra (SN) del sistema nervoso centrale. I pazienti affetti da tale malattia, sviluppano dal punto di vista clinico bradicinesia, tremore e rigidita', mentre dal punto di vista neurologico e' possibile notare la perdita dei neuroni dopaminergici nella SN e, nella maggior parte dei casi, la presenza di incursioni intraneuronali chiamate Lewy bodies.

Nella maggior parte dei casi, questa malattia insorge sporadicamente, rendendo difficile trovare un'eziologia comune per questo disturbo, ed individuare quindi una possibile terapia. Tuttavia, il 10% dei casi di morbo di Parkinson risultano avere un'origine genetica, e lo studio di mutazioni geniche relazionate all'insorgenza di forme familiari di Parkinson ha aiutato a comprendere meglio le cause e i meccanismi molecolari alla base di questa malattia. Ad esempio, mutazioni nel gene PARK2, che codifica per Parkin, sono la causa piu' comune di una forma Autosomica Recessiva Precoce di Parkinson. Parkin codifica per un'E3-ubiquitin ligasi, con un ruolo fondamentale nel riconoscimento ed eliminazione dei mitocondri non funzionali tramite autofagia. La sua funzione e' strettamente legata ad un'altra proteina le cui mutazioni sono legate all'insorgenza di forme precoci di Parkinson, PINK1.

La struttura di Parkin e' caratterizzata da un dominio Ubiquitin-like nella regione N-terminale e quattro domini zinc-finger RING-like: RING0, RING1, IBR e RING2. Inoltre, la struttura di Parkin contiene una regione chiamata linker, che non presenta alcuna similitudine con altri domini noti, unisce i due domini IBR e RING2. Questa regione e' anche chiamata Motivo Unico di Parkin. La funzione principale di Parkin e' trasferire molecole di ubiquitina da un'E2-ubiquitin ligasi alle proteine target. E' stato dimostrato che Parkin, la cui localizzazione subcellulare e' prevalentemente citoplasmatica, e' selettivamente recrutato ai mitocondri danneggiati e ne promuove l'eliminazione tramite mitofagia (una forma selettiva di autofagia). Come accennato, la traslocazione di Parkin dipende dalla Serina/Treonina protiein chinasi PINK1. Tuttavia il

meccanismo tramite cui PINK1 attiva Parkin rimane poco chiaro. Una volta ai mitocondri, Parkin ubiquitina vari target, tra cui la proteina pro-fusione Mitofusina, portando alla fissione dei mitocondri. Tuttavia, ancora non e' noto quali modificazioni post-trascrizionali avvengano su Parkin, regolandone il reclutamento, o da che condizioni cellulari sia influenzata la sua traslocazione. In questa tesi dimostriamo come il Calcio e la Calcineurina abbiano un ruolo fondamentale nel reclutamento di Parkin e nella mitofagia.

La concentrazione di Calcio nel citoplasma e' fondamentale per la regolazione di molti processi metabolici e per la trasduzione di segnale. Infatti, il pool citoplasmatico di Calcio e' molto limitato e finemente regolato dai mitocondri e dall'ER. Tra i vari processi regolati da Calcio, e' importante ricordare che l'attivazione di fosfatasi e di chinasi dipendenti da Calcio modifica non solo lo stato di fosforilazione di varie proteine, ma anche la loro localizzazione subcellulare, la conformazione e le interazioni con altre proteine. Ad esempio, la fosforilazione e defosforilazione della proteina pro-fissione mitocondriale Drp1, regola la sua traslocazione ai mitocondri, evento fondamentale per l'attivita' pro-fissione di Drp1. In particolare, la traslocazione di Drp1 a mitocondri a seguito di depolarizzazione indotta da CCCP e' regolata dalla defosforilazione del residuo Serina 637 da parte della Calcio-Calmodulina dipendente fosfatasi Calcineurina (CaN). Al contrario, la fosforilazione di Drp1 dipendende dalla protein chinasi A (PKA), che blocca Drp1 nel citoplasma nel caso di starvation della cellula e porta ad un allungamento dei mitocondri, che in questo caso non potranno essere eliminati tramite mitofagia. E' chiaro come la fosforilazione e defosforilazione di Drp1, dipendente da Calcio, funziona come una modificazione post-trascrizionale reversibile che regola la traslocazione e l'azione di questa proteina in funzione delle necessita' metaboliche della cellula.

Considerando il ruolo fondamentale del Calcio nella regolazione dell'attivita' delle proteine, abbiamo voluto capire se la traslocazione di Parkin e la conseguente mitofagia indotta da CCCP dipendesse da Calcio e fosse regolata da modifiche post-trasduzionali simile a quelle che controllano Drp1. Abbiamo utilizzato, quindi, un vettore codificante per una sonda fluorescente targettata ai mitocondri (mito-YFP) ed un vettore codificante per una sonda fluorescente legata a Parkin (mCherry-Parkin) per monitorare la localizzazione subcellulare di Parkin. Come ci aspettavamo in base a studi gia' pubblicati, Parkin si trova principalmente nel citoplasma, ma trasloca ai mitocondri in seguito a trattamento con CCCP. Tuttavia, il trattamento con BAPTA, un chelatore di Calcio, prima del trattamento con CCCP, bocca la traslocazione di Parkin. Lo stesso si puo' notare a seguito del trattamento con FK506, un inibitore della CaN.

Abbiamo quindi utilizzato un approccio genetico, utilizzando i mutanti dominante negativo (ΔCnA^{H151Q}) e costitutivamente attivo (ΔCnA) della CaN, gia' presenti nel laboratorio. La cotrasfezione di cellule overesprimenti mCherry-Parkin con il dominante negativo della CaN, inibiva la traslocazione di Parkin in seguito a trattamento con CCCP. Inoltre, tramite saggio di mitofagia e' stato possibile vedere come il dominante negativo della CaN inibisse anche la mitofagia indotta da CCCP. Quindi, l'inibizione farmacologica o genetica della CaN e' sufficiente per bloccare la traslocazione di Parkin e la mitofagia indotta da CCCP.

La traslocazione e l'attivita' di Parkin dipende fortemente da PINK1. Vari studi hanno infatti dimostrato che PINK1 fosforila direttamente Parkin e l'Ubiquitina a livello della Serina 65, e questa fosforilazione e' necessaria per l'attivita' di Parkin. Abbiamo quindi generato mutanti fosfo-mimetici di Parkin e dell'Ubiquitina per la Serina 65 (rispettivamente Parkin S65E e Ub S65E) e abbiamo analizzato l'effetto della coespressione espressione di questi mutanti con ΔCnA o ΔCnA^{H151Q} sulla traslocazione di Parkin. In cellule wildtype, l'espressione dei fosfo-mutanti non induceva la traslocazione di Parkin ai mitocondri *per se*, indice del fatto che la fosforilazione da parte di PINK1 di Parkin e dell'Ubiquitina non e' sufficiente ad indurre la traslocazione di Parkin.

Come atteso, in cellule PINK1 -/- trasfettate con Ub S65E, la localizzazione di Parkin S65E era citoplasmatica, ma traslocava in seguito ad trattamento di CCCP. In presenza del dominante negativo della CaN, inoltre, Parkin S65E non traslocava ai mitocondri in seguito a trattamento con CCCP, mentre in presenza del dominante costitutivamente attivo della CaN, Parkin S65E colocalizzava con i mitocondri. Questi dati sono a supporto con l'ipotesi che la CaN, in aggiunta a PINK1, e' necessaria per la traslocazione di Parkin.

Abbiamo quindi deciso di analizzare la traslocazione di Parkin in assenza di PINK1, utilizzando delle cellule PINK1 -/-. In questo modello, Parkin non trasloca ai mitocondri a seguito di trattamento con CCCP. Tuttavia, in presenza del mutante costitutivamente attivo della CaN, ΔCnA, Parkin era costitutivamente localizzato ai mitocondri. Inoltre, la mitofagia indotta da CCCP risultava essere significativamente aumentata. Quindi, i nostri dati suggeriscono che l'attivazione della CaN e' sufficiente alla traslocazione di Parkin, anche in assenza di PINK1.

Abbiamo quindi utilizzato un modello *in vivo* comunemente usato negli studi sul Parkinson, la *Drosophila melanogaster*, per valutare il significato fisiologico dell'inibizione o attivazione della CaN in un modello in cui PINK1 non fosse funzionale. L'overespressione di Parkin in un background PINK1 mutante (knock out) di questo modello e' in grado di migliorarne il fenotipo. Abbiamo pensato che un aumento dell'attivita' della CaN avrebbe potuto migliorare il fenotipo in un modo simile, favorendo la traslocazione di Parkin. Infatti, l'espressione di un mutante costitutivamente attivo della CaN in un background PINK1 mutante migliora significativamente il fenotipo delle mosche da noi analizzate. L'inibizione della CaN tramite FK506 e' in grado di bloccare anche l'effetto dell'overespressione di Parkin nelle mosche con un background PINK1 mutante, sottolineando il ruolo fondamentale della CaN nell'attivazione di Parkin in un assenza di PINK1.

Al momento, sono necessari ulteriori esperimenti per identificare i residui specifici su Parkin che sono defosforilati dalla CaN. Tramite un'analisi *in silico* abbiamo identificato la Serina 407 e la Treonina 410 come possibili siti di azione della CaN. Infatti i mutanti fosfomimetici per questi siti non traslocano ai mitocondri in seguito a trattamento con CCCP. Inoltre, stiamo procedendo con un'analisi di spettrometria di massa per Parkin, che e' stata isolata da cellule in condizioni di non trattamento, trattamento con CCCP e trattamento con FK506. Al momento abbiamo isolato Fla-Parkin da una linea stabile overesprimente la proteina, analizzando tramite Westernblot e Silver stain la purezza della proteina isolata.

La ricerca scientifica al momento sta esplorando l'effetto delle modifiche post-traduzionali come punto di partenza per la terapia di malattie. Ad esempio, sono stati trovati dei composti chimici che sono essere utili per pazienti affetti da leucemia e topi modelli del morbo di Alzheimer. Parkin risulta essere insolubile e formare aggregati, in questi modelli e Imatinib e Nilotib sono in grado di migliorarne la solubilita' e le funzioni. Questi studi risultano importanti per dimostrare come la manipolazione dell'attivita' di Parkin possa avvenire tramite modifiche post-trascrizionali ed essere usata come approccio terapeutico.

Anche l'ubiquitinazione sta emergendo come modificazione importante per regolare l'attivita' di proteine, tramite la localizzazione subcellulare o l'abilita' d interagire con altre proteine. L'attivita' delle ubiquitin ligasi e delle deubiquitinasi regola l'ubiquitinazione specifica di preoteine target. Nello specifico, gli enzimi in grado di contrastare l'attivita' ubiquitin ligasica di Parkin sono risultati candidati estremamente importanti per una terapia farmacologica.

Quindi, il nostro lavoro mostra come la CaN potrebbe essere utilizzata come terapia nel morbo di Parkinson, per regolare la traslocazione di Parkin e la mitofagia.

2. Summary

Mitochondria are crucial organelles in life and death of eukaryotic cells. Mitochondria are complex, double membrane-bound organelles, forming an extremely dynamic subcellular network. Their structure is fundamental for their function and their morphology undergoes continuous changes, sparing from an interconnected network to single units. Key components which regulate mitochondrial dynamics are the pro-fission proteins Drp1 and Fis1 and the pro-fusion proteins Mfn1/2 and Opa1.

Mitochondria not only are the main site of energy conversion, but also have a crucial role in apoptosis and autophagy regulation. Besides that, they also regulate Ca^{2+} and red-ox homeostasis, cellular differentiation and growth, and amplification of signaling cascades. Moreover, mitochondria dysfunctions have been implicated in the onset of several human diseases; in more details, accumulation of dysfunctional mitochondria has been linked to neurodegenerative disorders, such as Parkinson's disease.

Parkinson's disease (PD) is the second most common disorder after Alzheimer's disease. Pathologically, this disease is characterized by the progressive loss of dopaminergic (DA) neurons in the Substantia Nigra pars compacta (SNpc) in the midbrain and, in most of the cases, by the presence of proteinaceous cytoplasmic inclusions called Lewy bodies. However, the etiology of this disorder is still unclear.

PD is mainly a sporadic disorder, making it difficult, to find the etiology of the disease. However, 10% of PD cases are genetic and the analysis of familiar forms of PD resulted in a deeper understanding of the causes and molecular mechanisms of this disease. More in details, mutations in the PARK2 gene, encoding for Parkin, are the most common cause of Autosomal-Juvenile Recessive-Parkinsonism. PARK2 encodes for an E3-ubiquitin ligase which has a fundamental role in the recognition and elimination of dysfunctional mitochondria via autophagy. Its function is tightly related to another gene which has been found to be mutated in early PD, PINK1.

Parkin structure consists of an N-terminal Ubiquitin-like domain (Ubl) and four zinc-finger RING-like domains: RING0, RING1, IBR and RING2. A linker region connects the former segments. This region has no similarity with any known protein, so it is also called unique Parkin domain (UPD). Parkin main function is to transfer ubiquitin from an E2-ubiquitin ligase to the target protein. It has been demonstrated that Parkin subcellular localization is mainly cytoplasmic and then it is selectively recruited to dysfunctional mitochondria and it promotes their elimination through mitophagy. Parkin translocation is PINK1 dependent, even if the mechanism is still not clear. Once on mitochondria, Parkin ubiquitinates different proteins, including Mitofusin, which results in mitochondrial fission. However, it is still unclear which post-transcriptional modifications occur on Parkin, regulating its recruitment to mitochondria. Here, we show how Calcium and Calcineurin have a fundamental role in Parkin recruitment and mitophagy.

Free Ca^{2+} concentration is important in the regulation of metabolic processes and for signal transduction. Accordingly, the cytoplasmic pool of Ca^{2+} is very limited and is tightly regulated by mitochondria and ER. Besides all the other processes, Ca^{2+} dependent phosphorylation and dephosphorylation of targeted proteins affect their

activity by impinging on subcellular localization, conformation and protein-protein interaction. For example, phosphorylation and de-phosphorylation of mitochondria pro fission protein Drp1 regulates Drp1 translocation to mitochondria, an indispensable event for Drp1 dependent fission activity. In particular, translocation of Drp1 to mitochondria upon CCCP-induced depolarization is mediated by selective dephosphorylation of residue Serine 637, which is controlled by Ca^{2+} dependent phosphatase Calcineurin (CaN). On the other hands, protein kinase A (PKA) -dependent phosphorylation of Drp1 is retaining Drp1 in the cytoplasm during starvation, leading to elongated mitochondria, which cannot be eliminated by autophagy. Therefore, Ca^{2+} dependent regulation of phosphorylation and de-phosphorylation of Drp1 operates as a reversible post-transcriptional modification that impinges on Drp1 translocation, in response to metabolic changes.

Considering the fundamental role of Ca^{2+} in the regulation of proteins activity, we evaluated whether Parkin translocation and CCCP-dependent mitophagy depended on Ca^{2+} and was regulated by similar post-transcriptional modifications that control Drp1 translocation.

In our experiments, we transfected cells with fluorescent mCherry-Parkin and with mitochondrial targeted YFP (mito-YFP) constructs and looked at Parkin subcellular localization. Consistent with previous studies, we found that overexpressed Parkin mostly located in the cytosol of wild-type MEFs. Upon CCCP treatment, Parkin translocates to fragmented mitochondria in the 80% of the cells. Pre-treating cells with BAPTA, a Ca^{2+} chelator, abolished Parkin translocation, suggesting that its recruitment depended on Ca^{2+}. Moreover, chemical inhibition of CaN with FK506 blocked Parkin translocation.

Taking advantage of the already existing CaN dominant negative (ΔCnA^{H151Q}) and constitutive active mutants (ΔCnA), we turned to a genetic approach to evaluate the effect of CaN inhibition upon Parkin recruitment. We cotransfected MEFs with mCherry-Parkin and ΔCnB plus ΔCnA^{H151Q} and looked at Parkin localization. Interestingly, CCCP-induced Parkin translocation was impaired when in presence of the dominant negative mutant of CaN. To assess whether CaN played also a role in Parkin-dependent mitophagy[1,2], we next performed a mitophagy assay in presence of the dominant negative mutant of CaN. ΔCnA^{H151Q} expression significantly delayed CCCP-induced mitophagy. Taken together these data showed that pharmacological and genetic inhibition of CaN is sufficient to prevent Parkin translocation and CCCP-induced mitophagy.

Different studies linked Parkin translocation and mitophagy to PINK1 activity. Indeed, PINK1 directly phosphorylates Parkin and Ubiquitin (Ub) at Serine 65, which is required for Parkin activity. We generated phopsho-mimetic Parkin and phopsho-mimetic Ub at Serine 65 (Parkin S65E and Ub S65E, respectively) and investigated the effect of CaN constitutive active ΔCnA, and dominant negative ΔCnA^{H151Q} expression upon Parkin translocation. In wildtype MEFs transfected with Ub S65E, Parkin S65E localized to the cytosol and translocated to impaired mitochondria upon CCCP treatment. In this condition, we did not observe a constitutive localization of Parkin S65E on mitochondria in untreated cells, indicating that PINK1-dependent phosphorylation of Parkin and Ubiquitin might not be sufficient for Parkin recruitment.

As expected, in PINK1 -/- MEFs transfected with Ub S65E, Parkin S65E also localized to the cytosol and translocated to impaired mitochondria upon CCCP treatment. In presence of CaN dominant negative ΔCnA^{H151Q}, Parkin S65E did not translocate to mitochondria upon CCCP intoxication. Taken together, these observations supported the hypothesis that CaN, in addition to PINK1, is required for Parkin translocation.

Then, we looked at Parkin translocation in PINK1 -/- cells. As already reported, wildtype Parkin did not translocate to mitochondria upon CCCP treatment. Interestingly, in the presence of the constitutive active CaN mutant ΔCnA, Parkin constitutively co-localized with mitochondria. Moreover, CCCP-induced mitophagy was significantly enhanced.

As already reported, Parkin does not translocate to mitochondria upon CCCP treatment in the absence of PINK1. However, expression of constitutive active CaN was sufficient to promote Parkin translocation in PINK1 -/- cells, even in the absence of CCCP intoxication. Accordingly, mitophagy was enhanced in PINK1 -/- cells expressing CaN constitutive active. To conclude our data suggest that CaN activation can bypass PINK1 requirement in the induction of Parkin translocation and CCCP-induced mitophagy.

Comforted by the findings that CaN plays a role in regulating stress induced mitophagy *in vitro*, we next turned to a well-established *in vivo* model system to evaluate the physiological significance of CaN inhibition and/or activation in a PINK1 deficient model of PD. Parkin overexpression in a PINK1 mutant (knock out) background is able to rescue PINK1 mutant phenotype, as previously reported. We reasoned that enhancement of CaN activity *in vivo* would also ameliorate PINK1 mutant phenotype by enhancing Parkin translocation and activity. Indeed, CaN constitutive active expression in PINK1 mutant background rescues PINK1 mutant flies climbing deficiency. This effect was specific for CaN, as chemical inhibition of CaN with FK506, abolished the rescue. Importantly, FK506 administration partially blocked the rescuing effect of Parkin overexpression in PINK1 mutant flies, further suggesting an indispensable role for CaN in Parkin activation downstream PINK1.

Further experiments are required to identify the specific Parkin residue/s that is/are de-phosphorylated by CaN. An *in silico* analysis identified Serine 407 and Threonine 410 as potential candidates for CaN dependent de-phosphorylation. Indeed, translocation of phospho-mimetic Ser407Asp Parkin mutant was partially impaired, whereas phospho-mimetic Thr410Asp Parkin completely failed to translocate upon CCCP treatment. To convincingly prove that these are the residues that are de-phosphorylated by CaN, we are in the process of performing mass-spectrometry analysis of Parkin protein that has been pulled down from untreated cells and following CCCP intoxication.

Scientific research is exploring the effects of post-translational modifications as starting point for the developing of novel therapeutic targets for human diseases. In particular, Tyrosine Kinases Inhibitors (TKIs) such as Imatinib and Nilotib, are used as effective therapy for patients affected by leukemia. Recently, this drug resulted to be effective also in mouse models of Alzheimer's Disease (AD). Administration of TKIs to AD mice increases soluble Parkin leading to amyloid clearance and cognitive

improvement. Although no Parkin mutations are found in AD, these studies demonstrate how manipulation of Parkin activity through the modulation of post-transcriptional modifiers can be used as powerful therapeutic approach.

Ubiquitination is also emerging as a powerful tool to modulate proteins activity, via regulation of protein subcellular localization and/or ability to interact with other proteins. The counteracting activity of ubiquitin ligases and deubiquitinating enzymes (DUBs) mediate and regulate protein ubiquitination. Specifically in the context of PINK1/Parkin pathway, much effort has been put to identify specific DUBs that counter-act the ubiquitin-ligase activity of Parkin and impact mitophagy. These enzymes are therefore emerging as extremely attractive druggable candidates.

By exploring the role of CaN in Parkin translocation and stress induced mitophagy and *in vivo* in a PINK1 model of PD, this work ultimately identified a novel druggable target and has the potential to widen up medical intervention for the treatment of PD.

3. Introduction

3.1 Mitochondria

Mitochondria are crucial organelles in life and death of eukaryotic cells. These organelles reside in almost every eukaryotic cell and participate in the regulation of many cellular processes. They not only are the main site of energy conversion, but also have a crucial role in apoptosis and autophagy regulation. Besides that, they also regulate Ca^{2+} and red-ox homeostasis, cellular differentiation and growth, and amplification of signaling cascades. Moreover, mitochondria dysfunctions have been implicated in the onset of several human diseases; in more details, accumulation of dysfunctional mitochondria have been linked to neurodegenerative disorders, such as Parkinson's disease.

Mitochondria are complex, double membrane-bound organelles, forming a subcellular network which occupies almost 25% of the cytoplasmic volume. Their structure is fundamental for their function and their morphology undergoes continuous changes, sparing from an interconnected network to single units. Mitochondrial shape is regulated by an increasing number of proteins, called mitochondria shaping proteins, which belong to the large family of dynamin-related GTPases. These proteins control mitochondrial ultrastructure, which organization is also extremely complex. Mitochondria have an outer mitochondrial membrane (OMM) and an inner mitochondrial membrane (IMM). The IMM can be further divided into an inner boundary membrane and the cristae compartment, in which reside the complexes of the respiratory chain. Changes in both mitochondrial ultrastructure and cytosolic organization have a strong effect in the different process that mitochondria regulate within a cell.

3.1.1 Mitochondrial morphology and dynamics

According to the endosymbiontic theory developed around 1960s, mitochondria have an extracellular origin as free-living prokariotes and they became organelles of eukaryotic cells[1]. Important evidences supporting this theory come from mitochondrial genome, as well as from biochemical and physiological symilarities of these organelles with prokariotes. A corollary to the endosymbiontic theory also explains how the reduced number of proteins encoded by mitochondrial genome is the consequence of a gene transfer to the nucleus, known as endosymbiontic gene transfer (EGT). During evolution, mitochondria became fundamental for the life of eukaryotic cells and vice-versa[2].

Mitochondria were firstly described by the anatomist Rudolf Albrecht von Koelliker, who called them "sarcosome" in 1857. In 1950s Sjöstrand and Palade were the first to use electron microscopy to analyze mitochondrial internal structure. The interpretation of their micrograph led to different models for mitochondrial structure, nevertheless they both recognized the presence of two different membranes. However, Palade's model is the one that evolved to the currently accepted by the scientific community. According to this model, also called baffle model, mitochondria

have two membranes, an outer membrane (OMM) and an high convoluted inner membrane (IMM), and two soluble compartments, the intermembrane space (IMS) between the OMM and the IMM and the matrix, the central electrondense space[3,4]. While the OMM structure is quite simple and follows the shape of mitochondria, the IMM presents invaginations called *cristae*, which broadly open on the IMS on one side through the so called *cristae juctions*, and protrude across the matrix on the other side. The *cristae* house the complexes of the electron transport chain and the ATP-synthase, thus are the sites of ATP production. The IMM is a continue surface, nevertheless new advances in technologies allowed to further subdivided it into inner boundary membrane and *cristae* compartment[5].

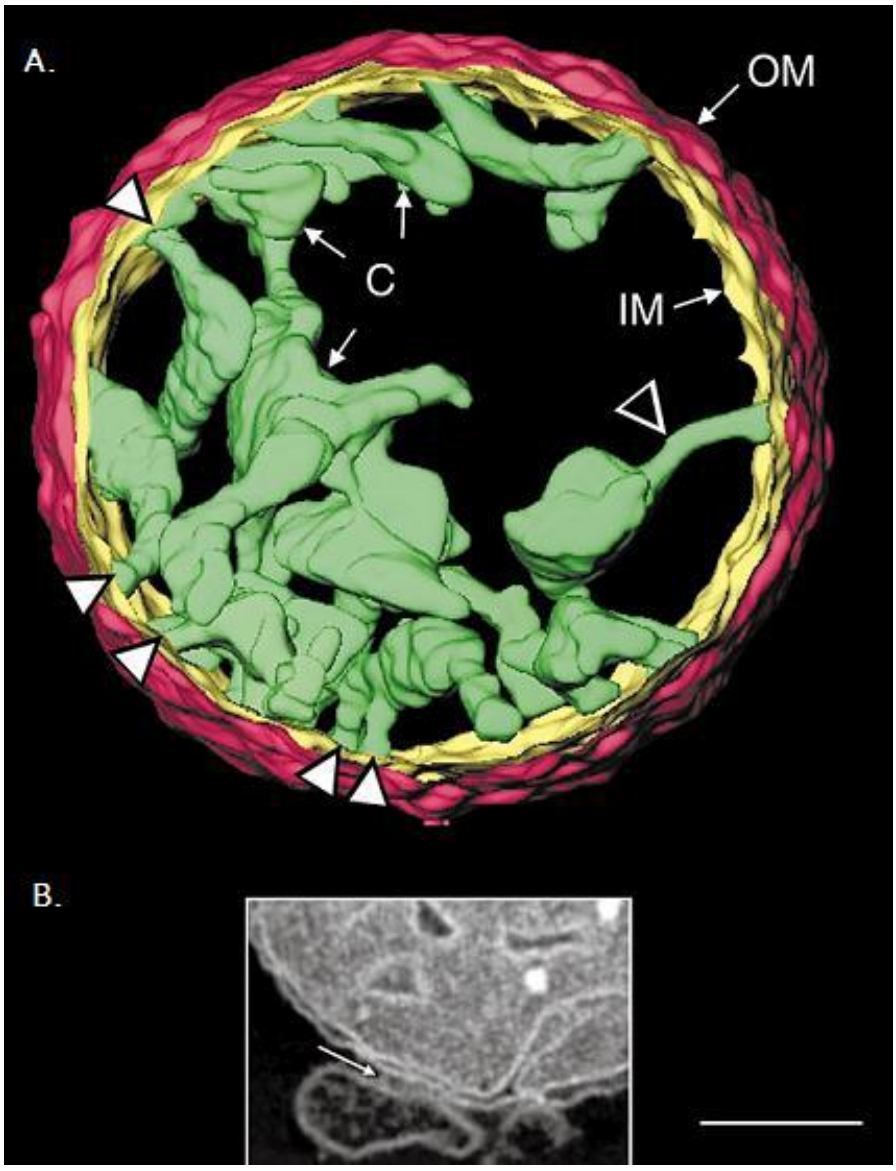

Figure 1. Tomography of a rat-liver mitochondrion. (A.) Three-dimensional reconstruction of an isolated rat-liver mitochondrion obtained by high-voltage electron microscopic tomography. OM: outer membrane; IM: inner membrane; C: cristae. **(B.)** Region of a 5-nm slice from the same tomogram. There are a lot of contact sites between OM and IM. Arrow points to binding site of the OM with putative endoplasmic reticulum. Bar, 0.4 mm[5].

The OMM contains a large number of *porins*, which are integral proteins acting as non-specific pores and allow the access in the IMS to ions and metabolites smaller than 10KDa. The composition of this membrane is similar to the other eukaryotic membrane and it is enriched in protein of the import machinery, through which the nuclear-encoded proteins can be imported into the mitochondria. In comparison, the IMM is less permeable and only small molecules or metabolite are allowed to cross it and enter into the matrix. Metabolites and proteins can cross the IMM only through specific carriers, since it is permeable only to oxygen, carbon-dioxide and water.

Another fundamental feature of the IMM consists in the restricted diffusion allowed between the internal compartments. This has profound implications since cristae, besides the site of ATP production, also store cytochrome c, which is released upon apoptosis.

Both the IMM and the OMM can change shape in response to different metabolic requirements of the cell. Mitochondria continuously undergo fission and fusion events, which processes regulate their functions and maintenance[6]. Fusion and fission are events which involve two membranes, thus they are fine regulated by specific proteins. Changes in mitochondria shape and ultrastructure can be easily monitored through confocal or electron microscopy.

3.1.1.1 Mitochondrial fission proteins

Dinamin-related protein 1 (Drp1) is a largely cytoplasmic GTPases which plays a major role in mitochondrial fission. It is a protein with an N-terminal GTPase domain, a dynamin-like middle domain and a GTPase effector domain (GED) located in the C-terminal region. Its translocation and activity is regulated by post-transcriptional modifications, in particular by phosphorylations at specific residues[7]. Drp1 can translocate to mitochondria in a Ca^{2+} and Calcineurin dependent manner[8], where it forms oligomers and binds to its adaptors on the OMM (Fis1, Mff and MiDs), thus causing mitochondrial constriction and fragmentation. Therefore, mitochondrial fragmentation depends on a loop involving sustained Ca2+ rise, activation of Calcineurin, dephosphorylation of Drp1 and its translocation to the organelle.

Other important players in fission are Drp1 receptors and adaptors on the OMM. Drp1 anchors to the OMM receptors to form the fission complex, which is an essential initial step for the fission process[9]. The role of the different receptors in Drp1 recruitment and stabilization on the OMM is not completely clear. However, it has been shown a role for both Fis1 and Mff in Drp1 recruitment and mitochondrial fission, with Mff playing a bigger role in both events[10]. Moreover, MiD49 and MiD51 play a role in Drp1 recruitment independently from Fis1 and Mff, and they can maintain it at an inactive state until a cellular signal triggers mitochondrial fission.

Many recent studies focused on identifying the sites where Drp1 is recruited and fission occurs. These sites are called fission foci, and seem to be marked by ER tubules[11]. Nevertheless, the complete mechanism of mitochondrial fission regulation is still far from being completely understood.

3.1.1.2 Mitochondrial fusion proteins

Two families of proteins regulate mitochondrial fusion in mammals: Mfns and Opa1.

The first identified mediator of mitochondrial fusion has been the large GTPase Fuzzy onion protein 1 (Fzo1), the *D. melanogaster* homologues of Mfns expressed during spermatogenesis[12]. Mutations in this gene cause male sterility in flies, and mitochondria cannot fuse normally. Besides Fzo1, *D. melanogaster* also possesses another Mfns homologue, the Mitochondrial assembly regulatory factor (Marf) which is ubiquitously expressed in both males and females[13]. In mammals, two Fzo1 homologues, Mfn1 and Mfn2, are widely expressed in many tissues and acting in *trans* they promote mitochondrial fusion[14]. Mfn1 and Mfn2 possess a similar structure, with an N-terminal GTPase domain, two transmembrane domains spanning the OMM separated by two heptad repeat regions (HR1 and HR2)[14-16]. Despite their high similarity, Mfn1 and Mfn2 seem to have different roles within the cell. Functionally, Mfn1 has a higher GTPase activity compared to Mfn2 and it shows and higher capacity to induce fusion[17-19]. Moreover, recent works form our lab showed that Mfn2 is also present on the ER, and it is enriched on the ER/mitochondria interface[19]. Interestingly, Mfn2 on the ER forms omo- and etero-typic complexes with Mfn1 and Mfn2 on the mitochondria, controlling the tether between the two organelles. By regulating mitochondrial/ER contacts, Mfn2 levels can affect Ca^{2+} exchange and signaling between the two organelles. Thus, it has been suggested that, while Mfn2 mainly regulates fusion, Mfn2 has additional regulatory functions[15]

Optic Atrophy 1 (Opa1) is the other player in the process of mitochondrial fusion of the IMM[20-22]. The Opa1 gene produces different isoforms, in human there are eight different splice variants. The role of Opa1 in fusion or fission is controversial. Interestingly, Opa1 overexpression causes mitochondrial elongation in cells[23], but high Opa1 levels can promote mitochondria fission[24]. Moreover, Opa1 has been found to have a fundamental role in apoptosis and cristae remodeling[25,26].

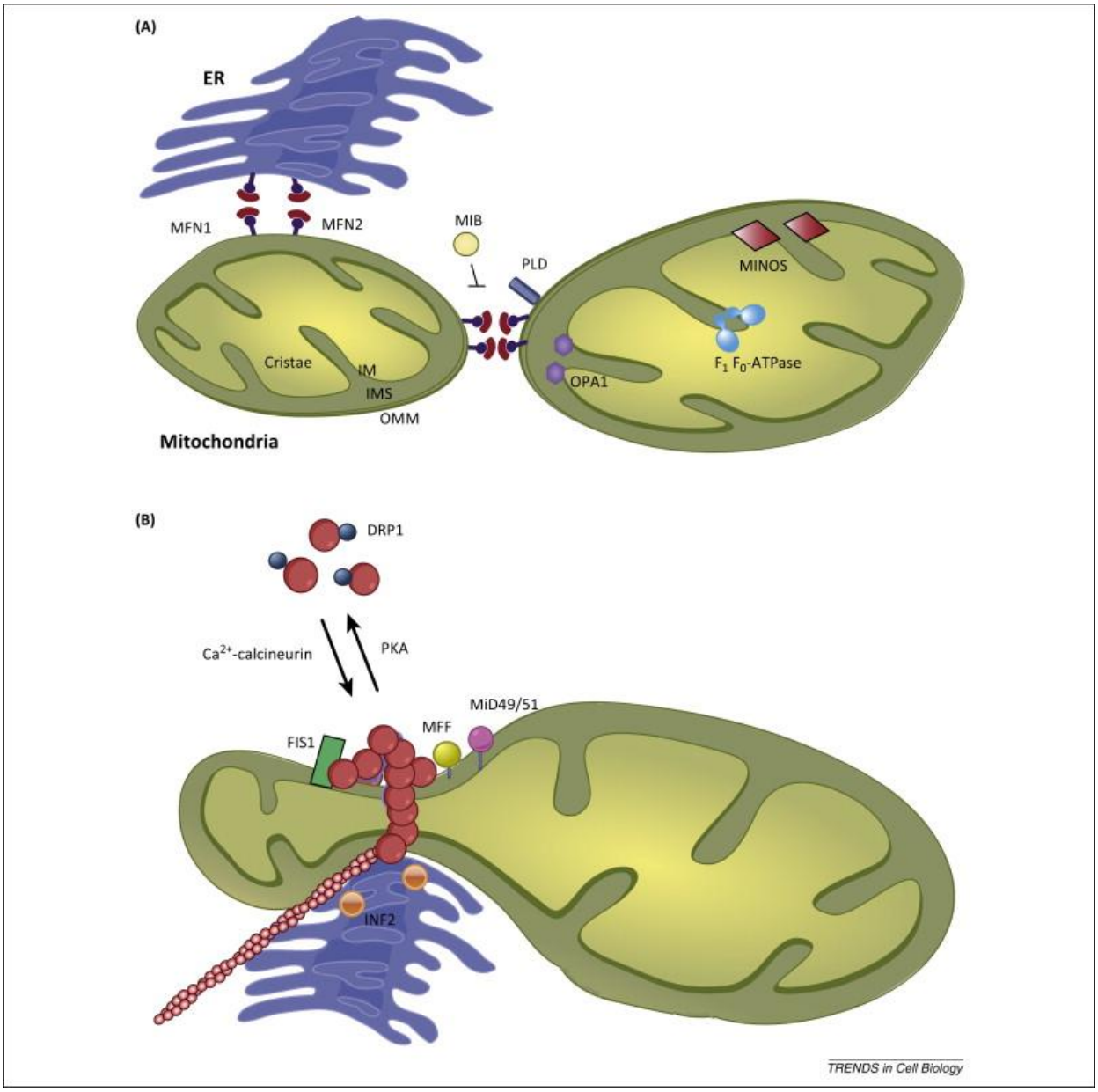

Figure 2. Regulation of mitochondrial dynamics. (A.) Cartoon showing the roles of mitochondrial fusion proteins. Mfns, besides their role in OMM fusion, also have an important role in the mitochondria-ER tethering. MIB inhibits MFNs fusion activity, while phospholipase D (PLD), by providing lipid remodeling, facilitates it. Opa1 is the regulator of IMM fusion and of cristae biogenesis and remodeling, together with the prohibitin-MINOS complex which controls the formation of cristae junction and of F_0F_1ATPdimers that contribute to curve the cristae. **(B.)** Rappresentation of mitochondrial fission mechanism. Drp1 is normally phosphorylated and sequestered in the cytoplasm. Dephosphorylated Drp1 is recruited at the ER-mitochondria contact sites, where ER tubules cross over and wrap around mitochondria. Once translocated, Drp1 oligomerizes and interacts with MFF, MiD49/51, and FIS1, promoting mitochondrial fission[27].

3.1.1.3 Mitochondrial shape and physiological function

Recently, many studies focused on the intimate connection between mitochondrial morphology and their function, specifically in the context of mitochondria quality control. For instance, mitochondrial fragmentation is a pre-requisite for elimination of defective mitochondria, which are engulfed by

autophagosomes and eventually degraded[28]. Moreover, mutations in fusion genes have been found to be linked to neurodegenerative disorders, Charcot-Marie Tooth type 2A (CMT2A) and autosomal dominant optic atrophy (ADOA)[29,30]. Indeed, mitochondrial motility along the axon in neurons is linked to mitochondrial fragmentation[31]. Thus, mitochondrial distribution on neuronal synapsis is intimately related to their ability to fuse and divide, and these processes control also the density and plasticity of the synapsis[32]. Moreover, Drp1 -/- mice show developmental abnormalities, especially in the forebrain, which can be associated to abnormalities in synapsis formation[33]. It is not surprising how, in the last years, mitochondrial dynamics was reported to participate in the pathophysiology of different neuronal disorders and mutations in genes involved in this process have been shown to cause neuronal disorders.

Opa1, which is genetically linked to ADOA, has been found to have different roles besides mitochondrial IMM fusion, such as cristae remodeling, supercomplex formation and regulation of mitochondrial autophagy[25,34]. These different functions seem to depend on the different isoforms and cleavage processes of Opa1.

Mutations in Mfn2 are linked to the onset of CMT2A. Besides its role in mitochondria fusion, Mfn2 levels are correlated with oxidative metabolism of skeletal muscle[35] and the proliferative ability of vascular smooth muscle cells by sequestering the protooncogene Ras[36]. Moreover, through its role in the regulation of ER-mitochondrial tether, Mfn2 allows the regulation of many different processes, such as lipid synthesis, mitochondria energy metabolism, Ca^{2+} transfer between the two organelles and Ca^{2+} dependent cell death[19]. Finally, Mfn2 has a fundamental role in mitophagy, being the receptor for Parkin on the OMM[37,38] and eventually getting ubiquitinated by Parkin. We will discuss its role in mitophagy in the following paragraphs.

Both mitochondrial fusion and fission events are pro-survival for the cell: fusion helps overcoming stress by sharing the contents of damaged mitochondria, and fission regulates the segregation of unfunctional mitochondria so that they can be eliminated[9,39].

3.1.2 Self-eating and self-killing: autophagy and apoptosis regulation

Autophagy and apoptosis are two main processes which regulate cell fate. Both are self-destructive processes which in the last years gained considerable interest in the context of different human pathologies. Autophagy is a highly regulated process involved in the turnover of long-lived proteins as well as whole organelles, and it can occur in a generalized fashion or can specifically target distinct organelles (f.i. mitochondria). There is a basal level of autophagy, which allows the turnover of cellular components. However, in some cases autophagy can be activated in response to stress conditions which can lead to apoptosis. Apoptosis is the best characterized programmed cell death process, which causes the rapid demolition of cellular components, upon the activation of a signaling cascade involving proteases mainly. Autophagy and apoptosis often occur in the same cell, in most of the cases with

autophagy preceding apoptosis[40-42]. The crosstalk between autophagy and apoptosis can determine cell fate. Similar stimuli can induce either autophagy or apoptosis in the cell. This is because a stress often stimulates an autophagy response first. When it exceeds, in terms of duration or intensity, apopototic lethal programs get activated.

It is still not clear whether autophagy represents a mechanism for preventing apoptosis or for enacting non-apoptotic programmed cell death.

3.1.2.1 Mechanism and regulation of autophagy

Autophagy (literally meaning "self-eating") is a catabolic process during which long-lived proteins and damaged cytoplasmic organelles are engulfed by double-membrane structures named autophagosomes and eventually get degraded to basic components, which can then be recycled by the cell itself. Three different form of autophagy can be recognized (Figure 3): chaperone-mediated autophagy (CMA), microautophagy and macroautophagy. Macroautophagy (hereafter called autophagy) is a conserved pathway in eukaryotic cells, from yeast to mammals that enables the degradation of cytoplasmic components. Compared to microautophagy, autophagy is a more complex process, since the target engulfed by autophagosomes which then fuse with lysosomes, while microautophagy involves only lysosomes[43]. Finally, CMA is a specific form of autophagy which removes individual proteins containing a specific sequence recognized by the chaperone Hsp70 (70 kDa heat shock cognate protein). This chaperone recognizes and binds to the target, then translocates to the lysosome where it binds to LAMP-2A (lysosome-associated membrane protein 2A). Thus, the target protein gets unfolded and imported to the lysosome where eventually gets degraded[44,45]

As already mentioned, autophagy is present at basal levels in every eukaryotic cell, but it can be strongly up-regulated by certain stimuli, such as starvation, hypoxia or pharmacological treatments, allowing the specific study of the pathways involved[43]. Autophagy can be divided into different stages, from the nucleation of the autophagosome, to its elongation and fusion with the lysosome, followed by engulfment and degradation of the target cargoes.

Three main pathways can control the process of autophagy. In mammals, the major regulator of autophagy is the Target Of Rapamycin (mTOR). mTOR is a Threonin/Serine kinase which binding to RAPTOR, PRAS40 and mLST8 forms the Target Of Rapamycin Complex I (mTORC1) and acts as a nutrient sensor. In presence of nutrients, class I phosphatidylinositol 3-kinase (PI3K) activates protein kinase B (Akt) and TORC1, inhibiting autophagy. In case of nutrient restriction, mTOR is inhibited, leading to autophagy pathway activation. The autophagosome forms as consequence of Ubiquitin Like Kinase 1 (ULK1) activation, which is usually inhibited by mTOR. ULK1 phosphorylates different substrates, among which mAtg13, FIP200 and AMBRA1[46]. Moreover, ULK1 forms a complex with mAtg13 and FIP200, which is fundamental for organization and maturation of the autophagosome.

A second pathway regulating autophagy is mediated by mammalian Beclin1. Activation of ULK1-mAtg13-FIP200 complex leads to Beclin1-Vsp34 complex formation on the lipid membrane of the forming autophagosome. Vsp34 is a class III PI3K, which

once in a complex with Beclin1 produces phosphatidylinositol 3-phosphate (PI3P), inducing the recruitment of other proteins of the autophagy complex[47]. Beclin1-regulated autophagy protein- 1 (Ambra-1), ultraviolet radiation resistance-associated gene (UVRAG), and Bax interacting factor-1 (Bif-1) promote the interaction through Vps34 and Beclin1. This pathway is an important link between autophagy and apoptosis, since the anti-apoptotic proteins Bcl-1 and Bcl-XL binding Beclin1 inhibit autophagy[48].

Finally, two ubiquitin-like conjugation processes mediate the elongation of the autophagosome, the Atg5-Atg12-Atg16L and the Atg4-Atg8 (LC3). LC3 is constitutively cleaved by Atg4 to produce LC3-I. When autophagy is induced, LC3-I binds to the membrane lipid phosphatidylethanolamine, to form LC3-II, which is recruited selectively on the expanding autophagosome membranes and mediates its closure[49]. LC3-II then gets degraded after autophagosome fusion with lysosome.

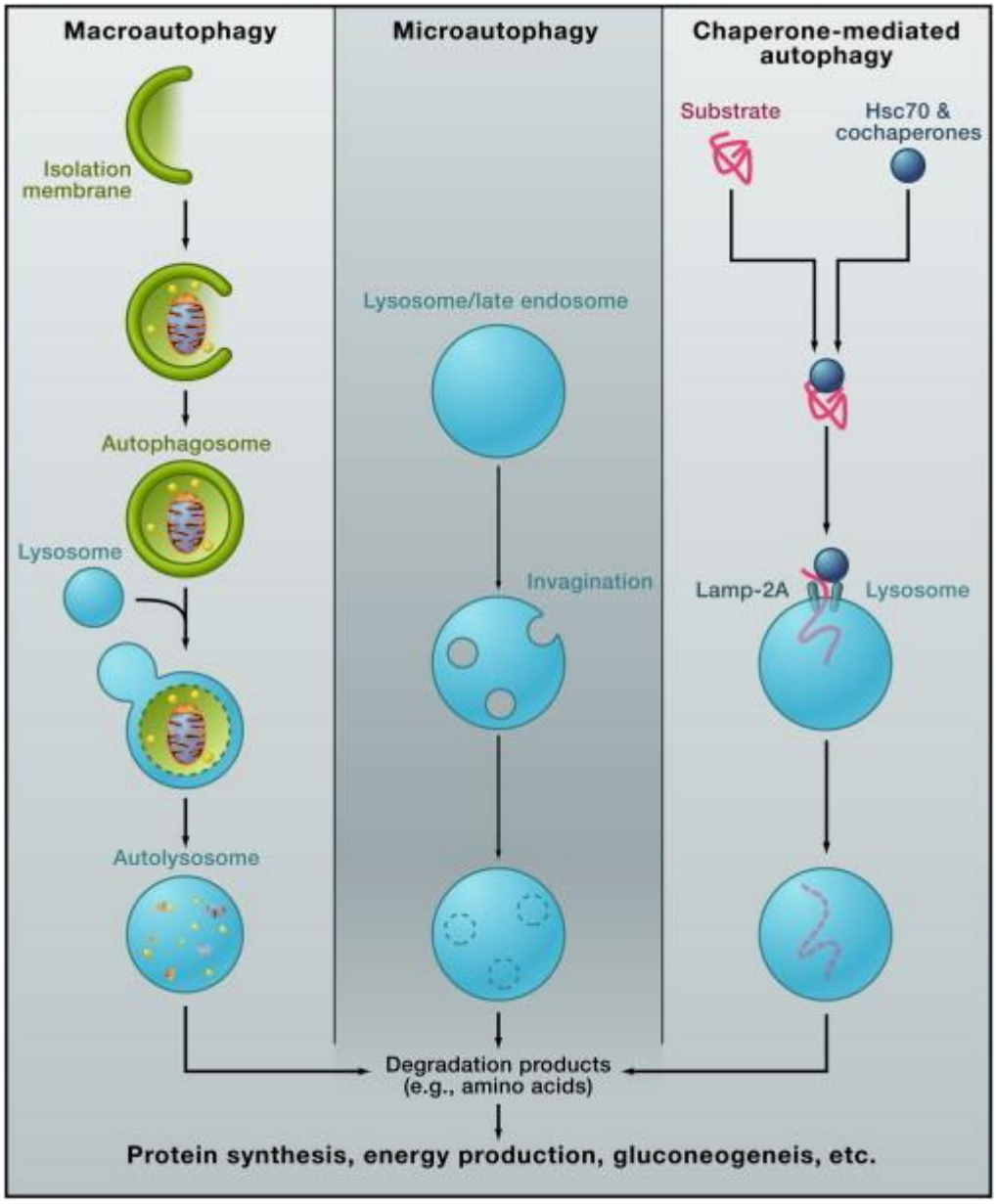

Figure 3. Different types of autophagy. Macroautophagy: a portion of cytoplasm is engulfed by a double membrane structure, the phagophore, to form an autophagosome. The outer membrane of the autophagosome fuses with the lysosome for degradation of the cargoes in the autolysosome. Microautophagy: small parts of the cytoplasm are enclosed in an invagination of the lysosomal or late endosomal membrane. Chaperone-mediated autophagy: first, cytosolic Hsc70 and cochaperones recognize substrate proteins containing a KFERQ-like pentapeptide sequence. Then they are translocated into the lysosomal lumen after binding with lysosomal Lamp-2A. All the different type of autophagy result in degradation of the cargoes and recycling[50].

3.1.2.1.1. Mitophagy: a selective form of autophagy

As previously mentioned, organelles-specific autophagy also exist as quality control mechanism to eliminate damaged or dysfunctional components. In the case of mitochondria, this process is defined mitophagy and depends on PINK1/Parkin pathway. In mammalian cells, mitophagy strongly depends on mitochondrial fission, during which mitochondria get divided into smaller pieces to get encapsulated by autophagosomes[28,51].

The PTEN induced kinase 1 (PINK1) is a Serine/Threonine kinase, which normally is imported into mitochondria and rapidly degraded. However, in presence of depolarized mitochondria, PINK1 is stabilized on the OMM were it recruits the E3 ubiquitin-ligase Parkin[52]. Once on mitochondria, Parkin leads to the ubiquitination of mitochondrial proteins (Mfn2, VDAC, TOM and other), as a signal for targeting selected mitochondria to the autophagosome[53,54]. It has been widely demonstrated that Parkin translocation is PINK1 dependent, but the mechanism is still unclear. Recent works show that PINK1-mediated phosphorylation of Parkin and Ubiquitin at residue Serine 65 (Ser65) is necessary but not sufficient for Parkin translocation to defective mitochondria, and it is indeed fundamental for its E3-ubiquitin ligase activity[RW.ERROR - Unable to find reference:182]. The ubiquitinated proteins on the OMM recruit, in turn, p62, which targets the organelle to the autophagosome through its interaction with LC3[58].

Interestingly, recent publications show a strong connection between Bcl-2 family members and mitophagy. In particular, Bcl-2 family member antagonizes mitophagy through inhibition of Parkin translocation to mirochondria[59]. Moreover, AMBRA1, a pool of which in basal condition is localized at the mitochondria interacting with BCL-2, is able to interact directly with Parkin, facilitating mitophagy[60]. Surprisingly, upon mitophagy induction, AMBRA1 is also able to directly interact with LC3, inducing mitochondrial degradation regardless of PINK1/Parkin pathway[61].

Autophagy and mitophagy are two similar processes which occurs for different reasons. Autophagy occurs when cells are deprived of nutrients or in response to specific metabolic requirements. Proteins and organelles get degraded to be recycled and for providing ATP. Interestingly, during autophagy mitochondria elongate, to be spared from degradation and provide membranes for the autophagosome[62]. By contrast, mitophagy occurs to eliminate dysfunctional mitochondria, which fragment and separate from the functional ones to be degraded.

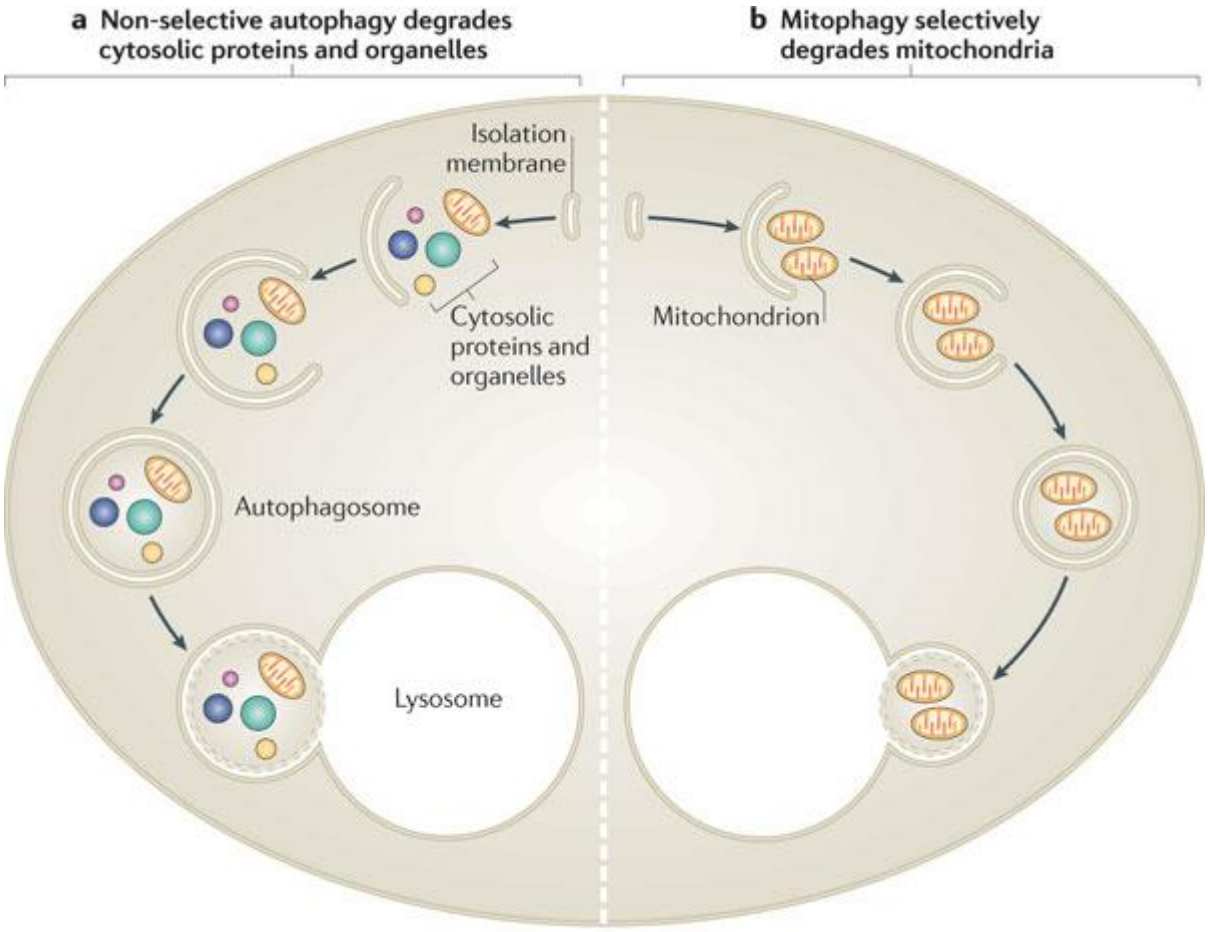

Figure 4. Selective and non-selective autophagy have different roles. (A.) Non selective autophagy occurs when cells are in starvation. The degradation of components of the cytoplasm supplies building blocks for re-use and for metabolism to provide ATP. **(B.)** Mitophagy occurs to eliminate defective mitochondria[28].

3.2. Parkinson's Disease

PD has been firstly described in 1817 by James Parkinson in his ''Essay on the Shaking Palsy'' as an "Involuntary tremulous motion, with lessened muscular power, in parts not in action and even when supported; with a propensity to bend the trunk forward, and to pass from a walking to a running pace: the senses and intellects being uninjured". However only over 60 years after the term ''Parkinson's disease'' was actually coined, by J. M. Charcout, expanding also the list of the symptoms.

Pathologically, this disease is characterized by the progressive loss of dopaminergic (DA) neurons in the Substantia Nigra pars compacta (SNpc) in the midbrain and, in most of the cases, by the presence of proteinaceous cytoplasmic inclusions called Lewy bodies[63]. However, the etiology of this disorder is still unclear even though it is one of the most common progressive movement disorders in the elderly[64].

Mitochondrial dysfunction has been found to be a main contributing factor to PD development, with evidences coming from observations on MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), rotenone and paraquat[65-68]. All of these chemicals are mitochondrial toxins, acting on Complex I, which have also been associated to PD.

Moreover, a reduction in Complex I activity was found in the substantia nigra, in platelets, lymphocytes and muscle tissue of PD patients[69-71].

However, the first familial PD gene was discovered a decade after these findings. A mutation in α-synuclein gene was found to be associated with autosomal dominant familial parkinsonism[72]. This protein was found to be the major component of Lewy bodies both in sporadic PD. Since then, more genetic factors have been linked to PD and today there are 16 loci identified which are associated to heritable forms of PD (PDGene database).

3.2.2. Clinical characteristic, symptoms and treatment

PD is a progressive neurodegenerative disorder with average onset at 55 years old, with incidence increasing with age. About 90% of the cases have no apparent genetic linkage (called as "sporadic" PD cases), but the disease is inherited in the rest of the cases. Moreover, some genetic linked forms of PD show an earlier manifestation, thus complicating the diagnosis of the disease. Due to the slower progression of the disease, the first symptoms occur when at least 60% of DA neurons in the Substantia Nigra pars compacta (SNpc) are dead and dopamine release is reduced by about 80%. Current research on treatments is directed through the prevention of DA neuronal loss.

Clinically, the term "parkinsonism" is used to describe a syndrome characterized by tremor at rest, rigidity, slowness or absence of voluntary movement, postural instability, and freezing, caused by striatal DA deficiency or damage.

PD is characterized by both motor and non-motor features. Four symptoms are considered the cardinal ones, and they are known with the acronym of TRAP: Tremor at rest, Rigidity, Akinesia (or bradykinesia) and Postural instability. In addition, flexed posture and motor blocks have been included among classic features of parkinsonism. Non-motor features are abnormalities of affect and cognition, indeed some patients show a loss of initiative, anhedonia, slowed cognitive processes, depression, and, especially in older patients, also dementia[73].

As already mentioned, the neuropathological hallmarks underlying these clinical features are the loss of DA neurons and the presence of intraneuronal proteinacious cytoplasmic inclusions termed "Lewy bodies" (Figure 6 A.). These neurons normally contain conspicuous amounts of neuro-melanin and their loss causes the classic SNpc depigmentation in PD patients[74]. However, the neurodegeneration in PD affects also a lot of different neurochemical systems, beyond the DA neurons. As regards for the Lewy bodies, these are not specifically found in PD, but also in some forms of Alzheimer's disease, in a condition called "dementia with LB disease" and also in a an incidental pathologic finding in people of advanced age[75]. Lewy bodies are spherical eosinophilic cytoplasmic proteins aggregates composed of numerous proteins, including α-synuclein, Ubiquitin, heat shock proteins, neurofilaments and Parkin, and they are found in all affected brain regions[72]. The role of Lewy bodies in neuronal cell death remains controversial.

PD is diagnosed on clinical criteria; there is no definitive test for diagnosis. Diagnostic criteria have been developed by the UK Parkinson's Disease Society Brain

Bank and the National Institute of Neurological Disorders and Stroke (NINDS), but the reliability and validity of these criteria have not been clearly established.

The selection of the treatment for PD patients is highly individualized. It should take into account different factors, including the patient's age, symptoms, symptom severity, occupational status, lifestyle, cognitive, behavioral and psychiatric status, and other medical characteristics. In the case of early Parkinson's disease, one of the most common therapy is based on the administration of L-Dopa to patients, a precursor of dopamine. Usually this occurs in combination with Carbidopa/Levodopa administration, that inhibits DA Decarboxylase (DDC) present in the periphery, increasing the dose of L-Dopa that reaches the brain[76]. The administration of Levodopa can also be used in the case of advanced PD[77].

Other strategies involve the inhibition of dopamine catabolism[76]. These include inhibition of Monoamine Oxidase B (MAO-B) or of Catechol-O-methyl-transferase (COMT). Through the inhibition MAO-B, DA would no more be converted to DOPAC (3,4-Dihydroxyphenyl acetic acid) resulting in higher concentrations of DA and in prolonged effect of L-DOPA. To treat severe symptoms, COMT inhibitors (Entacapone) are given together with L-DOPA. COMT reduces DA levels by methylating DA to 3-Methoxytyramine, and also acts in the periphery, resulting in too small amounts of L-Dopa reaching the brain. In some other, more severe cases, tremor is treated with anticholinergics[77].

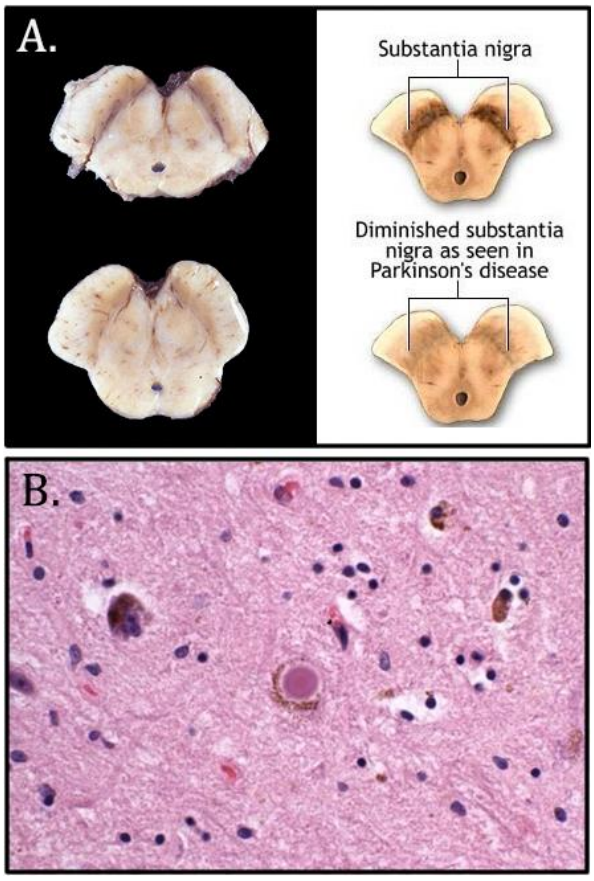

Figure 6. Anatomy and histology of brain of Parkinson's disease patients. (A.) Midbrain section showing normal substantia nigra (on the top); midbrain section showing loss of pigmented cells of the substantia nigra in PD patients' brain (on the bottom). From: CNS Pathology. **(B.)** Lewy bodies are the round eosinophilic inclusions composed of α-synuclein which can be clearly seen in this histology slide of the brain of a PD patient.

In case the patients cannot be treated using conventional oral medication, three invasive options can be used to ameliorate motor symptoms. The first option consists in continuous subcutaneous apomorphine infusion, the second in continuous duodenal levodopa carbidopa pump, or the third one in deep brain stimulation (DBS).

It is important to underline that all available treatment strategies can alleviate the symptoms of the disease, but, at now, the neuronal degeneration cannot be stopped or slowed down. Further research is needed to identify a definitely neuroprotective agent, to develop new treatment strategies with consistent benefits and fewer side effects.

3.2.2. Genetics of Parkinson's Disease

In recent years, major advantages have been made in the understanding of the genetic basis of PD, after the identification of SNCA gene encoding for α-synuclein, which mutations are linked to familial PD[72,78]. Indeed until 1997, PD was considered to be a non-genetic disorder, since it was thought to be caused by environmental factors[79]. Until now, many loci have been identified to be linked with PD, through linkage analysis, genome sequencing and genetic association. Although mutations and loci were identified in a relatively small number of families and only 10% of PD cases have a genetic cause, it is likely that the molecular pathways causing DA neurons loss are conserved among monogenic and sporadic forms of the disease.

Moreover, recent findings allowed the development of novel genetic animal models, providing the basis for a better understanding of PD pathogenesis and for the development of new therapeutic strategies[73]. So far, six are the most common genes identified in familial PD, and can be divided into genes causing autosomal dominant or autosomal recessive form of the disease [80] (see below).

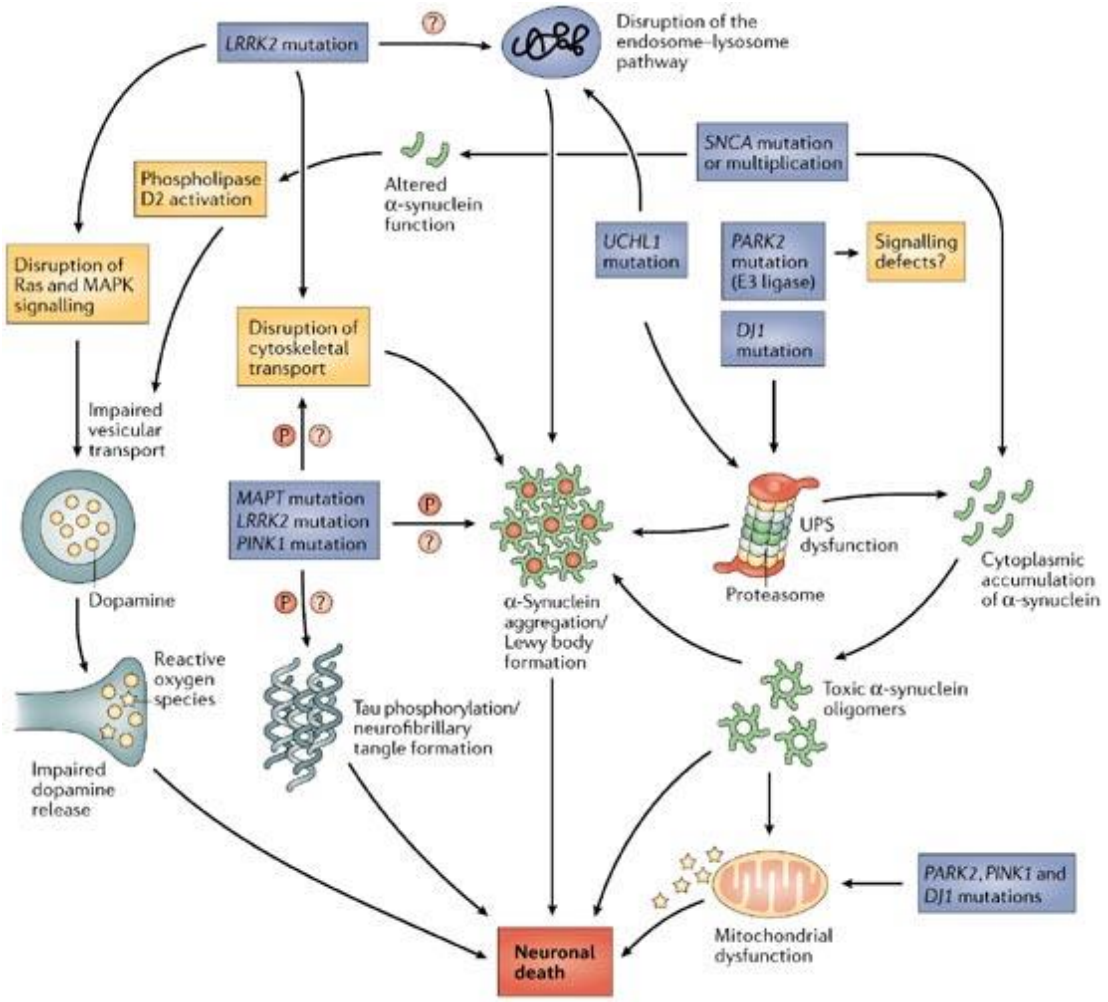

Figure 7. Pathways to PD. General rappresentation of the most common PD-related genes mutations. Mutations and genomic moltiplications of SNCA lead to a cytoplasmic accumulation of α -synuclein monomer and its subsequent oligomerization. α-synuclein is involved in protein misfolding and aggregation, one of the main pathway of cell toxicity. Parkin and DJ-1 interact and are involved in the UPS function. Mutations in these proteins could lead to accumulation of misfolded protein and its aggregation. The mitochondrial pathway that involves impaired oxidative phosphorylation and decreased complex I activity is also crucial in PD progress. UPS function and aggregate clearance requires ATP synthesis by mitochondria, and normal mitochondrial function is notably compromised by loss of PINK1, DJ1 and parkin activities, resulting in early-onset parkinsonism. ROS accumulation and oxidative stress, in parallel with loss of mitochondrial membrane potential, trigger to opening of the PTP and then apoptosis. Dysfunction of both pathways leads to oxidative stress, which causes further dysfunction of these pathways by feedback and feedforward mechanisms, ultimately leading to irreversible cellular damage and death[78].

3.2.2.1. Autosomal dominant forms of Parkinson's disease

Three loci have been identified as associated with late-onset dominant forms of PD: PARK1/4, PARK3, and PARK8. No corresponding gene is known for PARK3 yet. PARK1/4 corresponds to SNCA gene, and PARK8 to LRRK2.

SNCA encodes for α-synuclein and it has been the first gene to be associated to the onset of PD. PD associated mutations of this gene include both missense mutations and amplification. In particular, three mutations have been identified, which segregate with familial PD: Ala53Thr, Ala30Pro, Glu46Lys[81]. In addition to these missense mutations, also genomic duplication or triplication have been found to cause autosomal dominant, early-onset PD[82]. The protein is small and contains an N-terminal

a-helical region, a hydrophobic central component and an acidic C-terminal region. This protein is normally expressed throughout the brain, with highest levels in deeper layers of the cerebral neocortex, the hippocampus and the SN[83,84] The role of α-synuclein is not well understood, however its function correlates with signal transduction, membrane vesicle trafficking and cytoskeletal dynamics.

α-synuclein seems to be predominantly cytoplasmic in the brain, nevertheless it has been shown that it associates with native membranes and phospholipid vesicles in vitro, though its N-terminal region. The wild type protein is a potent inhibitor of phospholipase D2 and phospholipase C, which are enzymes involved in signal transduction, membrane vesicle trafficking and cytoskeletal dynamics. It is also a competitive inhibitor of tyrosine hydroxylase, the rate-limiting step in tyrosine to L-dopa biosynthesis. This protein has naturally an high propensity to aggregate, due to its hydrophobic central region[72].

Strong evidences associate α-synuclein accumulation with the early step of the pathogenesis of both sporadic and inherited PD. Pathogenic mutations in α-synuclein easily aggregates both in vitro and in vivo. Initially there is the formation of an intermediate annular structure, and ultimately they give rise to insoluble polymers or fibrils, which are the main constituent of the Lewy bodies. It is still under debate whether fibril, the protofibril or the soluble species is the most toxic species in neurons[85].

PARK8 has been identified as the leucine rich repeat kinase 2 gene (LRRK2) ad it has been linked to autosomal dominant late onset of parkinsonism[86]. It is the most common form of inherited PD in the world and the clinical features are similar to those of sporadic PD. Until now, 20 missense or nonsense mutations have been reported. The most common mutation is the Glu2019Ser substitution, which accounts for 5% of familial PD and 1.5% of sporadic cases[87]. LRRK2 is an extremely large protein of 285 kDa containing many different domains and highly expressed in the brain, even if the precise tissue and intracellular expression is not completely clear[88]. The function and the effects of the pathogenic mutations of LRRK2 also remain unclear. However, purified LRRK2 demonstrate a kinase activity in vivo, which increases in some pathogenic mutations[89]. It was reported to interact with Parkin, but also a genetic interaction with PINK1 and DJ-1 has been described. Other mutations in these gene promote dysregulation of mitochondrial function and oxidative damage[90].

3.2.2.2. Autosomal recessive forms of Parkinson's disease

Mutations in *parkin* (PARK2), PINK1 (PARK6) and DJ-1 (PARK7) genes can cause recessive forms of early-onset parkinsonism, usually with a relatively typical parkinsonian phenotype[91]. Among these genes, mutations in *parkin* are the most common and explain up to half of familial PD, compatible with recessive inheritance and early onset, and also ~15% of the sporadic cases with early-onset[92]. There are no specific clinical features that can distinguish patients with *parkin* mutations from the ones with PINK1 or DJ-1 mutations or from other early-onset PD forms.

Mutations in these genes, which include point or small mutations, but also large genomic rearrengements, leading to deletions or multiplications, have been identified worldwide. The probability of mutations in these genes is inversely proportional to the onset age of the symptoms: the earlier the onset, the higher the chance of finding mutations.

More than 40 mutations in the *parkin* gene have been found so far, and it was the first gene to be associate to recessive PD. However, only a weak correlation between clinical manifestation and the type of mutation has been pointed out[92]. Interestingly, LBs have not been detected in *parkin* disease-causing mutations, suggesting pathogenetic differences between the autosomal recessive and the typical forms of PD[93]. A more detailed summary on parkin is given in the following paragraph on the role of mitochondria in PD and on Parkin-dependent PD.

PARK6 has been identified as the gene coding for PINK1 (PTEN induced kinase 1), a mitochondrial targeted protein. This protein is an highly conserved putative serine/threonine kinase ubiquitously expressed, which has been demonstrated to protect neurons from undergoing oxidative stress[94]. Depending on mitochondrial membrane potential, this protein can be localized on the OMM or IMM and regulate mitochondrial dynamics and respiratory functions[95,96]. In *Drosophila*, mitochondrial fission is accelerated upon PINK1 overexpression[13]. However its specific role in regulating mitochondrial dynamic is still under debate, after the observation that mammalian PINK1 knockout cells showed a more fragmented network, suggesting a pro-fusion role for PINK1[96,97]. However, it is clear that this proteins plays a vital role in the balance of fission a fusion processes. Mutations in PINK1 have differential effects on protein stability, localization and kinase activity. Recently, a first patient with pathogenic PINK1 mutations was recently reported with Lewy-body pathology[98]. Moreover, PINK1 associated PD cases has been studied in a smaller number of patients, due to its infrequence but patitents show a broad phenotypic spectrum of symptoms, spanning from an early manifestation with atypical symptoms to late manifestation with the typical clinical PD symptoms.

DJ-1 is a multifactorial protein which participates in cells protection against oxidative stress[99,100]. Mutations in this gene were firstly found in an Italian and a Dutch family and linked to autosomal-recessive parkinsonism. After that, only another case of PD patient carrying DJ-1 mutation has been identified in an Uruguayan family. DJ-1 may regulate different cellular functions depending on its localization. It has been found to be in the cytosol, in the nucleus and on mitochondria, as well as at synaptic terminals. It has many diverse biological functions. The normal function of DJ-1 and its role in dopamine cell degeneration is unknown, but this protein is linked to oxidative stress response and mitochondrial function[101-103]. It has also been reported to have a role in oncogenesis and male fertility[102]. Moreover, it was recently reported that DJ-1 is involved in the protection of neurons from dopamine toxicity[104]. It has also been found around the Lewy bodies[105]. Several evidences suggested that DJ-I function as a

dimer. Analysis of the pathogenic Lys166Pro mutation showed that the dimer is less stable and an ectopic expression of these mutant is rapidly degraded[106].

When mitochondrial membrane depolarizes, PINK1 accumulates on the outer membrane of damaged mitochondria to achieve selective recruitment of Parkin and promote mitophagy. The complete pathway will be deeply discussed later on. However, these findings support the idea that the same molecular pathways underlie different forms of early-onset PD forms. Moreover, mitochondria quality control and function are fundamental events in this scenario[91,107]. The role of DJ-1 mutations in autophagy remain less clear.

Moreover, this mechanism may reflect a more general process in neurodegeneration[108].

3.2.3. Molecular pathogenesis of Parkinson's disease: mitochondrial quality control

In the late 1970s, accidental exposure to 1-methyl-4-phenyl- 1,2,3,6-tetrahydropyridine (MPTP) was found to cause parkinsonism and DA degeneration[65]. This has been the first evidence linking mitochondrial dysfunctions to neurodegeneration. Indeed, MPTP oxidized to MPP+ is taken up by the DA neurons and inhibits complex I[109-111]. Pesticides and herbicides, such as rotenone and paraquat, also inhibit complex I activity and cause parkinsonism in animal models and possibly also in human [67, 114]. All these findings suggested that DA neurons were particularly sensitive to mitochondrial dysfunction. Moreover, pathogenic mtDNA mutations are associated with PD. However, two are the main hypothesis on PD pathogenesis. One hypothesis suggests that the onset of PD is caused by mitochondrial dysfunction and subsequent oxidative stress. The other one suggests misfolding and aggregation of proteins as the primary cause[108]. Both hypotheses are plausible and perhaps interconnected.

The first hypothesis is based on the finding that complex I inhibition could reproduce parkinsonism. The inhibition of complex I can have two major effects: depletion of ATP and ROS accumulation. This hypothesis is supported by the fact that reduction of complex I activity has been described in organs of idiopathic PD patients and that oxidative stress was found in the brain of patients with idiopathic and familial forms of PD. PINK1 and DJ-1 seem to be the proteins linking PD and oxidative stress[112].

On the other hand, accumulation of misfolded proteins has been found in many neurodegenerative diseases. Characteristic of PD is the polymerization and altered conformation of proteins, which results in the presence of intracellular protein aggregation. As already mentioned, the main protein known to form aggregates in PD is α-synuclein, which gives rise to the Lewy bodies[72,93]. The significance of these inclusions remains unclear. Recent evidences suggest that Parkin protects neurons from these pathogenic protein accumulations, targeting them to the ubiquitin-proteasome system (UPS) and promoting their elimination. Thus, dysfunction of the UPS system could be responsible for the accumulation of cytosolic damaged proteins, and also could be important for the formation of Lewy bodies.

Both mitochondria damage and protein aggregation have to be taken into account for PD dependent degeneration, and because these processes are likely to be interconnected.

3.3. Parkin dependent Parkinson's disease

Mutations in *parkin* gene are the most common cause of Autosomal Juvenile Recessive Parkinsonism (AJ-RP)[113]. AJ-RP patients show the same clinical symptoms of patients affected by other forms of PD, except for earlier onset, dystonic features, brisk reflexes and sleep benefit. The normal gene product, the E3 ubiquitin ligase parkin, has a neuroprotective role, and acts together with the Serine/Threonine kinase PINK1 in the mitochondrial quality control pathway.

There is considerable disparity in age of onset, presentation, progression and response to drug treatment in Parkin-associated PD case[114]. Moreover, there is only a weak correlation between clinical manifestation and type of mutation, even if recent findings show that patient with point mutations tend to be less affected than patient with deletion.

Also the pathology of Parkin-related disease is under discussion, since only few Parkin-associated PD cases have been neuropathologically examined. All of these have PARK2 gene and show at selective loss of dopaminergic neurons in the SN and the locus coeruleus. In most cases no Lewy bodies are seen, just recently they have been found in some Parkin-associated cases[115]. These findings indicate that pathology in Parkin-positive cases seem to be variable, probably due to the "cross-talk" between the different pathways involved in dopaminergic neurons degeneration.

3.3.1. Parkin structure and mechanism of activation

Parkin is a member of ring-between-ring (RBR) E3 ubiquitin ligases, and is able to ubiquitinate a wide variety of cytosolic and OMM proteins upon mitochondria depolarization[116]. It is encoded by a 1.3 Mb gene, localized on chromosome 6q25.2-q27 and consists of 12 exons, and gives rise to the protein of 465 amino acids[113]. The Parkin locus (PRKN) is hyper-recombinable and it lies within FRA6E, the third most common fragile site in tumor issue, even if the potential role of Parkin in cancer is yet to be determined.

Probably due to its fundamental role for cell survival, this protein is well conserved during evolution, allowing the characterization of its function and molecular mechanism. It is conserved not only in mammals, but also in invertebrates[117].

Ubiquitination has firstly been found as a post-translational modification which tipically marks proteins for degradation by the proteasome[118]. However, in the last years it emerged as a powerful tool to modulate proteins activity, via regulation of their subcellular localization and ability to interact with other proteins. In the process of ubiquitination, enzymes called ubiquitin ligases covalently attach ubiquitin (Ub) and/or Ub chains to Lysine (Lys) residues or the N-terminal amino group (so-called "linear ubiquitination") of a substrate protein. Ubiquitination is carried through the sequential action of three enzymes: E1 (Ub-activating), E2 (Ub-conjugating) and E3

(Ub-ligase). Ub itself contains different Lys residues, thus allowing the formation of different Ub chains[119]. In particular, Ub can form polyubiquitin chains of eight different linkages that mediate distinct biological functions[120]. The most common chain types are Lys48 and Lys63. In the ubiquitination pathway, usually the E3 enzymes show a higher specificity and tighter regulation in recognition of the substrates and control of its activity, compared to E1 and E2[121]. In this context, a common regulatory mechanism for many E3 ligases is the ability to self-catalyze the ubiquitination of themselves, through the so called "auto-ubiquitination". There are three different E3 ligases families, which differ both on general structure and chemistry: RING-type (including U-box ligases), HECT-type and RING-HECT hybrids.

Post-translational modifications can influence each other and cooperate in regulating signaling pathways[122]. Phosphorylation is a reversible post-transcriptional modification which plays a regulatory role in almost every aspect of cell life, modulating the activity and the subcellular localization of proteins. Phosphorylation on Threonine, Serine and Tyrosine are the most common, however, recently also Histidine, Arginine and Lysine have found to undergo phosphorylation[123]. Phosphorylation and dephoaphorylation are catalyzed by protein kinases and phosphatases, respectively. More than five hundred different kinases have been identified in humans, and are classified into broad groups by their target substrate: protein kinases, lipid kinases, carbohydrate kinases[124]. Rukariotic protein phosphatase are represented by three distinct gene families: the PPP and PPM families, dephosphorylate dephosphorylate phosphoserine and phosphothreonine residues; protein tyrosine phosphatases (PTPs) dephosphorylate phosphotyrosine amino acids[125]. Interestingly, Tyrosin Kinases Inhibitors (TKIs) such as Imatinib and Nilotib, are used as effective therapy for patients affected by leukemia[126,127]. Recently, this drug resulted to be effective also in mouse models of Alzheimer's Disease (AD)[128]. AD is a neurodegenerative disease characterized by the by accumulation of β-amyloid (plaques) and hyper-phosphorylated Tau (tangles). AD animals present high levels of insoluble Parkin and decreased Parkin-Beclin-1 interaction. Administration of TKIs to AD mice increases soluble Parkin leading to amyloid clearance and cognitive improvement. Although no Parkin mutations are found in AD, these studies demonstrate how manipulation of Parkin activity through the modulation of post-transcriptional modifiers can be used as powerful therapeutic approach.

Parkin structure consists of an N-terminal Ubiquitin-like domain (Ubl) and four zinc-finger RING-like domains: RING0, RING1, IBR and RING2[129,130]. A linker region connects the former segments. This region has no similarity with any known protein, so it is also called unique Parkin domain (UPD)[131]. Similar to other RBR enzymes, Parkin possesses a catylitic cystein within its RING2 domain (Cys431) that acts as an intermediate ubiquitin acceptor between E2 and the substrate[132]. It can form Lys63, Lys48, Lys11 and Lys6 Ub chains on its targets[133]. As other E3 ligases, Parkin can be ubiquitinated and ubiquitinates itself, by the attachment of Lys6 chains, which seem to play a role in its degradation[134].

Recently, several groups reported high resolution crystal structures for a Parkin fragment consisting of the RING0, RING1, IBR and RING2 domains and low resolution crystallography of the Ubl domain[129]. This structures allowed to better understand

how this protein, which is usually kept inhibited, gets activated. Moreover, it provides a rationale for many of the PD associated mutations. It seems that certain mutations compromise the structural integrity of the protein, whereas others interfere with the binding of the substrate or affect the cathalytic activity directly. We will deeper discuss about Parkin mutations in the next paragraph.

Parkin structure shows how the RING0, RING1 and the IBR domains interact while the linker domain wraps around RING0-RING1 interface to reach the RING2. Normally, the Cys431is occluded in the RING0, and the E2-binding site on RING1 is blocked by the linker region, thus it results auto-inhibited[130,135,136]. Recent studies found that Parkin activation occurs upon PINK1 phosphorylation at Serine 65, which together with phospho-ubiquitin binding, causes a dramatical conformational change in the protein. In this context, the ubiquitin-E2-binding site on RING1 gets probably shifted proximal to the Cys431 acceptor site on RING2, activating Parkin[55,137,138].

Similar to other post-translational modifications, such as phosphorylation and acetylation, ubiquitination is also a reversible modification, mediated by a large family of deubiquitinating enzymes (DUBs). Interestingly, a large set of DUBs has opposite role of the E1/E2/E3 activity. Recent works, identified DUBs interacting with and regulating proteins associated with familial forms of PD, such as a-synuclein[139] and Parkin[134,140-142]. In a recent review, we focused on how this proteins impact on Parkin function and actvity, and how they could be a target for PD treatments[143]. In particular, ataxin-3, USP8, USP15 and USP30 have been found to have a role in modulating Parkin auto-ubiquitination and Parkin-mediated mitophagy. By modulating the activity of these enzymes, it might be possible to modulate Parkin dependent mitophagy, and interfere with the pathogenic pathway.

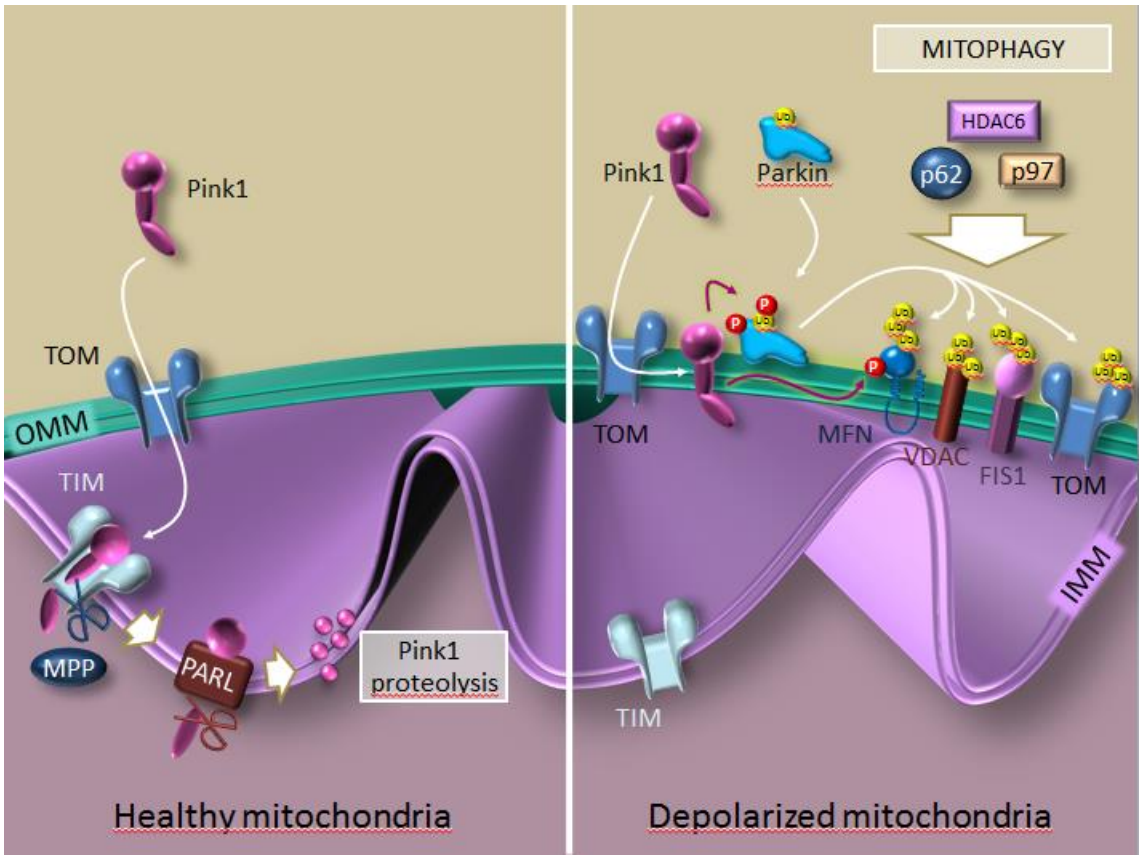

Figure 7. The Pink/Parkin pathway in mitophagy. In healthy miochondria, Pink1 is targeted to the outer mitochondrial membrane (OMM) owing to its mitochondrial target sequencing (MTS). It is then imported to the inner mitochondrial membrane (IMM) through the TOM/TIM complex and cleaved by the TIM-associated mitochondrial processing peptidase (MPP). This MPP-cleaved form of PINK1 is thereafter cleaved within its membrane-spanning domain by the presenilin associated rhomboid-like protease (PARL), and then rapidly undergo a proteolysis in a proteasome-dependent manner, allowing very low level of endogenous PINK1 levels to prevent mitophagy of healthy mitochondria. However in depolarized mitochondria (after damages, or CCCP uncoupler treatment), TIM-mediated import of mitochondria is reduced, and Pink1 accumulates in the OMM. The OMM-accumulation of Pink1 will lead to the selective recruitment of Parkin, its phosphorylation, and the phosphorylation of its ubiquitin at ser65. PINK1-dependent phosphorylation of both Parkin and ubiquitin is sufficient for full activation of Parkin E3 activity, and numerous OMM-located proteins will be target for Parkin-mediated ubiquitination (among them Mfn, TOM, VDAC or Fis1). These ubiquitinated proteins serve to recruit essential adapter proteins such as p62, HDAC6 or p97, which will tether the phagophore membrane and induce the mitophagic process[143].

3.3.2. Parkin mutations

The first mutations identified in the *parkin* gene was found in 1998, carried by a Japanese family with AJ-RP[113,144]. In these patients were detected large homozygous deletions of the gene. After that, a lot of other *parkin* mutations were detected, both in cases of parkinsonism and PD: in cases of autosomal recessive early onset (<45 years) parkinsonism, in isolates young onset cases presenting as fairly typical Parkinson's disease and in familial cases with an age at onset of symptoms as late as 64 years[145]. Nowadays, over 170 disease-linked mutations are known, spread trhoughout the *parkin* domains, and include multiplications, small deletions/insertions and a variety of point mutations in different ethnic groups. However the pathogenic relevance of these mutations is still controversial for several of these mutations. All these mutations have been listed in the Parkinson's disease Mutation Database[146].

Mutations have different effects on the function and structure of the protein. Recent bioinformatic advances allowed the modelling and the study of Parkin structure and function in the presence of several pathogenic mutations[145]. Mutations on the Ubl domain can affect Parkin activation and enzymatic function. Mutations in RING0 were disrupting the folding and stability of the protein structure and exhibited a reduction in free poly-Ub chain formation. RING1 harbours the E2 binding site, which usually is blocked by the linker region. Mutations in this domain affect Parkin ability to interact with E2. Other mutations affect Parkin ability to bind Ub or to translocate to damaged mitochondria.

3.3.3. Mitochondria and Parkin interacting proteins

A lot of proteins have been found to interact with Parkin, and include several E2-ubiquitin conjugating enzymes and many different Parkin substrates. Not all of Parkin interactors are involved in Parkin-dependent mitophagy, but they are linked to other Parkin functions within the cell. The first proteins found to interact with Parkin were UbcH7 and UbcH8[147]. In addition, also Ubc6 and Ubc7 on the ER were identified to interact with Parkin[148]. These protein link Parkin with the Ub-dependent proteasome machinery, allowing its ubiquitination and degradation. Accordingly, neuropathologic examination of Parkin-associated PD patients brains show accumulation of these substrates that cannot be ubiquitinated and subsequently degraded.

Starting from 2006, many studies focused on PINK1/Parkin interaction. Indeed, three independent groups showed that these two proteins function in the same molecular pathway, with PINK1 acting upstream of Parkin[149-151]. A couple of years after, an elegant work by Poole et al. showed a strong genetic interaction between PINK1/Parkin pathway and mitochondrial fission/fusion machinery[152]. Using a fruit fly genetic interaction screening, a strong interaction has been found between fission protein Drp1 and PINK1/Pakin. Indeed, Drp1 loss-of-function mutations were lethal in absence of functional PINK1 or Parkin. Also, PINK1 and Parkin mutant phenotypes were rescued by increased Drp1 gene dosage or decreased Mfn gene dosage. These results were the first evidence of a genetic interaction between PINK1/Parkin pathway and of mitochondrial dynamics, and suggested that this pathway might promote mitochondrial fission (or inhibit mitochondrial fusion). After this study, Parkin has been demonstrated to directly interact with Drosophila Mfn, mediating its ubiquitination on the outer mitochondrial membrane[54]. Loss of *Drosophila* Parkin or PINK1 leads to an increased abundance of Mfn in vivo and hyperfused mitochondria. Increased protein level of Mfn *in vivo* and hyperfused mitochondria. Moreover, ubiquitination of Mfn operates as a molecular tag for targeting dysfunctional mitochondria for degradation[54]. On the other hand, Parkin was found to regulate the protein levels of Opa1 and Drp1, supporting the physiological relevance of Parkin in mitochondrial morphology regulation at different levels[153,154].

Recently, PINK1/Parkin pathway function and regulation became clearer, even if the scenario is not yet complete. The mitochondrial uncoupler carbonyl cyanide m-chlorophenyl hydrazine (CCCP) causes mitochondrial depolarization, and has been

found to induce Parkin translocation[155]. Thus, this chemical has been broadly used to study Parkin translocation and Parkin-dependent mitophagy in living cells. Upon CCCP treatment, PINK1 is stabilized on impaired mitochondria where, through an unknown mechanism, recruits Parkin, which, in turn, promotes their elimination via mitophagy[52,156,157]. Although the signal triggering Parkin translocation is still not known, it has been recently demonstrated that PINK1 once stabilized on the OMM phosphorylates Parkin at Ser65, stimulating Parkin E3 ubiquitin ligase activity[57]. At the same time, PINK1 also phosphorylates Ub at Ser65, which binds Parkin and fully activates its Ub ligase function[137,158]. Mfn2 is another fundamental protein which gets phosphorylated by PINK1, and function as a mitochondrial receptor for Parkin on the OMM[37]. Once recruited to impaired mitochondria and activated by PINK1, Parkin selectively ubiquitinates pro-fusion protein Mfns[54,159]. Currently, the functional meaning of Parkin-dependent Mfns ubiquitination is unclear. Mfns ubiquitination might act as a molecular tag, which targets impaired mitochondria for degradation, thus providing an elegant biochemical mechanism for Parkin mediated mitophagy. Another possibility is that Parkin regulates Mfns steady state levels by targeting Mfns for proteasome dependent degradation, decreasing Mfn levels and mitochondrial fusion, and resulting ultimately in the accumulation of fragmented non-functional mitochondria, which eventually get eliminated by mitophagy.

3.4. Animal models of Parkinson's disease

While *in vitro* studies are widely used for understanding molecular pathways in biologic systems, animal models allow the investigation of disease pathophysiology *in vivo*. *In vivo* models provide useful information and the possibility to test new therapeutic approaches. In the case of PD, initially, only toxin-based models were used, however recently also transgenic models are available[160].

Toxin-based models of PD aim to reproduce the phenotypical changes of human diseases in rodents or primates using pharmacological agents called neurotoxins, which induce selective loss of dopaminergic neurons. These toxins can be sistematically or locally administrated. 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and pesticites are the most common sistematically administrated neurotoxins. 6-hydroxydopamine (6-OHDA) and lypopolisacharide are locally administrated to cause PD similar phenotypes.

MPTP was the first drug identified to be linked to the loss of dopaminergic neurons and to parkinsonism features[65]. When MPTP gets metabolized, it gets converted into its active form 1-methyl-4-phenylpyridinium (MPP+), which is then carried by DA transporters into dopaminergic neurons of the SNpc, where it blocks mitochondrial complex I activity. Primates and mice were broadly treated with these toxins to study their effects and correlation with PD, since they show a reaction to the drug, which is similar to human.

MPTP shares a high structural similarity with some pesticides like paraquat and rotenone[66]. Moreover epidemiological analysis showed that chronical exposure to this pesticides resulted in higher probability of developing PD. Rotenone is able to directly inhibit complex I activity, causing massive formation of ROS species. In rats, rotenone

was observed to cause selective degeneration of dopaminergic neurons as well as the formation of cytoplasmic inclusions similar to Lewy bodies. However, the effects of rotenone in animal models are variable, and the high toxicity of this drug often causes death of the animal. Paraquat is also a widely-used pesticide able toinduce PD phenotypes both in humans and animals. Once entered the cell through a transporter, paraquat acts as a redox cycling compound and generates massive oxidative stress.

The first animal model ever generated which was reproducing PD hallmarks was based on local (i.e. intracerebral) administraton of 6-OHDA[161]. This compound is a hydroxylated analogue of DA, with an higher affinity for DA transporters, which transport the toxin inside dopaminergic neurons. Once in the neurons, 6-OHDA accumulates in the cytosol and generates highly reactive products, suggested to be peroxides, superoxides, hydroxyindoles, and quinones. Additionally, it can accumulate on mitochondria, where it inhibits complex I. The effects of this drug are highly reproducible, which represent an important aspect for the developing of new therapeutic approaches.

Lipopolisaccharide (LPS) injection was the newest toxin-based model of PD, since inflammation has been found to be a key player in PD pathogenesis. LPS is a bacterial endotoxin, which causes intense tissue inflammation and usually is directly injected into the brain[162]. LPS *per se* is not toxic in neurons, its neurotoxicity is mediated by microglia activation and the release of cytotoxic molecules.

These toxin-based models have been powerful tools for the understanding of PD pathogenesis, however the lack of age-dependent, slowly progressive lesions and the fact that Lewy bodies were not observed forced the studies to move to genetic models. The most used PD animals models have been *Mus musculus* and *Drosophila melanogaster*.

3.4.1. Mouse genetic models of Parkinson's disease

Several genetic models of PD have been generated in mice. Mutations in the SNCA gene were the first associated to PD pathogenesis. To date, various transgenic α-synuclein mice have been generated, no significant nigrostriatal degeneration has been observed. Expression of wild-type, mutated or truncated α-synuclein in catecholaminergic neurons of mice didn't show any neuronal death[163]. However expressing the protein in other region of the brain, led to time-dependent striatal DA content decrease, reduced tyrosine hydroxilase expression as well as some PD typical beahavioural hallmarks in those mice[164]. Interestingly, some of these models also show non-motor phenotypes associated with early PD stages, such as gastrointestinal alterations and olfactory deficits.

Overexpression of LRRK2 in mice induces increased DA release in the striatum and motor hyperactivity, whereas the overexpression of a PD-linked mutant form causes age-dependent reduction of the striatal content, release and uptake of DA, suggesting a role for this protein in DA transmission[165]. However, overexpression of wild-type LRRK2 or of its mutated form does not induce neuronal cell death in mice. LRRK2 KO mice are viable and do not show any increased susceptibility to MPTP.

Several Parkin or PINK1 embryonic KO mice have been generated. In both cases, no changes in DA neurons number or DA levels have been observed. Nevertheless, in PINK1 KO mice a mild mitochondrial and nigrostriatal deficit can be present, and they show increased susceptibility to oxidative stress and ROS production[96,166]. Parkin mice show higher susceptibility both to neurotoxins and inflammatory stimuli[167,168]. Similarly, DJ-1 KO mice do not show any dopaminergic neuronal loss, although they show increased susceptibility to toxins and oxidative stress[169].

The lack of evindent phenotypes and potential compensatory mechanisms preventing neurodegeneration in conventional KO models, , promted scientists to generate conditional KO mouse models. By using tissue-specific Cre-expressing lines, exons or genes flanked by loxP sites in conditional mice can be deleted in desired tissues. By conditionally knocking out Parkin in adult mice, this approach allowed the generation of the first genetic model of PD showing progressive neurodegeneration[170]. Parkin flox/flox mice show progressive nigral neuron death 10 months after lentiviral-Cre nigral injection. The pathways revealed in this adult conditional parkin KO model may be quite relevant to PD pathogenesis and may represent a promising model to test new therapies.

3.4.2. Drosophila as a model for human diseases

In the past years, *Drosophila melanogaster* has emerged as one of the most effective model to study PD-related neurodegeneration *in vivo*[171]. Not only diseases-related genes are conserved between fruit fly and human[172], but in flies it is also possible to detect clusters of dopaminergic neurons and the metabolic pathways for DA syntesis are conserved. Compared to higher model organisms, *Drosophila* presents some attractive features, which make it suitable for studying complex biological pathways. First, flies can be maintained easily in large numbers in stock and populations without specialized instrumentation. *Drosophila* has a short life cycle, thus a large number of progeny can be easily produced in a short period. The fruit fly is widely used for genetic screens, due to its reduced 4-pairs chromosomes genome[173]. Moreover, genetic mutants can be easily generated through some well-known techniques, such as the P-element transposons for loss-of-functions studies[174,175], the UAS-GAL4 system for tissue-specific downregulation or overexpression of proteins[176] or site-specific gene integration via specific donor plasmids[177]. Furthermore, using X-ray or other mutagenic agents, gives the possibility to rapidly generate large number of mutant stocks[173]. The already established disease models are also used for the screen of chemical compounds, which ameliorate the phenotype, in order to point out putative drugs to use as treatment in humans[178].

3.4.2.1. Drosophila as a model for Parkinson's disease

In 2000, the neuronal overexpression of wild-type or mutant human α-synuclein, which has no homologue in flies, has been induced using GAL4/upstream activation system, thus generating the first PD-like *Drosophila* model[179]. Transgenic flies showed age-dependent selective degeneration of dopaminergic neurons, as well as α-synuclein-positive inclusions. Moreover they showed a progressive loss of

climbing ability, and they were positively responding to L-DOPA or DA-antagonists treatments. Exept for α-synuclein, all the other PD-related genes have at least one homologue in flies. Loss of function mutations in PINK1, parkin, DJ-1 or LRRK2 fly homologues induce selective dopaminergic neuronal loss and motor deficits.

Unlike humans, Drosophila has two DJ-1 homologues: DJ-1α, which is expressed only in male germline, and DJ-1β, which is ubiquitously expressed. DJ-1β KO flies show an increased susceptibility to cytotoxins, supporting the idea of the redox function of DJ-1. Mutations in these protein can cause accumulation of ROS in the brain. The results on LRRK2 KO and mutant flies are inconsistent, they do not show dopaminergic neurodegeneration.

The first *in vivo* evidences showing that PINK1 and Parkin work in the same pathway, with PINK1 upstream of Parkin, came from studies in flies[151,180]. Parkin loss-of-function mutants showed reduced life span, male sterility and severe defects in flight and climbing ability. They also show mitochondrial defects and indirect flight muscle degeneration, as well as reduced 26S proteasomal activity[181,182]. Drosophila mutant flies share phenotypic hallmarks with mammalian Parkin mutants, including dopaminergic neurodegeneration and mitochondrial defects[151,180]. Both mutants show disrupted cristae, resulting in reduced ATP levels and mtDNA, which then leads to flight muscle degeneration. It has been shown that transgenic overexpression of Parkin can compensate for PINK1 loss, whereas PINK1 overexpression cannot compensate for Parkin loss. Furthermore, double mutants show the same phenotypes as the single. All these data provided the first *in vivo* evidence that PINK1 and Parkin act in the same pathway, with Parkin downstream of PINK1[13,152,155].

Genetic interaction experiments revealed putative additional components of the PINK1/Parkin pathway, like Rhomboid-7 and HtrA2/Omi[183-185]. Rhomboid-7, which is a mitochondrial protease homologue of PARL, could act as an . upstream component of the PINK1/Parkin pathway. Cleaving the mitochondrial target sequence of PINK1, Rhomboid-7 seems to allow PINK1 activity in the cytosol. Differently, the mitochondrial protease HtrA2/Omi acts downstream of PINK1, independently of Parkin. 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.

Interestingly, overexpression of human PINK1 or Parkin in mutant flies, abolishes the typical PD-like phenotypes, underlining the functional conservation of this pathway among species. This is supported by the facts that also patients' fibroblasts display mitochondrial alteration and the neurons show Parkin translocation impairment[186,187].

Studies in *Drosophila* also provided the first evidence that PINK1 and Parkin promote mitophagy *in vivo*. This has long been debated, since all studies on the PINK1/Parkin mitophagic pathway have always been based on toxin-treated cell models and on PINK1 or Parkin overexpression. Through a proteomic in vivo approach, it has been proved that the turnover of mitochondrial proteins in wild-type flies was higher than in Parkin or PINK1 mutants, giving the final proof that this pathway induces

mitophagy *in vivo*[188]. Along with that, PINK1 was also found to phosphorylate Complex I on NDUFA10/ND42 subunit *in vivo,* thus regulating Complex I activity[189].

Interestingly, overexpression of Drp1 or downregulation of Marf, the fly homologue of mammalian Mfns, can rescue defects in mitochondrial morphology, cell death, muscle degeneration and locomotor deficits in PINK1 and Parkin loss of function *Drosophila* models[13,152]. Furthermore, increased Drp1 gene dosage can rescue the alterations in assembly of electron transport chain complexes and heterozygosity of Drp1 is lethal in a PINK1 or Parkin mutant background[190]. All these data support the hypothesis that manipulation of mitochondrial dynamics could provide a novel therapeutic strategy.

4. Results

Enhancement of Ca^{2+}-dependent phosphatase Calcineurin corrects a PINK1 model of Parkinson's disease.

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Abstract

PARK2 gene encodes for an E3 ubiquitin ligase called Parkin. Loss-of-function mutations in this gene cause early onset of Parkinson's disease, a neurodegenerative disorder of unknown etiology. The role of Parkin in neuron maintenance is still unknown, however it has been linked to the regulation of mitochondria dynamic. Recent works show that Parkin is selectively recruited to dysfunctional mitochondria, where it mediates their elimination via autophagy. Parkin translocation and Parkin-mediated autophagy depend on the Serine/Threonine-protein kinase PINK1 (PTEN-induced putative kinase 1), which is selectively stabilized on dysfunctional mitochondria where it recruits Parkin. However, it remains unknown which cellular signals or covalent modifications directly regulate Parkin translocation.

Confocal microscopy of EYFP-Parkin transfected MEFs shows that Parkin has a cytoplasmic localization. After treatment with the mitochondrial uncoupling agent Carbonyl cyanide m-chlorophenyl hydrazine (CCCP), Parkin appears as spots that co-localize with mitochondria. We found that pretreatment with the Ca^{2+} selective chelator BAPTA completely blocked Parkin translocation. We also found that inhibition of Ca^{2+} dependent phosphatase Calcineurin A (CnA) impaired Parkin recruitment and that CaN constitutive active expression was able to induce Parkin translocation *per se*, independently from PINK1. In accordance to the *in vitro* data, we showed that in the *Drosophila* fruit fly, Calcineurin constitutive active expression is able to rescue the climbing ability of a PINK1 *in vivo* model of Parkinson's Disease.

Introduction

Mutations in the PARK2 gene, encoding for the E3-ubiquitin ligase Parkin, is the most common cause of Autosomal-Juvenile Recessive-Parkinsonism[1]. The normal gene product has a neuroprotective role and it is involved in the degradation of dysfunctional mitochondria, through a process called mitophagy. Its action is closely related to the activity of another PD-related gene, PARK6, which encodes for a protein called PINK1[2-4].

PINK1 is a Serine/Threonine kinase that is imported into mitochondria, where it gets cleaved by the inner membrane protease PARL and then eliminated by the proteasome[5-7]. On depolarized mitochondria, PINK1 accumulates on the outer mitochondrial membrane, where it recruits Parkin and activates its activity[8-11]. These events lead to proteasomal degradation of OMM proteins and eventually to selective autophagy of damaged mitochondria, a process called mitophagy[12-15].

In flies, PINK1 and Parkin mutants (knock out) develop reduced life span, male sterility, flight muscles degeneration, dopaminergic neurons loss, locomotor deficits and mitochondrial defects[16,19]. Combined mutation of both PINK1 and Parkin genes do not result in the worsening of the phenotype. Moreover, transgenic overexpression of Parkin can compensate for PINK1 loss, whereas PINK1 overexpression cannot compensate for Parkin loss. These data provided *in vivo* evidence that PINK1 and Parkin act in the same molecular pathway, with Parkin downstream of PINK1[16-18]. In 2008, Poole et al. showed a strong genetic interaction between PINK1/Parkin pathway and mitochondrial fission/fusion machinery[20]. By using a fruit fly-based genetic interaction approach, they found that loss-of-function mutations of Drp1, a pro mitochondrial fission protein, are lethal in a PINK1 or Parkin mutant (knock out) background. Conversely, PINK1 and Parkin mutant phenotype is suppressed by increased Drp1 gene dosage or by decreasing gene dosage of pro-fusion proteins OPA1 and Mfn[20]. The biochemical explanation for the genetic interaction evidences illustrated by Poole et al. became clear when a direct biochemical interaction between Parkin ubiquitin activity and Mfn was discovered. In particular, Parkin was found to physically interact with and ubiquitinate the pro-mitochondrial fusion protein Mfn, providing the biochemical mechanism by which damaged mitochondria are labeled and sequestered by mitophagy[21].

Indeed, mitochondrial dysfunctions have been largely implicated in the onset of several human diseases. In particular, accumulation of dysfunctional mitochondria has been linked to neurodegenerative disorders, such as Parkinson's disease, which is the second most common neurodegenerative disorder after Alzheimer's disease and for which there is no cure.

It has been widely demonstrated that Parkin translocation is PINK1 dependent, but the molecular mechanism behind PINK1 dependent activation of Parkin is still unclear. Recent works show that PINK1-mediated phosphorylation of Parkin and Ubiquitin at residue Serine 65 (Ser65) is necessary but not sufficient for Parkin translocation to defective mitochondria, and it is indeed fundamental for its E3-ubiquitin ligase activity[23-25]. Moreover, PINK1 phosphorylates the pro-fusion protein

Mfn 2, which in turn gets stabilized on depolarized mitochondria and works as Parkin receptor[26].

All together these evidences suggest a tightly regulated control of the mitophagic process that operates at the post-transcriptional level via reversible modifications of proteins in the form of phosphorylation[25,27-29], ubiquitination[30-33], de-ubiquitination[34-39], sumoylation[40,41] and nitrosylation[42,43] and possibly other not yet characterized post-transcriptional events.

Free Ca^{2+} concentration is important in the regulation of metabolic processes and for signal transduction. Accordingly, the cytoplasmic pool of Ca^{2+} is very limited and is tightly regulated by mitochondria and ER[44-46]. Ca^{2+} controls apoptosis and the opening of the permeability transition pore (PTP)[47,48], a process linked to mitochondrial dysfunctions that increases the permeability of the mitochondrial inner membrane eventually leading to the release of pro-apoptotic factors from mitochondria[47]. Furthermore, Ca^{2+} dependent phosphorylation and dephosphorylation of targeted proteins affect their activity by impinging on subcellular localization, conformation and protein-protein interaction. For example, phosphorylation and de-phosphorylation of mitochondria pro fission protein Drp1 regulates Drp1 translocation to mitochondria, an indispensable event for Drp1 dependent fission activity. In particular, translocation of Drp1 to mitochondria upon CCCP-induced depolarization is mediated by selective dephosphorylation of residue Serine 637, which is controlled by Ca^{2+} dependent phosphatase Calcineurin (CaN)[49]. On the other hands, protein kinase A (PKA) -dependent phosphorylation of Drp1 is retaining Drp1 in the cytoplasm during starvation, leading to elongated mitochondria, which cannot be eliminated by autophagy. Therefore, Ca^{2+} dependent regulation of phosphorylation and de-phosphorylation of Drp1 operates as a reversible post-transcriptional modification that impinges on Drp1 translocation, in response to metabolic changes.

Here, we show that Parkin translocation depends on Ca^{2+} and on the Ca^{2+}/Calmodulin dependent phosphatase Calcineurin. Calcineurin activity is required for Parkin translocation, and it regulates Parkin-dependent mitophagy regardless of PINK1 presence. By using an *in vitro* and *in vivo* approach, we demonstrated that Calcineurin is indispensable and sufficient for Parkin translocation. Accordingly, we showed that Calcineurin constitutive active expression in the D. Melanogaster fruit fly rescues PINK1 deficiency *in vivo*.

Results

Parkin translocation and dependent mitophagy requires Calcineurin. Considering the fundamental role of Ca^{2+} in the regulation of proteins activity, we evaluated whether Parkin translocation and CCCP-dependent mitophagy depended on Ca^{2+} and was regulated by similar post-transcriptional modification that control Drp1 translocation.

We transfected cells with fluorescent mCherry-Parkin and with mitochondrial targeted YFP (mito-YFP) constructs and looked at Parkin subcellular localization. Consistent with previous studies[14,50], we found that overexpressed Parkin mostly

located in the cytosol of wild-type MEFs (Fig. 1A). Upon CCCP treatment, Parkin translocates to fragmented mitochondria in the 80% of the cells (Fig. 1B-C). Pre-treating cells with BAPTA, a Ca^{2+} chelator, abolished Parkin translocation, suggesting that its recruitment depended on Ca^{2+} (Suppl. Fig. 1A). Moreover, chemical inhibition of CaN with FK506 blocked Parkin translocation (Suppl. Fig. 1B).

Taking advantage of the already existing CaN dominant negative (ΔCnA^{H151Q}) and constitutive active (ΔCnA)[51,52] mutants, we turned to a genetic approach to evaluate the effect of CaN inhibition upon Parkin recruitment. CaN exists as an heterodimer, composed of a catalytic subunit (CnA) that binds calmodulin and a regulatory subunit (CnB) that binds Ca^{2+}. Ca2+/calmodulin activate CaN upon binding to the calmodulin-binding domain of CnA and inducing the dissociation of the autoinhibitory domain from the catalytic domain. ΔCnA lacks the calmodulin binding domain in the catalytic subunit, due to the introduction of a stop codon at position 392. Previous reports showed that ΔCnA increased fragmentation per se, by dephosphorylating Drp1 and inducing its translocation to mitochondria[49]. On the other hand, ΔCnA^{H151Q} misses the calmodulin binding domain and the autoinhibitory domain and harbors an inactivating His-151 to Gln point mutation. It has been previously demonstrated that the inhibition of CaN efficiently blocked CCCP-induced fragmentation[49]. We cotransfected MEFs with mCherry-Parkin and ΔCnB plus ΔCnA^{H151Q} and looked at Parkin localization. Interestingly, CCCP-induced Parkin translocation was impaired when in presence of the dominant negative mutant of CaN (Fig. 1D-E).

To assess whether CaN played also a role in Parkin-dependent mitophagy[9,53], we next performed a mitophagy assay in presence of the dominant negative mutant of CaN. ΔCnA^{H151Q} expression significantly delayed CCCP-induced mitophagy (Fig. 1F).

Taken together these data showed that pharmacological and genetic inhibition of CaN is sufficient to prevent Parkin translocation and CCCP-induced mitophagy.

Parkin-dependent mitophagy is regulated by Calcineurin and PINK1-dependent phosphorylation. Different studies linked Parkin translocation and mitophagy to PINK1 activity[11,21,54]. Ideed, PINK1 directly phosphorylates Parkin and Ubiquitin (Ub) at Serine 65, which is required for Parkin activity[23,28,29,55]. We generated phopsho-mimetic Parkin and phopsho-mimetic Ub at Serine 65 (Parkin S65E and Ub S65E, respectively) and investigated the effect of CaN constitutive active ΔCnA, and dominat negative ΔCnA^{H151Q} expression upon Parkin translocation. In wildtype MEFs transfected with Ub S65E, Parkin S65E localized to the cytosol and translocated to impaired mitochondria upon CCCP treatment (Suppl. Fig. 2A). In this condition, we did not observe a constitutive localization of Parkin S65E on mitochondria in untreated cells, indicating that PINK1-dependent phosphorylation of Parkin and Ubiquitin might not be sufficient for Parkin recruitment.

As expected, in PINK1 -/- MEFs transfected with Ub S65E, Parkin S65E also localized to the cytosol and translocated to impaired mitochondria upon CCCP treatment (Fig. 2C-D). In presence of CaN dominant negative ΔCnA^{H151Q}, Parkin S65E did not translocate to mitochondria upon CCCP intoxication (Fig 2C-D). Interestingly, in cells transfected with Ub S65E and CaN constitutive active ΔCnA, Parkin S65E

translocated to mitochondria 24hrs after transfection without CCCP intoxication (Fig. 2A-B). Also, 48 hours after transfection, mitochondria were clumped and localized around the perinuclear area, a structural feature preceding mitophagy (Fig. 2A).

Taken together, these observations supported the hypothesis that CaN, in addition to PINK1, is required for Parkin translocation.

Parkin translocation is induced by Calcineurin, in a PINK1-independent manner.

In order to further investigate the role of CaN in the regulation of Parkin translocation in the context of PINK1 deficiency, we looked at Parkin translocation in PINK1 -/- cells. Interestingly, in the presence of the constitutive active CaN mutant ΔCnA, Parkin constitutively co-localized with mitochondria in almost 80% of the analyzed cells (Fig. 3A-B). Moreover, CCCP-induced mitophagy was significantly enhanced (Fig. 3C).

As already reported, Parkin does not translocate to mitochondria upon CCCP treatment in the absence of PINK1 (Fig. 3D-E). However, expression of constitutive active CaN was sufficient to promote Parkin translocation in PINK1 -/- cells, even in the absence of CCCP intoxication (Fig. 3D-E). Accordingly, mitophagy was enhanced in PINK1 -/- cells expressing CaN constitutive active (Fig. 3F).

To conclude, our data suggest that CaN activation can bypass PINK1 requirement in the induction of Parkin translocation and CCCP-induced mitophagy.

Constitutive active Calcineurin in *Drosophila* rescues PINK1B9 climbing ability.

PINK1 and Parkin flies mutants (knock out) recapitulate several features of PD, including characteristic locomotor defects in flight and climbing ability, as well as degeneration of thorax muscle and of dopaminergic neurons. Combined mutation of both PINK1 and Parkin genes induce the same overall phenotype. Also, PINK1 mutant phenotype can be rescued by overexpression of Parkin, whereas Parkin mutant phenotype cannot be rescued by PINK1 overexpression. Together these evidences have been interpreted as PINK1 and Parkin interacting functionally in a linear pathway with PINK1 operating upstream Parkin[16,17,56]. With that in mind and to assess the role of CaN in this pathway, we evaluated the effect of CaN costitutive activation and inhibition *in vivo* in PINK1 mutant background. We therefore turned to a well-established locomotor assay previously used to study PINK1-Parkin genetic interactions *in vivo.* In such assay, 10 flies for each strain were collected in a vertical-positioned plastic tube positioned with a line drawn at 6 cm from the bottom of the tube and under a light source. After tapping the flies to the bottom of the tube, the flies that successfully climbed above the mark after 10 seconds were counted (Fig. 4A).

As already reported, $PINK1^{B9}$ mutant (knock out) flies performed poorly in the climbing assay compared to wild type (Fig. 4B). Parkin overexpression (Pk OE) partially rescued $PINK1^{B9}$ mutants climbing defects (Fig. 4B), as expected. Interestingly, overexpression of CaN constitutive active (CanA-14F) almost completely rescued $PINK1^{B9}$ mutant climbing defects (Fig. 4B) and inhibition of CaN with specific inhibitor FK506 abolished the protective effect of CaN (Fig. 4C). Finally, FK506 administration partially blocked the effect of Parkin overexpression in PINK1 mutant phenotype (Fig.

4D), further indicating that CaN is an indispensable requirement for Parkin activation downstream PINK1.

Thus, our results suggest that CaN plays a fundamental, indispensable role in the control of Parkin translocation and Parkin-dependent mitophagy *in vitro* and that its activation has a physiological impact in an *in vivo* PINK1 model of PD.

Conclusion and discussions

Mitophagy, a selective kind of autophagy during which defective mitochondria are recognized and degraded, depends on PINK1/Parkin, two genes which mutations have been linked to the onset of an autosomal recessive juvenile form of Parkinson's Disease. PINK1 is stabilized on depolarized mitochondria where it recruits Parkin. Once on mitochondria, Parkin ubiquitinates a subset of outer mitochondrial membrane (OMM) resident proteins, thus selecting unfunctional, depolarized mitochondria for autophagy dependent degradation. Up to now, it is still unclear how Parkin translocation to depolarized mitochondria is regulated.

Here we show a fundamental role for the Ca^{2+}-Calmodulin dependent Calcineurin in the regulation of this pathway.

As shown in previous studies[14,21], Parkin is mostly cytosolic, but it translocates to a subset of depolarized mitochondria upon CCCP intoxication (Fig 1A-B). CCCP has additional effects beside mitochondrial depolarization, which includes increasing in cytoplasmic Ca^{2+}. Ca^{2+} regulates many processes spanning from cells origin at fertilization to cellular differentiation and reprogramming, to cell demise. The pleiotropic role of Ca^{2+} led to the present concept as an essential messenger in cell life. The many ways by which Ca^{2+} impinges on cell physiology include the activation of proteins that are required for post-transcriptional modifications of molecular targets, which further amplify the signaling cascade. For instance, Ca^{2+}-activated phosphatase CaN is required for Drp1 mitochondria recruitment and subsequent mitochondria fission.

With that in mind, we embarked on a project that aimed at investigating the potential role of Ca^{2+} and Ca^{2+}-dependent post-transcriptional modifications in the regulation of Parkin translocation and Parkin-dependent mitophagy. The investigation of such topic is extremely relevant at present, as the identification of novel regulators of Parkin translocation and activity might be instrumental in characterizing novel therapeutic targets that enhance mitophagy downstream PINK1/Parkin. Indeed accumulation of unprocessed, undegraded proteins and organelles is a common hallmark in different neurodegenerative diseases, including PD.

In this study, we found that both chemical and genetic inhibition of CaN results in impaired Parkin recruitment, indicating that CaN is an indispensable requirement for Parkin translocation (Suppl. Fig. 1A-B and Fig. 1D-E). Furthermore, the phospho-mimetic mutant Parkin S65E does not translocate to mitochondria in untreated cells (Suppl. Fig. 2A) and CCCP-induced translocation is blocked in presence of dominant negative CaN (Suppl. Fig. 2B).

We also found that constitutive active CaN is able to induce Parkin translocation *per se*, independently of CCCP intoxication (Fig. 3A-B). Accordingly, CaN

inhibition results in impairment CCCP-induced mitophagy (Fig. 1F), while its activation enhances mitochondrial degradation (Fig. 3C). Different studies underlined the importance of PINK1 for Parkin translocation and activation[9,11,21,24]. PINK1 phosphorylates Parkin and ubiquitin in order to regulate Parkin E3-ubiquitin ligase activity[28,55,57]. Indeed, in line with published data, we found that Parkin does not translocate in PNK1 -/- MEFs upon CCCP treatment (Fig. 3D-E). However, the presence of CaN constitutive active is sufficient to induce Parkin translocation in the absence of PINK1 (Fig. 3D-E).

These findings suggest that CaN activity is necessary and sufficient to induce Parkin translocation to mitochondria and that CaN activation can bypass PINK1 requirement in the induction of Parkin translocation and CCCP-induced mitophagy.

According to the current model, PINK1 gets sequentially imported into the IMM of healthy mitochondria, where it is cleaved by proteases and eventually degraded in a proteasome-dependent manner[58,59]. In this scenario, Parkin stays cytosolic and it is not active. However, upon mitochondrial depolarization, PINK1 is stabilized on the OMM where it recruits and phosphorylates Parkin[21,60]. Parkin phosphorylation leads to its activation and subsequent mitophagy[23,25]. However, the mechanism by which Parkin was recruited to mitochondria was still not clear. Our data suggest that mitochondrial depolarization, followed by mitochondrial Ca^{2+} release, activates Calcineurin, which dephosphorylates Parkin, triggering its translocation to mitochondria (Fig. 5).

Comforted by the findings that CaN plays a role in regulating stress induced mitophagy *in vitro*, we next turned to a well-established *in vivo* model system to evaluate the physiological significance of CaN inhibition and/or activation in a PINK1 deficient model of PD (Fig. 4A). Parkin overexpression in a PINK1 mutant (knock out) background is able to rescue PINK1 mutant phenotype, as previously reported ([16,17] and Fig. 4B). We reasoned that enhancement of CaN activity *in vivo* would also ameliorate PINK1 mutant phenotype by enhancing Parkin translocation and activity. Indeed, CaN constitutive active expression in PINK1 mutant background rescues PINK1 mutant flies climbing deficiency (Fig. 4B). This effect was specific for CaN, as chemical inhibition of CaN with FK506, abolished the rescue (Fig. 4C). Importantly, FK506 administration partially blocked the rescuing effect of Parkin overexpression in PINK1 mutant flies (Fig. 4D), further suggesting an indispensable role for CaN in Parkin activation downstream PINK1.

Further experiments are required to identify the specific Parkin residue/s that is/are de-phosphorylated by CaN. An *in silico* analysis identified Serine 407 and Threonine 410 as potential candidates for CaN dependent de-phosphorylation (Suppl. Fig. 4A). Encouraged by this analysis, we generated two phospho-mimetic Parkin mutants (Ser407Asp and Thr410Asp, respectively), and assessed their ability to translocate to mitochondria upon CCCP intoxication. In this analysis, translocation of phospho-mimetic Ser407Asp Parkin mutant was partially impaired, whereas phospho-mimetic Thr410Asp Parkin completely failed to translocate upon CCCP treatment (Suppl. Fig. 4B). To convincingly prove that these are the residues that are de-phosphorylated by CaN, we are in the process of performing mass-spectrometry analysis of Parkin protein that has been pulled down from untreated cells and following CCCP intoxication (Suppl. Fig. 4C). At the present we were able to pull down

flag-tagged Parkin from MEFs stably expressing pMSCV-Flag-Parkin vector. Western blot and Silver stain analysis (Suppl. Fig. 4C) revealed the purity of the isolated protein. Mass-spectrometry analysis of these samples will be instrumental to reveal Parkin post-transcriptional modifications involved in the regulation of its translocation.

Protein functions can be regulated by post-translational modifications (phosphorylation Ubiquitination, Acethylation, Nitrosylation, Sumoylation). In that respect, scientific research is exploring the effects of post-translational modifications as starting point for the developing of novel therapeutic targets for human diseases. In particular, Tyrosine Kinases Inhibitors (TKIs) such as Imatinib and Nilotib, are used as effective therapy for patients affected by leukemia[61,62]. Recently, this drug resulted to be effective also in mouse models of Alzheimer's Disease (AD)[63]. AD is a neurodegenerative disease characterized by the accumulation of β-amyloid (plaques) and hyper-phosphorylated Tau (tangles). AD animals present high levels of insoluble Parkin and decreased Parkin-Beclin-1 interaction. Administration of TKIs to AD mice increases soluble Parkin leading to amyloid clearance and cognitive improvement. Although no Parkin mutations are found in AD, these studies demonstrate how manipulation of Parkin activity through the modulation of post-transcriptional modifiers can be used as powerful therapeutic approach.

Ubiquitination is also emerging as a powerful tool to modulate proteins activity, via regulation of protein subcellular localization and/or ability to interact with other proteins. The counteracting activity of ubiquitin ligases and deubiquitinating enzymes (DUBs) mediate and regulate protein ubiquitination. Specifically in the context of PINK1/Parkin pathway, much effort has been put to identify specific DUBs that counter-act the ubiquitin-ligase activity of Parkin and impact mitophagy[35]. These enzymes are therefore emerging as extremely attractive druggable candidates.

By exploring the role of CaN in Parkin translocation and stress induced mitophagy and *in vivo* in a PINK1 model of PD, this work ultimately identified a novel druggable target and has the potential to widen up medical intervention for the treatment of PD.

Materials and Methods

Cells

Mouse embryonic fibroblast cells (MEFs) were cultured in Dulbecco's modified Eagle medium (DMEM) (Gibco) supplemented with 1% penicillin/streptomycin, with 10% Fetal Bovine Serum (FBS), with L-glutammine, and 1% non-essential amino acids solution and incubated at 37°C, in a humidified 5% CO2 atmosphere. PINK1 -/- MEFs cell line was gently provided by Prof. F. Cecconi, IRCSS F. Santa Lucia and Department of Biology, University of Rome Tor Vergata.
Transfection was performed using Transfectin™ Lipid Reagent (BIO-RAD) following manufacturer instruction. 4-6 hours after transfection the medium was changed and cells were processed for the indicated experiment 24/48 hours after. This protocol has been used both for confocal microscope analysis and for protein assays. When indicated, cells were treated with 10 µM CCCP for 3 hours.

Constructs and Molecular Biology

mCherry-Parkin and HA-Ubiquitin plasmids were obtained from Addgene. Site directed mutagenesis, using QuickChange II XL kit (Agilent) and the following primers were used to generate a point mutation on Serine 65 in Parkin (S65E): F-MutpkSer65E (5'- GAC CTG GAT CAG CAG GCC ATT GTT CAC ATT GT- 3') and R-MutpkSer65E (5'-ACA ATG TGA ACA ATG GCC TGC TGA TCC AGG TC- 3'). The same protocol was used for Ubiquitin point mutation at Serine65, and the following primers were used: F-MutUbSer65E (5'-ATC CAG AAG GAG GAG ACC CTG CAC CT- 3') and R-MutUbSer65E (5'- AGG TGC AGG GTC TCC TCC TTC TGG AT- 3'). These constructs were named Parkin S65E and Ub-S65E.
The following primers were used to generate a point mutation on Threonine 410 and Serine 407, using the same Agilent mutagenesis kit: Thr410Ala forward (5'- GAA GCA GCC TCC AAA GAA GCC ATC AAG AAA ACC ACC AAG- 3') and reverse (5' -CTT GGT GGT TTT CTT GAT GGC TTC TTT GGA GGC TGC TTC- 3'); Thr410Asp forward (5'- GAA GCA GCC TCC AAA GAA GAC ATC AAG AAA ACC ACC AAG- 3') and reverse (5'- CTT GGT GGT TTT CTT GAT GTC TTC TTT GGA GGC TGC TTC- 3'); Ser407Ala forward (5'- GCT CGT TGG GAA GCA GCC GCC AAA GAA ACC ATC AAG AAA- 3') and reverse (5'- TTT CTT GAT GGT TTC TTT GGC GGC TGC TTC CCA ACG AGC- 3'); Ser407Asp forward (5'- GCT CGT TGG GAA GCA GCC GAC AAA GAA ACC ATC AAG AAA- 3') and reverse (5'- TTT CTT GAT GGT TTC TTT GTC GGC TGC TTC CCA ACG AGC- 3'). These constructs were named Parkin Thr410Ala, Thr410Asp, Ser407Ala and Ser407Asp, respectively.
Flag-tagged Parkin was inserted into pMSCV vector by using the pCR®8/GW/TOPO® TA Cloning® Kit (Invitrogen). To perform the pCR®8/GW/TOPO® cloning, Flag-Parkin construct was PCR amplified from pEYFP-C1-Parkin vector (available in the lab) using the following primers: Parkin-forward-BglII-Flag (5' -AGCT AGATCT ATG GAT TAC AAG GAT GAC GAC GAT AAG ATG ATA GTG TTT GTC AGG- 3') and EYFP-reverse (5' -ACC ATG GTG AGC AAG GGC GAG- 3').
pcDNA3.1-ΔCnA^{H151Q} (ΔCnA^{H151Q}), pDCR-HA-ΔCnA (ΔCnA), pDCR-HA-CnB and mito-YFP were plasmids already available in the lab and described in [49,64,65].

Imaging

For confocal imaging experiments of Parkin localization, transfected MEFs cells were seeded onto 24 mm-round glass coverslips in 6-well culture plates. Cells were co-transfected with one of the mCherry-constructs together with mito-YFP. When indicated, cells where cotransfected with CnB, the regulatory Calcineurin (Cn) domain, plus ΔCnA (constitutively active Cn) or ΔCnAH151Q (dominant negative mutant of Cn), and/or one of the Ubiquitin constructs (Ub or UbS65E). Image analysis was performed using ImageJ. These constructs were then excited using 561 or 488 laser and using a *UPlanSApo 60x/1.35* objective (iMIC Andromeda). Stack of

images separated by 0.25 0.4µm along 410 the z axis were acquired. The quantification was performed as calculation of the percentage of cells with Parkin puncta on mitochondria or through an ImageJ plugin for colocalization quantification (see following paragraph for details).

Image analysis using Squassh

To quantify Parkin colocalization with mitochondria, we created maximum-intensity projections of z-series with 0.25 µm increments. Quantification was then performed by using 'Squassh' (Segmentation and QUAntification of Subcellular SHapes), a new plugin compatible with the imaging processing softwares ImageJ or Fiji, freely available from http://mosaic.mpi-cbg.de/?q=downloads/imageJ[66]. Squassh is a segmentation method that enables both colocalization and shape analyses of subcellular structures in fluorescence microscopy images. For our analysis, segmentation was performed with the minimum intensity threshold for the first channel set to 0.35, for the second to 0.15 and the regularization weight to 0.015. Among the three different colocalization coefficients (C_{signal}, C_{number} and C_{size}), we preferentially used C_{number}.

Immunoblotting

At the established time points, the medium was removed and MEFs washed with PBS. After withdrawing PBS, cells were scraped off the wells using a plastic cell scraper, they were resuspended in 1,5 ml of cold PBS and they were centrifuged at 4'000 rpm at 4 °C for 5 min. Supernatant was discarded and then the pellet was resuspended in an appropriate volume of radioimmunoprecipitation assay (RIPA) buffer (RIPA buffer for 100 ml: 0,79 g of Tris base, 0,9 g of NaCl, 10 ml of NP-40 10%, 2,5 ml of Na-deoxycholate 10%, 1 ml of EDTA 0,1 M, and distilled water to 100 ml volume; adjust pH to 7.4) with freshly added protease inhibitor (PIC). Cells were kept on ice for 30 mins. The lysate was then centrifuged at 14'000 x g for 15 mins at 4 °C and then stored at -20 °C.

Protein concentrations of samples have been quantified using Pierce™ BCA Protein Assay Kit (ThermoFisher SCIENTIFIC).

NuPAGE® LDS Sample Buffer (Invitrogen) and 2-Mercaptoethanol (SIGMA) were mixed to samples and proteins were then denaturated at 95°C for 15 min. Samples were then centrifuged at 13300 rpm for 8 sec and separated on NuPAGE 4-12% Bis-Tris gels (NuPAGE®, Invitrogen) applying a constant voltage of 135 mV for 1 hour and 30 min. After electrophoresis run, proteins were electroblotted from gel matrix onto polyvinylidene fluoride transfer membranes (Thomas Scientific). The protein transfer was performed applying a constant voltage of 100 mV for 1 hours at 4 °C.

The following antibodies were used: anti-PMP70 (Sigma, 1:1000), anti-ATP5A (Abcam, 1:1000), anti-Actin (Chemicon, 1:20'000), anti-PINK1 (Cell Signalling, 1:100), anti-HA (Roche, 1:1000), anti-Parkin (Santa Cruz Biosience, 1:400).

Statistical analysis

All data are expressed as mean ± SE from at least three different experiments unless specified otherwise (Microsoft Office Excel and Origin 7.0 Professional). Statistical significance was measured by an unpaired t-test and p-values are specifically.

Fly stocks and breeding conditions

Flies were raised on standard cornmeal medium and were maintained at 23° C, 70% relative humidity, on a 12 h light : 12 h dark cycle.

We used ActGal4 standard lines, generous gifts from Dr. Alexander Whitworth (University of Sheffield) as control. PINK1B9 and PK OE lines were already described before[16,19] and were a

kind gift from Dr. Alexander Whitworth. CanA-14F line was already described before[67] and was a kind gift by Prof. Pascal Dijkers.

Climbing assay

The climbing assay (negative geotaxis assay) was used to assess locomotor ability (Fig . 4A). Climbing data were obtained from groups of untreated wildtype, untreated PINK1B9, FK506-treated wildtype, and FK506-treated PINK1B9. Briefly, 10 flies for each strain were collected in a vertically-positioned plastic tube (length 12 cm; diameter 5 cm) with a line drawn at 6 cm from the bottom of the tube. Flies were gently tapped to the bottom of the tube, and the number of flies that successfully climbed above the 6-cm mark after 10 seconds was noted. Fifteen separate and consecutive trials were performed for each experiment, and the results were averaged. At least 30 flies were tested for each genotype or condition.
The number of flies that could climb unto, or above, this line within 10 or 20 seconds was recorded and expressed as percentage of total flies.

In silico analysis

In silico analysis were performed by using Motif Scan (http://myhits.isb-sib.ch/cgi-bin/motifscan) and NetPhos 2.0 (http://www.cbs.dtu.dk/ services/NetPhos/). Human, murine and Drosophila Parkin protein sequences were aligned and compared for highly conserved residues.

Immunoprecipitation

For immunoprecipitation, cells were lysed in the following buffer: 10 mM Tris HCl pH 7.9, 340 mM Sucrose, 3 mM $CaCl_2$, 2 mM Mg(OAc), 0.1 mM EDTA, 1 mM DTT, 0.5% NP-40, protease inhibitors, phosphatase inhibitors. Anti-FlagM2 (Sigma) or anti-IgG Mouse (Sigma) were incubated overnight with the lysates. Protein A agarose beads (Roche) were conjugated overnight with the lysates. Beads were washed using the following washing buffer: 20 mM HEPES pH 7.9, 250 mM KOAc, 1% Tryton, 10% Glycerol, 3 mM EDTA, 1 mM DTT. The protein was eluted using the following buffer: 20 mM HEPES pH 7.9, 100 mM KOAc, 10% glycerol, 3mM EDTA, 200ug/ml Flag peptide. The samples were then analyzed by westernblot or Silver stain (SilverQuest™ Silver Staining Kit, Invitrogen).

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Conflict of Interest

The authors declare that they have no conflict of interest.

Figure legends

Figure 1: Parkin translocation to mitochondria is blocked upon Calcineurin genetic inhibition. (A) Wildtype MEF cells transfected with mCherry-Parkin and mito-YFP as described in Materials and methods, treated with DMSO as control or 10 μM CCCP (B). The panels at the right show enlarged views of the boxed areas. Arrows indicate mitochondria that colocalize with overexpressed Parkin. (C) mCherry-Parkin

colocalization with mitochondria expressed as percentage of cells with Parkin on mitochondria (upper panel) for at least ≥ 300 cells or Squassh colocalization parameter for at least ≥ 50 images (bottom panel). At least 4 independent experiments were performed for both quantifications. (D) MEF cells transfected with mCherry-Parkin and mito-YFP plus empty vector (top) or ΔCnB and ΔCnA^{H151Q} (bottom). Cells were treated with DMSO or CCCP when indicated. The panels on the right show enlarged views of the boxed areas. Arrows indicate mitochondria that colocalize with overexpressed mCherry-Parkin. (E) mCherry-Parkin colocalization with mitochondria were scored as in Fig. 1 C. (F) Wildtype MEFs transfected with mCherry-Parkin plus empty vector or ΔCnB and ΔCnA^{H151Q} as indicated, were treated with 10 μM CCCP at different time points and lysed. Lysates were separated by SDS–PAGE and immunoblotted using the indicated antibodies. Ratio between the densitometric levels of ATP5 and those of PMP70 in MEFs transfected as indicated on the upper panel. One representative experiment at least three independent repetitions performed is shown in the lower panels.

Figure 2: Parkin translocation is induced by Calcineurin constitutive active mutant, in a PINK1-independent manner. (A) Wildtype MEFs transfected with mCherry-Parkin and mito-YFP plus empty vector (top) or ΔCnB and ΔCnA (two bottom panels). Cells were treated with DMSO or CCCP when indicated. The panels on the right show enlarged views of the boxed areas. Arrows indicate mitochondria that colocalize with mCherry-Parkin. (B) mCherry-Parkin colocalization with mitochondria were scored as in Fig. 1 C. (C) Wildtype MEFs transfected with mCherry-Parkin plus empty vector or ΔCnB and ΔCnA as indicated, were treated with 10 μM CCCP at different time points and lysed. Lysates were separated by SDS–PAGE and immunoblotted using the indicated antibodies. Ratio between the densitometric levels of ATP5 and those of PMP70 in MEFs transfected as indicated on the upper panel. One representative experiment at least three independent repetitions performed is shown in the lower panels. (D) PINK1 -/- MEFs transfected with mCherry-Parkin and mito-YFP plus empty vector (left) or ΔCnB and ΔCnA (right) as indicated. Cells were treated with DMSO or CCCP when indicated. The panels on the bottom show enlarged views of the boxed areas. Arrows indicate mitochondria that colocalize with mCherry-Parkin. (E) mCherry-Parkin colocalization with mitochondria in PINK1 -/- MEFs were scored as in Fig. 1 C. (F) PINK1 -/- MEFs transfected with mCherry-Parkin plus empty vector or ΔCnB and ΔCnA as indicated, were treated with 10 μM CCCP at different time points and lysed. Lysates were separated by SDS–PAGE and immunoblotted using the indicated antibodies. Ratio between the densitometric levels of ATP5 and those of PMP70 in MEFs transfected as indicated on the upper panel. One representative experiment at least three independent repetitions performed is shown in the lower panels.

Figure 3: Parkin-dependent mitophagy is regulated by Calcineurin and PINK1-dependent phosphorylation. (A) Wildtype MEFs transfected with mCherry-Parkin S65E mutant and mito-YFP plus Ub S65E mutant, ΔCnB and ΔCnA. Cells were imaged 24 or 48 hours after transfection, as indicated. The panels on the right show enlarged views of the boxed areas. (B) mCherry-Parkin colocalization with mitochondria were scored

as in Fig. 1 C. (C) PINK1 -/- MEFs transfected with mCherry-Parkin S65E, Ub S65E and mito-YFP plus empty vector (left) or ΔCnB and ΔCnA^{H151Q} (right) as indicated. Cells were treated with DMSO or CCCP when indicated. The panels on the bottom show enlarged views of the boxed areas. Arrows indicate mitochondria that colocalize with mCherry-Parkin. (D) mCherry-Parkin colocalization with mitochondria were scored as in Fig. 1 C.

Figure 4: Constitutive active CnA in Drosphila rescues PINK1B9 climbing ability. (A) Schematic representation of the climbing assay. 10 flies were put into a tube in a dark room. A light was put on the top of the tube. After tapping the flies on the bottom of the tube, the number of flies that successfully climbed above the 6-cm mark after 10 seconds was noted. (B) Evaluation of the climbing ability of flies of the indicated genotype. Climbing test was performed as described. Bar graph represents the number of flies that successfully climbed above the 6-cm mark in 10 seconds. (C-D) Quantification of the climbing ability of flies of the indicated genotype. Flies were treated as indicated for 48 hours with DMSO or different FK506 concentrations.

Figure 5: Schematic representation of the pathway regulating Parkin translocation and mitophagy. Mitochondrial membrane potential drives PINK1 import into healthy mitochondria through the TOM and TIM complexes. Once on the IMM, PINK1 gets cleaved by MPP and PARL and eventually degraded by the ubiquitin-proteasome system. In this scenario, CaN is not active and Parkin is kept in the cytosol (left panel). Mitochondria depolarization is followed by cytosolic Ca^{2+} rise, which in turn activates CaN. CaN dephosphorylates Parkin which then translocates to depolarized mitochondria. Here, PINK1 is stabilized on the OMM where it phosphorylates Parkin and Ubiquitin. Phospho-Parkin undergoes a closed-to-open conformational change, binds to phospho-ubiquitin, and becomes fully active. In this context, Parkin ubiquitinates its targets on the outer mitochondrial membrane and leads to mitochondrial autophagy (right panel)

Suppl. Fig. 1: Parkin translocation is Ca^{2+}/CaN-dependent. (A) Wildtype MEFs transfected with mCherry-Parkin and mito-YFP. Cells were treated with 40 μM BAPTA for 30 min or with 40 μM BAPTA for 30 min prior to 3 hours CCCP treatment as indicated. The panels on the right show enlarged views of the boxed areas. (B) Wildtype MEFs transfected with mCherry-Parkin and mito-YFP. Cells were pretreated with 0.6 μM FK506 for 30 min before imaging or before 3 hours CCCP treatment, as indicated. The panels on the right show enlarged views of the boxed areas.

Suppl. Fig. 2: In wildtype MEFs, Parkin S65E behaves as wildtype Parkin. (A) Wildtype MEFs were cotransfected with mito-YFP plus mCherry-Parkin or mCherry-Parkin S65E and Ub S65E. When indicated, cells were treated with CCCP. mCherry-Parkin colocalization with mitochondria were scored as in Fig. 1 C. (B) Wildtype MEFs were cotransfected with mito-YFP plus mCherry-Parkin or mCherry-Parkin S65E together with Ub S65E, ΔCnB and ΔCnA^{H151Q}. When indicated, cells were treated with CCCP. mCherry-Parkin colocalization with mitochondria were scored as in Fig. 1 C.

Suppl. Fig. 3: In PINK1 -/- MEFs Parkin translocation is regulated by CaN. (A) Western blot analysis of PINK1-/- levels in wildtype and PINK1 knock out (KO) MEFs. In the first lane, PINK1 is detectable as two bands, with a molecular weight of 63 kDa and 52 kDa. In the third lane a positive control was loaded. (B) PINK1 -/- MEFs transfected with mito-YFP and mCherry-Parkin plus empty vector or ΔCnB and ΔCnA^{H151Q} as indicated. mCherry-Parkin colocalization with mitochondria were scored as in Fig. 1 C. (C) PINK1 -/- MEFs transfected with mCherry-Parkin plus empty vector or ΔCnB and ΔCnA^{H151Q} as indicated, were treated with 10 μM CCCP at different time points and lysed. Lysates were separated by SDS–PAGE and immunoblotted using the indicated antibodies. Ratio between the densitometric levels of ATP5 and those of PMP70 in MEFs transfected as indicated on the upper panel. (D) PINK1 -/- MEFs transfected with mito-YFP and mCherry-Parkin or plus mCherry-Parkin S65E together with Ub S65E, ΔCnB and ΔCnA as indicated. mCherry-Parkin colocalization with mitochondria were scored as in Fig. 1 C.

Suppl. Fig. 4: Target sites of CaN on Parkin. (A) Motif Scan and NetPhos 2.0 indicate Threonine 410 and Serine 407 as evolutionary conserved residues, which are likely to be phosphorylated. (B) Wildtype MEFs transfected with mito-YFP and mCherry-Parkin or Parkin phospho-mutant on Threonine 410 (Thr410Asp) and Serine 407 (Ser407Asp). mCherry-Parkin colocalization with mitochondria were scored as in Fig. 1 C. (C) Immunoprecipitation was performed in order to isolate and sequence Parkin through mass spectrometry. Western blot on top shows Parkin isolated from Flag-Parkin overexpressing MEFs. In the bottom, Silver Stain (SilverQuest™ Silver Staining Kit, Invitrogen) was performed, following the manufacturer's instruction.

References

1. Kitada T, Asakawa S, Hattori N, et al. Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. *Nature*. 1998;392(6676):605-608.

2. Narendra D, Walker JE, Youle R. Mitochondrial quality control mediated by PINK1 and parkin: Links to parkinsonism. *Cold Spring Harb Perspect Biol*. 2012;4(11):10.1101/cshperspect.a011338.

3. Ashrafi,G., Schwarz,T.L. The pathways of mitophagy for quality control and clearance of mitochondria. .

4. Winklhofer KF. Parkin and mitochondrial quality control: Toward assembling the puzzle. *Trends Cell Biol*. 2014;24(6):332-341.

5. Lin W, Kang UJ. Characterization of PINK1 processing, stability, and subcellular localization. *J Neurochem*. 2008;106(1):464-474.

6. Jin SM, Lazarou M, Wang C, Kane LA, Narendra DP, Youle RJ. Mitochondrial membrane potential regulates PINK1 import and proteolytic destabilization by PARL. *J Cell Biol*. 2010;191(5):933-942.

7. Yamano K, Youle RJ. PINK1 is degraded through the N-end rule pathway. *Autophagy*. 2013;9(11):1758-1769.

8. Narendra DP, Jin SM, Tanaka A, et al. PINK1 is selectively stabilized on impaired mitochondria to activate parkin. *PLoS Biol*. 2010;8(1):e1000298.

9. Vives-Bauza C, Zhou C, Huang Y, et al. PINK1-dependent recruitment of parkin to mitochondria in mitophagy. *Proc Natl Acad Sci U S A*. 2010;107(1):378-383.

10. Geisler S, Holmstrom KM, Skujat D, et al. PINK1/parkin-mediated mitophagy is dependent on VDAC1 and p62/SQSTM1. *Nat Cell Biol*. 2010;12(2):119-131.

11. Matsuda N, Sato S, Shiba K, et al. PINK1 stabilized by mitochondrial depolarization recruits parkin to damaged mitochondria and activates latent parkin for mitophagy. *J Cell Biol*. 2010;189(2):211-221.

12. Tanaka A, Cleland MM, Xu S, et al. Proteasome and p97 mediate mitophagy and degradation of mitofusins induced by parkin. *J Cell Biol*. 2010;191(7):1367-1380.

13. Chan NC, Salazar AM, Pham AH, et al. Broad activation of the ubiquitin-proteasome system by parkin is critical for mitophagy. *Hum Mol Genet*. 2011;20(9):1726-1737.

14. Narendra D, Tanaka A, Suen DF, Youle RJ. Parkin is recruited selectively to impaired mitochondria and promotes their autophagy. *J Cell Biol*. 2008;183(5):795-803.

15. Yoshii SR, Kishi C, Ishihara N, Mizushima N. Parkin mediates proteasome-dependent protein degradation and rupture of the outer mitochondrial membrane. *J Biol Chem*. 2011;286(22):19630-19640.

16. Park J, Lee SB, Lee S, et al. Mitochondrial dysfunction in drosophila PINK1 mutants is complemented by parkin. *Nature*. 2006;441(7097):1157-1161.

17. Clark IE, Dodson MW, Jiang C, et al. Drosophila pink1 is required for mitochondrial function and interacts genetically with parkin. *Nature*. 2006;441(7097):1162-1166.

18. Clark IE, Dodson MW, Jiang C, et al. Drosophila pink1 is required for mitochondrial function and interacts genetically with parkin. *Nature*. 2006;441(7097):1162-1166.

19. Tan JM, Dawson TM. Parkin blushed by PINK1. *Neuron*. 2006;50(4):527-529.

20. Poole AC, Thomas RE, Andrews LA, McBride HM, Whitworth AJ, Pallanck LJ. The PINK1/parkin pathway regulates mitochondrial morphology. *Proc Natl Acad Sci U S A*. 2008;105(5):1638-1643.

21. Ziviani E, Tao RN, Whitworth AJ. Drosophila parkin requires PINK1 for mitochondrial translocation and ubiquitinates mitofusin. *Proc Natl Acad Sci U S A*. 2010;107(11):5018-5023.

22. Caulfield TR, Fiesel FC, Moussaud-Lamodiere EL, Dourado DF, Flores SC, Springer W. Phosphorylation by PINK1 releases the UBL domain and initializes the conformational opening of the E3 ubiquitin ligase parkin. *PLoS Comput Biol*. 2014;10(11):e1003935.

23. Kondapalli C, Kazlauskaite A, Zhang N, et al. PINK1 is activated by mitochondrial membrane potential depolarization and stimulates parkin E3 ligase activity by phosphorylating serine 65. *Open Biol*. 2012;2(5):120080.

24. Kazlauskaite A, Kondapalli C, Gourlay R, et al. Parkin is activated by PINK1-dependent phosphorylation of ubiquitin at Ser65. *Biochem J*. 2014;460(1):127-139.

25. Chen Y, Dorn GW,2nd. PINK1-phosphorylated mitofusin 2 is a parkin receptor for culling damaged mitochondria. *Science*. 2013;340(6131):471-475.

26. Okatsu K, Koyano F, Kimura M, et al. Phosphorylated ubiquitin chain is the genuine parkin receptor. *J Cell Biol*. 2015.

27. Koyano F, Okatsu K, Kosako H, et al. Ubiquitin is phosphorylated by PINK1 to activate parkin. *Nature*. 2014;510(7503):162-166.

28. Shiba-Fukushima K, Arano T, Matsumoto G, et al. Phosphorylation of mitochondrial polyubiquitin by PINK1 promotes parkin mitochondrial tethering. *PLoS Genet*. 2014;10(12):e1004861.

29. Ziviani E, Whitworth AJ. How could parkin-mediated ubiquitination of mitofusin promote mitophagy? *Autophagy*. 2010;6(5):660-662.

30. Sarraf SA, Raman M, Guarani-Pereira V, et al. Landscape of the PARKIN-dependent ubiquitylome in response to mitochondrial depolarization. *Nature*. 2013;496(7445):372-376.

31. Escobar-Henriques M. Mitofusins: Ubiquitylation promotes fusion. *Cell Res*. 2014;24(4):387-388.

32. Koyano F, Matsuda N. Molecular mechanisms underlying PINK1 and parkin catalyzed ubiquitylation of substrates on damaged mitochondria. *Biochim Biophys Acta*. 2015.

33. Durcan TM, Fon EA. USP8 and PARK2/parkin-mediated mitophagy. *Autophagy*. 2015:0.

34. Nardin A, Schrepfer E, Ziviani E. Counteracting PINK/parkin deficiency in the activation of mitophagy: A potential therapeutic intervention for parkinson's disease. *Curr Neuropharmacol*. 2015.

35. Dikic I, Bremm A. DUBs counteract parkin for efficient mitophagy. *EMBO J*. 2014;33(21):2442-2443.

36. Bingol B, Tea JS, Phu L, et al. The mitochondrial deubiquitinase USP30 opposes parkin-mediated mitophagy. *Nature*. 2014;510(7505):370-375.

37. Cornelissen T, Haddad D, Wauters F, et al. The deubiquitinase USP15 antagonizes parkin-mediated mitochondrial ubiquitination and mitophagy. *Hum Mol Genet*. 2014;23(19):5227-5242.

38. Durcan TM, Tang MY, Perusse JR, et al. USP8 regulates mitophagy by removing K6-linked ubiquitin conjugates from parkin. *EMBO J*. 2014;33(21):2473-2491.

39. Um JW, Chung KC. Functional modulation of parkin through physical interaction with SUMO-1. *J Neurosci Res*. 2006;84(7):1543-1554.

40. Eckermann K. SUMO and parkinson's disease. *Neuromolecular Med*. 2013;15(4):737-759.

41. Yao D, Gu Z, Nakamura T, et al. Nitrosative stress linked to sporadic parkinson's disease: S-nitrosylation of parkin regulates its E3 ubiquitin ligase activity. *Proc Natl Acad Sci U S A*. 2004;101(29):10810-10814.

42. Chung KK, Thomas B, Li X, et al. S-nitrosylation of parkin regulates ubiquitination and compromises parkin's protective function. *Science*. 2004;304(5675):1328-1331.

43. van Vliet AR, Verfaillie T, Agostinis P. New functions of mitochondria associated membranes in cellular signaling. *Biochim Biophys Acta*. 2014;1843(10):2253-2262.

44. Kaufman RJ, Malhotra JD. Calcium trafficking integrates endoplasmic reticulum function with mitochondrial bioenergetics. *Biochim Biophys Acta*. 2014;1843(10):2233-2239.

45. Krebs J, Agellon LB, Michalak M. Ca(2+) homeostasis and endoplasmic reticulum (ER) stress: An integrated view of calcium signaling. *Biochem Biophys Res Commun*. 2015;460(1):114-121.

46. Bonora M, Bravo-San Pedro JM, Kroemer G, Galluzzi L, Pinton P. Novel insights into the mitochondrial permeability transition. *Cell Cycle*. 2014;13(17):2666-2670.

47. De Marchi E, Bonora M, Giorgi C, Pinton P. The mitochondrial permeability transition pore is a dispensable element for mitochondrial calcium efflux. *Cell Calcium*. 2014;56(1):1-13.

48. Cereghetti GM, Stangherlin A, Martins de Brito O, et al. Dephosphorylation by calcineurin regulates translocation of Drp1 to mitochondria. *Proc Natl Acad Sci U S A*. 2008;105(41):15803-15808.

49. Shimura H, Hattori N, Kubo S, et al. Immunohistochemical and subcellular localization of parkin protein: Absence of protein in autosomal recessive juvenile parkinsonism patients. *Ann Neurol*. 1999;45(5):668-672.

50. Zou Y, Hiroi Y, Uozumi H, et al. Calcineurin plays a critical role in the development of pressure overload-induced cardiac hypertrophy. *Circulation*. 2001;104(1):97-101.

51. Zou Y, Yao A, Zhu W, et al. Isoproterenol activates extracellular signal-regulated protein kinases in cardiomyocytes through calcineurin. *Circulation*. 2001;104(1):102-108.

52. Kwon KY, Viollet B, Yoo OJ. CCCP induces autophagy in an AMPK-independent manner. *Biochem Biophys Res Commun*. 2011;416(3-4):343-348.

53. Okatsu K, Kimura M, Oka T, Tanaka K, Matsuda N. Unconventional PINK1 localization mechanism to the outer membrane of depolarized mitochondria drives parkin recruitment. *J Cell Sci*. 2015.

54. Kane LA, Lazarou M, Fogel AI, et al. PINK1 phosphorylates ubiquitin to activate parkin E3 ubiquitin ligase activity. *J Cell Biol*. 2014;205(2):143-153.

55. Yang Y, Ouyang Y, Yang L, et al. Pink1 regulates mitochondrial dynamics through interaction with the fission/fusion machinery. *Proc Natl Acad Sci U S A*. 2008;105(19):7070-7075.

56. Sauvé V, Gehring K. Phosphorylated ubiquitin: A new shade of PINK1 in parkin activation. *Cell Res*. 2014.

57. Jin SM, Lazarou M, Wang C, Kane LA, Narendra DP, Youle RJ. Mitochondrial membrane potential regulates PINK1 import and proteolytic destabilization by PARL. *J Cell Biol*. 2010;191(5):933-942.

58. Yamano K, Youle RJ. PINK1 is degraded through the N-end rule pathway. *Autophagy*. 2013;9(11):1758-1769.

59. Sauve V, Gehring K. Phosphorylated ubiquitin: A new shade of PINK1 in parkin activation. *Cell Res*. 2014;24(9):1025-1026.

60. de Lavallade H, Apperley JF, Khorashad JS, et al. Imatinib for newly diagnosed patients with chronic myeloid leukemia: Incidence of sustained responses in an intention-to-treat analysis. *J Clin Oncol*. 2008;26(20):3358-3363.

61. Deremer DL, Ustun C, Natarajan K. Nilotinib: A second-generation tyrosine kinase inhibitor for the treatment of chronic myelogenous leukemia. *Clin Ther*. 2008;30(11):1956-1975.

62. Lonskaya I, Hebron ML, Desforges NM, Franjie A, Moussa CE. Tyrosine kinase inhibition increases functional parkin-beclin-1 interaction and enhances amyloid clearance and cognitive performance. *EMBO Mol Med*. 2013;5(8):1247-1262.

63. Cecconi C, Shank EA, Bustamante C, Marqusee S. Direct observation of the three-state folding of a single protein molecule. *Science*. 2005;309(5743):2057-2060.

64. Costa V, Giacomello M, Hudec R, et al. Mitochondrial fission and cristae disruption increase the response of cell models of huntington's disease to apoptotic stimuli. *EMBO Mol Med*. 2010;2(12):490-503.

65. Rizk A, Paul G, Incardona P, et al. Segmentation and quantification of subcellular structures in fluorescence microscopy images using squassh. *Nat Protoc*. 2014;9(3):586-596.

66. Dijkers PF, O'Farrell PH. Drosophila calcineurin promotes induction of innate immune responses. *Curr Biol*. 2007;17(23):2087-2093.

Figure 1

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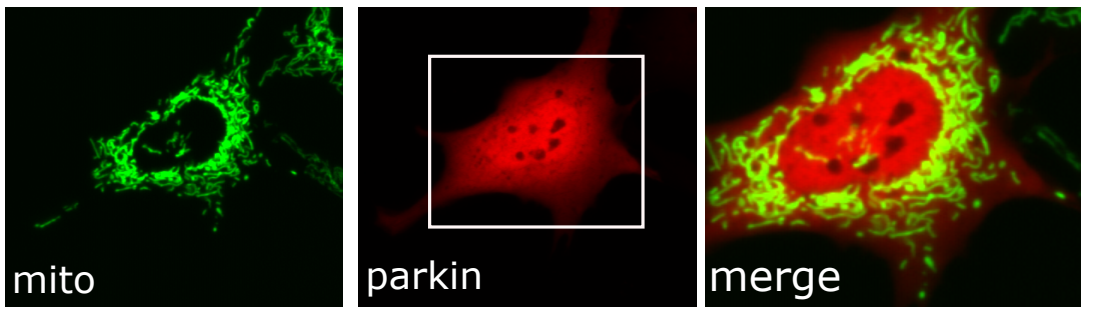

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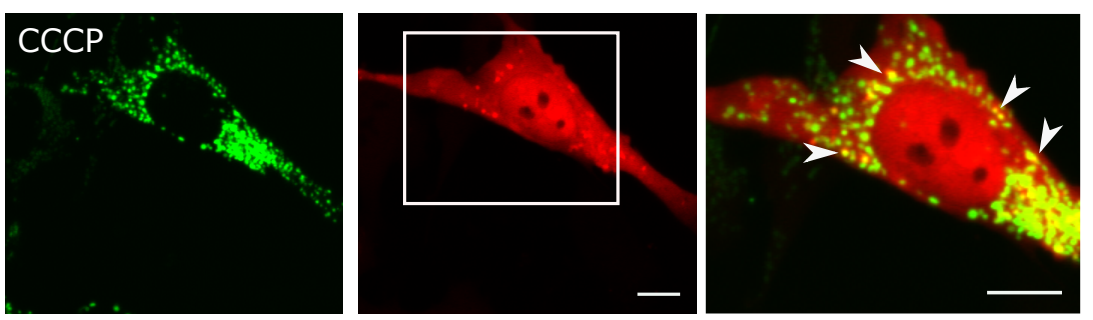

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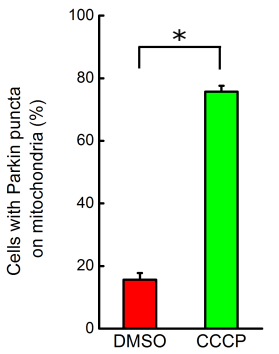

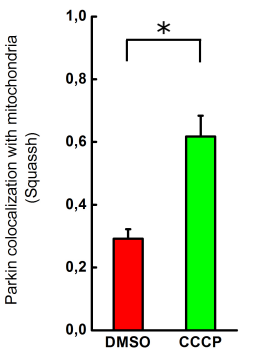

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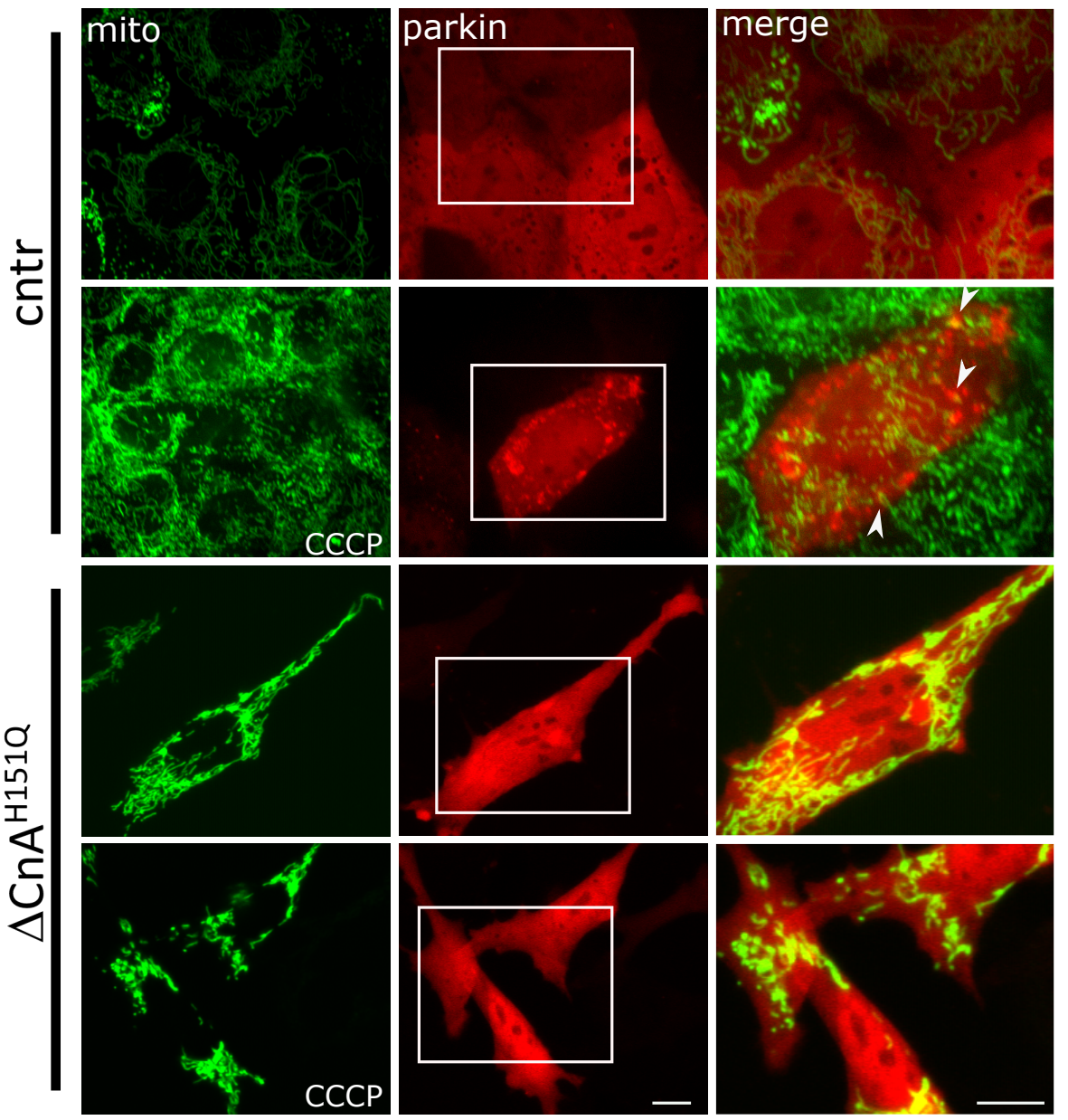

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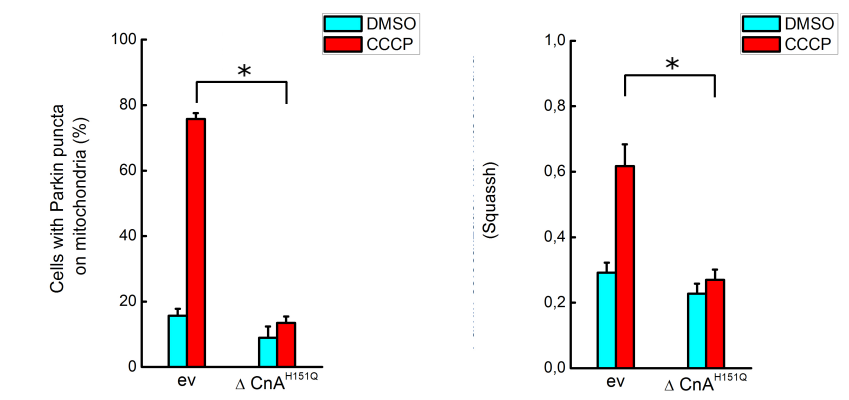

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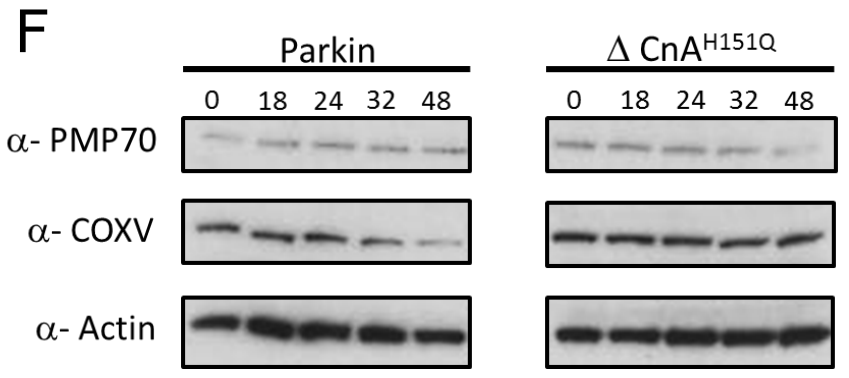

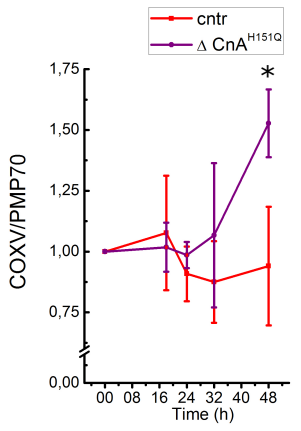

Figure 2

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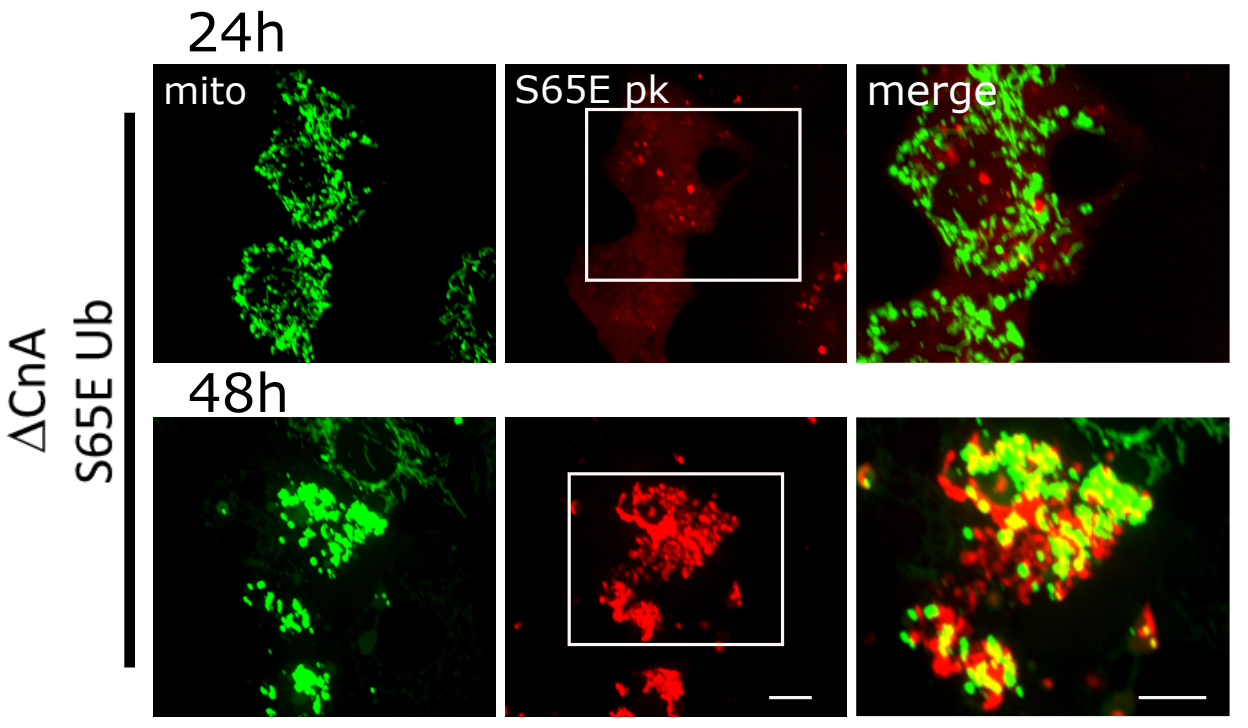

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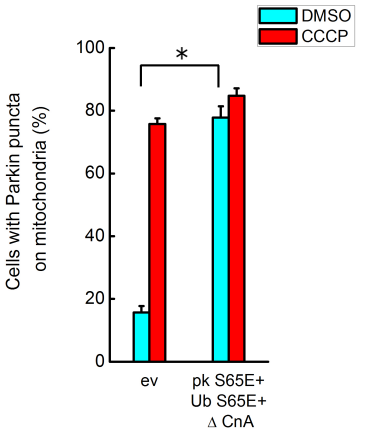

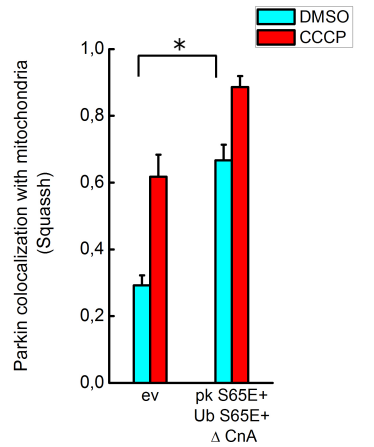

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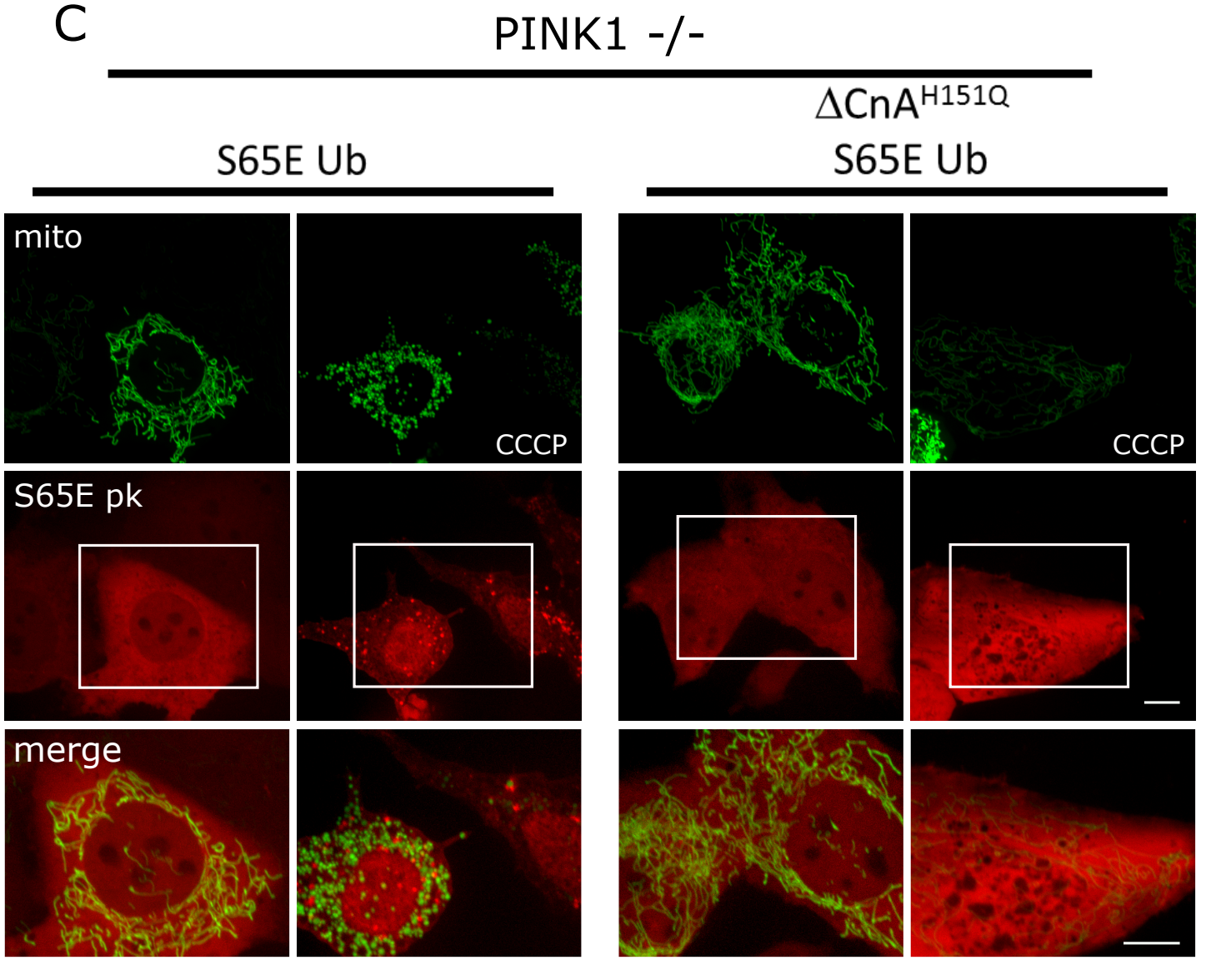

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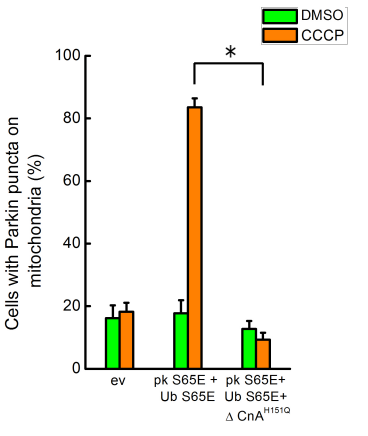


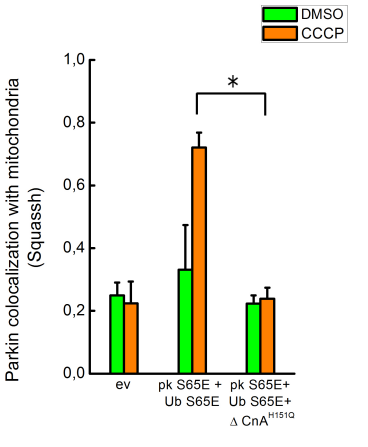
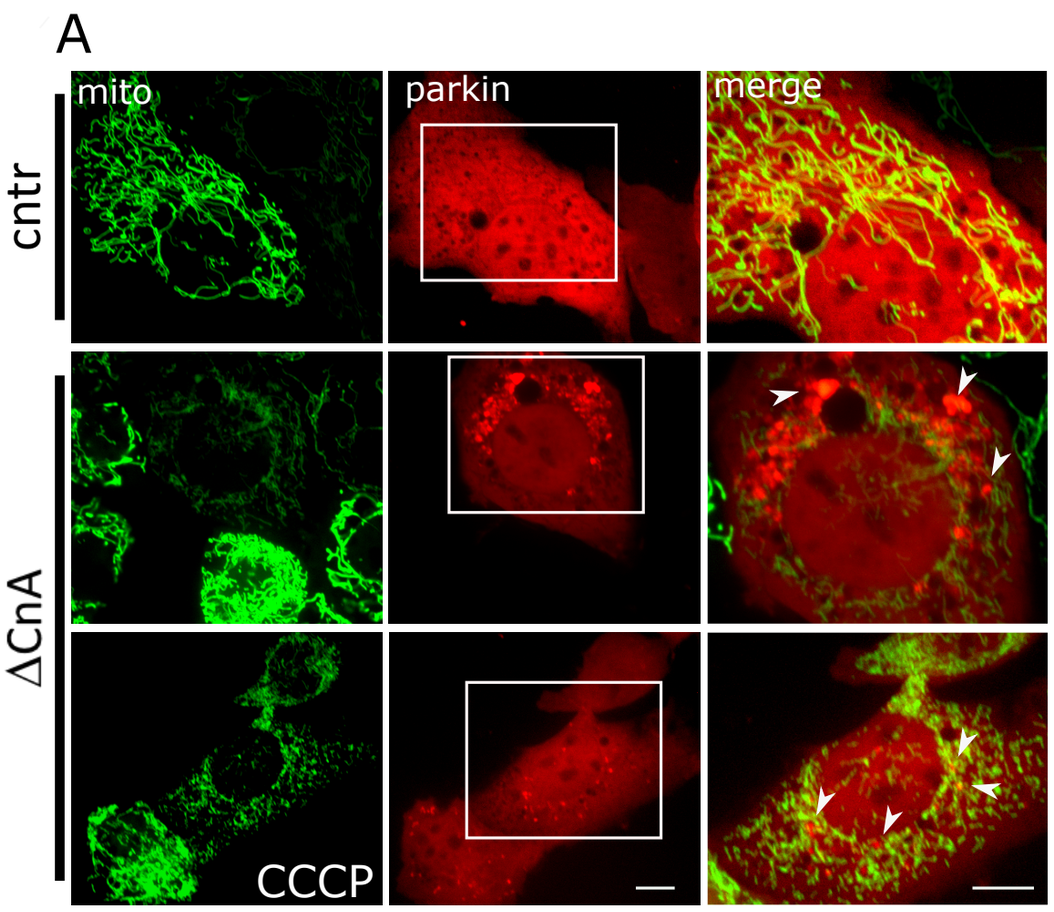
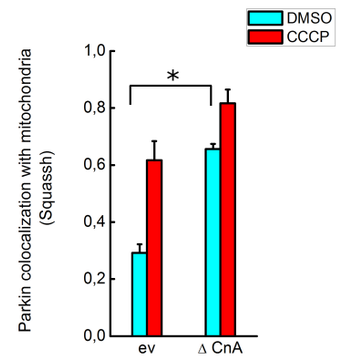


Figure 3


A
mito
parkin
merge
cntr
ΔCnA
CCCP


B
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CCCP
Parkin colocalization with mitochondria (Squassh)
1,0
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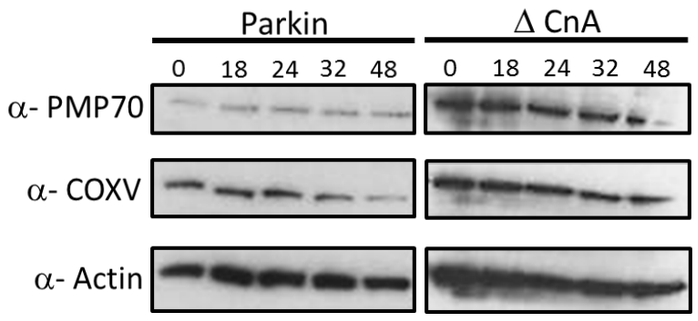
0,6
0,4
0,2
0,0
*
ev
Δ CnA

DMSO
CCCP
Parkin colocalization with mitochondria (Squassh)
1,0
0,8
0,6
0,4
0,2
0,0
*
ev
Δ CnA


C
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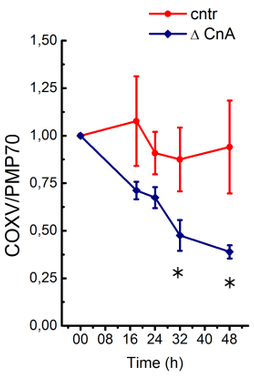
0 18 24 32 48
α- PMP70
α- COXV
α- Actin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COXV/PMP70
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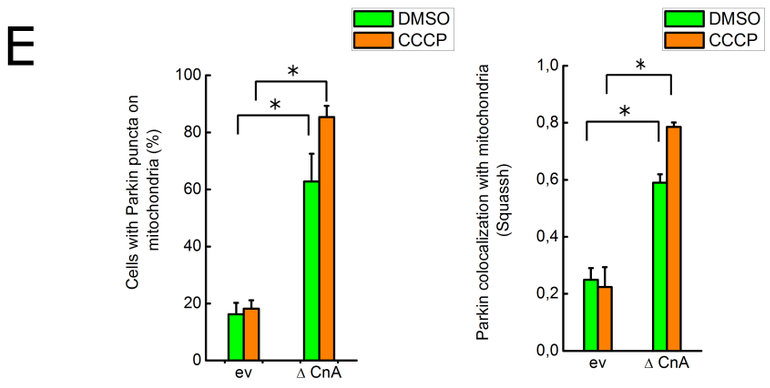
Time (h)


E
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Cells with Parkin puncta on mitochondria (%)
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60
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Δ CnA
DMSO
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Parkin colocalization with mitochondria (Squassh)
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0,8
0,6
0,4
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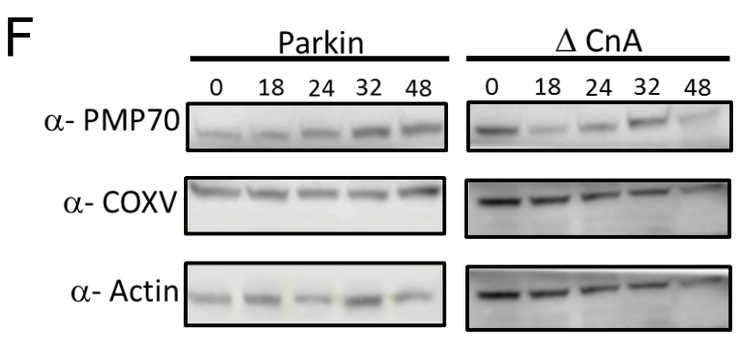
F
Parkin
Δ CnA
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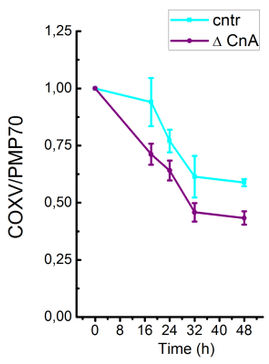
α- PMP70
α- COXV
α- Actin


cntr
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COXV/PMP70
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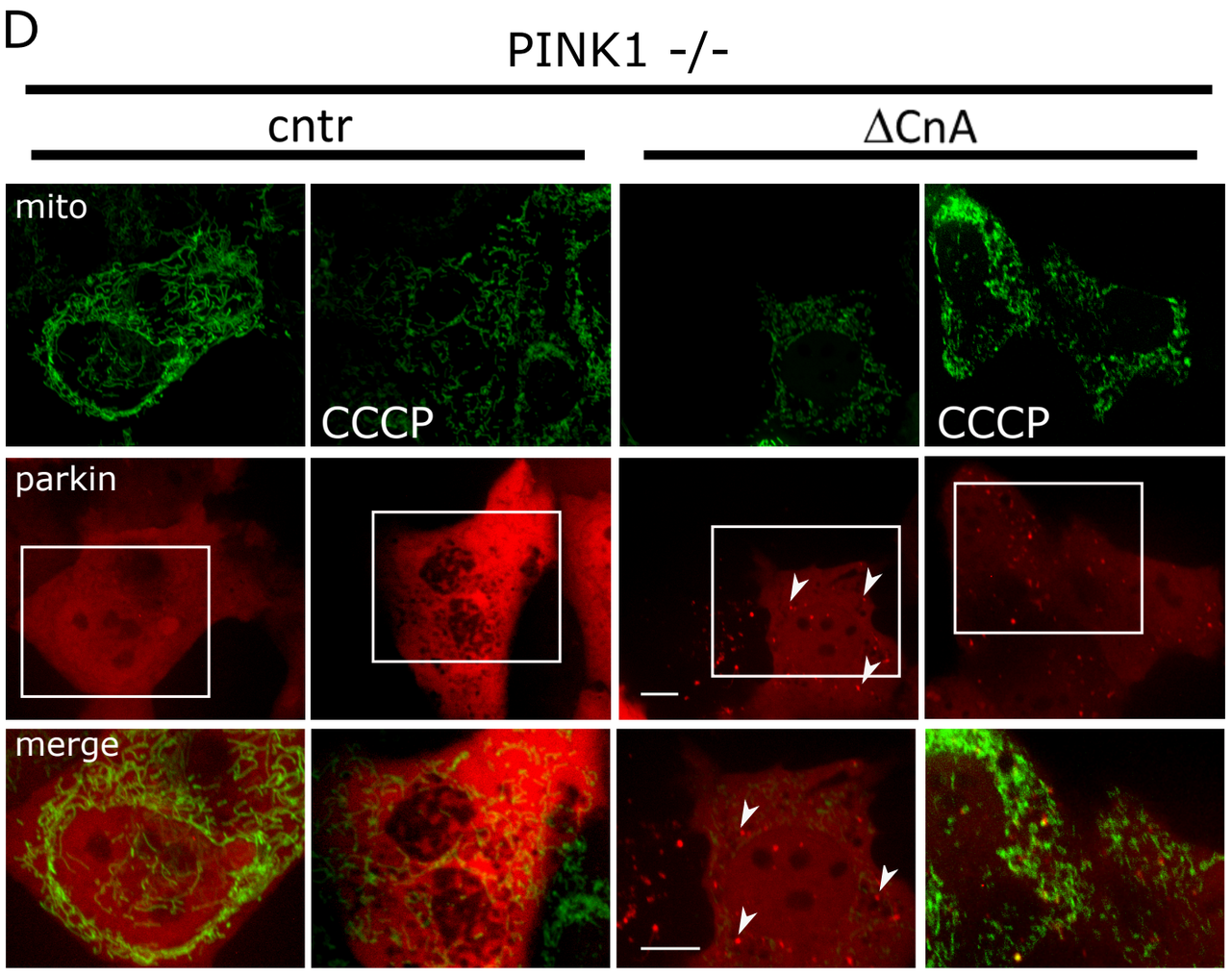
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PINK1 -/-
cntr
ΔCnA
mito
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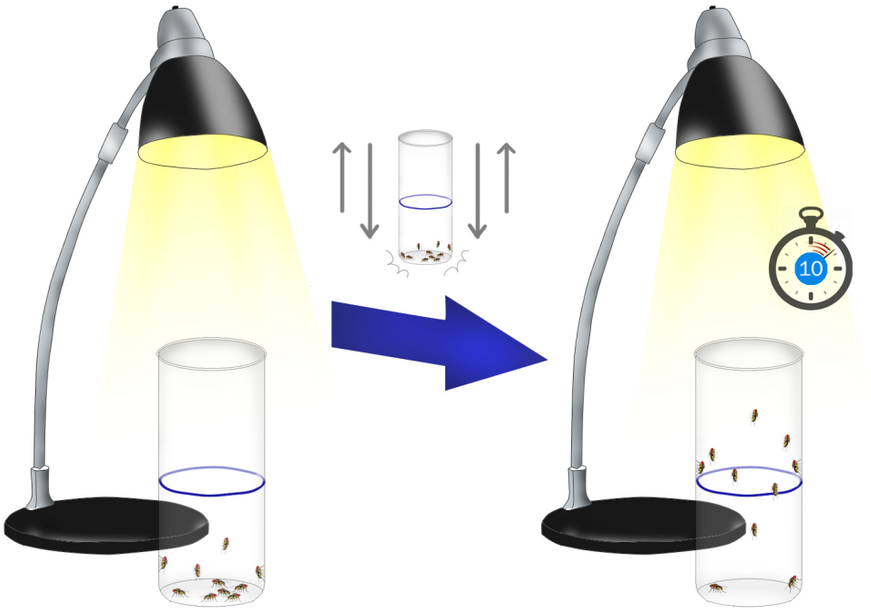
CCCP
parkin
merge

Figure 4

A



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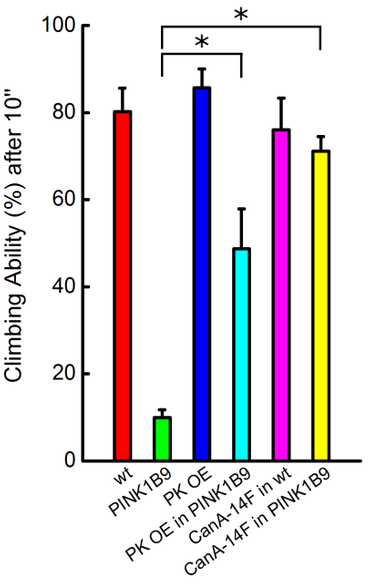

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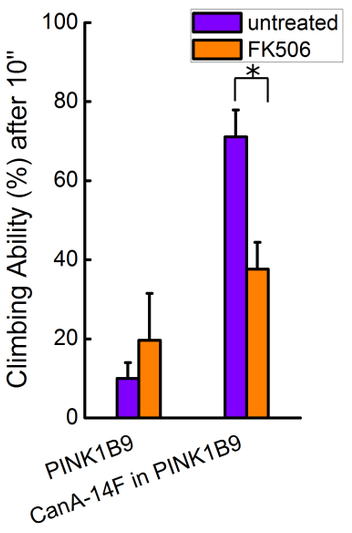
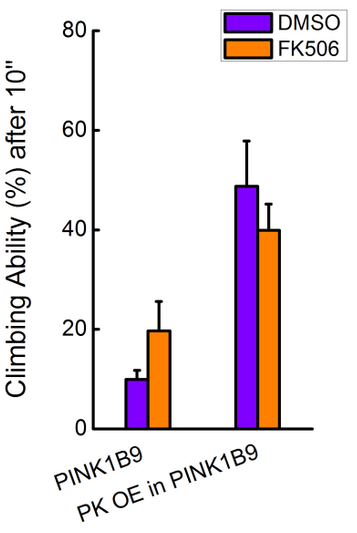


D



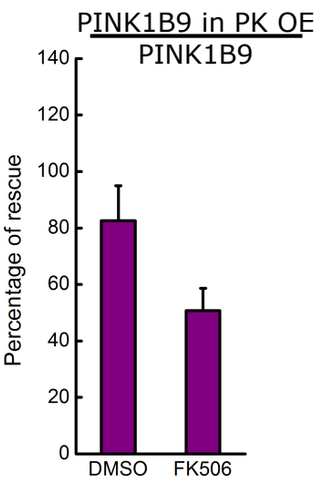


Figure 5

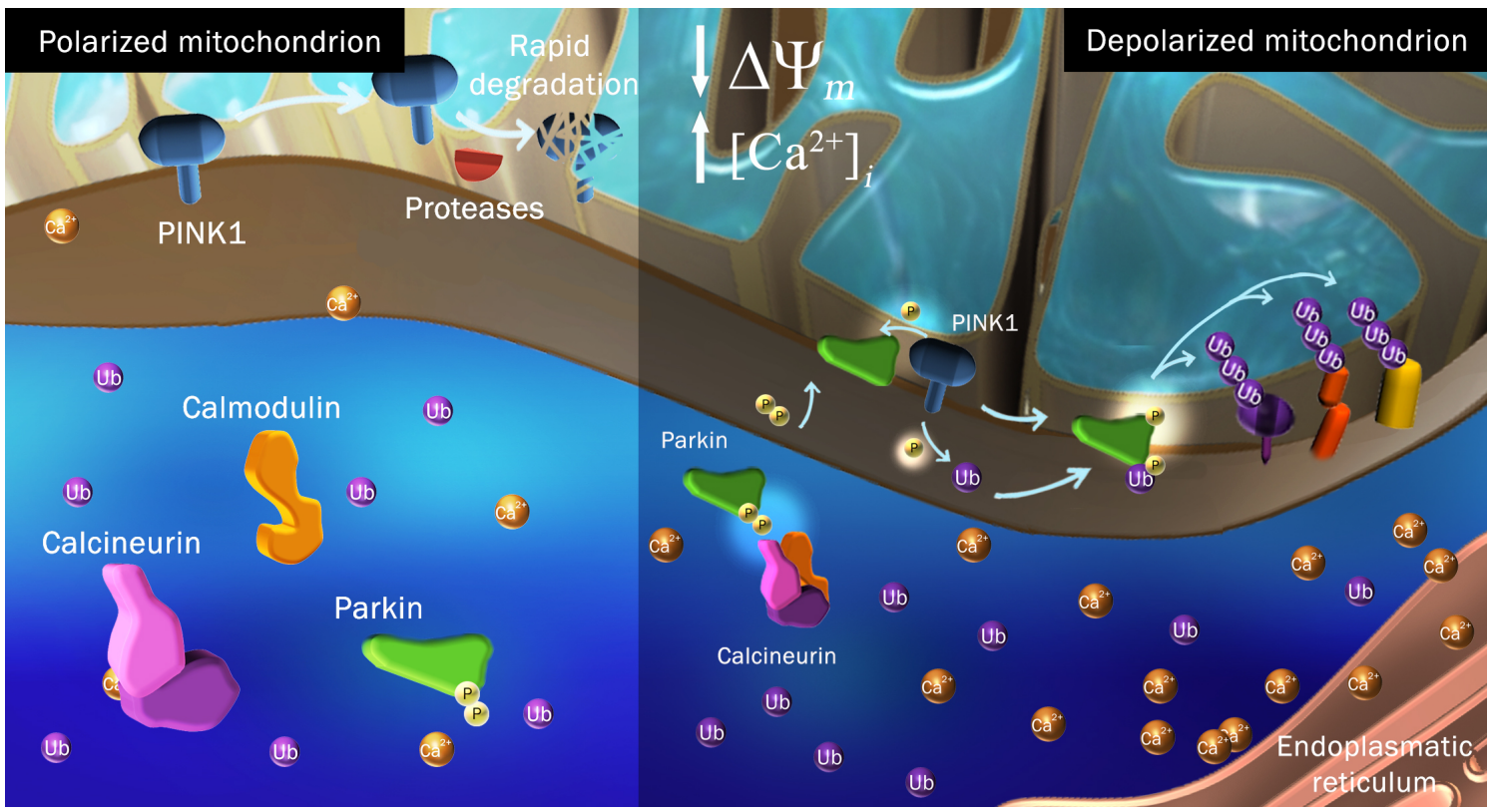
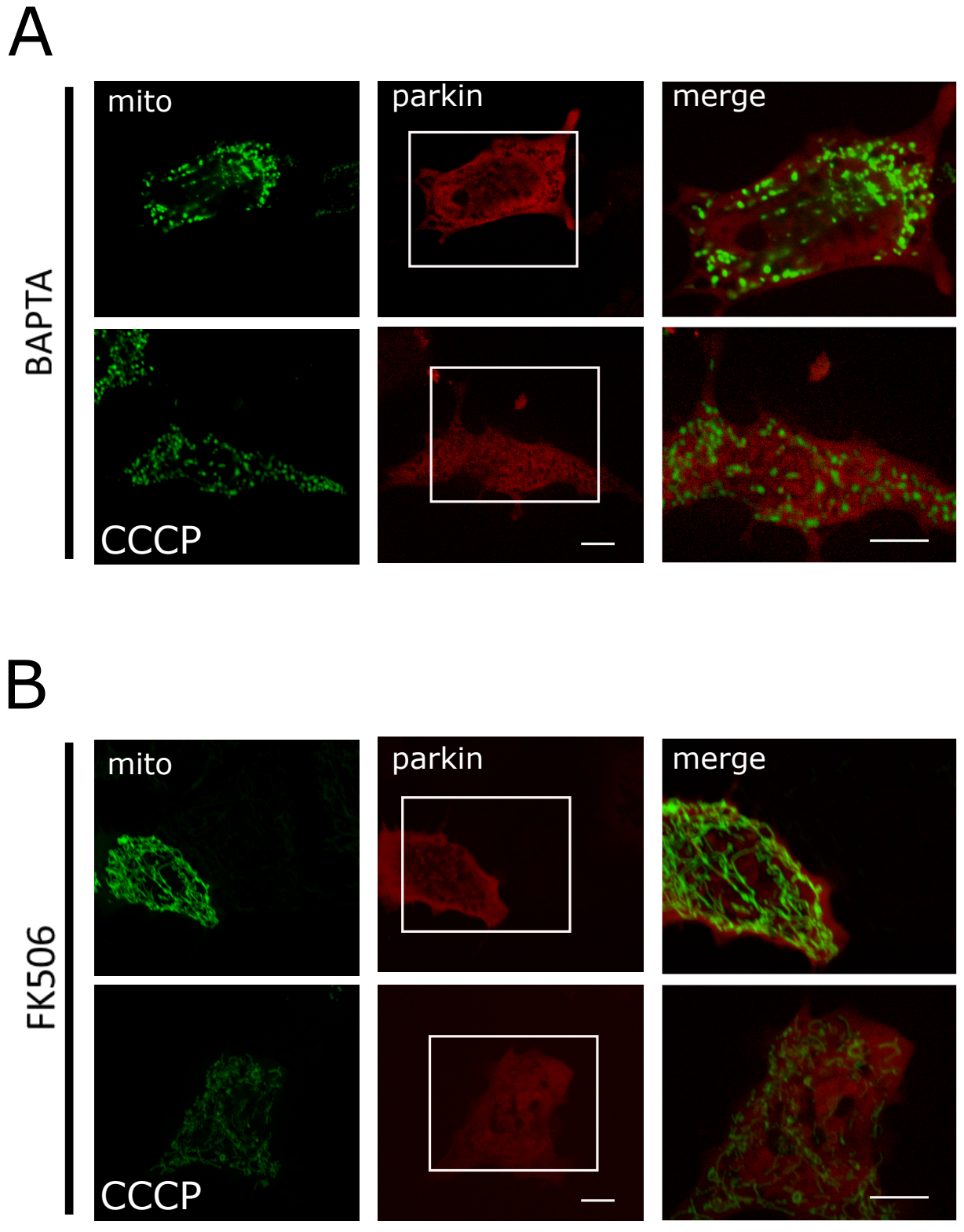
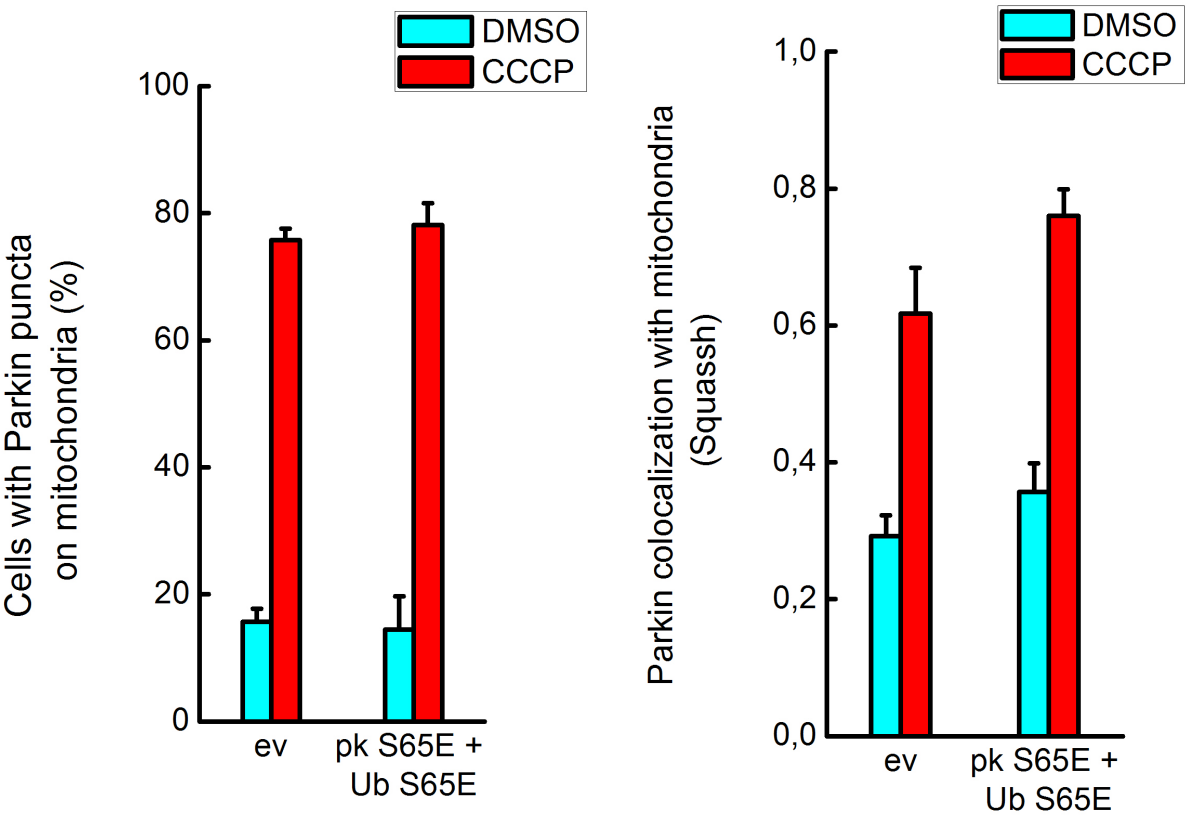

Polarized mitochondrion
Rapid degradation
PINK1
Proteases
Calmodulin
Calcineurin
Parkin
Depolarized mitochondrion
$\Delta\Psi_m$
$[Ca^{2+}]_i$
PINK1
Parkin
Calcineurin
Endoplasmatic reticulum

Fig. Suppl. 1

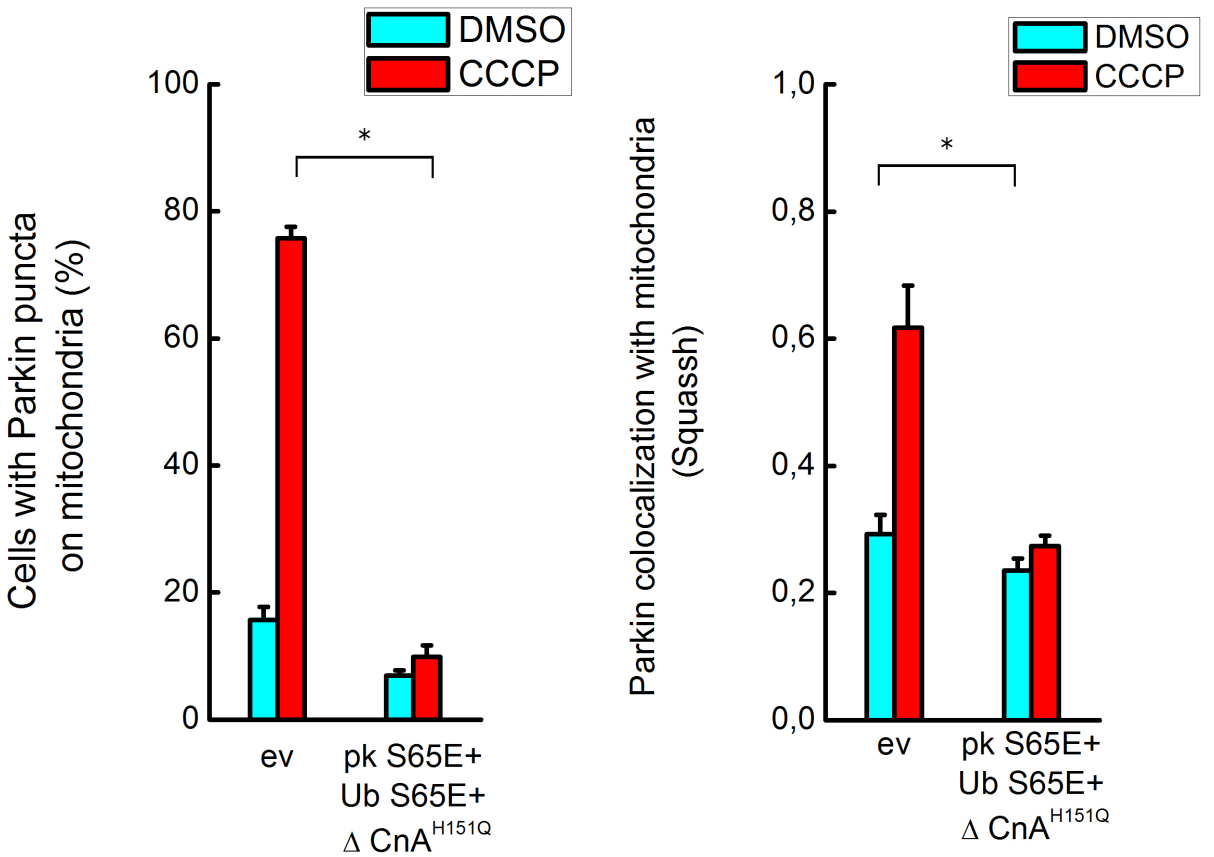

A
mito
parkin
merge
BAPTA
CCCP
B
mito
parkin
merge
FK506
CCCP

Suppl. Fig. 2

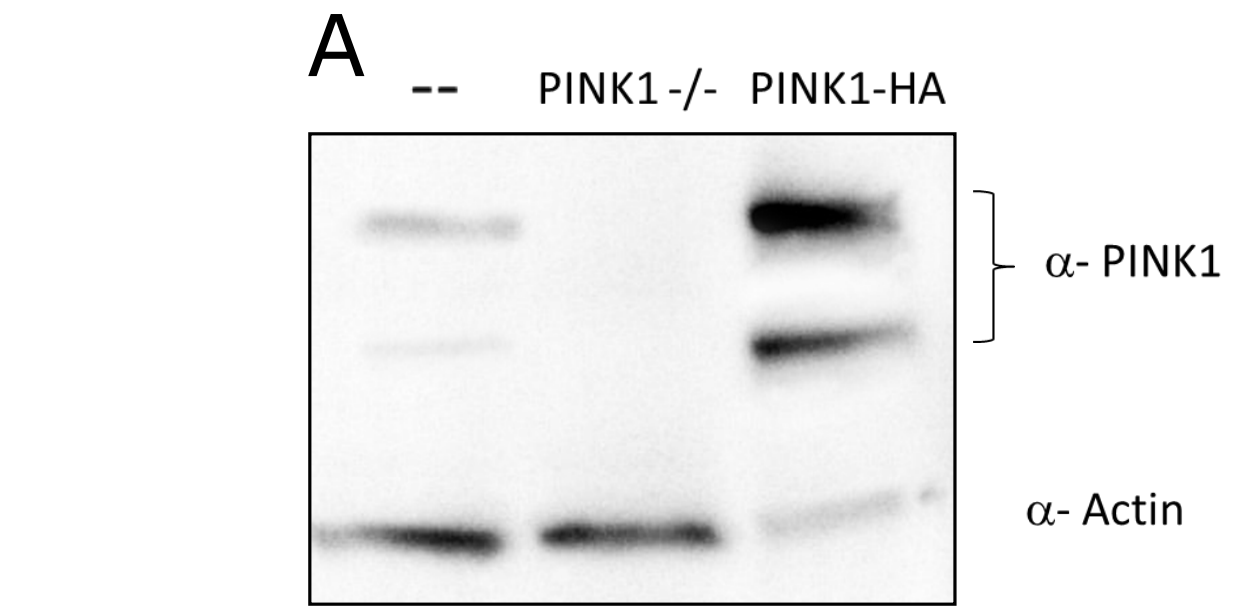
A



B



Suppl. Fig. 3


A
--
PINK1 -/-
PINK1-HA
α- PINK1
α- Actin
B
DMSO
CCCP
Cells with Parkin puncta on mitochondria (%)
100
80
60
40
20
0
ev
Δ CnA H151Q
Parkin colocalization with mitochondria (Squassh)
1,0
0,8
0,6
0,4
0,2
0,0
C
cntr
Δ CnA H151Q
COXV/PMP70
2,00
1,75
1,50
1,25
1,00
0,75
0,50
0,00
0
8
16
24
32
40
48
Time (h)
D
Cells with Parkin puncta on mitochondria (%)
ev
pk S65E+ Ub S65E+ Δ CnA
Parkin colocalization with mitochondria (Squassh)
pk S65E + Ub S65E

Suppl. Fig. 4

A

CENTERFO RBIOLOGI CALSEQU ENCEANA LYSIS CBS

NetPhos 2.0 Server - prediction results

Technical University of Denmark

Threonine predictions

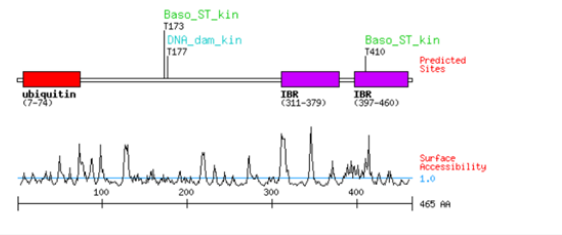
Name	Pos	Score	Pred
Sequence	21	0.012	.
Sequence	55	0.012	.
Sequence	83	0.181	.
Sequence	103	0.278	.
Sequence	125	0.010	.
Sequence	168	0.498	.
Sequence	173	0.512	*T*
Sequence	175	0.089	.
Sequence	177	0.107	.
Sequence	204	0.777	*T*
Sequence	217	0.846	*T*
Sequence	222	0.159	.
Sequence	231	0.030	.
Sequence	237	0.012	.
Sequence	240	0.111	.
Sequence	242	0.029	.
Sequence	270	0.009	.
Sequence	351	0.136	.
Sequence	386	0.243	.
Sequence	387	0.051	.
Sequence	388	0.117	.
Sequence	410	0.901	*T*
Sequence	414	0.399	.
Sequence	415	0.129	.

Serine predictions

Name	Pos	Score	Pred
Sequence	9	0.730	*S*
Sequence	10	0.247	.
Sequence	19	0.771	*S*
Sequence	22	0.475	.
Sequence	65	0.062	.
Sequence	101	0.812	*S*
Sequence	108	0.930	*S*
Sequence	109	0.070	.
Sequence	110	0.327	.
Sequence	116	0.060	.
Sequence	127	0.998	*S*
Sequence	131	0.996	*S*
Sequence	136	0.402	.
Sequence	141	0.881	*S*
Sequence	145	0.306	.
Sequence	167	0.167	.
Sequence	181	0.486	.
Sequence	193	0.996	*S*
Sequence	198	0.373	.
Sequence	205	0.130	.
Sequence	218	0.997	*S*
Sequence	223	0.310	.
Sequence	233	0.007	.
Sequence	246	0.027	.
Sequence	255	0.004	.
Sequence	286	0.009	.
Sequence	296	0.032	.
Sequence	378	0.004	.
Sequence	384	0.095	.
Sequence	407	0.970	*S*

Motif Scan Graphic Results: PARKIN_HUMAN

Description: User-entered sequence
Motifs scanned: All
Stringency: High
Show domains: Yes



Basophilic serine/threonine kinase group (Baso_ST_kin)

Calmodulin dependent Kinase 2 — Gene Card CAMK2G

Site	Score	Percentile	Sequence	SA
T173	0.3804	0.039 %	CSTCRQATLTLTQGP	0.568

PKC alpha/beta/gamma — Gene Card PRKCA

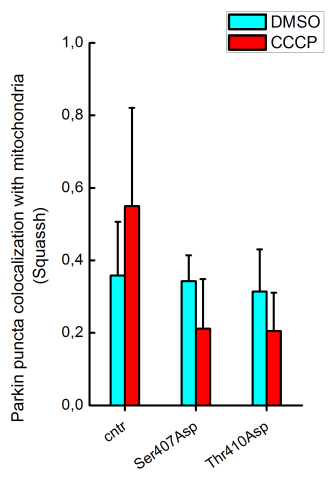
Site	Score	Percentile	Sequence	SA
T410	0.3849	0.133 %	WEAASKETIKKTTKP	3.211

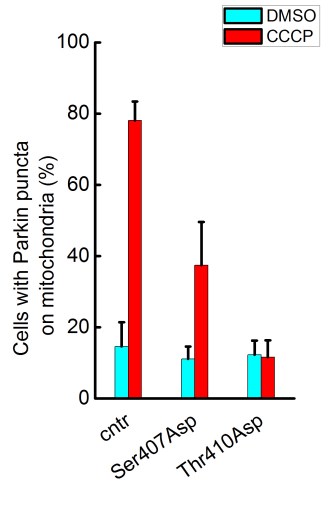
DNA damage kinase group (DNA_dam_kin)

ATM Kinase — Gene Card ATM

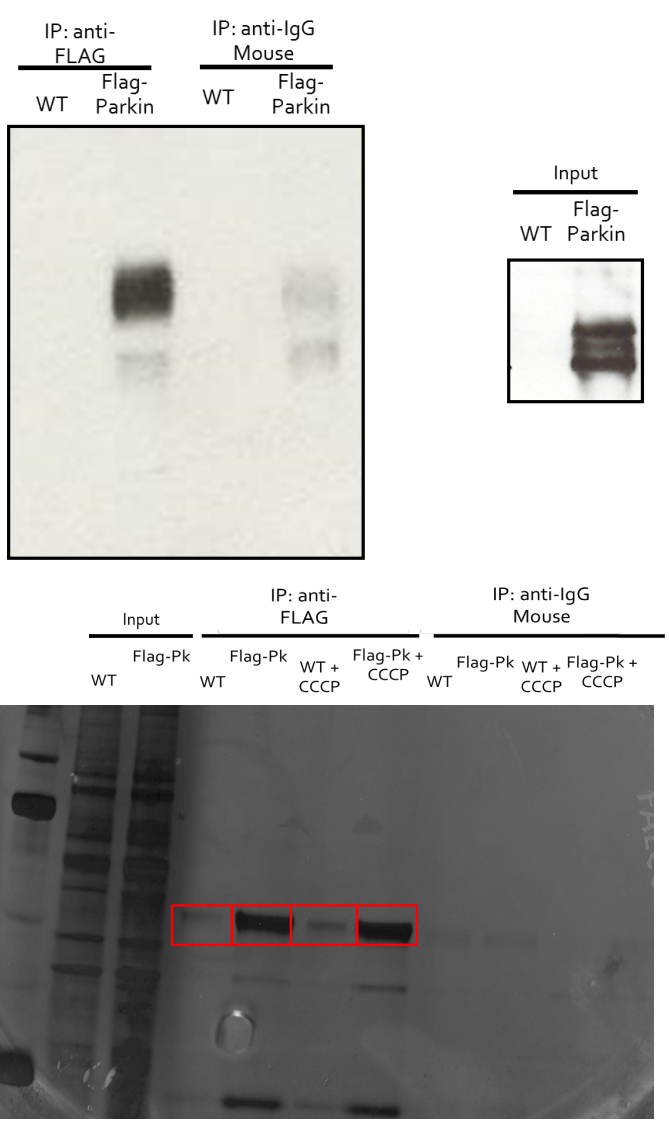
Site	Score	Percentile	Sequence	SA
T177	0.4482	0.191 %	RQATLTLTQGPSCWD	1.043

B





C



5. Conclusions

The molecular events which are responsible for neurodegeneration in Parkinson's disease are still mostly unclear. Recently, progresses in molecular research on genes linked to the disease provided new insights into its pathogenesis, thus facilitating the understanding of the molecular mechanism underlying PD. Loss of function mutations in the Parkin gene (PARK2) are linked to the majority of Autosomal-Recessive Juvenile-Parkinsonism (AR-JP)[1]. Parkin is an E3 Ubiquitin-ligase with an important neuroprotective role. Recent works have linked Parkin to the mitochondrial quality control pathway, nevertheless its role in neuron maintenance is still unknown[2]. Parkin translocates to unfunctional, uncoupled mitochondria, leading ton their elimination via autophagy[3-5]. Its role in PD could be linked to the accumulation of dysfunctional mitochondria, which fail to be eliminated as consequence of PD-related Parkin mutations. The aim of this thesis is to understand which molecular signals influence Parkin translocation and Parkin-dependent mitophagy.

Consistent with previous studies[6], we found that in MEFs Parkin was mostly located in the cytosol and was accumulating to mitochondria upon 3 hours of CCCP treatment (Fig. 1A-B). CCCP is a mitochondrial uncoupler commonly used to induce Parkin translocation to mitochondria. CCCP also induces microtubule depolymerization and increase in cytoplasmic Ca^{2+} concentration. Since Ca^{2+} regulates many processes in the cell, including protein trafficking and localization, we adressed whether Parkin translocation could be influenced by Ca^{2+}. Indeed, we found that treatment with Ca^{2+} chelator BAPTA prior to CCCP treatment, impaired Parkin translocation (Suppl. 1A), suggesting that Ca^{2+} is required for Parkin recruitment to mitochondria.

Sustained cytoplasmic Ca^{2+} concentration activates CaN, a Ca^{2+} dependent protein phosphatase which activity has been reported to regulate mitochondrial translocation of profission protein Drp1[7]. With that in mind, we addressed whether this could also be the case for Parkin translocation to. Indeed, both chemical and genetic inhibition of CaN prevented Parkin recruitment to mitochondria upon CCCP treatment (Suppl. 1B, Fig. 1C-D). Moreover, expression of a constitutive active mutant of CaN triggered Parkin translocation *per se*, independently of CCCP intoxication (Fig. 3A-B) or PINK1 (Fig. 3D-E). This evidence suggests that CaN activity is necessary and sufficient to induce Parkin recruitment downstream PINK1 (Fig. 2A-B).

The role of PINK1 in Parkin activation and translocation is still unclear. It has been shown that they act within the same genetic pathway, with PINK1 upstream of Parkin, but it is still unclear if PINK1 is sufficient for Parkin translocation[8]. Recent works, demonstrated that Parkin and Ubiquitin are PINK1 substrates and their interaction is necessary for Parkin ubiquitin ligase activity on its mitochondrial targets[9-11]. We demonstrated that PINK1 mediated phosphorylation of Parkin and Ubiquitin is necessary but not sufficient to trigger Parkin translocation. Indeed, in cells expressing phospho-mimetic Ubiquitin, phospho-mimetic Parkin is not constitutively expressed on mitochondria (Fig. Suppl. 2A), indicating that PINK1-dependent phosphorylation of Parkin and Ubiquitin might not be sufficient for Parkin recruitment. On the other hands, we showed that expression of a constitutive active form of CaN is sufficient to induce Parkin translocation, independently of CCCP intoxication or PINK1 (Fig. 3D-E).

Based on this, we hypothesized that either a pharmacological or a genetic approach that enhance CaN activity would be beneficial in ameliorating PINK1 knock out phenotype by bypassing PINK1 loss and activating Parkin dependent mitochondria quality control.

As previously reported, Parkin overexpression in PINK1 mutant (knock out) background suppress PINK1 mutant phenotype *in vivo*. Likewise, enhancement of CaN activity would rescue PINK1 mutant phenotype *in vivo*, by enhancing Parkin recruitment and activity downstream PINK1. Indeed, we found that PINK1 mutant (knock out) climbing ability was rescued upon expression of CaN constitutive active mutant (Fig. 4B).

Thus, our results indicate that CaN plays a fundamental, indispensable role in the control of Parkin translocation and Parkin-dependent mitophagy *in vitro* and that its activation has a physiological impact in an *in vivo* PINK1 model of PD.

By exploring the role of CaN in Parkin translocation and stress induced mitophagy and *in vivo* in a PINK1 model of PD, this work ultimately identified a novel druggable target and has the potential to widen up medical intervention for the treatment of PD.

6. References

1. Kwon KY, Viollet B, Yoo OJ. CCCP induces autophagy in an AMPK-independent manner. *Biochem Biophys Res Commun*. 2011;416(3-4):343-348.

2. Vives-Bauza C, Zhou C, Huang Y, et al. PINK1-dependent recruitment of parkin to mitochondria in mitophagy. *Proc Natl Acad Sci U S A*. 2010;107(1):378-383.

3. Margulis L. Symbiotic theory of the origin of eukaryotic organelles; criteria for proof. *Symp Soc Exp Biol*. 1975;(29)(29):21-38.

4. Martin W, Herrmann RG. Gene transfer from organelles to the nucleus: How much, what happens, and why? *Plant Physiol*. 1998;118(1):9-17.

5. PALADE GE. The fine structure of mitochondria. *Anat Rec*. 1952;114(3):427-451.

6. PALADE GE. An electron microscope study of the mitochondrial structure. *J Histochem Cytochem*. 1953;1(4):188-211.

7. Frey TG, Mannella CA. The internal structure of mitochondria. *Trends Biochem Sci*. 2000;25(7):319-324.

8. Bereiter-Hahn J, Voth M. Dynamics of mitochondria in living cells: Shape changes, dislocations, fusion, and fission of mitochondria. *Microsc Res Tech*. 1994;27(3):198-219.

9. Smirnova E, Griparic L, Shurland DL, van der Bliek AM. Dynamin-related protein Drp1 is required for mitochondrial division in mammalian cells. *Mol Biol Cell*. 2001;12(8):2245-2256.

10. Cereghetti GM, Stangherlin A, Martins de Brito O, et al. Dephosphorylation by calcineurin regulates translocation of Drp1 to mitochondria. *Proc Natl Acad Sci U S A*. 2008;105(41):15803-15808.

11. Otera H, Ishihara N, Mihara K. New insights into the function and regulation of mitochondrial fission. *Biochim Biophys Acta*. 2013;1833(5):1256-1268.

12. Loson OC, Song Z, Chen H, Chan DC. Fis1, mff, MiD49, and MiD51 mediate Drp1 recruitment in mitochondrial fission. *Mol Biol Cell*. 2013;24(5):659-667.

13. Friedman JR, Lackner LL, West M, DiBenedetto JR, Nunnari J, Voeltz GK. ER tubules mark sites of mitochondrial division. *Science*. 2011;334(6054):358-362.

14. Hales KG, Fuller MT. Developmentally regulated mitochondrial fusion mediated by a conserved, novel, predicted GTPase. *Cell*. 1997;90(1):121-129.

15. Deng H, Dodson MW, Huang H, Guo M. The parkinson's disease genes pink1 and parkin promote mitochondrial fission and/or inhibit fusion in drosophila. *Proc Natl Acad Sci U S A*. 2008;105(38):14503-14508.

16. Rojo M, Legros F, Chateau D, Lombes A. Membrane topology and mitochondrial targeting of mitofusins, ubiquitous mammalian homologs of the transmembrane GTPase fzo. *J Cell Sci*. 2002;115(Pt 8):1663-1674.

17. Chen H, Detmer SA, Ewald AJ, Griffin EE, Fraser SE, Chan DC. Mitofusins Mfn1 and Mfn2 coordinately regulate mitochondrial fusion and are essential for embryonic development. *J Cell Biol*. 2003;160(2):189-200.

18. Legros F, Lombes A, Frachon P, Rojo M. Mitochondrial fusion in human cells is efficient, requires the inner membrane potential, and is mediated by mitofusins. *Mol Biol Cell*. 2002;13(12):4343-4354.

19. Ishihara N, Eura Y, Mihara K. Mitofusin 1 and 2 play distinct roles in mitochondrial fusion reactions via GTPase activity. *J Cell Sci*. 2004;117(Pt 26):6535-6546.

20. Eura Y, Ishihara N, Yokota S, Mihara K. Two mitofusin proteins, mammalian homologues of FZO, with distinct functions are both required for mitochondrial fusion. *J Biochem*. 2003;134(3):333-344.

21. de Brito OM, Scorrano L. Mitofusin 2 tethers endoplasmic reticulum to mitochondria. *Nature*. 2008;456(7222):605-610.

22. Delettre C, Lenaers G, Griffoin JM, et al. Nuclear gene OPA1, encoding a mitochondrial dynamin-related protein, is mutated in dominant optic atrophy. *Nat Genet*. 2000;26(2):207-210.

23. Delettre C, Lenaers G, Pelloquin L, Belenguer P, Hamel CP. OPA1 (kjer type) dominant optic atrophy: A novel mitochondrial disease. *Mol Genet Metab*. 2002;75(2):97-107.

24. Alexander C, Votruba M, Pesch UE, et al. OPA1, encoding a dynamin-related GTPase, is mutated in autosomal dominant optic atrophy linked to chromosome 3q28. *Nat Genet*. 2000;26(2):211-215.

25. Cipolat S, Martins de Brito O, Dal Zilio B, Scorrano L. OPA1 requires mitofusin 1 to promote mitochondrial fusion. *Proc Natl Acad Sci U S A*. 2004;101(45):15927-15932.

26. Misaka T, Miyashita T, Kubo Y. Primary structure of a dynamin-related mouse mitochondrial GTPase and its distribution in brain, subcellular localization, and effect on mitochondrial morphology. *J Biol Chem*. 2002;277(18):15834-15842.

27. Frezza C, Cipolat S, Martins de Brito O, et al. OPA1 controls apoptotic cristae remodeling independently from mitochondrial fusion. *Cell*. 2006;126(1):177-189.

28. Ishihara N, Fujita Y, Oka T, Mihara K. Regulation of mitochondrial morphology through proteolytic cleavage of OPA1. *EMBO J*. 2006;25(13):2966-2977.

29. Kasahara A, Scorrano L. Mitochondria: From cell death executioners to regulators of cell differentiation. *Trends Cell Biol*. 2014;24(12):761-770.

30. Youle RJ, Narendra DP. Mechanisms of mitophagy. *Nat Rev Mol Cell Biol*. 2011;12(1):9-14.

31. Olichon A, Landes T, Arnaune-Pelloquin L, et al. Effects of OPA1 mutations on mitochondrial morphology and apoptosis: Relevance to ADOA pathogenesis. *J Cell Physiol*. 2007;211(2):423-430.

32. Lawson VH, Graham BV, Flanigan KM. Clinical and electrophysiologic features of CMT2A with mutations in the mitofusin 2 gene. *Neurology*. 2005;65(2):197-204.

33. Schwarz TL. Mitochondrial trafficking in neurons. *Cold Spring Harb Perspect Biol*. 2013;5(6):10.1101/cshperspect.a011304.

34. Li Z, Okamoto K, Hayashi Y, Sheng M. The importance of dendritic mitochondria in the morphogenesis and plasticity of spines and synapses. *Cell*. 2004;119(6):873-887.

35. Ishihara N, Nomura M, Jofuku A, et al. Mitochondrial fission factor Drp1 is essential for embryonic development and synapse formation in mice. *Nat Cell Biol*. 2009;11(8):958-966.

36. Cogliati S, Frezza C, Soriano ME, et al. Mitochondrial cristae shape determines respiratory chain supercomplexes assembly and respiratory efficiency. *Cell*. 2013;155(1):160-171.

37. Bach D, Pich S, Soriano FX, et al. Mitofusin-2 determines mitochondrial network architecture and mitochondrial metabolism. A novel regulatory mechanism altered in obesity. *J Biol Chem*. 2003;278(19):17190-17197.

38. Chen KH, Guo X, Ma D, et al. Dysregulation of HSG triggers vascular proliferative disorders. *Nat Cell Biol*. 2004;6(9):872-883.

39. Chen Y, Dorn GW,2nd. PINK1-phosphorylated mitofusin 2 is a parkin receptor for culling damaged mitochondria. *Science*. 2013;340(6131):471-475.

40. Gegg ME, Cooper JM, Chau KY, Rojo M, Schapira AH, Taanman JW. Mitofusin 1 and mitofusin 2 are ubiquitinated in a PINK1/parkin-dependent manner upon induction of mitophagy. *Hum Mol Genet*. 2010;19(24):4861-4870.

41. Chen H, Chan DC. Physiological functions of mitochondrial fusion. *Ann N Y Acad Sci*. 2010;1201:21-25.

42. Marino G, Niso-Santano M, Baehrecke EH, Kroemer G. Self-consumption: The interplay of autophagy and apoptosis. *Nat Rev Mol Cell Biol*. 2014;15(2):81-94.

43. Mukhopadhyay S, Panda PK, Sinha N, Das DN, Bhutia SK. Autophagy and apoptosis: Where do they meet? *Apoptosis*. 2014;19(4):555-566.

44. Maiuri MC, Zalckvar E, Kimchi A, Kroemer G. Self-eating and self-killing: Crosstalk between autophagy and apoptosis. *Nat Rev Mol Cell Biol*. 2007;8(9):741-752.

45. Glick D, Barth S, Macleod KF. Autophagy: Cellular and molecular mechanisms. *J Pathol*. 2010;221(1):3-12.

46. Cuervo AM. Chaperone-mediated autophagy: Selectivity pays off. *Trends Endocrinol Metab*. 2010;21(3):142-150.

47. Orenstein SJ, Cuervo AM. Chaperone-mediated autophagy: Molecular mechanisms and physiological relevance. *Semin Cell Dev Biol*. 2010;21(7):719-726.

48. Chan EY. mTORC1 phosphorylates the ULK1-mAtg13-FIP200 autophagy regulatory complex. *Sci Signal*. 2009;2(84):pe51.

49. Cheong H, Yorimitsu T, Reggiori F, Legakis JE, Wang CW, Klionsky DJ. Atg17 regulates the magnitude of the autophagic response. *Mol Biol Cell*. 2005;16(7):3438-3453.

50. Levine B, Sinha S, Kroemer G. Bcl-2 family members: Dual regulators of apoptosis and autophagy. *Autophagy*. 2008;4(5):600-606.

51. Ichimura Y, Kirisako T, Takao T, et al. A ubiquitin-like system mediates protein lipidation. *Nature*. 2000;408(6811):488-492.

52. Mizushima N, Komatsu M. Autophagy: Renovation of cells and tissues. *Cell*. 2011;147(4):728-741.

53. Kageyama Y, Hoshijima M, Seo K, et al. Parkin-independent mitophagy requires Drp1 and maintains the integrity of mammalian heart and brain. *EMBO J*. 2014;33(23):2798-2813.

54. Narendra DP, Jin SM, Tanaka A, et al. PINK1 is selectively stabilized on impaired mitochondria to activate parkin. *PLoS Biol*. 2010;8(1):e1000298.

55. Koyano F, Matsuda N. Molecular mechanisms underlying PINK1 and parkin catalyzed ubiquitylation of substrates on damaged mitochondria. *Biochim Biophys Acta*. 2015.

56. Ziviani E, Tao RN, Whitworth AJ. Drosophila parkin requires PINK1 for mitochondrial translocation and ubiquitinates mitofusin. *Proc Natl Acad Sci U S A*. 2010;107(11):5018-5023.

57. Caulfield TR, Fiesel FC, Moussaud-Lamodiere EL, Dourado DF, Flores SC, Springer W. Phosphorylation by PINK1 releases the UBL domain and initializes the conformational opening of the E3 ubiquitin ligase parkin. *PLoS Comput Biol*. 2014;10(11):e1003935.

58. Kazlauskaite A, Kondapalli C, Gourlay R, et al. Parkin is activated by PINK1-dependent phosphorylation of ubiquitin at Ser65. *Biochem J*. 2014;460(1):127-139.

59. Kondapalli C, Kazlauskaite A, Zhang N, et al. PINK1 is activated by mitochondrial membrane potential depolarization and stimulates parkin E3 ligase activity by phosphorylating serine 65. *Open Biol*. 2012;2(5):120080.

60. Narendra D, Kane LA, Hauser DN, Fearnley IM, Youle RJ. p62/SQSTM1 is required for parkin-induced mitochondrial clustering but not mitophagy; VDAC1 is dispensable for both. *Autophagy*. 2010;6(8):1090-1106.

61. Hollville E, Carroll RG, Cullen SP, Martin SJ. Bcl-2 family proteins participate in mitochondrial quality control by regulating parkin/PINK1-dependent mitophagy. *Mol Cell*. 2014;55(3):451-466.

62. Van Humbeeck C, Cornelissen T, Hofkens H, et al. Parkin interacts with Ambra1 to induce mitophagy. *J Neurosci*. 2011;31(28):10249-10261.

63. Strappazzon F, Nazio F, Corrado M, et al. AMBRA1 is able to induce mitophagy via LC3 binding, regardless of PARKIN and p62/SQSTM1. *Cell Death Differ*. 2015;22(3):419-432.

64. Gomes LC, Di Benedetto G, Scorrano L. During autophagy mitochondria elongate, are spared from degradation and sustain cell viability. *Nat Cell Biol*. 2011;13(5):589-598.

65. Samii A, Nutt JG, Ransom BR. Parkinson's disease. *Lancet*. 2004;363(9423):1783-1793.

66. Huang Y, Halliday GM. Aspects of innate immunity and parkinson's disease. *Front Pharmacol*. 2012;3:33.

67. Langston JW, Ballard P, Tetrud JW, Irwin I. Chronic parkinsonism in humans due to a product of meperidine-analog synthesis. *Science*. 1983;219(4587):979-980.

68. Betarbet R, Sherer TB, MacKenzie G, Garcia-Osuna M, Panov AV, Greenamyre JT. Chronic systemic pesticide exposure reproduces features of parkinson's disease. *Nat Neurosci*. 2000;3(12):1301-1306.

69. Richardson JR, Quan Y, Sherer TB, Greenamyre JT, Miller GW. Paraquat neurotoxicity is distinct from that of MPTP and rotenone. *Toxicol Sci*. 2005;88(1):193-201.

70. Day BJ, Patel M, Calavetta L, Chang LY, Stamler JS. A mechanism of paraquat toxicity involving nitric oxide synthase. *Proc Natl Acad Sci U S A*. 1999;96(22):12760-12765.

71. Mizuno Y, Ohta S, Tanaka M, et al. Deficiencies in complex I subunits of the respiratory chain in parkinson's disease. *Biochem Biophys Res Commun*. 1989;163(3):1450-1455.

72. Parker WD,Jr, Boyson SJ, Parks JK. Abnormalities of the electron transport chain in idiopathic parkinson's disease. *Ann Neurol*. 1989;26(6):719-723.

73. Schapira AH, Cooper JM, Dexter D, Clark JB, Jenner P, Marsden CD. Mitochondrial complex I deficiency in parkinson's disease. *J Neurochem*. 1990;54(3):823-827.

74. Spillantini MG, Schmidt ML, Lee VM, Trojanowski JQ, Jakes R, Goedert M. Alpha-synuclein in lewy bodies. *Nature*. 1997;388(6645):839-840.

75. Dauer W, Przedborski S. Parkinson's disease: Mechanisms and models. *Neuron*. 2003;39(6):889-909.

76. Marsden CD. Neuromelanin and parkinson's disease. *J Neural Transm Suppl*. 1983;19:121-141.

77. Gibb WR, Lees AJ. The relevance of the lewy body to the pathogenesis of idiopathic parkinson's disease. *J Neurol Neurosurg Psychiatry*. 1988;51(6):745-752.

78. Pahwa R, Lyons KE. Treatment of early parkinson's disease. *Curr Opin Neurol*. 2014;27(4):442-449.

79. Giugni JC, Okun MS. Treatment of advanced parkinson's disease. *Curr Opin Neurol*. 2014;27(4):450-460.

80. Farrer M, Gwinn-Hardy K, Hutton M, Hardy J. The genetics of disorders with synuclein pathology and parkinsonism. *Hum Mol Genet*. 1999;8(10):1901-1905.

81. Farrer MJ. Genetics of parkinson disease: Paradigm shifts and future prospects. *Nat Rev Genet*. 2006;7(4):306-318.

82. Wood-Kaczmar A, Gandhi S, Wood NW. Understanding the molecular causes of parkinson's disease. *Trends Mol Med*. 2006;12(11):521-528.

83. Cookson MR. The biochemistry of parkinson's disease. *Annu Rev Biochem*. 2005;74:29-52.

84. Farrer M, Kachergus J, Forno L, et al. Comparison of kindreds with parkinsonism and alpha-synuclein genomic multiplications. *Ann Neurol*. 2004;55(2):174-179.

85. Lotharius J, Brundin P. Pathogenesis of parkinson's disease: Dopamine, vesicles and alpha-synuclein. *Nat Rev Neurosci*. 2002;3(12):932-942.

86. Sidhu A, Wersinger C, Moussa CE, Vernier P. The role of alpha-synuclein in both neuroprotection and neurodegeneration. *Ann N Y Acad Sci*. 2004;1035:250-270.

87. Conway KA, Harper JD, Lansbury PT,Jr. Fibrils formed in vitro from alpha-synuclein and two mutant forms linked to parkinson's disease are typical amyloid. *Biochemistry*. 2000;39(10):2552-2563.

88. Zimprich A, Biskup S, Leitner P, et al. Mutations in LRRK2 cause autosomal-dominant parkinsonism with pleomorphic pathology. *Neuron*. 2004;44(4):601-607.

89. Trinh J, Guella I, McKenzie M, et al. Novel LRRK2 mutations in parkinsonism. *Parkinsonism Relat Disord*. 2015;21(9):1119-1121.

90. Higashi S, Biskup S, West AB, et al. Localization of parkinson's disease-associated LRRK2 in normal and pathological human brain. *Brain Res*. 2007;1155:208-219.

91. Gloeckner CJ, Kinkl N, Schumacher A, et al. The parkinson disease causing LRRK2 mutation I2020T is associated with increased kinase activity. *Hum Mol Genet*. 2006;15(2):223-232.

92. Li JQ, Tan L, Yu JT. The role of the LRRK2 gene in parkinsonism. *Mol Neurodegener*. 2014;9:47-1326-9-47.

93. Bonifati V. Autosomal recessive parkinsonism. *Parkinsonism Relat Disord*. 2012;18 Suppl 1:S4-6.

94. Lucking CB, Durr A, Bonifati V, et al. Association between early-onset parkinson's disease and mutations in the parkin gene. *N Engl J Med*. 2000;342(21):1560-1567.

95. Doherty KM, Hardy J. Parkin disease and the lewy body conundrum. *Mov Disord*. 2013;28(6):702-704.

96. Wang D, Qian L, Xiong H, et al. Antioxidants protect PINK1-dependent dopaminergic neurons in drosophila. *Proc Natl Acad Sci U S A*. 2006;103(36):13520-13525.

97. Yang Y, Ouyang Y, Yang L, et al. Pink1 regulates mitochondrial dynamics through interaction with the fission/fusion machinery. *Proc Natl Acad Sci U S A*. 2008;105(19):7070-7075.

98. Dagda RK, Cherra SJ,3rd, Kulich SM, Tandon A, Park D, Chu CT. Loss of PINK1 function promotes mitophagy through effects on oxidative stress and mitochondrial fission. *J Biol Chem*. 2009;284(20):13843-13855.

99. Exner N, Treske B, Paquet D, et al. Loss-of-function of human PINK1 results in mitochondrial pathology and can be rescued by parkin. *J Neurosci*. 2007;27(45):12413-12418.

100. Samaranch L, Lorenzo-Betancor O, Arbelo JM, et al. PINK1-linked parkinsonism is associated with lewy body pathology. *Brain*. 2010;133(Pt 4):1128-1142.

101. Yokota T, Sugawara K, Ito K, Takahashi R, Ariga H, Mizusawa H. Down regulation of DJ-1 enhances cell death by oxidative stress, ER stress, and proteasome inhibition. *Biochem Biophys Res Commun*. 2003;312(4):1342-1348.

102. Taira T, Saito Y, Niki T, Iguchi-Ariga SM, Takahashi K, Ariga H. DJ-1 has a role in antioxidative stress to prevent cell death. *EMBO Rep*. 2004;5(2):213-218.

103. Miller DW, Ahmad R, Hague S, et al. L166P mutant DJ-1, causative for recessive parkinson's disease, is degraded through the ubiquitin-proteasome system. *J Biol Chem*. 2003;278(38):36588-36595.

104. Kahle PJ, Waak J, Gasser T. DJ-1 and prevention of oxidative stress in parkinson's disease and other age-related disorders. *Free Radic Biol Med*. 2009;47(10):1354-1361.

105. Zhang L, Shimoji M, Thomas B, et al. Mitochondrial localization of the parkinson's disease related protein DJ-1: Implications for pathogenesis. *Hum Mol Genet*. 2005;14(14):2063-2073.

106. Lev N, Barhum Y, Pilosof NS, et al. DJ-1 protects against dopamine toxicity: Implications for parkinson's disease and aging. *J Gerontol A Biol Sci Med Sci*. 2013;68(3):215-225.

107. Wakabayashi K, Tanji K, Odagiri S, Miki Y, Mori F, Takahashi H. The lewy body in parkinson's disease and related neurodegenerative disorders. *Mol Neurobiol*. 2013;47(2):495-508.

108. Deeg S, Gralle M, Sroka K, Bahr M, Wouters FS, Kermer P. BAG1 restores formation of functional DJ-1 L166P dimers and DJ-1 chaperone activity. *J Cell Biol*. 2010;188(4):505-513.

109. van der Merwe C, Jalali Sefid Dashti Z, Christoffels A, Loos B, Bardien S. Evidence for a common biological pathway linking three parkinson's disease-causing genes: Parkin, PINK1 and DJ-1. *Eur J Neurosci*. 2015;41(9):1113-1125.

110. Schon EA, Przedborski S. Mitochondria: The next (neurode)generation. *Neuron*. 2011;70(6):1033-1053.

111. Ramsay RR, Singer TP. Energy-dependent uptake of N-methyl-4-phenylpyridinium, the neurotoxic metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, by mitochondria. *J Biol Chem*. 1986;261(17):7585-7587.

112. Nicklas WJ, Vyas I, Heikkila RE. Inhibition of NADH-linked oxidation in brain mitochondria by 1-methyl-4-phenyl-pyridine, a metabolite of the neurotoxin, 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine. *Life Sci*. 1985;36(26):2503-2508.

113. Javitch JA, D'Amato RJ, Strittmatter SM, Snyder SH. Parkinsonism-inducing neurotoxin, N-methyl-4-phenyl-1,2,3,6 -tetrahydropyridine: Uptake of the metabolite N-methyl-4-phenylpyridine by dopamine neurons explains selective toxicity. *Proc Natl Acad Sci U S A*. 1985;82(7):2173-2177.

114. Moore DJ, Zhang L, Troncoso J, et al. Association of DJ-1 and parkin mediated by pathogenic DJ-1 mutations and oxidative stress. *Hum Mol Genet*. 2005;14(1):71-84.

115. Kitada T, Asakawa S, Hattori N, et al. Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. *Nature*. 1998;392(6676):605-608.

116. Thompson AJ, Scholz SW, Singleton AB, Hardwick A, McFarland NR, Okun MS. Variability in clinical phenotypes of heterozygous and homozygous cases of parkin-related parkinson's disease. *Int J Neurosci*. 2013;123(12):847-849.

117. Sharp ME, Marder KS, Cote L, et al. Parkinson's disease with lewy bodies associated with a heterozygous PARKIN dosage mutation. *Mov Disord*. 2014;29(4):566-568.

118. Sarraf SA, Raman M, Guarani-Pereira V, et al. Landscape of the PARKIN-dependent ubiquitylome in response to mitochondrial depolarization. *Nature*. 2013;496(7445):372-376.

119. Horowitz JM, Vernace VA, Myers J, et al. Immunodetection of parkin protein in vertebrate and invertebrate brains: A comparative study using specific antibodies. *J Chem Neuroanat*. 2001;21(1):75-93.

120. Ciechanover A. The ubiquitin-mediated system for intracellular protein degradation. *J Basic Clin Physiol Pharmacol*. 1991;2(3):141-159.

121. Kleiger G, Mayor T. Perilous journey: A tour of the ubiquitin–proteasome system. *Trends Cell Biol*. 2014;24(6):352-359.

122. Komander D. Mechanism, specificity and structure of the deubiquitinases. *Subcell Biochem*. 2010;54:69-87.

123. Dye BT, Schulman BA. Structural mechanisms underlying posttranslational modification by ubiquitin-like proteins. *Annu Rev Biophys Biomol Struct*. 2007;36:131-150.

124. Herhaus L, Dikic I. Expanding the ubiquitin code through post-translational modification. *EMBO Rep*. 2015;16(9):1071-1083.

125. Ciesla J, Fraczyk T, Rode W. Phosphorylation of basic amino acid residues in proteins: Important but easily missed. *Acta Biochim Pol*. 2011;58(2):137-148.

126. Manning G, Whyte DB, Martinez R, Hunter T, Sudarsanam S. The protein kinase complement of the human genome. *Science*. 2002;298(5600):1912-1934.

127. Barford D, Das AK, Egloff MP. The structure and mechanism of protein phosphatases: Insights into catalysis and regulation. *Annu Rev Biophys Biomol Struct*. 1998;27:133-164.

128. de Lavallade H, Apperley JF, Khorashad JS, et al. Imatinib for newly diagnosed patients with chronic myeloid leukemia: Incidence of sustained responses in an intention-to-treat analysis. *J Clin Oncol*. 2008;26(20):3358-3363.

129. Deremer DL, Ustun C, Natarajan K. Nilotinib: A second-generation tyrosine kinase inhibitor for the treatment of chronic myelogenous leukemia. *Clin Ther*. 2008;30(11):1956-1975.

130. Lonskaya I, Hebron ML, Desforges NM, Franjie A, Moussa CE. Tyrosine kinase inhibition increases functional parkin-beclin-1 interaction and enhances amyloid clearance and cognitive performance. *EMBO Mol Med*. 2013;5(8):1247-1262.

131. Seirafi M, Kozlov G, Gehring K. Parkin structure and function. *FEBS J*. 2015;282(11):2076-2088.

132. Riley BE, Lougheed JC, Callaway K, et al. Structure and function of parkin E3 ubiquitin ligase reveals aspects of RING and HECT ligases. *Nat Commun*. 2013;4:1982.

133. Moore DJ, West AB, Dawson VL, Dawson TM. Molecular pathophysiology of parkinson's disease. *Annu Rev Neurosci*. 2005;28:57-87.

134. Wenzel DM, Lissounov A, Brzovic PS, Klevit RE. UBCH7 reactivity profile reveals parkin and HHARI to be RING/HECT hybrids. *Nature*. 2011;474(7349):105-108.

135. Ordureau A, Sarraf SA, Duda DM, et al. Quantitative proteomics reveal a feedforward mechanism for mitochondrial PARKIN translocation and ubiquitin chain synthesis. *Mol Cell*. 2014;56(3):360-375.

136. Durcan TM, Tang MY, Perusse JR, et al. USP8 regulates mitophagy by removing K6-linked ubiquitin conjugates from parkin. *EMBO J*. 2014;33(21):2473-2491.

137. Trempe JF, Sauve V, Grenier K, et al. Structure of parkin reveals mechanisms for ubiquitin ligase activation. *Science*. 2013;340(6139):1451-1455.

138. Wauer T, Komander D. Structure of the human parkin ligase domain in an autoinhibited state. *EMBO J*. 2013;32(15):2099-2112.

139. Kane LA, Lazarou M, Fogel AI, et al. PINK1 phosphorylates ubiquitin to activate parkin E3 ubiquitin ligase activity. *J Cell Biol*. 2014;205(2):143-153.

140. Stolz A, Dikic I. PINK1-PARKIN interplay: Down to ubiquitin phosphorylation. *Mol Cell*. 2014;56(3):341-342.

141. Rott R, Szargel R, Haskin J, et al. Alpha-synuclein fate is determined by USP9X-regulated monoubiquitination. *Proc Natl Acad Sci U S A*. 2011;108(46):18666-18671.

142. Durcan TM, Kontogiannea M, Bedard N, Wing SS, Fon EA. Ataxin-3 deubiquitination is coupled to parkin ubiquitination via E2 ubiquitin-conjugating enzyme. *J Biol Chem*. 2012;287(1):531-541.

143. Durcan TM, Fon EA. Ataxin-3 and its e3 partners: Implications for machado-joseph disease. *Front Neurol*. 2013;4:46.

144. Dikic I, Bremm A. DUBs counteract parkin for efficient mitophagy. *EMBO J*. 2014;33(21):2442-2443.

145. Nardin A, Schrepfer E, Ziviani E. Counteracting PINK/parkin deficiency in the activation of mitophagy: A potential therapeutic intervention for parkinson's disease. *Curr Neuropharmacol*. 2015.

146. Hattori N, Kitada T, Matsumine H, et al. Molecular genetic analysis of a novel parkin gene in japanese families with autosomal recessive juvenile parkinsonism: Evidence for variable homozygous deletions in the parkin gene in affected individuals. *Ann Neurol*. 1998;44(6):935-941.

147. Fiesel FC, Caulfield TR, Moussaud-Lamodiere EL, et al. Structural and functional impact of parkinson disease-associated mutations in the E3 ubiquitin ligase parkin. *Hum Mutat*. 2015;36(8):774-786.

148. Cruts M, Theuns J, Van Broeckhoven C. Locus-specific mutation databases for neurodegenerative brain diseases. *Hum Mutat*. 2012;33(9):1340-1344.

149. Ardley HC, Tan NG, Rose SA, Markham AF, Robinson PA. Features of the parkin/ariadne-like ubiquitin ligase, HHARI, that regulate its interaction with the ubiquitin-conjugating enzyme, Ubch7. *J Biol Chem*. 2001;276(22):19640-19647.

150. Mengesdorf T, Jensen PH, Mies G, Aufenberg C, Paschen W. Down-regulation of parkin protein in transient focal cerebral ischemia: A link between stroke and degenerative disease? *Proc Natl Acad Sci U S A*. 2002;99(23):15042-15047.

151. Clark IE, Dodson MW, Jiang C, et al. Drosophila pink1 is required for mitochondrial function and interacts genetically with parkin. *Nature*. 2006;441(7097):1162-1166.

152. Tan JM, Dawson TM. Parkin blushed by PINK1. *Neuron*. 2006;50(4):527-529.

153. Park J, Lee SB, Lee S, et al. Mitochondrial dysfunction in drosophila PINK1 mutants is complemented by parkin. *Nature*. 2006;441(7097):1157-1161.

154. Poole AC, Thomas RE, Andrews LA, McBride HM, Whitworth AJ, Pallanck LJ. The PINK1/parkin pathway regulates mitochondrial morphology. *Proc Natl Acad Sci U S A*. 2008;105(5):1638-1643.

155. Muller-Rischart AK, Pilsl A, Beaudette P, et al. The E3 ligase parkin maintains mitochondrial integrity by increasing linear ubiquitination of NEMO. *Mol Cell*. 2013;49(5):908-921.

156. Wang H, Song P, Du L, et al. Parkin ubiquitinates Drp1 for proteasome-dependent degradation: Implication of dysregulated mitochondrial dynamics in parkinson disease. *J Biol Chem*. 2011;286(13):11649-11658.

157. Narendra D, Tanaka A, Suen DF, Youle RJ. Parkin is recruited selectively to impaired mitochondria and promotes their autophagy. *J Cell Biol*. 2008;183(5):795-803.

158. Jin SM, Lazarou M, Wang C, Kane LA, Narendra DP, Youle RJ. Mitochondrial membrane potential regulates PINK1 import and proteolytic destabilization by PARL. *J Cell Biol*. 2010;191(5):933-942.

159. Kim Y, Park J, Kim S, et al. PINK1 controls mitochondrial localization of parkin through direct phosphorylation. *Biochem Biophys Res Commun*. 2008;377(3):975-980.

160. Sauvé V, Gehring K. Phosphorylated ubiquitin: A new shade of PINK1 in parkin activation. *Cell Res*. 2014.

161. Ziviani E, Whitworth AJ. How could parkin-mediated ubiquitination of mitofusin promote mitophagy? *Autophagy*. 2010;6(5):660-662.

162. Blandini F, Armentero MT. Animal models of parkinson's disease. *FEBS J*. 2012;279(7):1156-1166.

163. Ungerstedt U, Ljungberg T, Steg G. Behavioral, physiological, and neurochemical changes after 6-hydroxydopamine-induced degeneration of the nigro-striatal dopamine neurons. *Adv Neurol*. 1974;5:421-426.

164. Hunter RL, Cheng B, Choi DY, et al. Intrastriatal lipopolysaccharide injection induces parkinsonism in C57/B6 mice. *J Neurosci Res*. 2009;87(8):1913-1921.

165. Chesselet MF. In vivo alpha-synuclein overexpression in rodents: A useful model of parkinson's disease? *Exp Neurol*. 2008;209(1):22-27.

166. Lam HA, Wu N, Cely I, et al. Elevated tonic extracellular dopamine concentration and altered dopamine modulation of synaptic activity precede dopamine loss in the striatum of mice overexpressing human alpha-synuclein. *J Neurosci Res*. 2011;89(7):1091-1102.

167. Li X, Patel JC, Wang J, et al. Enhanced striatal dopamine transmission and motor performance with LRRK2 overexpression in mice is eliminated by familial parkinson's disease mutation G2019S. *J Neurosci*. 2010;30(5):1788-1797.

168. Gautier CA, Kitada T, Shen J. Loss of PINK1 causes mitochondrial functional defects and increased sensitivity to oxidative stress. *Proc Natl Acad Sci U S A*. 2008;105(32):11364-11369.

169. Casarejos MJ, Solano RM, Menendez J, et al. Differential effects of l-DOPA on monoamine metabolism, cell survival and glutathione production in midbrain neuronal-enriched cultures from parkin knockout and wild-type mice. *J Neurochem*. 2005;94(4):1005-1014.

170. Casarejos MJ, Menendez J, Solano RM, Rodriguez-Navarro JA, Garcia de Yebenes J, Mena MA. Susceptibility to rotenone is increased in neurons from parkin null mice and is reduced by minocycline. *J Neurochem*. 2006;97(4):934-946.

171. Paterna JC, Leng A, Weber E, Feldon J, Bueler H. DJ-1 and parkin modulate dopamine-dependent behavior and inhibit MPTP-induced nigral dopamine neuron loss in mice. *Mol Ther*. 2007;15(4):698-704.

172. Shin JH, Ko HS, Kang H, et al. PARIS (ZNF746) repression of PGC-1alpha contributes to neurodegeneration in parkinson's disease. *Cell*. 2011;144(5):689-702.

173. Von Stockum S, Nardin A, Schrepfer E, Ziviani E. Mitochondrial dynamics and mitophagy in parkinson's disease: A fly point of view. *Neurobiol Dis*. 2015.

174. Reiter LT, Potocki L, Chien S, Gribskov M, Bier E. A systematic analysis of human disease-associated gene sequences in drosophila melanogaster. *Genome Res*. 2001;11(6):1114-1125.

175. St Johnston D. The art and design of genetic screens: Drosophila melanogaster. *Nat Rev Genet*. 2002;3(3):176-188.

176. Adams MD, Sekelsky JJ. From sequence to phenotype: Reverse genetics in drosophila melanogaster. *Nat Rev Genet*. 2002;3(3):189-198.

177. Rubin GM, Spradling AC. Genetic transformation of drosophila with transposable element vectors. *Science*. 1982;218(4570):348-353.

178. Brand AH, Perrimon N. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development*. 1993;118(2):401-415.

179. Venken KJ, Schulze KL, Haelterman NA, et al. MiMIC: A highly versatile transposon insertion resource for engineering drosophila melanogaster genes. *Nat Methods*. 2011;8(9):737-743.

180. Chang S, Bray SM, Li Z, et al. Identification of small molecules rescuing fragile X syndrome phenotypes in drosophila. *Nat Chem Biol*. 2008;4(4):256-263.

181. Feany MB, Bender WW. A drosophila model of parkinson's disease. *Nature*. 2000;404(6776):394-398.

182. Clark IE, Dodson MW, Jiang C, et al. Drosophila pink1 is required for mitochondrial function and interacts genetically with parkin. *Nature*. 2006;441(7097):1162-1166.

183. Whitworth AJ. Drosophila models of parkinson's disease. *Adv Genet*. 2011;73:1-50.

184. Greene JC, Whitworth AJ, Kuo I, Andrews LA, Feany MB, Pallanck LJ. Mitochondrial pathology and apoptotic muscle degeneration in drosophila parkin mutants. *Proc Natl Acad Sci U S A*. 2003;100(7):4078-4083.

185. Whitworth AJ, Lee JR, Ho VM, Flick R, Chowdhury R, McQuibban GA. Rhomboid-7 and HtrA2/omi act in a common pathway with the parkinson's disease factors Pink1 and parkin. *Dis Model Mech*. 2008;1(2-3):168-74; discussion 173.

186. Tain LS, Chowdhury RB, Tao RN, et al. Drosophila HtrA2 is dispensable for apoptosis but acts downstream of PINK1 independently from parkin. *Cell Death Differ*. 2009;16(8):1118-1125.

187. Chao JR, Parganas E, Boyd K, Hong CY, Opferman JT, Ihle JN. Hax1-mediated processing of HtrA2 by parl allows survival of lymphocytes and neurons. *Nature*. 2008;452(7183):98-102.

188. Seibler P, Graziotto J, Jeong H, Simunovic F, Klein C, Krainc D. Mitochondrial parkin recruitment is impaired in neurons derived from mutant PINK1 induced pluripotent stem cells. *J Neurosci*. 2011;31(16):5970-5976.

189. Mortiboys H, Thomas KJ, Koopman WJ, et al. Mitochondrial function and morphology are impaired in parkin-mutant fibroblasts. *Ann Neurol*. 2008;64(5):555-565.

190. Vincow ES, Merrihew G, Thomas RE, et al. The PINK1-parkin pathway promotes both mitophagy and selective respiratory chain turnover in vivo. *Proc Natl Acad Sci U S A*. 2013;110(16):6400-6405.

191. Pogson JH, Ivatt RM, Sanchez-Martinez A, et al. The complex I subunit NDUFA10 selectively rescues drosophila pink1 mutants through a mechanism independent of mitophagy. *PLoS Genet*. 2014;10(11):e1004815.

192. Liu W, Acin-Perez R, Geghman KD, Manfredi G, Lu B, Li C. Pink1 regulates the oxidative phosphorylation machinery via mitochondrial fission. *Proc Natl Acad Sci U S A*. 2011;108(31):12920-12924.

193. Narendra D, Walker JE, Youle R. Mitochondrial quality control mediated by PINK1 and parkin: Links to parkinsonism. *Cold Spring Harb Perspect Biol*. 2012;4(11):10.1101/cshperspect.a011338.

194. Chan NC, Salazar AM, Pham AH, et al. Broad activation of the ubiquitin-proteasome system by parkin is critical for mitophagy. *Hum Mol Genet*. 2011;20(9):1726-1737.

195. Winklhofer KF. Parkin and mitochondrial quality control: Toward assembling the puzzle. *Trends Cell Biol*. 2014;24(6):332-341.

196. Narendra D, Tanaka A, Suen DF, Youle RJ. Parkin is recruited selectively to impaired mitochondria and promotes their autophagy. *J Cell Biol*. 2008;183(5):795-803.

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Counteracting PINK/Parkin Deficiency in the Activation of Mitophagy: A Potential Therapeutic Intervention for Parkinson's Disease

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Abstract: Parkinson's Disease (PD) related genes PINK1, a protein kinase [1], and Parkin, an E3 ubiquitin ligase [2], operate within the same pathway [3-5], which controls, *via* specific elimination of dysfunctional mitochondria, the quality of the organelle network [6]. Parkin translocates to impaired mitochondria and drives their elimination *via* autophagy, a process known as mitophagy [6]. PINK1 regulates Parkin translocation through a not yet completely understood mechanism [7, 8]. Mitochondrial outer membrane proteins Mitofusin (MFN), VDAC, Fis1 and TOM20 were found to be targets for Parkin mediated ubiquitination [9-11]. By adding ubiquitin molecules to its targets expressed on mitochondria, Parkin tags and selects dysfunctional mitochondria for clearance, contributing to maintain a functional and healthy mitochondrial network. Abnormal accumulation of misfolded proteins and unfunctional mitochondria is a characteristic hallmark of PD pathology. Therefore a therapeutic approach to enhance clearance of misfolded proteins and potentiate the ubiquitin-proteosome system (UPS) could be instrumental to ameliorate the progression of the disease. Recently, much effort has been put to identify specific de-ubiquitinating enzymes (DUBs) that oppose Parkin in the ubiquitination of its targets. Similar to other post-translational modifications, such as phosphorylation and acetylation, ubiquitination is also a reversible modification, mediated by a large family of DUBs [12]. DUBs inhibitors or activators can affect cellular response to stimuli that induce mitophagy *via* ubiquitination of mitochondrial outer membrane proteins MFN, VDAC, Fis1 and TOM20. In this respect, the identification of a Parkin-opposing DUB in the regulation of mitophagy, might be instrumental to develop specific isopeptidase inhibitors or activators that can modulate the fundamental biological process of mitochondria clearance and impact on cell survival.

Keywords: Drosophila, DUB, mitofusin, mitophagy, parkin, parkinson's disease, PINK1, ubiquitination.

THE NUMBERS OF PD

Parkinson's disease (PD) is the second most common neurodegenerative disease for which there is no cure. It is characterized by selective loss of dopaminergic neurons (DA) in the *substantia nigra* (SN) *pars compacta* and specific hallmark include accumulation of aggregates and unfolded proteins in the form of Lewy bodies. PD is a movement disorders with patients developing resting tremors, bradikinesia, muscle rigidity, postural instability and gait problems. It affects 1-2% of the population over the age of 65 and this percentage increases by approximately 4% in those older that 85 years [13]. Life expectancy has risen in developed countries from about 47 at the beginning of the last century to about 80 today and it is likely to increase even more, thanks to improving medical care and intervention. However, with the increased life expectancy worldwide, an increasing number of people will develop PD, which will socially and economically impact public healthcare and the future of modern society.

Nowadays, most of the treatment strategies for PD are based on the administration of dopamine, to compensate the lack of dopamine release from DA neurons [14]. However, these treatment strategies can alleviate the symptoms of the disease, but they can not stop or slow down the neuronal degeneration.

FAMILIAR FORMS OF PD AND THEIR GENETICS

Although most PD cases are sporadic and the exact cause for the disease onset is unknown, a small percentage of PD cases is genetically linked and shows an earlier manifestation [15]. Since the phenotypes of both sporadic and familiar cases are indistinguishable at the level of DA degeneration, the genetic cases, although rare, can provide the basis for a better understanding of the molecular pathways underling the disease and be instrumental to tackle the disease and potentially find a cure [16].

After the identification of SNCA gene, encoding for α-synuclein, that causes familiar forms of PD, many other genes have been discovered, which cause inherited PD and account for 10% of PD cases. Until now, several loci responsible genes for PD have been identified, and for six of them, the corresponding genes have been characterized. Four loci (Park1/4, Park3, Park5 and Park8) have been associated with autosomal dominant forms of PD, whereas Park2, Park6, Park7 and Park9 have been associated with autosomal recessive forms. Although no corresponding gene is known

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for loci Park3 and Park9, the other loci have been associated to α-synuclein gene (Park1/4), Parkin (Park2), UCHL1 (Park5), PINK1 (Park6), DJ1 (Park7) and LRRK2 (Park8), respectively [17].

Dominant Genes

Park1/4, one of the most common inherited forms of PD, is linked to parkinsonism caused by missense mutations and amplifications of α-synuclein and has been associated with autosomal dominant forms of PD [18]. This protein is expressed throughout the brain and is involved in learning, synaptic plasticity, vesicle dynamics and dopamine synthesis. The wild type protein is a potent inhibitor of phospholipase D2, which is involved in signal transduction, membrane vesicle trafficking and cytoskeletal dynamics. Considering how neurons rely on vesicular trafficking for their survival, functional α-synuclein is crucial for neuronal survival. Interestingly, due to its hydrophobic central region, this protein has naturally a high propensity to aggregate that is accentuated in mutants. Mutant forms of these proteins easily aggregate in neuronal cells *in vitro* and *in vivo*, initially forming an intermediate annular structure, and ultimately forming insoluble polymers or fibrils, which are the main constituents of the Lewy bodies, one of the most common histological hallmarks of PD.

Park8 has been identified as the leucine rich repeat kinase 2 gene (LRRK2). This is the most common form of inherited PD and the clinical features are similar to those of sporadic PD, except for the earlier onset age [19]. Until now, 20 missense or nonsense mutations have been reported. This gene encodes for an extremely large protein of 250 kDa, containing many different functional domains and it is highly expressed in the brain. It was reported to interact with Parkin [20], but it also genetically interacts with PINK1 and DJ-1 [21]. The G2019S mutation in the LRRK2 gene has been reported a number of times and appears to be one of the most common LRRK2 related mutations, accounting for 3–7% familial PD and 1–1.6% of so-called 'sporadic PD'. Mutations in this gene inhibit an endogenous peroxidase promoting dysregulation of mitochondrial function and oxidative damage.

Recessive Genes

Recessive form of parkinsonism is known to be caused by mutations in parkin (Park2), PINK1 (Park6) and DJ-1 (Park7) genes. These are all relatively rare loss-of-function mutations that result in an early age of onset and slow disease progression.

DJ-1 mutations were firstly found in an Italian and a Dutch family and linked to autosomal-recessive forms of PD. After that, only one other mutation in a Uruguayan family has been identified [22]. DJ-I is almost ubiquitously expressed in organs, and it is present in synaptic terminals, mitochondria and membranous organelles [23]. The normal function of DJ-1 and its role in dopamine cell degeneration is unknown, but this protein is linked to oxidative stress response and mitochondrial function [24]. It has also reported that this gene has a role as tumor suppressor [25]. DJ-I protein was detected around Lewy bodies, but not as part of these. Several evidences suggested that DJ-I function as a dimer and analysis of the pathogenic Lys166Pro mutation showed that the dimer is less stable and an ectopic expression of this mutant is rapidly degraded [26].

Park6 gene was identified as PINK1 (PTEN induced kinase 1), a serine/threonine protein kinase that contains a Mitochondria Targeting Sequence (MTS) and localizes to mitochondria [27]. Mutations in this gene are much less common than mutations in the DJ1 or parkin gene. This protein is ubiquitously expressed and contains a serine/ threonine kinase domain. Its function is to regulate mitochondrial dynamics and respiratory functions [28]. Interestingly, mitochondrial shape and dynamic is affected in PINK1 lacking cells, although literature does not always agree on the effect of PINK1 downregulation or overexpression upon mitochondria shape. Mutations in PINK1 have differential effects on protein stability, localization and kinase activity [29]. PINK1 associated cases of PD show a broad phenotypic spectrum, spanning from an early manifestation with atypical symptoms to late manifestation with the typical clinical PD symptoms.

Mutations in the parkin gene (Park2) are the most common among the three recessive forms of parkinsonism, and this gene was the first associated with recessive form of PD [30]. The gene codifies for an E3 ubiquitin ligase that is normally expressed in the cytoplasm, but translocates to mitochondria upon specific stimulation. More than 40 mutations have been identified, but only a weak correlation between clinical manifestation and type of mutation has been pointed out [31]. Of note, mutations in Parkin are not typically associated with the formation of Lewy bodies and α-synuclein aggregates.

PARKINSON'S DISEASE AND MITOCHONDRIA DYSFUNCTION

Most of the proteins encoded by Parkinson's related genes, are linked to mitochondria and they have a role in protecting against some form of mitochondrial dysfunction and oxidative stress [32]. Some of them, like PINK1, are expressed on mitochondria and actively regulates mitochondria activity and fitness [1]. Others, like Parkin, are targeted to mitochondria upon specific stimulation, and select a subset of dysfunctional mitochondria for degradation [6]. DJ1 localizes to mitochondria during oxidative stress [33]. α-synuclein affects mitochondria function by interacting with mitochondria and enhancing mitochondria susceptibility to toxins, like rotenone, that interfere with electron transport chain creating build-up of electrons in the matrix and formation of reactive oxygen species (ROS) [34, 35].

Indeed, mitochondrial dysfunction is strongly implicated in the etiology of the disease and impaired mitochondria are found in animal and cellular models of PD. Body of evidences suggests that mitochondria dysfunction and subsequent oxidative stress causes the onset of PD.

Fission to Segregate; Fusion to Mitigate

Mitochondria are double membrane-bound organelles, which are responsible for multiple cellular events, including energy conversion [36, 37], Calcium (Ca^{2+}) signaling [38]

and amplification of programmed cells death cascade [39]. The most intriguing thing about mitochondria is that they are extremely dynamic and they frequently divide (mitochondria fission) and fuse (mitochondria fusion), changing size and shape and subcellular location [40]. Intuitively, mitochondria undergo division to populate new cells with new organelles; mitochondria fusion is on the other hands required to preserve the mitochondria network and allow intermixing of mitochondria matrix content (including mitochondria DNA) to preserve mitochondria function [41].

Mitochondria shape and localization are not random and directly correlated to mitochondria activity and fitness. In this respect, regulation of mitochondria fission and fusion events is required to respond to changes in metabolism. This is supported by the observations that mitochondria elongate in times of nutrient deprivation [42] or to boost oxidative phosphorylation.

Mitochondria fission and fusion is particularly important under stressful conditions: fusion between damaged mitochondria blends oxidative stress into the mitochondrial network and functionally compensate for potential damage [43]; fission is required to facilitate the removal of dysfunctional, damaged mitochondria. A pivotal study demonstrated how mitochondria fission often generates an asymmetric division where one daughter exhibits higher membrane potential and has better probability to undergoes fusion, while the other has lower membrane potential, does not fuse and it is more likely to be eliminated *via* mitophagy [41]. This work suggested that fission followed by selective fusion segregates dysfunctional mitochondria for degradation. In this respect, impairment of the fission machinery inhibits mitophagy.

Core components of the fission and fusion machinery are pro-fusion members dynamin related GTPases optic atrophy 1 (Opa1) and Mitofusin (Mfn), and pro-fission members dynamin like protein 1 (Drp1) and Fis1 [44]. Mitochondria fusion is achieved upon the coordinated activity of Mfn and Opa1 [45]. Mfn is a transmembrane GTPase embedded in the outer mitochondrial membrane, which is required on adjacent organelles to mediate the fusion of outer mitochondrial membrane. Opa1 is expressed on the inner mitochondrial membrane and regulates inner membrane fusion [46]. Mfn and Opa1 are eclectic proteins that have broader functions, despite their involvement in mitochondria fusion. For example, in mammals, while Mfn1 participates in the mitochondrial fusion reaction, in coordination with Opa1, Mfn2 forms complexes that are capable of tethering mitochondria to endoplasmic reticulum (ER), a structural feature essential for lipid synthesis, mitochondrial energy metabolism, Calcium (Ca^{2+}) transfer between the organelles and Ca^{2+} dependent cell death [47]. Opa1 also has genetically distinguishable functions in mitochondria fusion and mitochondria cristae remodeling [48, 49], an ultrastuctural feature that allows the intramitochondrial redistribution of cytochrome c that is contained inside the mitochondrial cristae pockets. Opa1 functions as a molecular staple that keeps the mitochondria cristae junctions tight and its activity is required in the control of cristae junctions size upon induction of apoptosis [48].

To oppose fusion, Drp1, MFF (mitochondrial fission factor) and Fis1 have been found to be key components of the mammalian mitochondrial fission machinery. The large GTPase Drp1 is a dynamin-related protein that is expressed in the cytosol. A fraction of this protein is localized in spots on mitochondria, and a subset of these spots mark future fission sites in coordination with the endoplasmic reticulum. Upon induction of mitochondria fission, intermolecular oligomerization of Drp1 into ring-like structures occurs at membrane constriction sites [50]. Fis1 and MFF operate as Drp1 receptor on mitochondria outer membrane [51-53].

Pathogenesis of the Disease: The Mitochondrial Hypothesis

The earliest hypothesis of PD pathogenesis was based on the finding that chemical inhibition of mitochondrial Complex I could reproduce Parkinsonism [54]. Indeed it results in selective dopaminergic neuron loss and it is widely used to create PD animal models. For instance, mitochondrial toxin rotenone that inhibits electron transfer from Complex I to ubiquinone, causes Parkinsonism [55]. Also, injection of MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), causes PD [56]: the product of metabolized MPTP, MPP+, inhibits Complex I of the electron transport chain and results in electron build up. The inhibition of Complex I has two major consequences: depletion of ATP and the generation of free radicals, with subsequent ROS formation that is toxic for the cell.

Supporting the hypothesis of a network of pathways converging to mitochondrial dysfunction, clear evidence of oxidative stress in postmortem PD brain and a reduction of Complex I activity has been described in organs of idiopathic PD patients [54]. Moreover, a primary role of mitochondrial respiratory chain impairment and consequent oxidative stress has also emerged from the study of rare familial forms of PD. PINK1 deficiency or disease-related PINK1 mutations, affects Complex I activity resulting in mitochondria depolarization and increased susceptibility to apoptotic stimuli [57]. General impaired respiration has also been observed in PINK1 deficient cells as a consequence of impaired delivery of respiratory chain substrates due to ROS dependent inhibition of glucose transporter [58]. Recently, a direct interaction between PINK1 and Complex I activity was described by Morais and co. workers, who showed that PINK1 dependent phosphorylation of Complex I on Serine-250 is a prerequisite for ubiquitinone reduction, thus unrevealing the biochemical link between PINK1 dysfunction and impaired respiration [59].

Of note, Parkin mutant fibroblasts from PD patients have also shown to have lower mitochondrial Complex I activity and ATP production, which was more markedly impaired when cells were forced to rely on oxidative phosphorylation rather than glycolysis to generate their ATP. These results are consistent with those seen in PINK1 deficient models and suggest that there might be a common pathway mediating recessive parkinsonism in humans as has been suggested from studies in *Drosophila* [60].

Interestingly, abnormalities in mitochondria shape, ultrastructure and subcellular localization, have been

described in models of both PINK1 and Parkin deficient cells [9, 60, 61] and enlarged and swollen mitochondria have been found in postmortem tissues from biopsy of PD patients. However, it is still not clear whether mitochondria structure abnormalities are a consequence of respiratory chain impairment, perhaps to compensate for electron leak and decreased ATP synthesis, or directly contribute to the etiology of the disease and precede respiration impairment. Of note, deficits in Complex I driven respiration and specific Complex I activity impairment are observed before any mitochondrial morphology alteration manifests, suggesting that mitochondria morphology abnormalities are a consequence of respiration defects [57]. Considering that mitochondria respond to metabolism change by modulating their shape and dynamics, it is plausible that to compensate PINK1/Parkin respiratory defects and impaired ATP production, mitochondria elongate to boost oxidative phosphorylation. This might at least partially explain why enlarged and hyperfused mitochondria are found in cellular and animal models of PINK1/Parkin deficiency.

PINK1 AND PARKIN AND THE MITOCHONDRIA QUALITY CONTROL PATHWAY: A HATE-LOVE RELATIONSHIP

In 2006, by using the *Drosophila Melanogaster* fruit fly model system, three independent groups showed that PD related genes PINK1 and Parkin operate within the same pathway, with PINK1 functioning upstream of Parkin [3-5]. These works highlighted the fruit fly as an extremely powerful model system to gain insight into PD etiology. A number of fly models have been developed (such as PINK1, Parkin and OMI mutant flies), which show dopaminergic neuronal loss, mitochondrial dysfunction and locomotor deficits. In 2008, by using a fruit fly-based genetic interaction screening, Poole *et al*. showed a strong genetic interaction between PINK1/Parkin pathway and mitochondrial fission and fusion machinery. In particular, in flies, loss of function mutations of pro-fission protein Drp1 is lethal in a PINK1 or Parkin mutant background. Furthermore, PINK1 and Parkin mutant phenotypes, such as muscle degeneration, locomotor deficits and mitochondrial morphology alterations, are suppressed by increased Drp1 gene dosage or decreased pro-fusion Mitofusin gene dosage [62]. This finding highlighted for the first time a potential role for mitochondrial dynamics in the PINK1/Parkin pathway and suggested that PINK1/ Parkin pathway might promote mitochondria fission (or inhibit mitochondria fusion).

Recently, new insights have emerged into the function of the PINK1/Parkin pathway. Upon prolonged mitochondrial intoxication, using CCCP, Parkin is selectively recruited to impaired mitochondria and promotes their elimination *via* autophagy, a process known as mitophagy [6]. Further, PINK1 is required for Parkin translocation through a yet not fully understood mechanism [8]. Extended studies to elucidate the potential molecular mechanisms of this pathway showed that in several cell model systems, upon mitochondria intoxication by CCCP, Parkin is recruited to impaired mitochondria where it selectively ubiquitinates pro fusion protein Mfn [9, 10]. Lack of Parkin (or PINK1, which acts upstream Parkin in a linear pathway) results in impaired Mfn ubiquitination and increased Mfn steady state levels in several *in vitro* cellular systems and *in vivo*. This finding provided a biochemical explanation for the *in vivo* genetic interaction observations that show how decreased pro- fusion Mitofusin gene dosage in flies could ameliorate PINK1 or Parkin mutant phenotype.

Extensive studies in recent years allowed the dissection of this pathway and further details of the molecular mechanism of action of PINK/Parkin in mitochondria quality control have emerged. In healthy mitochondria, PINK1 is sequentially imported to mitochondria through the TOM and TIM complexes, and then it is released to span the inner mitochondrial membrane [63]. It has been shown that the proteases PARL and MPP [64] are responsible for its cleavage and subsequent degradation in a proteasome dependent manner [45]. However, upon CCCP induced mitochondria depolarization, PINK1 fails to be cleaved, it is exposed on the outer mitochondrial membrane, where it drives the recruitment of Parkin [8]. Recent works suggest that PINK1 both phosphorylates Parkin [65], Ubiquitin [66] and Mfn [67]. PINK1-dependent phosphorylation of Parkin regulates Parkin E3 ubiquitin ligase activity [65], although phosphomimetic Parkin mutation does not bypass PINK1 requirement for Parkin recruitment. However, PINK1 dependent phosphorylation of Ubiquitin is a Parkin activator and, in combination with PINK1-dependent phosphorylation of Parkin, is sufficient to fully activate Parkin E3 activity [66]. PINK1-dependent phosphorylation of Mfn is required for Parkin translocation. In particular, phospho-Mfn works as a molecular tag for the recruitment of Parkin that, once on mitochondria, ubiquitinates its targets [67]. In 2013 the complete repertoire of Parkin targets (Parkin-dependent ubiquitylome) have been published, which includes Mfn, VDAC, TOM20, Fis1, the authophagy adaptor p62 and Miro [11]. Parkin dependent ubiquitination of Miro (a GTPase that senses Ca^{2+} and binds mitochondria to the cytoskeleton *via* Milton) results in proteasome dependent degradation of Miro and consequent disruption of mitochondrion mobility [68]. Also, Mfn ubiquitination has followed by chaperone-mediated extraction of the protein from the outer mitochondrial membrane and its degradation [10]. As a result of Parkin-dependent ubiquitination of its targets on the outer mitochondrial membrane, mitochondria both loose their ability to fuse and to move along the microtubules of the cytoskeleton. They are therefore isolated from the mitochondrial network and targeted for degradation *via* Parkin dependent recruitment of cytosolic factors, including p62, that are required for the activation of mitophagy.

Recently a new pathway in the regulation of mitochondria quality control has been described, that accounts for the formation of mitochondria-derived vesicles (MDVs) that bud off mitochondria. Depending on the cargoes, emerging MDVs promote the degradation of their contents by either fusing to a subpopulation of peroxisomes [69] or lysosomes [70]. The latter is independent of autophagy, and it is induced by oxidative stress. Latest works describe a role for Parkin/PINK1 in the biogenesis of these vesicles, suggesting an additional role for these two proteins in the control of damaged mitochondrial proteins degradation [71]. These data characterize a novel vesicles-based highway that direct

damaged mitochondrial proteins to lysosomes. This pathway is distinct from canonical mitophagy and it is primarily activated upon oxidative stress. Thus, PINK1 and Parkin promote mitochondrial quality control *via* at least two distinct pathways, either by tagging the entire organelle for autophagy-dependent degradation, or by shuttling specific cargoes to lysosome in an LC3/ATG-independent manner. The existence of an autophagy-independent pathway in the activation of mitochondria quality control is indeed supported by *in vivo* evidences [72].

UBIQUITINATION AND DE-UBIQUITINATION: A REVERSIBLE MODIFICATION THAT REGULATES SIGNALING PATHWAYS

Ubiquitination has recently emerged as a powerful tool to modulate proteins activity, *via* regulation of their subcellular localization and ability to interact with other proteins to form signaling complexes. E1 (ubiquitin-activating), E2 (ubiquitin-conjugating) and E3 (ubiquitin-ligase) enzymes can regulate the activity of proteins, through conjugating Ubiquitin (Ub) monomers. This event is called "ubiquitination" and consists in the formation of an isopeptide bond between the carboxyl group of the Ub C-terminus and the free amine group of a lysine side-chain of a target protein. In some cases, "linear ubiquitination" can also occur, that means the E1/E2/E3 cascade promotes the formation of a bond between Ub and the first methionine of a target, without the formation of a chain. However, Ub itself contain seven lysine residues, thus allowing ubiquitin chains formation [73]. Ub can form polyubiquitin chains of eight different linkages that mediate distinct biological functions [74]. Thus, the biological outcome of ubiquitin linkage can be modulated, depending on the ubiquitin chain that is formed. The best-characterized type of Ub conjugation is the Lys48 linked Ub chain that typically leads to degradation of the target by the proteasome. In contrast, chains linked *via* one of the other six lysines in Ub can function as regulatory signal in a variety of cellular pathways, including trafficking, signaling and autophagy. In this context, a common regulatory mechanism for many E3 ligases is the ability to self-catalyze their own ubiquitination, by a so-called "auto-ubiquitination" process.

Similar to other post-translational modifications, such as phosphorylation and acetylation, ubiquitination is also a reversible modification, mediated by a large family of deubiquitinating enzymes (DUBs). Interestingly, a large set of DUBs has opposite role of the E1/E2/E3 activity. Most DUBs recognize and remove ubiquitin from conjugated proteins and/or shorten ubiquitin chains, although some DUBs can be cross-reactive for some Ubs. Clearly, this class of enzymes not only can regulate E1/E2/E3 ubiquitinated targets, but also auto-ubiquitinating proteins. Therefore, DUBs have been found to play an important role in the regulation of multiple processes, such as regulation of receptor trafficking, cell cycle progression, regulation of cell migration, regulation of intracellular signaling and transcriptional control [12]. Two different DUB enzyme mechanisms have been described: metalloproteases, classified as the JAMN/MPN+ domain superfamily, and the Cys-proteases. The Cys-proteases DUBs are further divided into four subclasses: the USP (ubiquitin-specific protease) superfamily, the OUT (ovarian tumour) superfamily, the UCH (ubiquitin C-terminal hydrolase) superfamily, and the Machado-Joseph disease domain superfamily (MJDs) [75]. Accumulating evidences indicate that DUBs mutations are involved with human diseases development.

Recent works identified DUBs interacting with and regulating proteins associated with familial forms of PD, -synucleinαsuch as [76] and Parkin [77-84]. In more detail, ataxin-3, USP8, USP15 and USP30 have been found to have a role in modulating Parkin auto-ubiquitination and Parkin-mediated mitophagy.

The MJDs superfamily member ataxin-3 is the first DUB reported to have a role in Parkin deubiquitination and stability. This enzyme, which mutations cause MJD, has been found to deubiquitinate Parkin, but no evidences indicated a role of wild-type ataxin-3 in Parkin stability regulation. However, the MJF-associated form of ataxin-3 promotes Parkin degradation in a proteasome-independent manner [78, 85]. Moreover, ataxin-3 resulted to be unable to hydrolyze preassembled Ub-conjugates on Parkin. Ataxin-3 acts through an unusual mechanism, stabilizing the interaction between Parkin and E2 that now cannot dissociate. When ataxin-3 is present, it can also interact with E2-Ub complex and redirect the Ub transfer from E2 onto itself, rather than onto Parkin [77]. Patients with MJD can exhibit symptoms similar to those with PD and show neurodegeneration in many of the same brain region. The interaction between ataxin-3 and Parkin could, at least partially, explain the similarities between these diseases.

Although ataxin-3 came out as a regulator of Parkin stability and turnover, its role on Parkin-mediated mitophagy was not investigated. Furthermore, as E3s could be regulated by multiple DUBs, the same research group performed an RNAi-screen in U2OS cells to identify other enzymes that could be involved in Parkin deubiquitination and Parkin-dependent mitophagy [79]. They report that Usp8, which was known to be associated with endosomal trafficking, is necessary for Parkin recruitment to mitochondria and mitophagy. Knock-down of Usp8 delayed but not abolished Parkin translocation to depolarized mitochondria, upon CCCP treatment. Counterintuitively, Parkin steady-state levels were increasing in Usp8 RNAi conditions, advocating the hypothesis that Usp8 specifically acts on Parkin by functional ubiquitination rather than degradative ubiquitination. Of interest, they found that Usp8 specifically removes K6-linked Ub conjugates, which in turns promotes Parkin recruitment to mitochondria [80]. Therefore, by impinging on the transcriptional levels of a Parkin-specific DUB, the authors showed how mitophagy could be inhibited.

Another research group used a different approach to reveal Parkin interacting protein. Tandem affinity purification coupled to mass spectrometry identified Usp11 and Usp15 as DUBs binding Parkin [84]. Further experiments, confirmed an interaction between Usp15 and overexpressed Parkin. Usp15 has an opposite role on mitophagy compared to Usp8, since Usp15 knockdown enhanced mitochondria elimination in SH-SY5Y cells. Moreover, RNAi-mediated knockdown of this DUB rescued mitophagy defects in fibroblasts both from

PARK2 and PINK1 mutant PD patients. However, Usp15 does not act on Parkin protein itself, nor prevents Parkin translocation to impaired mitochondria. The targets of Usp15 are the Parkin-ubiquitinated proteins on mitochondria. Usp15 impairs mitophagy through deubiquitinating Parkin targets on depolarized mitochondria and in this respect, it counteracts Parkin ubiquitination activity over Parkin targets. These data were also confirmed *in vivo* in *Drosophila*, where silencing of Usp15 homolog rescued *parkin* mutant phenotype, thus showing a genetic interaction between the two genes [84].

Usp30 came out as a DUB inhibiting mitophagy during a human cDNA library screening in a mitochondrial degradation assay. Although more than 100 DUBs were tested, only two DUBs were able to robustly block mitophagy (*USP30* and *DUBA2*) [83]. However, only Usp30 was reported to be localized in the outer mitochondrial membrane. Co-expression of Usp30 did not alter Parkin expression levels or its translocation to depolarized mitochondria, nevertheless it was able to reduce CCCP-induced recruitment of autophagy markers and ubiquitin signal on GFP-Parkin positive mitochondria. The genuine target of Usp30 deubiquitinating activity was found to be TOM20. Thus, Usp30 overexpression opposes Parkin ubiquitination of TOM20, blocking mitophagy. On the other hands, Usp30 downregulation enhances mitochondrial degradation in neurons *via* stabilization of ubiquitinated forms of TOM20, which work as mitophagy signal. Fruit fly *in vivo* models of Parkinson disease showed that knockdown of Usp30 was able to rescue defective mitophagy caused by Parkin mutation. Moreover, it improves mitochondrial integrity in Parkin- or PINK1-deficient flies and protects flies against paraquat toxicity *in vivo*. Parkin synthesizes Lys 6, Lys 11 and Lys 63 Ub chains on depolarized mitochondria. Usp30 opposes the induction of mitophagy through preferentially hydrolyzing Lys 6- and Lys 11-linked Ub chains on Parkin target TOM20 [82]. Lys 6- and Lys 11-linked Ub chains are fundamental mitophagy signals, since other DUBs targeted to mitochondria which are able to specifically hydrolyze these Ub chains are blocking mitochondrial degradation

All these studies, suggest a potential role for DUBs in modulating mitochondrial quality control and impact on cell survival. Specific DUBs inhibition or enhancement could, for instance, compensate for PINK1 or Parkin loss-of-function mutations in PD patients.

DUBs AS THERAPEUTIC TARGETS

Due to their involvement in the regulation of important signaling pathways, DUBs are emerging as attractive druggable candidates [86]. Clinical trials for specific inhibitors of the ubiquitin-proteasome system have already been approved in cancer therapy for the treatment of multiple myeloma [87-89]. Moreover, high-throughput screening of small chemical libraries identified non selective DUBs inhibitors as potent inducers of apoptosis in various cancer cells. For example, G5, a small molecule inhibitor of DUBs, was recently identified as a strong inhibitor of NLRP3 signaling pathway, thus affecting the NLRP3-dependent inflammatory response [90].

Recently, a number of reports identified additional syntetic small molecule as effective DUB inhibitors. Chemically diverse molecules have been reported to inhibit one or more of the UCH and USP family members. However, drug discovery against the ubiquitin system is still emerging and effective compounds are relatively limited. Often, these compounds target more than one DUB and the most promising chemicals showing selective inhibitions still have not been tested for all full length human DUBs. Here, we report the most promising inhibitors for DUBs of the USP superfamily, which hopefully, in the next future, could be used as therapy for different pathologies, including PD. For detailed information about the biochemical mechanism of action and other available inhibitors for DUBs, the reader is referred to excellent previews [91, 92].

Usp7 is a deubiquitinase first identified as associated with a herpes-virus, and it facilitates lytic growth. It has also been shown as indirect regulator of p53, FOXO4 and PTEN proteins, able to destabilize and compromise the effectiveness of these tumor suppressor proteins [93]. Therefore, Usp7 can be considered as an oncogenic pro-survival protein. Since it was discovered, Usp7 antagonists have been deeply studied, as its inhibition could block tumor progression. At now, an high-throughput screening (HTS) identified HBX 19,818 and the related HBX 28,258 as inhibitors for Usp7 [94]. Using the same method, but in a parallel study, P5091 came out as selective antagonist of this DUB. These molecules were tested in multiple cell lines, thus proving their ability to stabilize p53, to inhibit tumor growth and to promote apoptosis in tumor cell lines. *In vivo* studies confirmed the effect of P22077, an optimized derivative of P5091, in several orthotopic neuroblastoma xenograft models [95].

Some DUBs have been found to have a role in DNA damage response, this is the case of Usp1. When it is associated to the co-factor UAF1 (USP1-associated factor 1), Usp1 deubiquitinates different targets involved in cell damage response and related to different pathologies. Usp1 deubiquitinates FANCD2 and FANCI in the Falconi anemia pathway, promoting DNA repair. This DUB participate in a similar process also in the translesion synthesis pathway, where deubiquitinates PCNA (proliferating cell nuclear antigen) in order to prevent low-fidelity polymerases recruitment and thereby preserve DNA integrity [96]. Through deubiquitinating inhibitors of DNA binding transcription factors, Usp1 has been found to maintain stem-cell characteristics in osteosarcoma cells [97]. An HTS using USP1/UAF1 complex identified different inhibitors for this deubiquitinating enzyme complex [98], and pimozide resulted to be the more efficient chemical against it. However, other DUBs resulted to be inhibited by this same compound, thus, the effects seen by using it in cells could not be imputed to the specific inhibition of USP1/UAF1 complex. However, other two independent HTS revealed other molecules that could be used as Usp1 inhibitors: ML323, and the two analogues SJB2-043 and SJB3-019A [99, 100]. This compounds resulted to be more selective for Usp1, interfering with the protein in the different pathways in which it controls DNA integrity maintenance. Different studies validated the potential therapeutic efficacy of Usp1

inhibition in different pathologies, such as leukemia and other cancers.

Usp14 and UCHL5 are two deubiquitinating enzymes found to be associated with the proteasome. These two proteins antagonize proteasomal degradation of substrate, by hydrolyzing poly-Ub chains from the substrate distal end, a process called trimming. Usp14 deletion is embryonic lethal in mice, since this enzyme is important in general homeostasis. Recently, IU1 has been reported as Usp14 inhibitor, confirming its role in Ub chain trimming. Inhibition of Usp14 has beneficial effects on cell viability, promoting proteasomal degradation of damaged proteins. On the other hand, overexpression of Usp14 is associated with cancer progression [101]. Moreover, *in vivo* and *in vitro* evidences showed the efficiency of inhibiting Usp14 and UCHL5 in tumor models, using b-AP15 [102].

Besides some exceptions, most of these inhibitor molecules are poorly characterized in terms of structure and mechanism of action. Further biochemical studies together with proteomic tools could better explain the features of these molecules, thus allowing to better understand their mechanism of action. Nevertheless, more DUBs inhibitors are under development, with the aim to discover new therapies for different human diseases.

AUREA MEDIOCRITAS: THE DESIRABLE MIDDLE BETWEEN TWO EXTREMES

In recent years, much effort has been put in order to identify specific DUBs that oppose Parkin in the ubiquitination of its targets. The identification of a Parkin-opposing DUB, counteracting Parkin activity in the regulation of mitophagy, might be instrumental to develop specific isopeptidase inhibitors or activators that can modulate the fundamental biological process of mitophagy and impact on cell survival.

Since one of the hallmark of PD consists of accumulation of misfolded proteins and unfunctional mitochondria as a result of impaired mitophagy, chemical or genetic intervention that suppress Parkin-opposing specific DUB, can potentially be used to eliminate these toxic compounds and improve viability (Fig. **1**).

Overall, the enhancement of proteasome activity may offer a strategy to reduce the levels of aberrant proteins in cells and in the whole organism under stress.

Of note, interference with the proteasome machinery has already been proved to be effective in cancer therapy and recently, high throughput screening resulted in the discovery of highly specific synthetic small molecules that target selective components of the proteasome machinery *via* enhancement or suppression of ubiquitin-conjugating enzymes and DUBs.

In vivo evidences suggest that suppression of specific DUBs is sufficient to promote mitophagy in the absence of Parkin or PINK1, *via* stabilization of the ubiquitinated forms of Parkin substrates. Suppression or enhancement of specific DUB activity might therefore be instrumental for the activation of mitochondria clearance pathway downstream

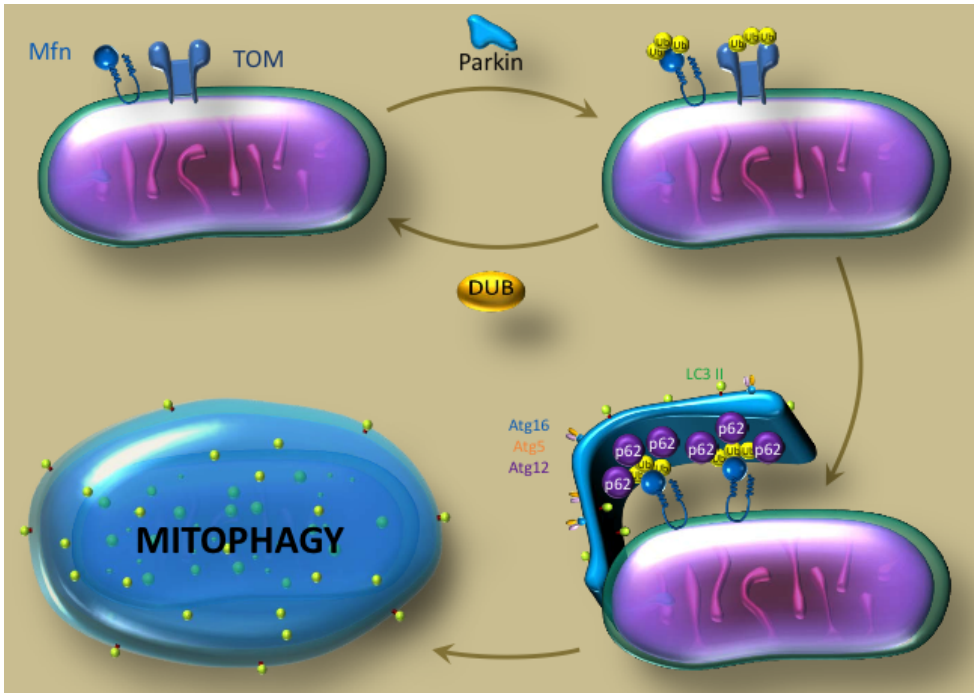

Fig. (1). Counteracting Parkin. The cartoon illustrates the rational behind the potential therapeutic advantages of identifying DUBs that oppose Parkin in the ubiquitination of its target. *In vivo* evidences suggest that suppression of specific DUBs is sufficient to promote mitophagy in the absence of Parkin or PINK1, *via* stabilization of the ubiquitinated forms of Parkin substrates. Therefore, suppression of specific DUB activity might be instrumental for the activation of mitochondria clearance pathway downstream PINK1/Parkin and can offer a therapeutic approach to ameliorate PD phenotype.

PINK1/Parkin and can potentially be beneficial in ameliorating phenotypic deficits in PD.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflict of interest.

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REFERENCES

[1] Silvestri, L. *et al.* Mitochondrial import and enzymatic activity of PINK1 mutants associated to recessive parkinsonism. *Hum Mol Genet* **14**, 3477-3492 (2005).

[2] Shimura, H. *et al.* Familial Parkinson disease gene product, parkin, is a ubiquitin-protein ligase. *Nat Genet* **25**, 302-305 (2000).

[3] Clark, I. E. *et al.* Drosophila pink1 is required for mitochondrial function and interacts genetically with parkin. *Nature* **441**, 1162-1166 (2006).

[4] Yang, Y. *et al.* Mitochondrial pathology and muscle and dopaminergic neuron degeneration caused by inactivation of Drosophila Pink1 is rescued by Parkin. *Proc Natl Acad Sci U S A* **103**, 10793-10798 (2006).

[5] Park, J. *et al.* Mitochondrial dysfunction in Drosophila PINK1 mutants is complemented by parkin. *Nature* **441**, 1157-1161 (2006).

[6] Narendra, D., Tanaka, A., Suen, D. F. & Youle, R. J. Parkin is recruited selectively to impaired mitochondria and promotes their autophagy. *J Cell Biol* **183**, 795-803 (2008).

[7] Ziviani, E., Tao, R. N. & Whitworth, A. J. Drosophila Parkin requires PINK1 for mitochondrial translocation and ubiquitinates Mitofusin. *Proc Natl Acad Sci U S A*.

[8] Narendra, D. P. *et al.* PINK1 is selectively stabilized on impaired mitochondria to activate Parkin. *PLoS Biol* **8**, e1000298.

[9] Ziviani, E., Tao, R. N. & Whitworth, A. J. Drosophila parkin requires PINK1 for mitochondrial translocation and ubiquitinates mitofusin. *Proc Natl Acad Sci U S A* **107**, 5018-5023, doi:10.1073/pnas.0913485107 (2010).

[10] Tanaka, A. *et al.* Proteasome and p97 mediate mitophagy and degradation of mitofusins induced by Parkin. *J Cell Biol* **191**, 1367-1380, doi:jcb.201007013 [pii]

[11] 10.1083/jcb.201007013.

[12] Sarraf, S. A. *et al.* Landscape of the PARKIN-dependent ubiquitylome in response to mitochondrial depolarization. *Nature* **496**, 372-376, doi:10.1038/nature12043 (2013).

[13] Burrows, J. F. & Johnston, J. A. Regulation of cellular responses by deubiquitinating enzymes: an update. *Frontiers in bioscience* **17**, 1184-1200 (2012).

[14] Moore, D. J., West, A. B., Dawson, V. L. & Dawson, T. M. Molecular pathophysiology of Parkinson's disease. *Annu Rev Neurosci* **28**, 57-87 (2005).

[15] Alobaidi, H. & Pall, H. The role of dopamine replacement on the behavioural phenotype of Parkinson's disease. *Behavioural neurology* **26**, 225-235, doi:10.3233/BEN-2012-120265 (2013).

[16] Gasser, T. Molecular pathogenesis of Parkinson disease: insights from genetic studies. *Expert Rev Mol Med* **11**, e22 (2009).

[17] Ryan, B. J., Hoek, S., Fon, E. A. & Wade-Martins, R. Mitochondrial dysfunction and mitophagy in Parkinson's: from familial to sporadic disease. *Trends in biochemical sciences* **40**, 200-210, doi:10.1016/j.tibs.2015.02.003 (2015).

[18] Corti, O., Lesage, S. & Brice, A. What genetics tells us about the causes and mechanisms of Parkinson's disease. *Physiological reviews* **91**, 1161-1218, doi:10.1152/physrev.00022.2010 (2011).

[19] Ozansoy, M. & Basak, A. N. The central theme of Parkinson's disease: alpha-synuclein. *Molecular neurobiology* **47**, 460-465, doi:10.1007/s12035-012-8369-3 (2013).

[20] Esteves, A. R., Swerdlow, R. H. & Cardoso, S. M. LRRK2, a puzzling protein: insights into Parkinson's disease pathogenesis. *Exp Neurol* **261**, 206-216, doi:10.1016/j.expneurol.2014.05.025 (2014).

[21] Smith, W. W. *et al.* Leucine-rich repeat kinase 2 (LRRK2) interacts with parkin, and mutant LRRK2 induces neuronal degeneration. *Proc Natl Acad Sci U S A* **102**, 18676-18681, doi:10.1073/pnas.0508052102 (2005).

[22] Samann, J. *et al.* Caenorhabditits elegans LRK-1 and PINK-1 act antagonistically in stress response and neurite outgrowth. *J Biol Chem* **284**, 16482-16491, doi:10.1074/jbc.M808255200 (2009).

[23] van Duijn, C. M. *et al.* Park7, a novel locus for autosomal recessive early-onset parkinsonism, on chromosome 1p36. *American journal of human genetics* **69**, 629-634, doi:10.1086/322996 (2001).

[24] Nagakubo, D. *et al.* DJ-1, a novel oncogene which transforms mouse NIH3T3 cells in cooperation with ras. *Biochemical and biophysical research communications* **231**, 509-513, doi:10.1006/bbrc.1997.6132 (1997).

[25] Kim, S. J. *et al.* Nuclear translocation of DJ-1 during oxidative stress-induced neuronal cell death. *Free radical biology & medicine* **53**, 936-950, doi:10.1016/j.freeradbiomed.2012.05.035 (2012).

[26] Cao, J., Lou, S., Ying, M. & Yang, B. DJ-1 as a human oncogene and potential therapeutic target. *Biochemical pharmacology* **93**, 241-250, doi:10.1016/j.bcp.2014.11.012 (2015).

[27] Repici, M. *et al.* Parkinson's disease-associated mutations in DJ-1 modulate its dimerization in living cells. *Journal of molecular medicine* **91**, 599-611, doi:10.1007/s00109-012-0976-y (2013).

[28] Narendra, D. P. *et al.* PINK1 is selectively stabilized on impaired mitochondria to activate Parkin. *PLoS Biol* **8**, e1000298, doi:10.1371/journal.pbio.1000298 (2010).

[29] Matsuda, S., Kitagishi, Y. & Kobayashi, M. Function and characteristics of PINK1 in mitochondria. *Oxidative medicine and cellular longevity* **2013**, 601587, doi:10.1155/2013/601587 (2013).

[30] Song, S. *et al.* Characterization of PINK1 (PTEN-induced putative kinase 1) mutations associated with Parkinson disease in mammalian cells and Drosophila. *J Biol Chem* **288**, 5660-5672, doi:10.1074/jbc.M112.430801 (2013).

[31] Kitada, T. *et al.* Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. *Nature* **392**, 605-608, doi:10.1038/33416 (1998).

[32] Morrison, K. E. Parkin mutations and early onset parkinsonism. *Brain : a journal of neurology* **126**, 1250-1251 (2003).

[33] Bueler, H. Impaired mitochondrial dynamics and function in the pathogenesis of Parkinson's disease. *Exp Neurol* **218**, 235-246 (2009).

[34] Bonifati, V. *et al.* DJ-1(PARK7), a novel gene for autosomal recessive, early onset parkinsonism. *Neurol Sci* **24**, 159-160, doi:10.1007/s10072-003-0108-0 (2003).

[35] Manning-Bog, A. B. *et al.* The herbicide paraquat causes up-regulation and aggregation of alpha-synuclein in mice: paraquat and alpha-synuclein. *J Biol Chem* **277**, 1641-1644, doi:10.1074/jbc.C100560200 (2002).

[36] Sherer, T. B. *et al.* An *in vitro* model of Parkinson's disease: linking mitochondrial impairment to altered alpha-synuclein metabolism and oxidative damage. *J Neurosci* **22**, 7006-7015, doi:20026721 (2002).

[37] Attardi, G. & Schatz, G. Biogenesis of mitochondria. *Annu Rev Cell Biol* **4**, 289-333 (1988).

[38] Saraste, M. Oxidative phosphorylation at the fin de siecle. *Science* **283**, 1488-1493 (1999).

[39] Butow, R. A. & Avadhani, N. G. Mitochondrial signaling: the retrograde response. *Mol Cell* **14**, 1-15 (2004).

[40] Danial, N. N. & Korsmeyer, S. J. Cell death: critical control points. *Cell* **116**, 205-219 (2004).

[41] Chen, H. & Chan, D. C. Critical dependence of neurons on mitochondrial dynamics. *Current opinion in cell biology* **18**, 453-459, doi:S0955-0674(06)00080-9 [pii]

[42] 10.1016/j.ceb.2006.06.004 (2006).

[43] Twig, G. *et al.* Fission and selective fusion govern mitochondrial segregation and elimination by autophagy. *The EMBO journal* **27**, 433-446, doi:10.1038/sj.emboj.7601963 (2008).

[44] Gomes, L. C., Di Benedetto, G. & Scorrano, L. During autophagy mitochondria elongate, are spared from degradation and sustain cell viability. *Nature cell biology* **13**, 589-598, doi:10.1038/ncb2220 (2011).

[45] Soubannier, V., Rippstein, P., Kaufman, B. A., Shoubridge, E. A. & McBride, H. M. Reconstitution of mitochondria derived vesicle formation demonstrates selective enrichment of oxidized cargo. *PloS one* **7**, e52830, doi:10.1371/journal.pone.0052830 (2012).

[46] Chan, D. C. Mitochondrial fusion and fission in mammals. *Annu Rev Cell Dev Biol* **22**, 79-99 (2006).
[47] Meeusen, S. L. & Nunnari, J. How mitochondria fuse. *Current opinion in cell biology* **17**, 389-394, doi:10.1016/j.ceb.2005.06.014 (2005).
[48] McNiven, M. A., Cao, H., Pitts, K. R. & Yoon, Y. The dynamin family of mechanoenzymes: pinching in new places. *Trends in biochemical sciences* **25**, 115-120 (2000).
[49] de Brito, O. M. & Scorrano, L. Mitofusin 2 tethers endoplasmic reticulum to mitochondria. *Nature* **456**, 605-610 (2008).
[50] Frezza, C. *et al.* OPA1 controls apoptotic cristae remodeling independently from mitochondrial fusion. *Cell* **126**, 177-189 (2006).
[51] Cogliati, S. *et al.* Mitochondrial cristae shape determines respiratory chain supercomplexes assembly and respiratory efficiency. *Cell* **155**, 160-171, doi:10.1016/j.cell.2013.08.032 (2013).
[52] Smirnova, E., Griparic, L., Shurland, D. L. & van der Bliek, A. M. Dynamin-related protein Drp1 is required for mitochondrial division in mammalian cells. *Molecular biology of the cell* **12**, 2245-2256 (2001).
[53] Yoon, Y., Krueger, E. W., Oswald, B. J. & McNiven, M. A. The mitochondrial protein hFis1 regulates mitochondrial fission in mammalian cells through an interaction with the dynamin-like protein DLP1. *Molecular and cellular biology* **23**, 5409-5420 (2003).
[54] Gandre-Babbe, S. & van der Bliek, A. M. The novel tail-anchored membrane protein Mff controls mitochondrial and peroxisomal fission in mammalian cells. *Molecular biology of the cell* **19**, 2402-2412, doi:10.1091/mbc.E07-12-1287 (2008).
[55] Otera, H. *et al.* Mff is an essential factor for mitochondrial recruitment of Drp1 during mitochondrial fission in mammalian cells. *J Cell Biol* **191**, 1141-1158, doi:10.1083/jcb.201007152 (2010).
[56] Schapira, A. H. *et al.* Mitochondrial complex I deficiency in Parkinson's disease. *Lancet* **1**, 1269 (1989).
[57] Betarbet, R. *et al.* Chronic systemic pesticide exposure reproduces features of Parkinson's disease. *Nature neuroscience* **3**, 1301-1306, doi:10.1038/81834 (2000).
[58] Langston, J. W., Ballard, P., Tetrud, J. W. & Irwin, I. Chronic Parkinsonism in humans due to a product of meperidine-analog synthesis. *Science* **219**, 979-980 (1983).
[59] Morais, V. A. *et al.* Parkinson's disease mutations in PINK1 result in decreased Complex I activity and deficient synaptic function. *EMBO molecular medicine* **1**, 99-111, doi:10.1002/emmm.200900006 (2009).
[60] Gandhi, S. *et al.* PINK1-associated Parkinson's disease is caused by neuronal vulnerability to calcium-induced cell death. *Mol Cell* **33**, 627-638, doi:10.1016/j.molcel.2009.02.013 (2009).
[61] Morais, V. A. *et al.* PINK1 loss-of-function mutations affect mitochondrial complex I activity *via* NdufA10 ubiquinone uncoupling. *Science* **344**, 203-207, doi:10.1126/science.1249161 (2014).
[62] Mortiboys, H. *et al.* Mitochondrial function and morphology are impaired in parkin-mutant fibroblasts. *Annals of neurology* **64**, 555-565, doi:10.1002/ana.21492 (2008).
[63] Klein, P. *et al.* Ret rescues mitochondrial morphology and muscle degeneration of Drosophila Pink1 mutants. *The EMBO journal* **33**, 341-355, doi:10.1002/embj.201284290 (2014).
[64] Poole, A. C. *et al.* The PINK1/Parkin pathway regulates mitochondrial morphology. *Proc Natl Acad Sci U S A* **105**, 1638-1643 (2008).
[65] Jin, S. M. *et al.* Mitochondrial membrane potential regulates PINK1 import and proteolytic destabilization by PARL. *J Cell Biol* **191**, 933-942, doi:10.1083/jcb.201008084 (2010).
[66] Greene, A. W. *et al.* Mitochondrial processing peptidase regulates PINK1 processing, import and Parkin recruitment. *EMBO reports* **13**, 378-385, doi:10.1038/embor.2012.14 (2012).
[67] Sha, D., Chin, L. S. & Li, L. Phosphorylation of parkin by Parkinson disease-linked kinase PINK1 activates parkin E3 ligase function and NF-kappaB signaling. *Hum Mol Genet* **19**, 352-363, doi:10.1093/hmg/ddp501 (2010).
[68] Koyano, F. *et al.* Ubiquitin is phosphorylated by PINK1 to activate parkin. *Nature* **510**, 162-166, doi:10.1038/nature13392 (2014).
[69] Chen, Y. & Dorn, G. W., 2nd. PINK1-phosphorylated mitofusin 2 is a Parkin receptor for culling damaged mitochondria. *Science* **340**, 471-475, doi:10.1126/science.1231031 (2013).
[70] Wang, X. *et al.* PINK1 and Parkin target Miro for phosphorylation and degradation to arrest mitochondrial motility. *Cell* **147**, 893-906, doi:10.1016/j.cell.2011.10.018 (2011).
[71] Neuspiel, M. *et al.* Cargo-selected transport from the mitochondria to peroxisomes is mediated by vesicular carriers. *Current biology : CB* **18**, 102-108, doi:10.1016/j.cub.2007.12.038 (2008).
[72] Soubannier, V. *et al.* A vesicular transport pathway shuttles cargo from mitochondria to lysosomes. *Current biology : CB* **22**, 135-141, doi:10.1016/j.cub.2011.11.057 (2012).
[73] McLelland, G. L., Soubannier, V., Chen, C. X., McBride, H. M. & Fon, E. A. Parkin and PINK1 function in a vesicular trafficking pathway regulating mitochondrial quality control. *The EMBO journal* **33**, 282-295, doi:10.1002/embj.201385902 (2014).
[74] Vincow, E. S. *et al.* The PINK1-Parkin pathway promotes both mitophagy and selective respiratory chain turnover *in vivo*. *Proc Natl Acad Sci U S A* **110**, 6400-6405, doi:10.1073/pnas.1221132110 (2013).
[75] Kleiger, G. & Mayor, T. Perilous journey: a tour of the ubiquitin-proteasome system. *Trends in cell biology* **24**, 352-359, doi:10.1016/j.tcb.2013.12.003 (2014).
[76] Komander, D. & Rape, M. The ubiquitin code. *Annual review of biochemistry* **81**, 203-229, doi:10.1146/annurev-biochem-060310-170328 (2012).
[77] Komander, D. Mechanism, specificity and structure of the deubiquitinases. *Sub-cellular biochemistry* **54**, 69-87, doi:10.1007/978-1-4419-6676-6_6 (2010).
[78] Rott, R. *et al.* alpha-Synuclein fate is determined by USP9X-regulated monoubiquitination. *Proc Natl Acad Sci U S A* **108**, 18666-18671, doi:10.1073/pnas.1105725108 (2011).
[79] Durcan, T. M., Kontogiannea, M., Bedard, N., Wing, S. S. & Fon, E. A. Ataxin-3 deubiquitination is coupled to Parkin ubiquitination *via* E2 ubiquitin-conjugating enzyme. *J Biol Chem* **287**, 531-541, doi:10.1074/jbc.M111.288449 (2012).
[80] Durcan, T. M. *et al.* The Machado-Joseph disease-associated mutant form of ataxin-3 regulates parkin ubiquitination and stability. *Hum Mol Genet* **20**, 141-154, doi:10.1093/hmg/ddq452 (2011).
[81] Durcan, T. M. *et al.* USP8 regulates mitophagy by removing K6-linked ubiquitin conjugates from parkin. *The EMBO journal* **33**, 2473-2491, doi:10.15252/embj.201489729 (2014).
[82] Durcan, T. M. & Fon, E. A. USP8 and PARK2/parkin-mediated mitophagy. *Autophagy*, 0, doi:10.1080/15548627.2015.1009794 (2015).
[83] Dikic, I. & Bremm, A. DUBs counteract parkin for efficient mitophagy. *The EMBO journal* **33**, 2442-2443, doi:10.15252/embj.201490101 (2014).
[84] Cunningham, C. N. *et al.* USP30 and parkin homeostatically regulate atypical ubiquitin chains on mitochondria. *Nature cell biology* **17**, 160-169, doi:10.1038/ncb3097 (2015).
[85] Bingol, B. *et al.* The mitochondrial deubiquitinase USP30 opposes parkin-mediated mitophagy. *Nature* **510**, 370-375, doi:10.1038/nature13418 (2014).
[86] Cornelissen, T. *et al.* The deubiquitinase USP15 antagonizes Parkin-mediated mitochondrial ubiquitination and mitophagy. *Hum Mol Genet* **23**, 5227-5242, doi:10.1093/hmg/ddu244 (2014).
[87] Durcan, T. M. & Fon, E. A. Mutant ataxin-3 promotes the autophagic degradation of parkin. *Autophagy* **7**, 233-234 (2011).
[88] Salmena, L. & Pandolfi, P. P. Changing venues for tumour suppression: balancing destruction and localization by monoubiquitylation. *Nature reviews. Cancer* **7**, 409-413, doi:10.1038/nrc2145 (2007).
[89] Brancolini, C. Inhibitors of the Ubiquitin-Proteasome System and the cell death machinery: How many pathways are activated? *Current molecular pharmacology* **1**, 24-37 (2008).
[90] Hussain, S., Zhang, Y. & Galardy, P. J. DUBs and cancer: the role of deubiquitinating enzymes as oncogenes, non-oncogenes and tumor suppressors. *Cell cycle* **8**, 1688-1697 (2009).
[91] Colland, F. The therapeutic potential of deubiquitinating enzyme inhibitors. *Biochemical Society transactions* **38**, 137-143, doi:10.1042/BST0380137 (2010).
[92] Py, B. F., Kim, M. S., Vakifahmetoglu-Norberg, H. & Yuan, J. Deubiquitination of NLRP3 by BRCC3 critically regulates

inflammasome activity. *Mol Cell* **49**, 331-338, doi:10.1016/j.molcel.2012.11.009 (2013).

[93] Lill, J. R. & Wertz, I. E. Toward understanding ubiquitin-modifying enzymes: from pharmacological targeting to proteomics. *Trends Pharmacol Sci* **35**, 187-207, doi:10.1016/j.tips.2014.01.005 (2014).

[94] Ndubaku, C. & Tsui, V. Inhibiting the Deubiquitinating Enzymes (DUBs). *Journal of medicinal chemistry* **58**, 1581-1595, doi:10.1021/jm501061a (2015).

[95] Li, M. *et al.* Deubiquitination of p53 by HAUSP is an important pathway for p53 stabilization. *Nature* **416**, 648-653, doi:10.1038/nature737 (2002).

[96] Colland, F. *et al.* Small-molecule inhibitor of USP7/HAUSP ubiquitin protease stabilizes and activates p53 in cells. *Molecular cancer therapeutics* **8**, 2286-2295, doi:10.1158/1535-7163.MCT-09-0097 (2009).

[97] Fan, Y. H. *et al.* USP7 inhibitor P22077 inhibits neuroblastoma growth *via* inducing p53-mediated apoptosis. *Cell death & disease* **4**, e867, doi:10.1038/cddis.2013.400 (2013).

[98] Garcia-Santisteban, I., Peters, G. J., Giovannetti, E. & Rodriguez, J. A. USP1 deubiquitinase: cellular functions, regulatory mechanisms and emerging potential as target in cancer therapy. *Molecular cancer* **12**, 91, doi:10.1186/1476-4598-12-91 (2013).

[99] Williams, S. A. *et al.* USP1 deubiquitinates ID proteins to preserve a mesenchymal stem cell program in osteosarcoma. *Cell* **146**, 918-930, doi:10.1016/j.cell.2011.07.040 (2011).

[100] Chen, J. *et al.* Selective and cell-active inhibitors of the USP1/UAF1 deubiquitinase complex reverse cisplatin resistance in non-small cell lung cancer cells. *Chemistry & biology* **18**, 1390-1400, doi:10.1016/j.chembiol.2011.08.014 (2011).

[101] Liang, Q. *et al.* A selective USP1-UAF1 inhibitor links deubiquitination to DNA damage responses. *Nature chemical biology* **10**, 298-304, doi:10.1038/nchembio.1455 (2014).

[102] Mistry, H. *et al.* Small-molecule inhibitors of USP1 target ID1 degradation in leukemic cells. *Molecular cancer therapeutics* **12**, 2651-2662, doi:10.1158/1535-7163.MCT-13-0103-T (2013).

[103] D'Arcy, P. & Linder, S. Proteasome deubiquitinases as novel targets for cancer therapy. *The international journal of biochemistry & cell biology* **44**, 1729-1738, doi:10.1016/j.biocel.2012.07.011 (2012).

[104] D'Arcy, P. *et al.* Inhibition of proteasome deubiquitinating activity as a new cancer therapy. *Nature medicine* **17**, 1636-1640, doi:10.1038/nm.2536 (2011).

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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.
This article is part of a Special Issue entitled: Role of mitochondria in physiological and pathophysiological functions in the central nervous system.

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 juxstaposition (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.

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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

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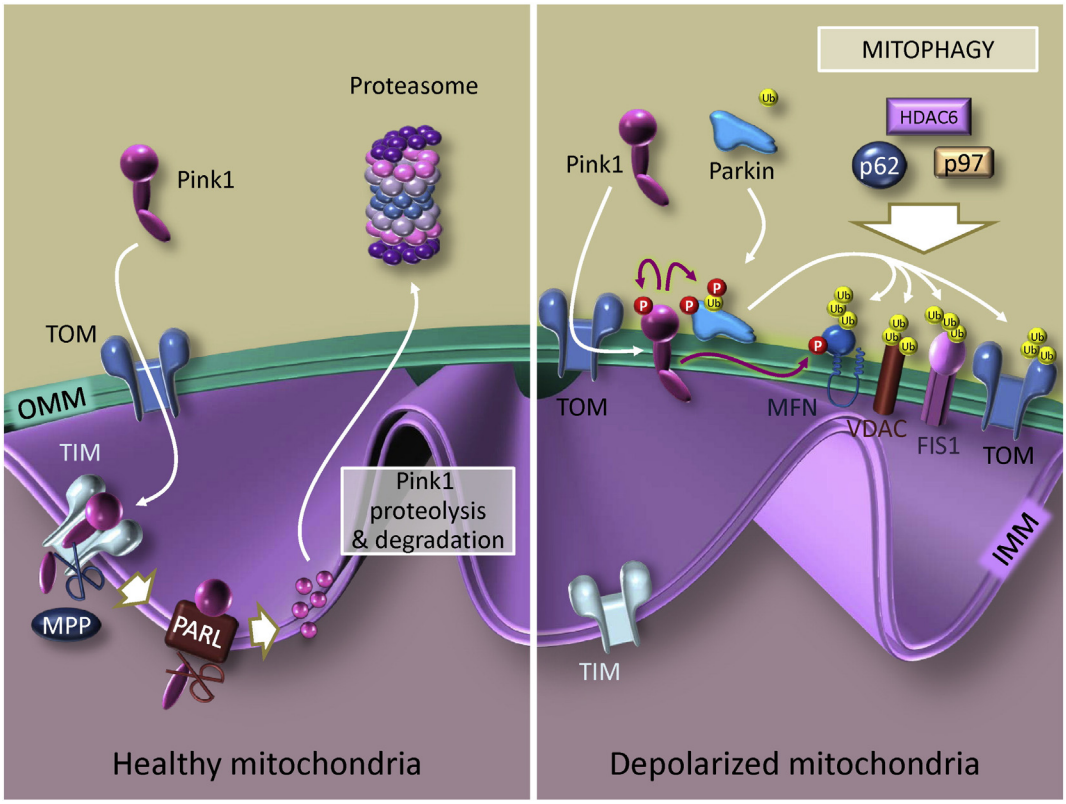

Fig. 1. The Pink/Parkin pathway in mitophagy. In healthy mitochondria (left panel), PINK1 is targeted to the outer mitochondrial membrane (OMM) owing to its mitochondrial target sequencing (MTS). It is then imported into the inner mitochondrial membrane (IMM) through the TOM/TIM complex and cleaved by 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

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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

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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/

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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

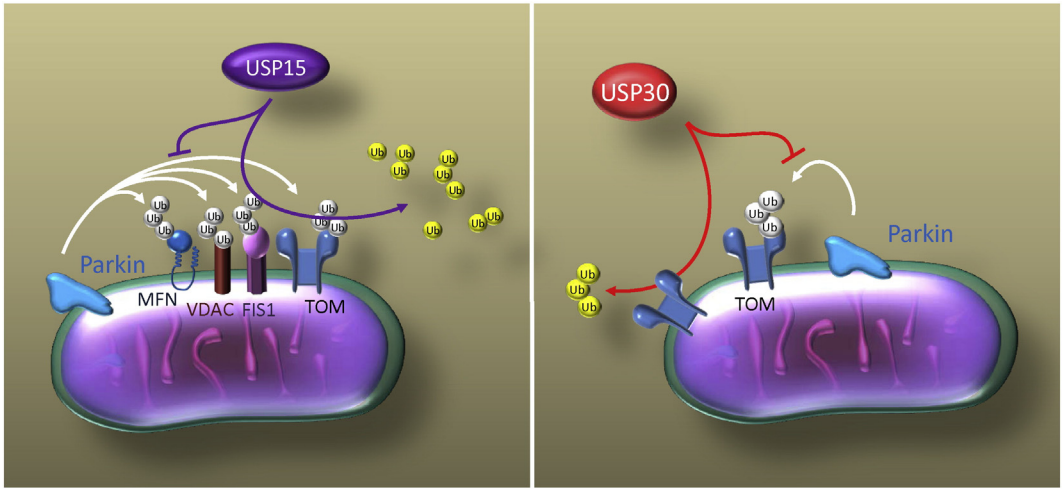

Fig. 2. DUBs role in mitochondria quality control. By impacting on the ubiquitination levels of Parkin targets, USP15 and USP30 affect Parkin translocation and Parkin-dependent mitophagy. USP15 deubiquitinates different Parkin targets on mitochondria, and knockdown of USP15 fly homolog CG8334, in a Parkin 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.

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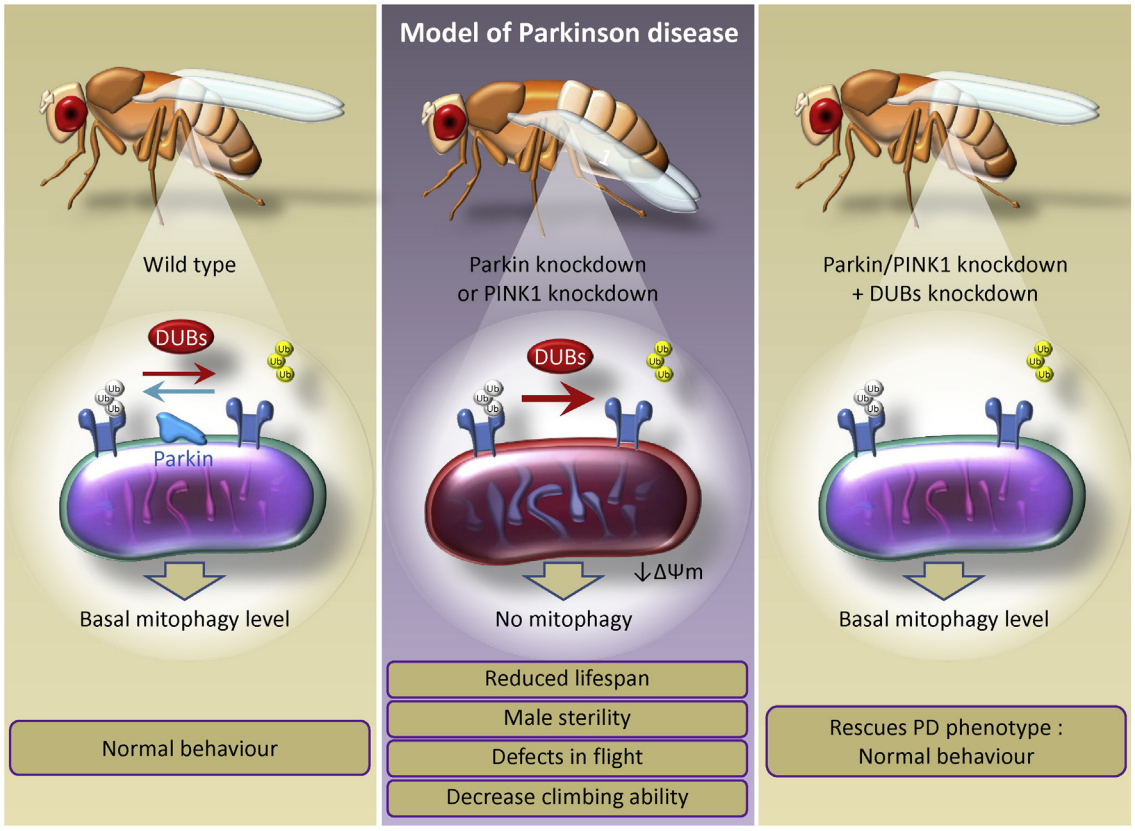

Fig. 3. Fly-based *in vivo* screening. The identification of specific DUB/DUBs that counteract Parkin activity in the ubiquitination of mitophagy substrates is emerging as one of the most promising approaches to promote mitophagy in PINK1/Parkin deficient system. In this respect, the fruit fly has proved 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).

References

Adams, M.D., Sekelsky, J.J., 2002. From sequence to phenotype: reverse genetics in *Drosophila melanogaster*. Nat. Rev. Genet. 3, 189–198. http://dx.doi.org/10.1038/nrg752.
Akepati, V.R., et al., 2008. Characterization of OPA1 isoforms isolated from mouse tissues. J. Neurochem. 106, 372–383. http://dx.doi.org/10.1111/j.1471-4159.2008.05401.x.
Amo, T., et al., 2011. Mitochondrial membrane potential decrease caused by loss of PINK1 is not due to proton leak, but to respiratory chain defects. Neurobiol. Dis. 41, 111–118. http://dx.doi.org/10.1016/j.nbd.2010.08.027.
Area-Gomez, E., et al., 2012. Upregulated function of mitochondria-associated ER membranes in Alzheimer disease. EMBO J. 31, 4106–4123. http://dx.doi.org/10.1038/emboj.2012.202.
Bach, D., et al., 2003. Mitofusin-2 determines mitochondrial network architecture and mitochondrial metabolism. A novel regulatory mechanism altered in obesity. J. Biol. Chem. 278, 17190–17197. http://dx.doi.org/10.1074/jbc.M212754200.
Bingol, B., et al., 2014. The mitochondrial deubiquitinase USP30 opposes parkin-mediated mitophagy. Nature 510, 370–375. http://dx.doi.org/10.1038/nature13418.
Bove, J., Prou, D., Perier, C., Przedborski, S., 2005. Toxin-induced models of Parkinson's disease. NeuroRx: The Journal of the American Society for Experimental NeuroTherapeutics 2, 484–494. http://dx.doi.org/10.1602/neurorx.2.3.484.
Boyer, P.D., et al., 1977. Oxidative phosphorylation and photophosphorylation. Annu. Rev. Biochem. 46, 955–966. http://dx.doi.org/10.1146/annurev.bi.46.070177.004515.
Brand, A.H., Perrimon, N., 1993. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development 118, 401–415.
Cali, T., Ottolini, D., Brini, M., 2011. Mitochondria, calcium, and endoplasmic reticulum stress in Parkinson's disease. Biofactors 37, 228–240. http://dx.doi.org/10.1002/biof.159.
Cali, T., Ottolini, D., Negro, A., Brini, M., 2013a. Enhanced parkin levels favor ER-mitochondria crosstalk and guarantee Ca(2+) transfer to sustain cell bioenergetics. Biochim. Biophys. Acta 1832, 495–508. http://dx.doi.org/10.1016/j.bbadis.2013.01.004.
Cali, T., Ottolini, D., Brini, M., 2013b. Calcium and endoplasmic reticulum-mitochondria tethering in neurodegeneration. DNA Cell Biol. 32, 140–146. http://dx.doi.org/10.1089/dna.2013.2011.
Cereghetti, G.M., et al., 2008. Dephosphorylation by calcineurin regulates translocation of Drp1 to mitochondria. Proc. Natl. Acad. Sci. U. S. A. 105, 15803–15808. http://dx.doi.org/10.1073/pnas.0808249105.

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Chan, D.C., 2006. Mitochondrial fusion and fission in mammals. Annu. Rev. Cell Dev. Biol. 22, 79–99.

Chang, C.R., Blackstone, C., 2007. Cyclic AMP-dependent protein kinase phosphorylation of Drp1 regulates its GTPase activity and mitochondrial morphology. J. Biol. Chem. 282, 21583–21587. http://dx.doi.org/10.1074/jbc.C700083200.

Chang, S., et al., 2008. Identification of small molecules rescuing fragile X syndrome phenotypes in *Drosophila*. Nat. Chem. Biol. 4, 256–263. http://dx.doi.org/10.1038/nchembio.78.

Chao, J.R., et al., 2008. Hax1-mediated processing of HtrA2 by Parl allows survival of lymphocytes and neurons. Nature 452, 98–102. http://dx.doi.org/10.1038/nature06604.

Chen, Y., Dorn 2nd., G.W., 2013. PINK1-phosphorylated mitofusin 2 is a Parkin receptor for culling damaged mitochondria. Science 340, 471–475. http://dx.doi.org/10.1126/science.1231031.

Chen, H., et al., 2003. Mitofusins Mfn1 and Mfn2 coordinately regulate mitochondrial fusion and are essential for embryonic development. J. Cell Biol. 160, 189–200. http://dx.doi.org/10.1083/jcb.200211046.

Chen, K.H., et al., 2004. Dysregulation of HSG triggers vascular proliferative disorders. Nat. Cell Biol. 6, 872–883. http://dx.doi.org/10.1038/ncb1161.

Chen, Y., et al., 2012. Mitofusin 2-containing mitochondrial-reticular microdomains direct rapid cardiomyocyte bioenergetic responses via interorganelle Ca(2+) crosstalk. Circ. Res. 111, 863–875. http://dx.doi.org/10.1161/CIRCRESAHA.112.266585.

Cipolat, S., Martins de Brito, O., Dal Zilio, B., Scorrano, L., 2004. OPA1 requires mitofusin 1 to promote mitochondrial fusion. Proc. Natl. Acad. Sci. U. S. A. 101, 15927–15932. http://dx.doi.org/10.1073/pnas.0407043101.

Clark, I.E., et al., 2006. Drosophila pink1 is required for mitochondrial function and interacts genetically with parkin. Nature 441, 1162–1166.

Cogliati, S., et al., 2013. Mitochondrial cristae shape determines respiratory chain supercomplexes assembly and respiratory efficiency. Cell 155, 160–171. http://dx.doi.org/10.1016/j.cell.2013.08.032.

Cornelissen, T., et al., 2014. The deubiquitinase USP15 antagonizes Parkin-mediated mitochondrial ubiquitination and mitophagy. Hum. Mol. Genet. 23, 5227–5242. http://dx.doi.org/10.1093/hmg/ddu244.

Cosson, P., Marchetti, A., Ravazzola, M., Orci, L., 2012. Mitofusin-2 independent juxtaposition of endoplasmic reticulum and mitochondria: an ultrastructural study. PLoS One 7, e46293. http://dx.doi.org/10.1371/journal.pone.0046293.

Costa, V., Scorrano, L., 2012. Shaping the role of mitochondria in the pathogenesis of Huntington's disease. EMBO J. 31, 1853–1864. http://dx.doi.org/10.1038/emboj.2012.65.

Cribbs, J.T., Strack, S., 2007. Reversible phosphorylation of Drp1 by cyclic AMP-dependent protein kinase and calcineurin regulates mitochondrial fission and cell death. EMBO Rep. 8, 939–944. http://dx.doi.org/10.1038/sj.embor.7401062.

Dawson, T.M., Ko, H.S., Dawson, V.L., 2010. Genetic animal models of Parkinson's disease. Neuron 66, 646–661. http://dx.doi.org/10.1016/j.neuron.2010.04.034.

de Brito, O.M., Scorrano, L., 2008. Mitofusin 2 tethers endoplasmic reticulum to mitochondria. Nature 456, 605–610.

De Stefani, D., Raffaello, A., Teardo, E., Szabo, I., Rizzuto, R., 2011. A forty-kilodalton protein of the inner membrane is the mitochondrial calcium uniporter. Nature 476, 336–340. http://dx.doi.org/10.1038/nature10230.

Deas, E., et al., 2011. PINK1 cleavage at position A103 by the mitochondrial protease PARL. Hum. Mol. Genet. 20, 867–879. http://dx.doi.org/10.1093/hmg/ddq526.

Deng, H., Dodson, M.W., Huang, H., Guo, M., 2008. The Parkinson's disease genes pink1 and parkin promote mitochondrial fission and/or inhibit fusion in *Drosophila*. Proc. Natl. Acad. Sci. U. S. A. 105, 14503–14508. http://dx.doi.org/10.1073/pnas.0803998105.

Durcan, T.M., et al., 2014. USP8 regulates mitophagy by removing K6-linked ubiquitin conjugates from parkin. EMBO J. 33, 2473–2491. http://dx.doi.org/10.15252/embj.201489729.

Ehses, S., et al., 2009. Regulation of OPA1 processing and mitochondrial fusion by m-AAA protease isoenzymes and OMA1. J. Cell Biol. 187, 1023–1036. http://dx.doi.org/10.1083/jcb.200906084.

Ernster, L., Schatz, G., 1981. Mitochondria: a historical review. The Journal of cell biology 91, 227s–255s.

Eura, Y., Ishihara, N., Yokota, S., Mihara, K., 2003. Two mitofusin proteins, mammalian homologues of FZO, with distinct functions are both required for mitochondrial fusion. J. Biochem. 134, 333–344.

Filadi, R., et al., 2015. Mitofusin 2 ablation increases endoplasmic reticulum-mitochondria coupling. Proc. Natl. Acad. Sci. U. S. A. 112, E2174–E2181. http://dx.doi.org/10.1073/pnas.1504880112.

Frey, T.G., Mannella, C.A., 2000. The internal structure of mitochondria. Trends Biochem. Sci. 25, 319–324.

Frezza, C., et al., 2006. OPA1 controls apoptotic cristae remodeling independently from mitochondrial fusion. Cell 126, 177–189. http://dx.doi.org/10.1016/j.cell.2006.06.025.

Friedman, J.R., et al., 2011. ER tubules mark sites of mitochondrial division. Science 334, 358–362. http://dx.doi.org/10.1126/science.1207385.

Gandhi, S., et al., 2009. PINK1-associated Parkinson's disease is caused by neuronal vulnerability to calcium-induced cell death. Mol. Cell 33, 627–638. http://dx.doi.org/10.1016/j.molcel.2009.02.013.

Geisler, S., et al., 2010. PINK1/Parkin-mediated mitophagy is dependent on VDAC1 and p62/SQSTM1. Nat. Cell Biol. 12, 119–131. http://dx.doi.org/10.1038/ncb2012.

Greene, J.C., et al., 2003. Mitochondrial pathology and apoptotic muscle degeneration in *Drosophila* parkin mutants. Proc. Natl. Acad. Sci. U. S. A. 100, 4078–4083. http://dx.doi.org/10.1073/pnas.0737556100.

Groth, G., Walker, J.E., 1996. ATP synthase from bovine heart mitochondria: reconstitution into unilamellar phospholipid vesicles of the pure enzyme in a functional state. Biochem. J. 318 (Pt 1), 351–357.

Hailey, D.W., et al., 2010. Mitochondria supply membranes for autophagosome biogenesis during starvation. Cell 141, 656–667. http://dx.doi.org/10.1016/j.cell.2010.04.009.

Hamasaki, M., et al., 2013. Autophagosomes form at ER-mitochondria contact sites. Nature 495, 389–393. http://dx.doi.org/10.1038/nature11910.

Han, X.J., et al., 2008. CaM kinase I alpha-induced phosphorylation of Drp1 regulates mitochondrial morphology. J. Cell Biol. 182, 573–585. http://dx.doi.org/10.1083/jcb.200802164.

Harder, Z., Zunino, R., McBride, H., 2004. Sumo1 conjugates mitochondrial substrates and participates in mitochondrial fission. Curr. Biol.: CB 14, 340–345. http://dx.doi.org/10.1016/j.cub.2004.02.004.

Ishihara, N., Fujita, Y., Oka, T., Mihara, K., 2006. Regulation of mitochondrial morphology through proteolytic cleavage of OPA1. EMBO J 25, 2966–2977. http://dx.doi.org/10.1038/sj.emboj.7601184.

James, D.I., Parone, P.A., Mattenberger, Y., Martinou, J.C., 2003. hFis1, a novel component of the mammalian mitochondrial fission machinery. J. Biol. Chem. 278, 36373–36379. http://dx.doi.org/10.1074/jbc.M303758200.

Jin, S.M., et al., 2010. Mitochondrial membrane potential regulates PINK1 import and proteolytic destabilization by PARL. J. Cell Biol. 191, 933–942. http://dx.doi.org/10.1083/jcb.201008084.

Kim, Y., et al., 2008. PINK1 controls mitochondrial localization of Parkin through direct phosphorylation. Biochem. Biophys. Res. Commun. 377, 975–980. http://dx.doi.org/10.1016/j.bbrc.2008.10.104.

Kitada, T., et al., 1998. Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. Nature 392, 605–608. http://dx.doi.org/10.1038/33416.

Koshiba, T., et al., 2004. Structural basis of mitochondrial tethering by mitofusin complexes. Science 305, 858–862. http://dx.doi.org/10.1126/science.1099793.

Koyano, F., et al., 2014. Ubiquitin is phosphorylated by PINK1 to activate parkin. Nature 510, 162–166. http://dx.doi.org/10.1038/nature13392.

Langston, J.W., Ballard, P., Tetrud, J.W., Irwin, I., 1983. Chronic Parkinsonism in humans due to a product of meperidine-analog synthesis. Science 219, 979–980.

Lee, Y.J., Jeong, S.Y., Karbowski, M., Smith, C.L., Youle, R.J., 2004. Roles of the mammalian mitochondrial fission and fusion mediators Fis1, Drp1, and Opa1 in apoptosis. Mol. Biol. Cell 15, 5001–5011. http://dx.doi.org/10.1091/mbc.E04-04-0294.

Liu, W., et al., 2011. Pink1 regulates the oxidative phosphorylation machinery via mitochondrial fission. Proc. Natl. Acad. Sci. U. S. A. 108, 12920–12924. http://dx.doi.org/10.1073/pnas.1107332108.

Loson, O.C., Song, Z., Chen, H., Chan, D.C., 2013. Fis1, Mff, MiD49, and MiD51 mediate Drp1 recruitment in mitochondrial fission. Mol. Biol. Cell 24, 659–667. http://dx.doi.org/10.1091/mbc.E12-10-0721.

Mannella, C.A., et al., 2001. Topology of the mitochondrial inner membrane: dynamics and bioenergetic implications. IUBMB life 52, 93–100. http://dx.doi.org/10.1080/15216540152845885.

Matsuda, N., et al., 2010. PINK1 stabilized by mitochondrial depolarization recruits Parkin to damaged mitochondria and activates latent Parkin for mitophagy. J. Cell Biol. 189, 211–221. http://dx.doi.org/10.1083/jcb.200910140.

McBride, H.M., Neuspiel, M., Wasiak, S., 2006. Mitochondria: more than just a powerhouse. Curr. Biol.: CB 16, R551–R560. http://dx.doi.org/10.1016/j.cub.2006.06.054.

McLelland, G.L., Soubannier, V., Chen, C.X., McBride, H.M., Fon, E.A., 2014. Parkin and PINK1 function in a vesicular trafficking pathway regulating mitochondrial quality control. EMBO J. 33, 282–295. http://dx.doi.org/10.1002/embj.201385902.

McNiven, M.A., Cao, H., Pitts, K.R., Yoon, Y., 2000. The dynamin family of mechanoenzymes: pinching in new places. Trends Biochem. Sci. 25, 115–120.

Mears, J.A., et al., 2011. Conformational changes in Dnm1 support a contractile mechanism for mitochondrial fission. Nat. Struct. Mol. Biol. 18, 20–26. http://dx.doi.org/10.1038/nsmb.1949.

Mitchell, P., 1977. A commentary on alternative hypotheses of protonic coupling in the membrane systems catalysing oxidative and photosynthetic phosphorylation. FEBS Lett. 78, 1–20.

Morais, V.A., et al., 2009. Parkinson's disease mutations in PINK1 result in decreased Complex I activity and deficient synaptic function. EMBO Molecular Medicine 1, 99–111. http://dx.doi.org/10.1002/emmm.200900006.

Mortiboys, H., et al., 2008. Mitochondrial function and morphology are impaired in parkin-mutant fibroblasts. Ann. Neurol. 64, 555–565. http://dx.doi.org/10.1002/ana.21492.

Mukhopadhyay, D., Riezman, H., 2007. Proteasome-independent functions of ubiquitin in endocytosis and signaling. Science 315, 201–205. http://dx.doi.org/10.1126/science.1127085.

Nakada, K., et al., 2001. Inter-mitochondrial complementation: mitochondria-specific system preventing mice from expression of disease phenotypes by mutant mtDNA. Nat. Med. 7, 934–940. http://dx.doi.org/10.1038/90976.

Naon, D., Scorrano, L., 2014. At the right distance: ER-mitochondria juxtaposition in cell life and death. Biochim. Biophys. Acta 1843, 2184–2194. http://dx.doi.org/10.1016/j.bbamcr.2014.05.011.

Narendra, D.P., Youle, R.J., 2011. Targeting mitochondrial dysfunction: role for PINK1 and parkin in mitochondrial quality control. Antioxid. Redox. Signal. 14, 1929–1938. http://dx.doi.org/10.1089/ars.2010.3799.

Narendra, D., Tanaka, A., Suen, D.F., Youle, R.J., 2008. Parkin is recruited selectively to impaired mitochondria and promotes their autophagy. J. Cell Biol. 183, 795–803. http://dx.doi.org/10.1083/jcb.200809125.

Narendra, D., Kane, L.A., Hauser, D.N., Fearnley, I.M., Youle, R.J., 2010a. p62/SQSTM1 is required for Parkin-induced mitochondrial clustering but not mitophagy; VDAC1 is dispensable for both. Autophagy 6, 1090–1106.

Narendra, D.P., et al., 2010b. PINK1 is selectively stabilized on impaired mitochondria to activate Parkin. PLoS Biol. 8, e1000298. http://dx.doi.org/10.1371/journal.pbio.1000298.

Narendra, D., Walker, J.E., Youle, R., 2012. Mitochondrial quality control mediated by PINK1 and Parkin: links to parkinsonism. Cold Spring Harb. Perspect. Biol. 4. http://dx.doi.org/10.1101/cshperspect.a011338.

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Nassel, D.R., Elekes, K., 1992. Aminergic neurons in the brain of blowflies and *Drosophila*: dopamine- and tyrosine hydroxylase-immunoreactive neurons and their relationship with putative histaminergic neurons. Cell Tissue Res. 267, 147–167.

Neuspiel, M., et al., 2008. Cargo-selected transport from the mitochondria to peroxisomes is mediated by vesicular carriers. Curr. Biol.: CB 18, 102–108. http://dx.doi.org/10.1016/j.cub.2007.12.038.

Okatsu, K., et al., 2012. PINK1 autophosphorylation upon membrane potential dissipation is essential for Parkin recruitment to damaged mitochondria. Nat. Commun. 3, 1016. http://dx.doi.org/10.1038/ncomms2016.

Olichon, A., et al., 2002. The human dynamin-related protein OPA1 is anchored to the mitochondrial inner membrane facing the inter-membrane space. FEBS Lett. 523, 171–176.

Ono, T., Isobe, K., Nakada, K., Hayashi, J.I., 2001. Human cells are protected from mitochondrial dysfunction by complementation of DNA products in fused mitochondria. Nat. Genet. 28, 272–275. http://dx.doi.org/10.1038/90116.

Otera, H., et al., 2010. Mff is an essential factor for mitochondrial recruitment of Drp1 during mitochondrial fission in mammalian cells. J. Cell Biol. 191, 1141–1158. http://dx.doi.org/10.1083/jcb.201007152.

Paisan-Ruiz, C., et al., 2004. Cloning of the gene containing mutations that cause PARK8-linked Parkinson's disease. Neuron 44, 595–600. http://dx.doi.org/10.1016/j.neuron.2004.10.023.

Park, J., et al., 2006. Mitochondrial dysfunction in *Drosophila* PINK1 mutants is complemented by parkin. Nature 441, 1157–1161.

Parone, P.A., et al., 2008. Preventing mitochondrial fission impairs mitochondrial function and leads to loss of mitochondrial DNA. PLoS One 3, e3257. http://dx.doi.org/10.1371/journal.pone.0003257.

Pimenta de Castro, I., et al., 2012. Genetic analysis of mitochondrial protein misfolding in *Drosophila melanogaster*. Cell Death Differ. 19, 1308–1316. http://dx.doi.org/10.1038/cdd.2012.5.

Pogson, J.H., et al., 2014. The complex I subunit NDUFA10 selectively rescues *Drosophila* pink1 mutants through a mechanism independent of mitophagy. PLoS Genet. 10, e1004815. http://dx.doi.org/10.1371/journal.pgen.1004815.

Polymeropoulos, M.H., et al., 1997. Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. Science 276, 2045–2047.

Poole, A.C., et al., 2008. The PINK1/Parkin pathway regulates mitochondrial morphology. Proc. Natl. Acad. Sci. U. S. A. 105, 1638–1643.

Ragland, M., Hutter, C., Zabetian, C., Edwards, K., 2009. Association between the ubiquitin carboxyl-terminal esterase L1 gene (UCHL1) S18Y variant and Parkinson's disease: a HuGE review and meta-analysis. Am. J. Epidemiol. 170, 1344–1357. http://dx.doi.org/10.1093/aje/kwp288.

Reiter, L.T., Potocki, L., Chien, S., Gribskov, M., Bier, E., 2001. A systematic analysis of human disease-associated gene sequences in *Drosophila melanogaster*. Genome Res. 11, 1114–1125. http://dx.doi.org/10.1101/gr.169101.

Rizzuto, R., Bernardi, P., Pozzan, T., 2000. Mitochondria as all-round players of the calcium game. J. Physiol. 529 (Pt 1), 37–47.

Rubin, G.M., Spradling, A.C., 1982. Genetic transformation of *Drosophila* with transposable element vectors. Science 218, 348–353.

Sarraf, S.A., et al., 2013. Landscape of the PARKIN-dependent ubiquitylome in response to mitochondrial depolarization. Nature 496, 372–376. http://dx.doi.org/10.1038/nature12043.

Scherz-Shouval, R., Elazar, Z., 2011. Regulation of autophagy by ROS: physiology and pathology. Trends Biochem. Sci. 36, 30–38. http://dx.doi.org/10.1016/j.tibs.2010.07.007.

Schrader, M., 2006. Shared components of mitochondrial and peroxisomal division. Biochim. Biophys. Acta 1763, 531–541. http://dx.doi.org/10.1016/j.bbamcr.2006.01.004.

Seibler, P., et al., 2011. Mitochondrial Parkin recruitment is impaired in neurons derived from mutant PINK1 induced pluripotent stem cells. The Journal of Neuroscience: The Official Journal of the Society for Neuroscience 31, 5970–5976. http://dx.doi.org/10.1523/JNEUROSCI.4441-10.2011.

Serrano, R., Kanner, B.I., Racker, E., 1976. Purification and properties of the proton-translocating adenosine triphosphatase complex of bovine heart mitochondria. J. Biol. Chem. 251, 2453–2461.

Sha, D., Chin, L.S., Li, L., 2010. Phosphorylation of parkin by Parkinson disease-linked kinase PINK1 activates parkin E3 ligase function and NF-kappaB signaling. Hum. Mol. Genet. 19, 352–363. http://dx.doi.org/10.1093/hmg/ddp501.

Shin, J.H., et al., 2011. PARIS (ZNF746) repression of PGC-1alpha contributes to neurodegeneration in Parkinson's disease. Cell 144, 689–702. http://dx.doi.org/10.1016/j.cell.2011.02.010.

Song, Z., Chen, H., Fiket, M., Alexander, C., Chan, D.C., 2007. OPA1 processing controls mitochondrial fusion and is regulated by mRNA splicing, membrane potential, and Yme1L. J. Cell Biol. 178, 749–755. http://dx.doi.org/10.1083/jcb.200704110.

Sood, A., et al., 2014. A Mitofusin-2-dependent inactivating cleavage of Opa1 links changes in mitochondria cristae and ER contacts in the postprandial liver. Proc. Natl. Acad. Sci. U. S. A. 111, 16017–16022. http://dx.doi.org/10.1073/pnas.1408061111.

Soubannier, V., et al., 2012a. A vesicular transport pathway shuttles cargo from mitochondria to lysosomes. Curr. Biol.: CB 22, 135–141. http://dx.doi.org/10.1016/j.cub.2011.11.057.

Soubannier, V., Rippstein, P., Kaufman, B.A., Shoubridge, E.A., McBride, H.M., 2012b. Reconstitution of mitochondria derived vesicle formation demonstrates selective enrichment of oxidized cargo. PLoS One 7, e52830. http://dx.doi.org/10.1371/journal.pone.0052830.

St Johnston, D., 2002. The art and design of genetic screens: *Drosophila melanogaster*. Nat. Rev. Genet. 3, 176–188. http://dx.doi.org/10.1038/nrg751.

Strappazzon, F., et al., 2011. Mitochondrial BCL-2 inhibits AMBRA1-induced autophagy. EMBO J. 30, 1195–1208. http://dx.doi.org/10.1038/emboj.2011.49.

Sugiura, A., et al., 2013. MITOL regulates endoplasmic reticulum-mitochondria contacts via Mitofusin2. Mol. Cell 51, 20–34. http://dx.doi.org/10.1016/j.molcel.2013.04.023.

Suzuki, M., Jeong, S.Y., Karbowski, M., Youle, R.J., Tjandra, N., 2003. The solution structure of human mitochondria fission protein Fis1 reveals a novel TPR-like helix bundle. J. Mol. Biol. 334, 445–458.

Taguchi, N., Ishihara, N., Jofuku, A., Oka, T., Mihara, K., 2007. Mitotic phosphorylation of dynamin-related GTPase Drp1 participates in mitochondrial fission. J. Biol. Chem. 282, 11521–11529. http://dx.doi.org/10.1074/jbc.M607279200.

Tain, L.S., et al., 2009a. Drosophila HtrA2 is dispensable for apoptosis but acts downstream of PINK1 independently from Parkin. Cell Death Differ. 16, 1118–1125. http://dx.doi.org/10.1038/cdd.2009.23.

Tain, L.S., et al., 2009b. Rapamycin activation of 4E-BP prevents parkinsonian dopaminergic neuron loss. Nat. Neurosci. 12, 1129–1135. http://dx.doi.org/10.1038/nn.2372.

Tufi, R., et al., 2014. Enhancing nucleotide metabolism protects against mitochondrial dysfunction and neurodegeneration in a PINK1 model of Parkinson's disease. Nat. Cell Biol. 16, 157–166. http://dx.doi.org/10.1038/ncb2901.

Twig, G., et al., 2008. Fission and selective fusion govern mitochondrial segregation and elimination by autophagy. EMBO J. 27, 433–446 doi:7601963 [pii]10.1038/sj.emboj.7601963.

Valente, E.M., et al., 2004. Hereditary early-onset Parkinson's disease caused by mutations in PINK1. Science 304, 1158–1160. http://dx.doi.org/10.1126/science.1096284.

Venken, K.J., et al., 2011. MiMIC: a highly versatile transposon insertion resource for engineering *Drosophila melanogaster* genes. Nat. Methods 8, 737–743.

Vilain, S., et al., 2012. The yeast complex I equivalent NADH dehydrogenase rescues pink1 mutants. PLoS Genet. 8, e1002456. http://dx.doi.org/10.1371/journal.pgen.1002456.

Vincow, E.S., et al., 2013. The PINK1–Parkin pathway promotes both mitophagy and selective respiratory chain turnover in vivo. Proc. Natl. Acad. Sci. U. S. A. 110, 6400–6405. http://dx.doi.org/10.1073/pnas.1221132110.

Vives-Bauza, C., et al., 2010. PINK1-dependent recruitment of Parkin to mitochondria in mitophagy. Proc. Natl. Acad. Sci. U. S. A. 107, 378–383. http://dx.doi.org/10.1073/pnas.0911187107.

Vos, M., et al., 2012. Vitamin K2 is a mitochondrial electron carrier that rescues pink1 deficiency. Science 336, 1306–1310. http://dx.doi.org/10.1126/science.1218632.

Wang, X., et al., 2011. PINK1 and Parkin target Miro for phosphorylation and degradation to arrest mitochondrial motility. Cell 147, 893–906. http://dx.doi.org/10.1016/j.cell.2011.10.018.

Wasilewski, M., et al., 2012. Optic atrophy 1-dependent mitochondrial remodeling controls steroidogenesis in trophoblasts. Curr. Biol.: CB 22, 1228–1234. http://dx.doi.org/10.1016/j.cub.2012.04.054.

Whitworth, A.J., et al., 2005. Increased glutathione S-transferase activity rescues dopaminergic neuron loss in a *Drosophila* model of Parkinson's disease. Proc. Natl. Acad. Sci. U. S. A. 102, 8024–8029. http://dx.doi.org/10.1073/pnas.0501078102.

Whitworth, A.J., et al., 2008. Rhomboid-7 and HtrA2/Omi act in a common pathway with the Parkinson's disease factors Pink1 and Parkin. Dis. Model. Mech. 1, 168–174 discussion 173, doi:10.1242/dmm.000109.

Yamano, K., Youle, R.J., 2013. PINK1 is degraded through the N-end rule pathway. Autophagy 9, 1758–1769. http://dx.doi.org/10.4161/auto.24633.

Yoon, Y., Krueger, E.W., Oswald, B.J., McNiven, M.A., 2003. The mitochondrial protein hFis1 regulates mitochondrial fission in mammalian cells through an interaction with the dynamin-like protein DLP1. Mol. Cell. Biol. 23, 5409–5420.

Yoshikawa, T., 1993. Free radicals and their scavengers in Parkinson's disease. Eur. Neurol. 33 (Suppl. 1), 60–68.

Youdim, M.B., Lavie, L., 1994. Selective MAO-A and B inhibitors, radical scavengers and nitric oxide synthase inhibitors in Parkinson's disease. Life Sci. 55, 2077–2082.

Youle, R.J., Narendra, D.P., 2011. Mechanisms of mitophagy. Nat. Rev. Mol. Cell. Biol. 12, 9–14. http://dx.doi.org/10.1038/nrm3028.

Zhu, J., Chu, C.T., 2010. Mitochondrial dysfunction in Parkinson's disease. Journal of Alzheimer's Disease: JAD 20 (Suppl. 2), S325–S334. http://dx.doi.org/10.3233/JAD-2010-100363.

Ziviani, E., Tao, R.N., Whitworth, A.J., 2010. *Drosophila* parkin requires PINK1 for mitochondrial translocation and ubiquitinates mitofusin. Proc. Natl. Acad. Sci. U. S. A. 107, 5018–5023. http://dx.doi.org/10.1073/pnas.0913485107.

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8. Abbreviations

ADP: Adenosine diphosphate
AKAP1: A kinase anchor protein 1
Ala: Alanine
APAF1: apoptotic protease-activating factor 1
Asp: Aspartatic Acid
ATP: Adenosine Tri-Phosphate
BAK: Bcl-2 homologous antagonist/killer
BAPTA: 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid
BAX: BCL (B Cell Lymphoma)-Associated X
Bcl-2: B-cell lymphoma 2
Bcl-xL: B-cell lymphoma-extra large
BH3: Bcl-2 homology 3
BID: BH3 interacting domain death agonist
Ca^{2+}: Calcium
CaN: calcineurin
CCCP: carbonyl cyanide *m*-chlorophenyl hydrazine
CD95: cluster of differentiation 95
CJs: cristae junctions
CM: cristae membrane
CnA: catalytic subunit of Calcineurin
CnB: regulatory subunit of Calcineurin
CO_2: Carbon dioxide
DA: dopamine
DMEM: Dulbecco's modified Eagle medium
DNA: Deoxyribonucleic acid
Drp1: dynamin-related protein 1
DUBs: deubiquitinating enzymes
EDTA: ethilendiammintetracetic acid
ER: endoplasmatic reticulum
FBS: Fetal Bovine Serum
Fis1: fission protein 1
FK506: tacrolimus
GTPase: GTP-binding proteins
H_2O_2: hydrogen peroxide
His: histidine
Hrs: hours
HtrA2: high temperature requirement A2 protease
ICS: intercrystal space
IMM: inner mitochondrial membrane
IMS: intermembrane space
KDa: kiloDalton
LB: Lewy bodies
LC3: light chain 3

L-DOPA: L-3,4-dihydroxyphenylalanine
L-OPA1: long OPA1 form
LRRK2: leucine-rich repeat kinase 2
L-strand: light strand
MAMs: mitochondria-associated-membranes
MAO-B: Monoamine Oxidase B
MAPK: mitogen-activated protein kinase
MCU: mitochondrial Ca^{2+} uniporter
MEFs: mouse embryonic fibroblasts
Mff: mitochondrial fission factor
MFN1: Mitofusin 1
MFN2: Mitofusin 2
Mg^{2+}: Magnesium
MIRO: mitochondrial Rho GTPases
Mito-YFP: mitochondrial yellow fluorescent protein
MPP: mitochondrial processing peptidase
MPTP: mitochondrial permeability transition pore
MtDNA: mitochondrial DNA
MTS: mitochondrial targeting sequence
NAD^+: nicotinamide adenine dinucleotide
NADH: reduced Nicotinamide adenine dinucleotide
NADPH: reduced Nicotinamide adenine dinucleotide phosphate
nM: nanoMolar
O_2: Oxygen
OMA1: overlapping activity with m-AAA protease
OMM: outer mitochondrial membrane
OPA1: Optic Atrophy 1
OriH: origin of heavy-strand replication
OriL: origin of light-strand replication
PARL: presenilin-associated rhomboid-like protease
PBS: phosphate saline buffer
PD: Parkinson's disease
PIC: protease inhibitor
PINK1: (PTEN)-induced putative kinase 1
PKA: protein kinase A
PP2A: Protein phosphatase 2
RaM: rapid mode of Ca^{2+} uptake
RBR: ring-between-ring fingers
REP: repressor element of Parkin
RIPA: radioimmunoprecipitation assay
RNAi: RNA interference
ROS: reactive oxygen species
rpm: revolutions per minute
RT: room temperature
s: second

Ser: Serine
SMAC: second mitochondria-derived activator of caspases
SNc: substantia nigra pars compacta
SNCA: synuclein alpha
SOD: superoxide dismutase
S-OPA1: short OPA1 form
Squassh: Segmentation and QUAntification of Subcellular Shapes
tBID: truncated BH3 interacting domain death agonist
TCA: tricarboxylic acid cycle
TFAM: transcription factor A of mitochondria
Thr: Threonine
TIM: translocase of the inner membrane
TK: tyrosine kinase
TNFR1: tumour necrosis factor receptor 1
TOM: translocase of the OMM
Ub: ubiquitin
UBL: ubiquitin-like
UPD: unique Parkin domain
USP: Ubiquitin-specific protease
VDAC1: Voltage-Dependent Anion Channel 1
ΔCnA: constitutively active mutant of Calcineurin
ΔCnA^{H151Q}: dominant negative mutant of Calcineurin
µM: microMolarPINK1: PTEN-induced putative kinase 1