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SERIES XXX

Regulation of ER-Mitochondria tethering in an *in vivo* animal model of Parkinson's disease.

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INDICE

Summary

Mitochondria form a tubular, reticulated network which shape is controlled by opposing fusion and fission events (Bereiter-Hahn and Voth, 1994). The mitofusins 1 and 2 (Mfn1 and Mfn2) are conserved, dynamin-like GTPases embedded in the outer mitochondrial membrane (OMM) that mediate mitochondrial fusion in coordination with OPA1 (Rojo et al., 2002; Santel and Fuller, 2001; Wong et al., 2000). Mitochondrial shaping proteins have pleiotropic functions. In particular, while Mfn1 seems primarily involved in organellar docking and fusion, Mfn2 is enriched at contact sites between ER and mitochondria where it is implicated in the formation of molecular linkers that are capable of organelles tethering (Chen et al., 2012; de Brito and Scorrano, 2008). Recent works attributed to these points of close contact between the OMM and the nearby ER, called MAMs (mitochondria-associated ER-membranes) or MERCs (mitochondria-ER contacts), an important role in the propagation of cellular signals, including those that control lipid metabolism, calcium (Ca^{2+}) homeostasis and cell death (Rowland et al., 2012; Rizzuto et al., 1998; Vance, 1990). Indeed, aberrations in ER-mitochondria juxtaposition have been described in cellular models of different neurodegenerative diseases, including Alzheimer's, Huntington's and Parkinson's disease (Krols et al., 2016; Calì et al., 2013; Ottolini et al., 2013; Area-Gomez et al., 2012; Calì et al., 2012; Panov et al., 2002). Although the exact cause for neuronal loss is not clear

Parkin, an E3-ubiquitin ligase mutated in familiar Parkinson's Disease (PD) is selectively recruited to dysfunctional mitochondria and promotes their elimination via autophagy, a process known as mitophagy (Narendra et al., 2008). PINK1, a protein kinase, also a PD related gene, is required for Parkin recruitment and stress induced mitophagy (Ziviani et al., 2010). In several model systems, Parkin selectively ubiquitinates the mitochondrial outer membrane profusion protein Mfn1 and Mfn2 and fly homologue Marf (Tanaka et al., 2010; Ziviani et al., 2010; Gegg et al., 2010). Accordingly, lack of Parkin or PINK1, which operates upstream Parkin in the same pathway, results in impaired ubiquitination of Mfn and increased levels of Mfn (Ziviani et al., 2010).

Given that Parkin affects Mfn steady state and ubiquitination levels, we propose to (i) address the ubiquitination levels of Mfn2 and whether Parkin downregulation affects it; (ii) investigate whether Parkin regulates ER-mitochondria tethering by impinging on Mfn2 steady

state and ubiquitination levels; (iii) evaluate the physiological significance of ER-mitochondria interaction in an *in vivo* animal model of Parkinson's disease.

Our hypothesis is that Parkin dependent Mfn ubiquitinatination controls ER-mitochondria tethering, thus impinging on Ca^{2+} transfer and Ca^{2+} homeostasis, which dysregulation has been described in a number of molecular pathways leading to PINK1 and Parkin loss of function dependent neurodegeneration (Calì et al., 2013; Ottolini et al., 2013; Calì et al., 2012).

In order to address the previously listed hypothesis we analysed the pattern of ubiquitination of Mfn2 in mouse embryonic fibroblasts (MEFs) upon downregulation of Parkin. To this aim, we (i) immunoprecipitated Mfn2 with specific anti Mfn2 antibody and performed western blotting analysis with specific anti HA antibody in cells overexpressing HA tagged Ubiquitin; (ii) measured the degree of tethering between ER and mitochondria in control and Parkin downregulating cells. We used two independent approaches to measure ER-mitochondria tethering: we first measured the percentage of ER co-localizing with mitochondria by using Mander's coefficient of co-localization upon volume-rendered 3D reconstruction of z-axis stacks of confocal images of cells expressing organelles targeted fluorescence probes (mito-RFP and ER-YFP, respectively) (Rizzuto et al., 1998). Secondly, we took advantage of a FRET based probe (Naon et al., 2016) to measure ER-mitochondria proximity. In this sensor, called FEMP, FRET intensity is inversely proportional to the distance between the two fluorophores (mito-YFP and ER-CFP) that are appropriately targeted to the two compartments. (iii) We investigated the physiological significance of ER-mitochondria tether in an *in vivo* animal model of PD that lacks PINK1 expression. To this aim we used the fruitfly *Drosophila melanogaster*, which has many advantages. First, fly mutants deriving from loss of function mutations of PINK1 have been extensively characterized and cause a robust phenotype represented by age-related degeneration of DA neuron loss and locomotor deficits (Poole et al., 2008; Clark et al., 2006; Park et al., 2006; Yang et al., 2006; Wang et al., 2006). Secondly, a variety of genetic modifications and epistasis experiments can be easily performed *in vivo* to dissect molecular pathways.

Our results showed that Parkin downregulation reduced Mfn ubiquitination and ER-mitochondria tethering in MEFs. Interestingly, we found that the pattern of Mfn2 ubiquitination and ER-mitochondria tethering is also impaired in CMT type 2A disease-associated Mfn2 mutations ($Mfn2^{R94Q}$, $Mfn2^{P251A}$ and $Mfn2^{R280H}$ respectively). Although

indirectly, these findings strongly suggested that ubiquitination of Mfn2, rather than its steady state levels, is important in the regulation of ER-mitochondria tethering.

To identify the precise site of Mfn2 ubiquitination and directly link lack of ubiquitination with impaired ER-mitochondria tether, we took advantage of a bioinformatics approach to identify among species-highly conserved lysine (K) residues. We identified twenty Lysine residues that were conserved between human, mouse and fly. We compared these residues with those identified by a mass spectrometry-based study published in 2014 (Bingol et al., 2014) that identified Parkin-dependent ubiquitination sites. We identified six Lysine residues that were likely to represent good candidates for Parkin-dependent ubiquitination of Mfn2. We generated non-ubiquitinatable mutants for those sites by substituting Lysine (K) with Arginine (R), a common procedure to impair ubiquitination and investigated the pattern of ubiquitination of the non-ubiquitinatable Mfn2 mutants by western blotting. Expression of non-ubiquitinable mutant K416R resulted in impaired Mfn2 ubiquitination. Of note this mutant was also unable to correct ER-mitochondrial contacts when expressed in *Mfn2* KO MEFs and only partial restored ER-mitochondrial Ca^{2+} transfer.

In summary, our results provided strong evidences that Mfn2 ubiquitination is a prerequisite for ER-mitochondria physical and functional interaction and that K416 in the HR1 domain of Mfn2 is a genuine site for Parkin dependent ubiquitination.

A number of studies have shown impaired Ca^{2+} homeostasis in cellular models lacking PINK1 or Parkin (Heeman et al., 2011; Sandebring et al., 2009). Although it is not clear why dopaminergic neurons specifically degenerate in PD, it is tempting to hypothesis that impaired Ca^{2+} homeostasis resulting from impaired Ca^{2+} cross talk at ER-mitochondria interface could lead or contribute to degeneration. Elegant studies have shown that artificial tether between ER and mitochondria can be used to modulate Ca^{2+} transfer (Csordás et al., 2010; Csordás et al., 2006). With that in mind, we addressed whether expressing an ER-mitochondria synthetic linker in a well-established *in vivo Drosophila* model of PINK1 loss of function could ameliorate PINK1 KO phenotypes by impinging on ER-mitochondria cross talk. We therefore generated a number of fly lines expressing the synthetic linker driven by a neuron-specific driver in the fly wing neurons. This linker was generated by Csordás et al. (Csordás et al., 2006) and consists of a monomeric fluorescent protein (RFP) fused to the outer mitochondrial membrane targeting sequence at the N terminus and fused to the ER targeting sequence at the C

terminus. We could observe a well-defined and easily quantifiable RFP-fluorescence spots throughout the L1 vein of the fly wing that perfectly matched the morphology seen when expressing mito-GFP or ER-GFP alone in the wing neurons (Vagnoni and Bullock, 2016), which indicated that the synthetic linker was appropriately expressed.

Interestingly, we found an amelioration of PINK1 KO climbing ability upon expression of the artificial synthetic linker. This result strong indicates that restoration of proper ER-mitochondrial communication in PINK1 KO background can be beneficial in ameliorating the phenotype associated to an *in vivo* animal model of PD, paving the way for novel approaches for medical intervention.

INTRODUCTION

1. Mitochondria

Mitochondria are key organelles that reside in almost every eukaryotic cell where they play a central role in ATP production through the oxidative phosphorylation of Krebs cycle substrates. In addition to their role as the cellular powerhouse (they generate 90% of the energy needed to maintain cellular homeostasis), mitochondria are also essential players in the regulation of apoptosis and autophagy, and in coordination with the endoplasmic reticulum (ER), they are responsible for maintaining calcium (Ca^{2+}) homeostasis within the cell.

They appeared about two million years ago following the engulfment of an α-proteobacterium by a precursor of the modern eukaryotic cell (Lane and Martin; 2010). As a consequence of that, mitochondria maintain similar compartments as their bacterial precursor: the outer and the inner mitochondrial membranes, an intermembrane space and the matrix. Over time, mitochondria acquired additional functions and in parallel, they lost or transferred most of their genomic material to the nuclear DNA of their guest. The mitochondrial genome (mtDNA) contains approximately 16,500 base pairs, and is organized in a circular, covalently closed, double-stranded DNA formation. mtDNA contains the genetic information essential for the codification of 13 proteins, that compose part of the four respiratory complexes localized to the inner mitochondrial membrane (complex I, II, III, IV). By using metabolites from the Krebs cycle, the respiratory complexes generate the electrochemical gradient essential for ATP synthesis and contribute to maintain cellular Ca^{2+} concentration (Baughman et al., 2011; De Stefani et al., 2011).

Mitochondria do not exist as isolated organelles, but rather form a highly interconnected tubular network which shape can change in response to external factors and metabolic inputs. Mitochondria shape is controlled by fission and fusion events that are highly orchestrated by a number of mitochondrial shaping proteins, which include large dynamin-related GTPases Mitofusin 1 and 2 (MFN1, MFN2), Optic Atrophy 1 (OPA1) and the cytosolic dynamin-related protein 1 (DRP1). Mitochondrial shape is intimately related to mitochondria function, and thus it is not surprising how perturbations in mitochondria

dynamic has been associated with several human pathologies, including neurodegenerative disorders such as Parkinson's disease (PD).

1.1. Mitochondrial structure and ultrastructure

In 1952, work from two different groups independently described mitochondria ultrastructure using electron microscopy. The two groups proposed two different models, the so-called baffle model and the septa model, by Palade and Sjostrand respectively (Sjostrand, 1953; Palade,1952). Both groups independently concluded that mitochondria are made of two membranes, the outer and the inner mitochondrial membrane, with a small space in between which was called the intermembrane space (IMS). According to the Palade model, the inner mitochondrial membrane (IMM) has many invaginations called cristae membranes (CM) in which the electron transport chain and the ATP synthase complex are localized. The space contained by the inner membrane is called matrix. Additional work using electron microscopic tomography showed that mitochondrial cristae are connected to the inner boundary membrane through tubular structures called cristae junctions, which have a diameter of approximately 28nm (Perkin et al., 1997; Mannella et al., 1994) (Fig.1). The cristae junctions keep the contents of the cristae secured within the intracristal space. Following activation of programmed cell death for example, mitochondria cristae undergo remodeling allowing the release of cytochrome c from the intracristal space, which is a prerequisite for the activation of the downstream effectors of apoptosis (Yamaguchi and Perkins, 2009). Of note, the process of cristae remodeling is under intense investigation by a number of research groups as its dysregulation can lead to severe consequences for cell.

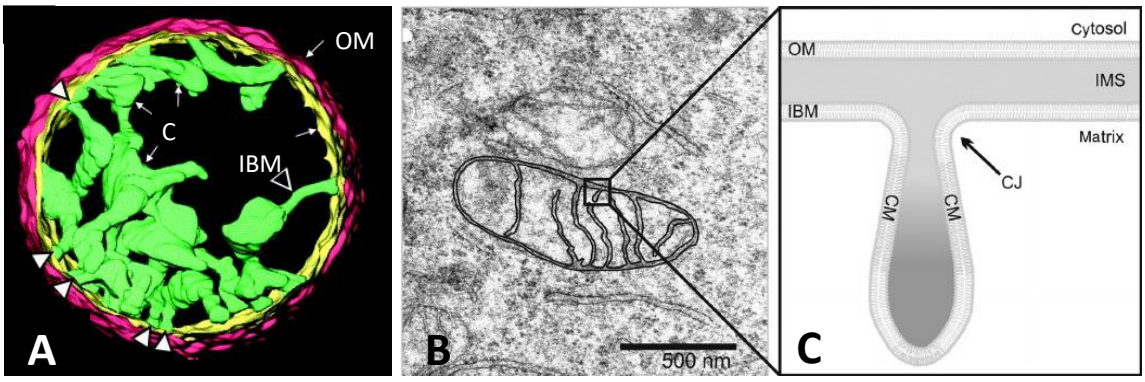

Figure 1. Mitochondrial ultrastructure and subcompartments. (A) 3D reconstruction of a rat-liver mitochondrion obtained by electron microscopy tomography (5 nm slides). OM: outer membrane; IBM: inner boundary membrane; C: cristae. (Frey and Mannella, 2000). (B) Transmission electron micrograph of a mitochondrion within human 143B cell. (C) Representation of a single cristae structure. IMS: intermembrane space; CJ: cristae junction; CM: cristae membrane (Koob and Reichert, 2014).

Both the inner mitochondria membrane and the outer mitochondrial membrane are subject to continuous conformational modifications in response to metabolic demands. These two membranes continuously undergo fission and fusion events, which are tightly controlled and are essential for maintaining mitochondrial function.

1.1.1. Mitochondrial fission

The complexity of mitochondrial fission machinery has been first elucidated in yeast. In 1999 following a yeast genetic screening, DNM1 was identified as a large dynamin-related GTPase that is required for mitochondria fission (Bleazard et al., 1999). Subsequent work identified dynamin-related protein 1 (DRP1), an orthologue to yeast DNM1, and FIS1 as components of the mammalian mitochondrial fission machinery.

The key fission mediator DRP1 is a soluble protein with an N-terminal GTPase domain and a central domain, which is able to self-assemble via its C-terminal GTPase effector (Smirnowa et al., 1998). In response to specific stimuli, DRP1 translocates from the cytosol to the outer mitochondria membrane. DRP1 translocates to specific mitochondrial fission sites that are marked by tubules of the endoplasmic reticulum (ER) that physically enclose and constrict mitochondria, thus facilitating the self-assembly of DRP1 into ring-like structures (Friedman et al., 2011). After binding dedicated adaptor proteins, DRP1 forms a homomultimeric complex that circumscribes the organelle and, after GTP binding and hydrolysis, it undergoes a morphological change that causes mitochondrial membrane constriction and scission (Mears et al., 2011). The deletion of *Drp1* in mouse is lethal due to developmental defects; in human, the dominant negative mutation in *Drp1* induces neonatal disorder (Ishihara et al., 2009; Waterham et al., 2007). FIS1 is a small adaptor protein that is localized to the outer mitochondrial membrane where it is anchored to a second set of adaptor proteins (called MDV and CAF4 in yeast), via its cytosolic N terminal region. Homologues of MDV and CAF4 have not been identified in mammals, although mammals appear to possess their own set of DRP1 adaptor proteins that operate in the same way. MDV and CAF4 are able to interact with DNM1, and allow the interaction between FIS1 and

DMN1 (Griffin et al., 2005; Tieu et al., 2002; Tieu et al., 2000) that is required for fission to proceed.

Another regulator of fission that was identified in *Drosophila melanogaster* that has no yeast homologues, is the mitochondrial fission factor (MFF) (Gandre-Babbe and van der Bliek, 2008). The mammalian homologue retains the same function. MFF is an adaptor protein for DRP1, and like FIS1 is anchored to the outer mitochondrial membrane. Ablation of *Mff* reduces mitochondrial division (Fig.2). However MFF and FIS1 do not belong to the same protein complex (Losón et al., 2013). Using *Fis1*-null, *Mff*-null, and *Fis1/Mff*-null cells it was observed that both FIS1 and MFF participate in mitochondrial fission. Interestingly two additional proteins, MiD49 and MiD51, can mediate DRP1 recruitment to mitochondria, usually localizes to the cytosol of cells, and mitochondrial fission in the absence of FIS1 and MFF suggesting a complex interplay between components of the fission machinery (Losón et al., 2013).

It is possible that additional components of the fission machinery that are involved in the fission of the inner mitochondrial membrane have yet to be identified. A potential candidate named MDM33 was identified in yeast, but no homologues have been found in higher eukaryotes. This protein assembles into an oligomeric complex in the inner membrane and induces its constriction and/or fission (Messerschmitt et al., 2003). Human MTP18 (mitochondrial fission process protein 1) is postulated to mediate the fission of the inner mitochondrial membrane in mammals (Tondera et al., 2005).

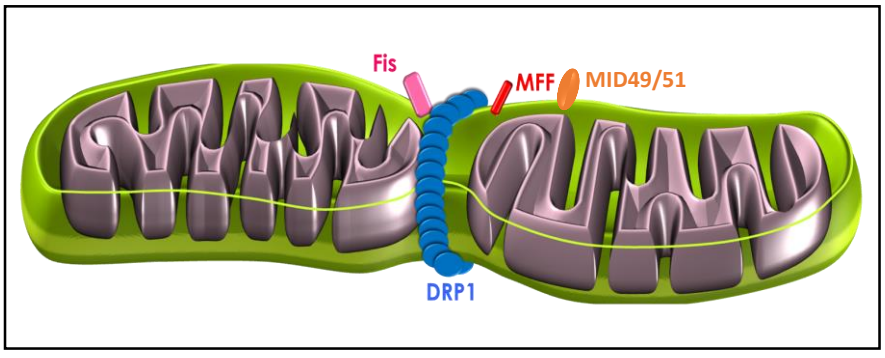

Figure 2. Schematic representation of molecular machine that mediate fission process in mammals. The key protein involved in mammalian mitochondrial fission is DPR1. Once translocated from cytosol to mitochondria, after its dephosphorylation, it self-oligomerizes, assembling a scission machine around the OMM, and interacts with MFF, MID49/51 and FIS1, promoting mitochondrial fission.

1.1.2. Mitochondrial fusion

The counterpart of mitochondria division is the process of mitochondria fusion. Mitochondrial fusion is dependent on large dynamin-related GTPases fusion proteins, the first of which was identified in 1997, following a genetic analysis in *Drosophila melanogaster*. This protein, called fuzzy onions (Fzo), regulates the fusion of mitochondria during spermatogenesis. The authors observed that during meiosis, the mitochondria of the cell wrapped around each other to form a structure called the Nebenken. This spherical organization that was observed by electron microscopy, appeared like an onion due to the concentric disposition of mitochondria (Hales et al., 1997). *Drosophila* also possesses an ubiquitous mitochondrial assembly regulatory factor called Marf that mediates fusion. Fzo and Marf are differentially expressed during development and show a tissue specific distribution, with Fzo mainly expressed in testis (Hwa et al., 2002). There is one Fzo homologe in yeast, called Fzo1, and two in mammals, Mitofusin 1 and 2 (MFN1 and MFN2) (Santel et al., 2001; Hermann et al., 1998). These proteins, like in *Drosophila*, induce the docking and the fusion of the outer mitochondria membranes of two adjacent mitochondria (Fig.3A). Mutations in Fzo1 are known to cause inhibition of mitochondrial fusion during yeast mating, while mice deficient in either *Mfn1* or *Mfn2* die in midgestation (Chen et al., 2003).

The fusion of the inner mitochondrial membrane in yeast is regulated by the dynamin-like GTPase Mgm1 (Wong et al., 2000). The mammalian orthologue of the yeast protein is called OPA1 (optic atrophy 1) (Fig.3B). Mutations in this protein are associated with autosomal dominant forms of optic atrophy (ADOA), a disease characterized by a progressive loss of the retina ganglion cells axon that causes vision loss (Olichon et al., 2002). In addition to regulating mitochondrial fusion, OPA 1 has many different functions, including the maintenance of mtDNA and cristae integrity. In human there are eight different isoforms which are generated by alternative splicing of the transcript (Del Dotto et al., 2017). Knockdown of *Opa1* induces mitochondrial fragmentation and an alteration of cristae structure (Griparic et al., 2004).

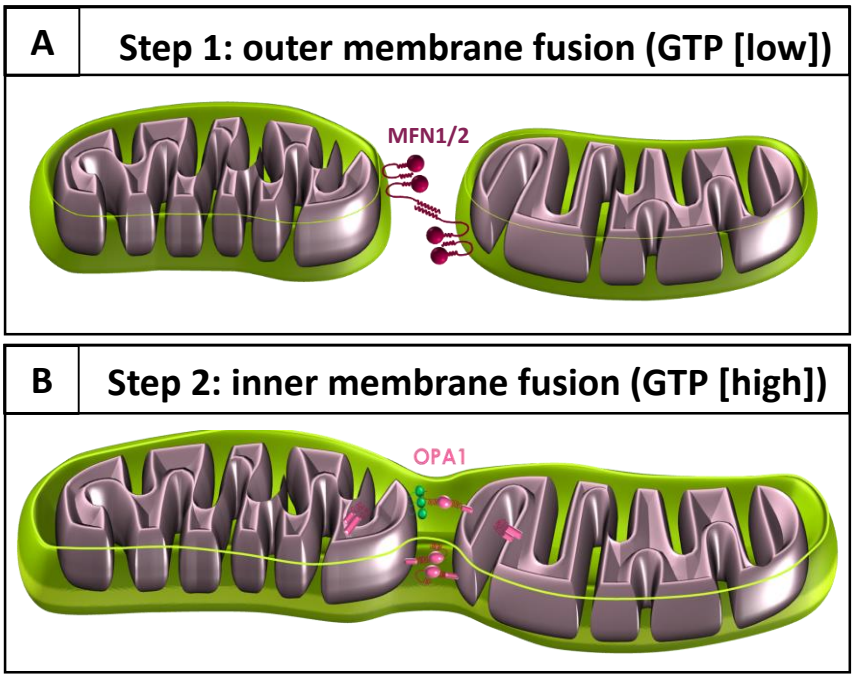

Figure 3. Schematic illustration of mitochondrial fusion in mammalian cells. (A) Mitochondrial fusion proteins MFN1 and MFN2 play an important role in the docking and fusion of OMM, through homo- or heterotypic interaction between MFN1 and MFN2 localize on the outer mitochondrial membrane. (B) OPA1 is localized at the IMM and in the intermembrane space and is required for the fusion of inner mitochondrial membrane as well as in the formation of cristae junction. OPA1 is processed into several cleavage isoforms, which have different roles in the fusion events.

1.1.2.1. Mitofusin

Mitofusin 1 and 2 are GTPase proteins localized at the outer mitochondrial membrane (Rojo et al., 2002; Santel and Fuller, 2001) and have essential roles in the induction of outer mitochondrial membrane fusion. In the absence of both *Mfn1* and *Mfn2*, mitochondrial fusion is lost (Chen et al., 2005). MFN1 and 2 are anchored at the outer mitochondrial membrane and have N- and C-terminal domains that protrude into the cytosol. At the N-terminus, there is a GTP binding domain, followed by a hydrophobic heptad repeat region (HR1) and a transmembrane domain (TM), which allows anchorage to the outer membrane. At the C-terminal, there is also a second hydrophobic heptad repeat (HR2) (Rojo et al., 2002) (Fig.4). Two mitofusins on adjacent mitochondria are able to engage in homo- or heterotypic interactions in *trans* through the HR2 domain and this process is dependent on GTP hydrolysis (Chen et al., 2005; Koshiba et al., 2004). The function of the two mitofusins appear partially redundant; cells lacking *Mfn1* can be rescued through the overexpression of *Mfn2*, and vice versa. However OPA1 requires only MFN1 to regulate mitochondrial fusion and not MFN2 (Cipolat et al., 2004). Another difference between the two orthologoes is that MFN1 has higher GTPase activity and larger affinity for GTP than MFN2 (Ishihara et al.,

2004). In 2008, De Brito et al. showed that MFN2 is enriched at the ER-mitochondrial interface, and the ablation of this protein induced an alteration of ER morphology and a reduction in the physical and functional interactions between ER and mitochondria (de Brito and Scorrano, 2008). This model has been recently challenged by two works (Filadi et al., 2015; Cosson et al., 2012) which, by using electron and confocal based microscopy techniques, produced the opposite result that Mfn2 removal results in increased ER-mitochondria interaction. Despite the controversy, both schools and a number of previous works attribute to these points of close contact between the OMM and the nearby ER, a fundamental role in the propagation of cellular signals, including those that control lipid metabolism, calcium (Ca^{2+}) homeostasis and cell death (Rowland et al., 2012; Rizzuto et al., 1998; Vance, 1990). Interestingly, expression of MFN2 but not MFN1 in Drosophila lacking Marf corrects the ER morphology and reduces the development and motor defects (Debattisti et al., 2014) suggesting that Marf has a specific role in ER functions. Mutations in MFN2 are known to cause an hereditary neuropathy called Charcot Marie Tooth disease type 2A (CMT 2A), which is characterized by a progressive impairment of motor and sensor neurons that causes atrophy of distal muscular nerves (Züchner et al., 2004). The CMT mutation R94Q, which is in the GTPase domain, fails to rescue the alteration of ER morphology and ER-mitochondrial interaction when expressed in *Mfn2* knockout mouse embryonic fibroblasts (MEFs) (de Brito and Scorrano, 2008).

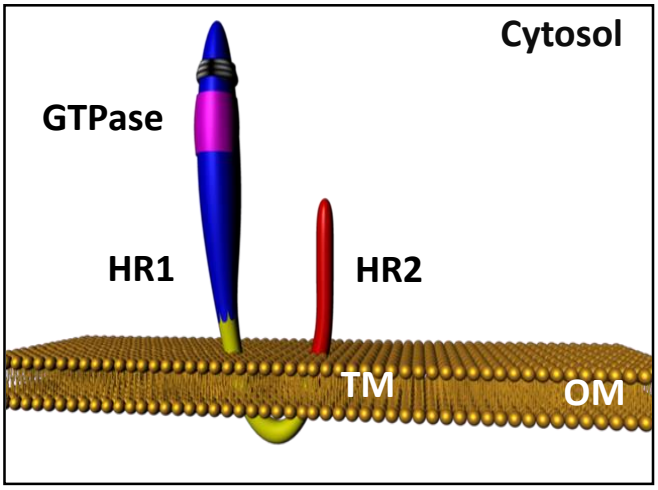

Figure 4. Mitofusin 2 structure. Illustration of the transmembrane GTPase protein Mitofusin 2. MFN 2 is a mitochondrial-shaping protein with both N- and C- termini exposed to the cytosol. It consists of one GTPase domain, 2 hydrophobic heptad repeat (HR1 and HR2) and a transmembrane domain.

2. Mitochondrial associated membranes (MAMs)

In 1959, Copeland and Dalton described for the first time the presence of particular regions where the mitochondria come in close proximity with the endoplasmic reticulum (ER). These ER domains are known as mitochondrial associated membranes (MAMs) (Copeland and Dalton, 1956). Interestingly, 5-20% of mitochondrial surface is associated with the ER, with an average distance of 10-30 nm (Csordás et al., 2006). The physical interaction between ER and mitochondria is indispensable for important cellular functions that are instrumental to cell survival. In particular ER-mitochondria cross talk regulate: calcium transfer, phospholipid synthesis and exchange, intracellular trafficking, ER stress and the unfolded protein response (UPS), autophagosome formation, mitochondrial fission and inflammasome formation.

In the following paragraph we provide a detailed description of the important physiological and pathological pathways that depends on ER-mitochondria interaction and how this is regulated.

2.1 ER-mitochondrial tethering structure

The mechanism and the molecular components that regulate ER-mitochondria communication are not fully understood. In yeast, Mmm1/Mdm10/Mdm12/Mdm34 complex called ERMES (ER mitochondria encounter structure) was identified following a genetic screening using a synthetic ER-mitochondria tether. Through this synthetic protein designed to artificially tether two organelles they screened a set of mutants, identifying that mutation of a single ERMES component causes complex disassembly that results in different growth phenotypes. Mdm34 and 10 are situated on the OMM, while Mdm12 and Mmm1 are localized on the ER. The ER-mitochondrial communication ERMES-mediated was found to be necessary for an efficiency phospholipid exchange between the ER and mitochondria, which is an essential step for phospholipid synthesis (Kornmann et al., 2009). ERMES homologoes is not found in mammals.

The first mammalian protein identified to be involved in ER-mitochondrial communication is voltage-dependent anion channel 1 (VDAC1). This protein, localized on the OMM, interacts through the chaperone Grp75 with the inositol 1,4,5-triphosphate receptor (IP3R), anchored on ER (Szabadkai et al., 2006; Rapizzi et al., 2002). The disruption of this

molecular bridge does not alter the proximity of the two organelles, but it has an impact on Ca^{2+} transfer (Csordás et al., 2006). As mentioned before, Mitofusin 2 is a candidate regulator of ER-mitochondrial communication (Filadi et al., 2015; Cosson et al., 2012; de Brito and Scorrano, 2008). The physical interaction between mitochondrial associated membrane protein B (VAPB) on the ER and the mitochondrial protein tyrosine phosphatase-interacting protein 51 (PTPIP51) also generates a molecular complex that bridges the two compartments (Stoica et al., 2014; De Vos et al., 2012). One more complex identified for its role in apoptotic signaling rather than for the maintenance of ER-mitochondria juxtaposition is composed of Bap31 resident on the ER and mitochondrial resident protein Fis1 (Iwasawa et al., 2011). Finally, it has to be mentioned that the vesicular sorting protein phosphofurin acidic cluster sorting protein 2 (PACS2) also contributes to the structural integrity of the ER-mitochondria communication, although the mechanism of interaction is not yet clear (Simmen et al., 2005) (Fig.5). All these complexes, and many yet to be identified, control and regulate the interaction between ER and mitochondria.

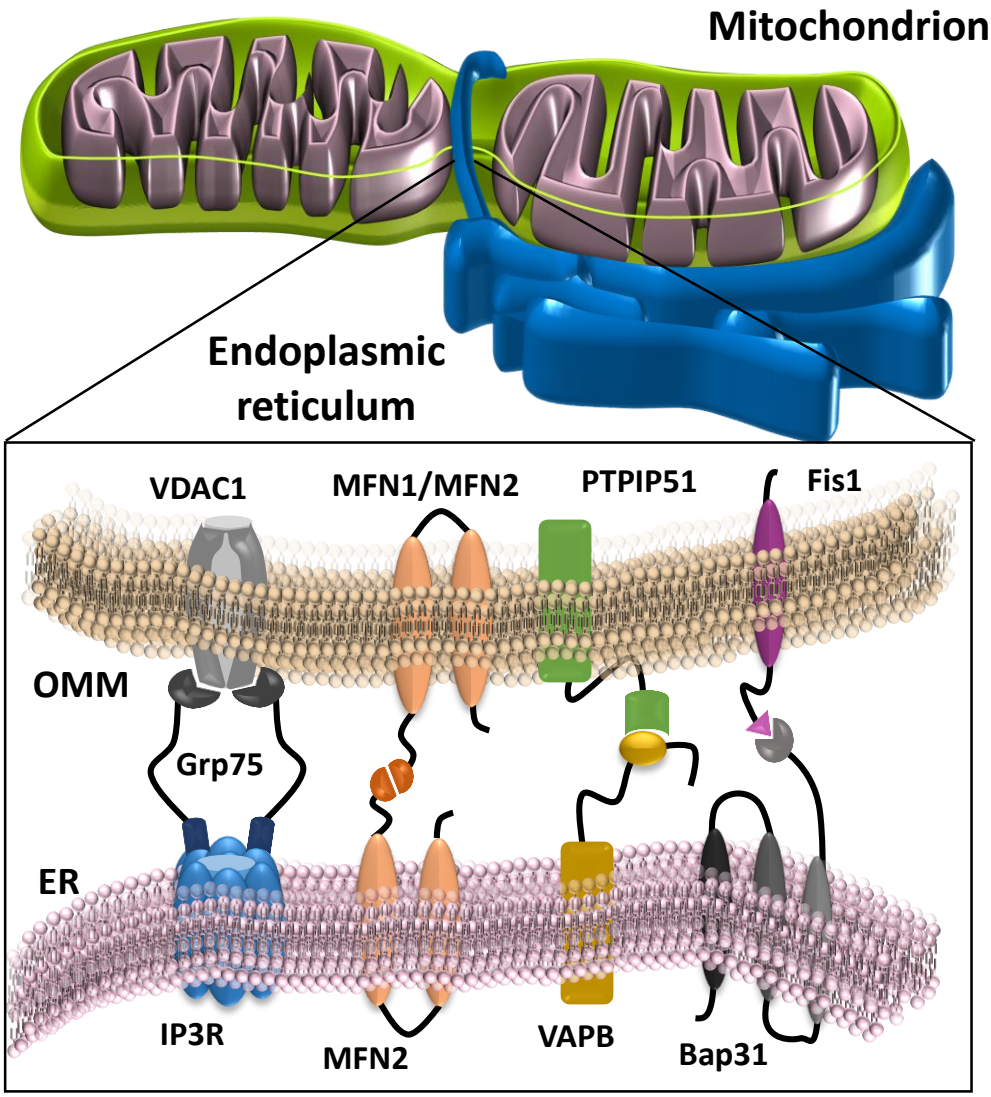

Figure 5. ER-mitochondria tethering protein complex. Inositol 1,4,5-trisphosphate receptor (IP3R) and voltage-dependent anion channel (VDAC) interact through the chaperone GRP75, forming a link that mediate the transport of Ca^{2+} from the ER to the mitochondria. Mitofusin 1 and 2 (MFN1 and MFN2) induce the physical tethering between ER and mitochondria. In particular MFN2 on the ER creates hetero- or homotypic complex with MFN1 or MFN2 respectively localize on the OMM, maintaining the organelles in close contact (de Brito and Scorrano, 2010). Tyrosine phosphatase interacting protein 51 (PTPIP51) situated on the mitochondria creates a complex with vesicle-associated membrane protein-associated protein B (VAPB) identified on the ER. This interaction is important for apoptotic signaling. Bap31 on the ER binds the mitochondrial fission 1 protein (Fis1).

2.2 ER-mitochondrial tethering functions

2.2.1. Ca^{2+} transfer

One of the most important roles of proper ER-mitochondria interaction is Ca^{2+} cross talk and the regulation of Ca^{2+} transfer. Mitochondria require Ca^{2+} for proper functioning of the enzymes of the Krebs cycles (McCormack and Denton, 1980). Too high mitochondrial Ca^{2+} concentration however, can lead to the opening of the mitochondrial permeability transition pore (MPTP), inducing cytochrome c release and activating apoptosis (Kroemer et al., 2007;

Boehning et al., 2003) (Fig.6A). Thus, mitochondria Ca^{2+} uptake needs to be tightly regulated. Of note, mitochondria have a very low affinity for Ca^{2+}, which raises the question of, how can they efficiently pick up Ca^{2+} that is required for proper activity? This enigma was solved after the discovery of the so-called Ca^{2+} "hot spot" domains that are generated at the MAMs. High Ca^{2+} domains that are forming at the MAMs allow the transfer of Ca^{2+} across the IMM, through the mitochondrial calcium uniporter (MCU), which has low affinity for Ca^{2+} (De Stefani et al, 2011; Baughman et al., 2011; Kirichok et al., 2004; Rizzuto et al., 1998). In this process, IP3 (inositol 1,4,5-triphosphate) activates the release of Ca^{2+} from the ER via IP3R that resides on the ER. Ca^{2+} released from the ER is transferred to mitochondria through VDAC1/Porin, and generates Ca^{2+} "hot spots" at the IMM, which allows the MCU channel to pick it up. Ca^{2+} "hot spots" that are generated at the MAMs allow ER-mitochondria Ca^{2+} transfer that results in an increase in mitochondrial Ca^{2+} levels of around 10-100 fold, without increasing cytosolic Ca^{2+} concentration (Várnai et al., 2005).

2.2.2. Phospholipid synthesis and exchange

Most of the lipids are synthesized in the ER, but their maturation occurs in the mitochondria. For this reason exchange between ER and mitochondria is an essential process for lipids synthesis (Vance, 1990). Lipid transport occurs at the MAM domains, where the enzymatic machinery that is required for lipid biosynthesis is enriched (Stone and Vance, 2000). For example, phosphatidylserine (PS) synthase 1 and 2 (Pss1 and Pss2) that are localized in the ER, are required for PS synthesis. PS needs to translocate to the mitochondria to be converted into phosphatidylethanolamine (PE) and is subsequently brought back to the ER where it is modified to phosphatidylcholine (PC) (Rowland and Voeltz, 2012) (Fig.6B).

2.2.3. Intracellular trafficking

Mitochondria are highly dynamic organelles that not only undergo continuous fission and fusion processes but also move along the cytoskeleton. In 2010, Friedman et al. shown that mitochondria and ER remain tethered to each other during intracellular trafficking, although it is not completely clear whether they both interact with the microtubules, or the ER is simply dragged along by the mitochondria that are known to be attached to the

microtubules (Friedman et al., 2010). Mitochondrial trafficking is a well characterized process, which largely depends on the function of the GTPase protein Miro. Miro localized on the OMM where it forms a molecular complex with MILTON, which binds to Kinesin, a protein belonging to a class of motor molecule that can move along microtubule (MT) filaments (Woźniak et al., 2009; Saotome et al., 2008; Glater et al., 2006). Interestingly, Miro can sense Ca^{2+} concentration so that its interaction with MILTON is disrupted when Ca^{2+} concentration becomes too high (Wang and Schwarz, 2009). One of the two mammalian paralogs of Miro (Miro-1) is found at ER-mitochondrial contact sites. Moreover the homologue in yeast is a regulatory subunit of the ERMES complex. These findings led to the intriguing hypothesis that mitochondrial trafficking and ER-mitochondria cross talk (via Ca^{2+} signaling) are intimately correlated (Kornmann et al., 2011; Stroud et al., 2011) (Fig.6C).

2.2.4. ER stress and the UPR

The ER is the primary site for protein synthesis. Proteins require chaperones for proper folding. When unfolded proteins accumulate inside the ER, the unfolded protein response (UPS) is activated, leading to a condition termed “ER stress” (Malhotra and Kaufman, 2007; Ron and Walter, 2007). There are several intra and extracellular factors inducing ER stress, which can cause ER Ca^{2+} depletion, oxidative stress, glucose deprivation and an alteration in ATP levels. The UPS is activated by at least three ER-localized stress sensors called inositol-requiring enzyme 1 (IRE1α), PKR-like ER kinase (PERK), and activating factor 6 (ATF6) (Hetz, 2012). Interestingly, the UPR transducer PERK is enriched at MAMs and has a direct role in ER-mitochondrial tethering. In details, the ablation of PERK reduces the ER-mitochondria communication and enhances protection from apoptosis caused by agents which mobilize Ca^{2+} from the ER and induce ROS production (Verfaillie et al., 2012). In addition, PERK has been reported to interact with MFN2 a key component of MAMs. In more detail under basal conditions MFN2 maintains PERK inactive (Muñoz et al., 2013). Other work has shown that IRE1, which is also found enriched at the MAM domains under stress conditions, controls ER-mitochondrial Ca^{2+} transfer through a negative regulation of IP3R (Son et al., 2014). These studies led to the conclusion that UPS that generates in the ER requires proper ER-mitochondria cross talk at the MAMs to propagate.

2.2.5. Autophagosome formation

The factors that cause ER stress and the activation of UPS can also activate autophagosome formation. For years the origin of the autophagosomal membranes has remained mysterious. In 2013, Hamasaki showed that ER-mitochondrial contacts site are the primary source of autophagosomal membranes (Hamasaki et al., 2013), opening a brand new niche of investigation which aims at understanding the relationship between autophagy, the main pathway involved in the recycle of intracellular components (Shintani and Klionsky, 2004), and ER-mitochondria cross talk.

2.2.6. Mitochondrial fission

As previously mentioned, DRP1 is a cytoplasmic protein that translocates to the OMM where it forms an oligomeric structure that circumscribes the mitochondrion. Following GTP hydrolysis, DRP1 promotes mitochondria fission. In 2011 Friedman et al. showed that the division of mitochondria does not occur at random sites but at ER-mitochondria contact sites. This model supports the hypothesis that ER tubules constrict the mitochondrion before DRP1 recruitment thus labelling sites of mitochondrial division (Friedman et al., 2011) (Fig.6C).

2.2.7. Inflammasome formation

ER-mitochondrial cross talk and physical interaction has been linked to the formation of inflammasome, a complex of proteins that allows the activation of caspase-1, the secretion of interleukin 1β (IL-1β) and the start of innate immune response (Gross et al., 2011). There are different types of inflammasome, but the most studied is the NLRP3 inflammasome (NOD-like receptor family, pyrin domain containing 3). A variety of alterations and dysregulations can trigger activation of the NLRP3 inflammasome. For instance an accumulation of the reactive oxygen species (ROS) is known to trigger the activation of NLRP3 inflammasome response. Interestingly, NLRP3 inflammasome activation by monosodium urate (MSU) or nigericin results in substantial association between inflammasome complexes and MAMs. Furthermore, the downregulation of VDAC, an important component of MAMs, causes a reduction of mitochondrial ROS production, caspase-1 activation and IL-1β formation (Zhou et al., 2011). These findings support the hypothesis of the contribution of MAMs in inflammation response.

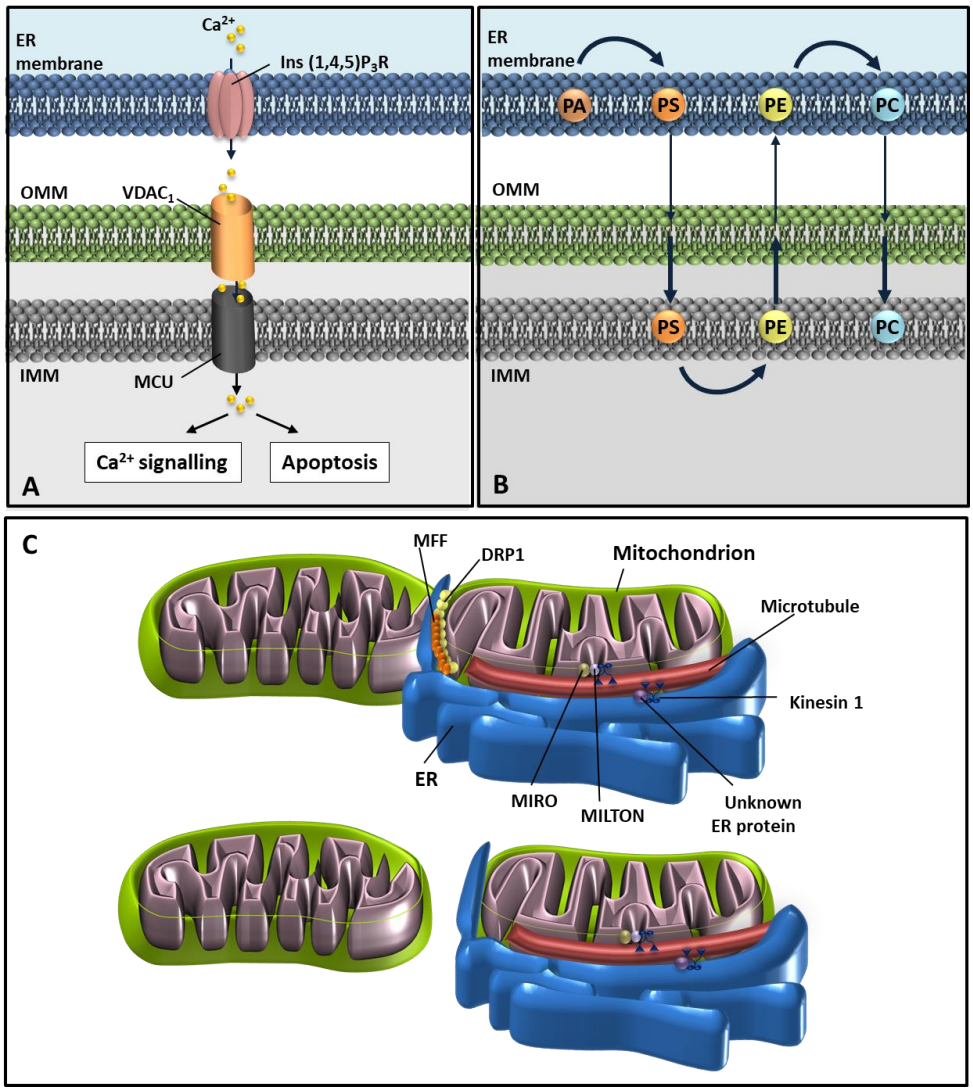

Figure 6. Functions of ER-mitochondria contacts. (A) **Ca^{2+} transfer.** Ca^{2+} released from the ER through the inositol 1,4,5-trisphosphate receptor (IP3R) is transferred in the mitochondrial across the voltage-dependent anion selective channel protein 1 (VDAC1) localized on the OMM. Calcium crosses the IMM through the mitochondrial calcium uniporter (MCU) due to the calcium gradient in the IMS. In the mitochondrion, Ca^{2+} has a dual function: allowing the production of ATP and, at high concentrations, inducing cell death by apoptosis. (B) **Lipid biosynthesis and exchange.** Biosynthesis of phosphatidylcholine acid (PC) requires steps in the ER and in mitochondria. The phosphatidylserine acid (PS) synthesized in the ER is transferred in the mitochondrion where it is converted to phosphatidylethanolamine (PE). It is retransferred in the ER where it undergoes a transformation to PC and is moved again to the mitochondrion. The enzymes that catalyze the conversion of PS in PE are localized in the ER, while the enzyme that transform the PA in PS and PE in PC are located in the mitochondrion. (C) **Intracellular trafficking.** Mitochondria and ER are linked to the microtubules by the motor kinesin 1. Specifically, mitochondria bind kinesin 1 through the Miro-MILTON complex and Miro works like a calcium sensor to regulate the mitochondrial dynamics. The initial mitochondrial constriction is mediated by ER, while the last step of the mitochondrial fission needs the recruitment and the oligomerization of DRP1 around the mitochondria, which circumscribes the mitochondria at the ER-mitochondria contact site. DRP1 requires the cofactors Fis1 and MFF to guarantee the mitochondrial scission.

3. ER-mitochondrial tethering and neurodegenerative disease

Considering the fundamental role of ER-mitochondria communication in a large number of physiological processes, it is not surprising that dysregulation of this interaction may be directly linked to pathological conditions, including those that are associated to the onset of

neurodegenerative disorders. Of note, α-synuclein, Parkin and DJ1, which are known to be mutated in familiar forms of Parkinson's disease (PD), are able to modulate ER-mitochondria cross talk (Ottolin et al., 2013; Calì et al., 2013; Calì et al., 2012). Specifically, Parkin overexpression increases ER-mitochondria tethering, favoring Ca^{2+} transfer from ER to mitochondria. Accordingly, Parkin genetic inhibition impairs Ca^{2+} uptake and reduces ER-mitochondrial communication (Calì et al., 2013).

Neurons are known to be more vulnerable to perturbations in Ca^{2+} homeostasis and lipid transfer, both affected upon impaired ER-mitochondrial juxtaposition (Paillusson et al., 2016; Krols et al.; 2016). In particular, it was shown that the vulnerability of dopaminergic neurons, that are specifically lost in PD patients, depends on their reliance on a specific subtype of Ca^{2+} channels that require Ca^{2+} to maintain their autonomous pacemaking activity and which expression is age dependent (Chan et al., 2007). Surmeier and co workers demonstrated that blocking these channels with isradipine has a neuroprotective effect because it allows neurons to "rejuvenile" and go back to a Ca^{2+} independent mechanism to generate their pacemaking activity (Chan et al., 2007). This work highlighted for the first time how important Ca^{2+} homeostasis regulation is for the survival of dopaminergic neurons.

4. Parkinson's disease

Parkinson's Disease (PD) was first described by Dr. James Parkinson in 1817 in his "Essay on the Shaking Palsy" as a "shock paralysis": *"Involuntary tremulous motion, with lessened voluntary power, in parts not in action, and even when supported; with a propensity to bend the trunk forwards, and to pass from a walking to a running pace: the senses and intellects being uninjured"* (Parkinson et al., 2002). In 1919 it was shown that PD patients specifically loose neurons of the substantia nigra. Later in 1957 Carlosson and co workers discovered that these neurons produce the neurotransmitter dopamine (Bjorklund et al., 2007). PD patients progressively lose their ability to release dopamine in the striatum, where the dopaminergic neurons of the substantia nigra pars compacta (SNpc) project (Fig.7). The imbalance that is generated between the cholinergic activity and the dopaminergic activity in the striatum causes a variety of neurological disorders such as PD and results in a number of complex motor symptoms that can be grouped under the acronym "TRAP": resting tremor, rigidity, akinesia (or bradykinesia) and postural instability. In addition, PD patients

develop flexed posture and motor blocks (Jankovic et al., 2008). Before the onset of motor symptoms, PD patients present non-motor symptoms, which include sleep disorder, depression, cognitive alteration (Schrag et al., 2015) as well as constipation and olfactory impairment (Magerkurth et al., 2005; Markopoulou et al.,1997). The pathological hallmark of PD is the presence of Lewy bodies composed of α-synuclein, neurofilaments and molecular chaperones (Spillantini et al., 1997). The average age of PD onset is 55 years and the risk for developing PD increases 5-fold after the age of 70 (Hornykiewicz et al., 1987).

Currently there is no cure for Parkinson's disease. Medical intervention consists of treating symptoms by administrating patients with Levodopa, a precursor of dopamine, dopamine agonists or inhibitors of dopamine catabolism (Marsden, 1983; Ehringer et al., 1960). In addition to the dopaminergic drugs, non-dopaminergic drugs, such as the anticholinergics and amantadine, ameliorate symptoms in early phases (Fox, 2013). However, dopamine replacement can only ameliorate some of the motor symptoms but does not prevent the progressive degeneration of the dopaminergic neurons.

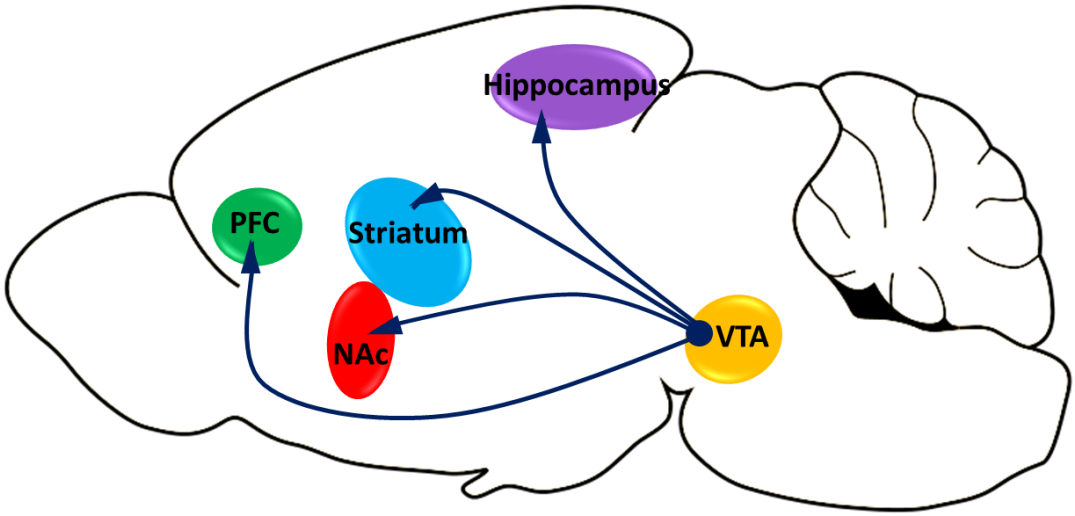

Figure 7. Schematic representation of the circuit of the mesolimbic dopamine system. The prefrontal cortex (PFC) is shown in green, the nucleus accumbens (NAc) in red, the hippocampus in purple and the ventral tegmental area (VTA) is shown in yellow. The dopamine projections are shown in blue. **T**he ventral tegmental area (VTA) is composed of two major cell types, the dopamine neurons and the γ-aminobutyricacid (GABA) neurons. The dopamine neurons (blue) project to the ventral striatum (light blue), the NAc, the PFC and the hippocampus (Tan et al., 2011).

4.1. Sporadic forms of PD

Most PD cases are sporadic and the exact cause for the disease onset is unknown, although both environmental and genetic factors are implicated in the onset of the disease. Several studies demonstrated a clear link between mitochondrial dysfunction and PD onset.

In the late 1970 a new drug of abuse appeared in the United States that caused early onset of irreversible parkinsonism and rapidly led to mortality. Its administration induces a degeneration of the substantia nigra pars compact without a clear presence of Lewy bodies (Varastet et al.,1994; Hantraye et al., 1993). The compound was identified to be 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Langston et al., 1983; Davis et al., 1979). MPTP is a lipophilic compound that is able to cross the blood-brain barrier and once in the brain is oxidized by monoamine oxidase (MAO B) into the toxic molecule cation 1-methyl-4-phenylpyridinium (MPP+) (Langston et al., 1984). Via a mechanism that is still unknown, MPP+ enters the dopaminergic neurons, where it concentrates in the mitochondria and inhibits complex I activity, causing an impairment in ATP production and in parallel an increase in reactive oxygen and nitrogen species (Fabre et al., 1999; Hantraye et al., 1996; Przedborski et al., 1996; Hasegawa et al., 1990; Javitch et al.,1985; Nicklas et al., 1985). These events also cause the activation of pro apoptotic factors, inflammation and cell death (Beal et al., 2003). Importantly, it was also observed that complex I activity is reduced in about 30% of post-mortem sporadic PD patients (Schapira et al., 1990). These findings provided evidence that mitochondrial dysfunction, and specifically complex I impairment, might be implicated in the pathophysiology of PD. Additional indication for this came from the observation that farmers, who routinely use the herbicide rotenone, a highly selective complex I inhibitor, have a much higher probability to develop PD. Indeed, prolonged administration of rotenone can reproduce the neuropathological features of PD in rats (Sherer et al., 2003; Betarbet et al., 2000). The herbicide Paraquat, which induces the Lewy bodies formation and dopaminergic neuronal loss (Mak et al., 2010), causes reactive oxygen species (ROS) production and toxicity mainly in the mitochondria (Castello et al., 2007), highlighting again the importance of mitochondria in the pathophysiology of PD.

4.2. Familial forms of PD

Although most PD cases are sporadic with no known cause, about 5-10% are genetically linked and show early manifestation (Thomas and Beal, 2007). In the 1997, α-synuclein was described as the first gene associated with an autosomal dominant form of PD. Later, four other genes linked with autosomal (LRRK2) or recessive (Parkin, PINK1, DJ-1) onset forms of PD were described. The identification and the study of these rare genetic forms identified

oxidative stress, alteration of UPS, and mitochondrial dysfunction as causal factors in the pathophysiology of PD (Bogaerts et al., 2008) (Fig.8). Genetic and sporadic forms manifest similar clinical symptoms and indistinguishable PD hallmarks (dopaminergic neuron loss, intracellular inclusions, mitochondria abnormalities). Therefore, sporadic PD will likely benefit from studies of the molecular pathways which are impaired in familiar cases.

In the next paragraphs we provide a detailed description of the genes, which mutations have been associated to the onset of familiar cases of PD, with a special focus on PINK1 and Parkin, the main protagonists of my phD studies.

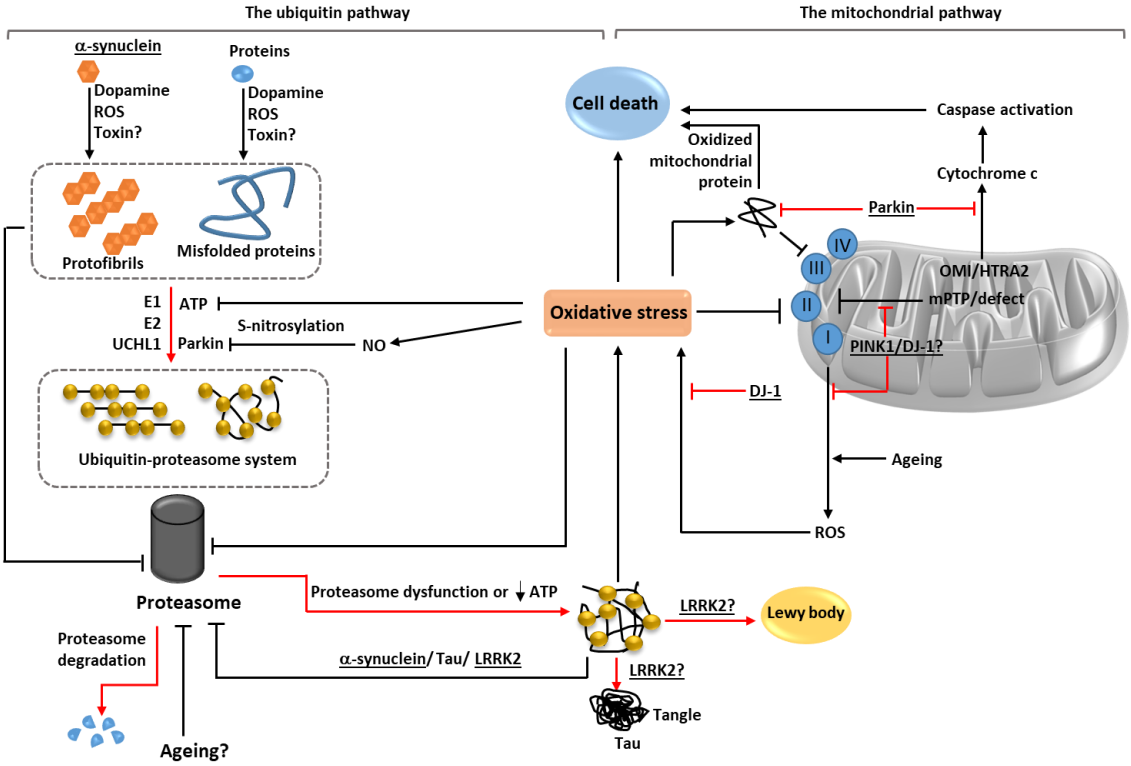

Figure 8. Pathway of PD. α-synuclein binds to phospholipids or it can be found in unfolded conformation in the cytoplasm. The unfolded forms are initially ubiquitinated and eliminated by the ubiquitin-protein system (UPS), in which Parkin has a crucial role. Mutations of α-syn or the alterations in the UPS lead to fibrillar α-syn aggregates and Lewy bodies formation that is one of the main cause of cell toxicity. Protofibrils can also induce directly toxicity causing oxidative stress, which leads to depletion in ATP levels, impairment in the UPS and in conclusion an additional accumulation of aggregates. Simultaneously a UPS pathway, also mitochondrial pathway is involved in the pathophysiology of PD. Reduction in the complex I activity, caused by PINK1 mutations, leads to reactive oxygen species formation (ROS), oxidative stress and lose of membrane potential which causes the opening of mPTP, cytochrome c release and at the end apoptosis. Several works demonstrated the neuroprotective effects of Parkin, DJ-1 and PINK1 against mitochondrial dysfunction. In particular DJ-1 executes its neuronal defense mechanism against oxidative insults. Parkin and DJ1 collaborate to maintain the normal UPS function. Moreover Parkin and PINK1 are involved in the mitochondrial quality control, inducing the degradation of damage mitochondria through proteasome (Abou-Sleiman et al., 2006).

4.2.1. SNCA

The α-synuclein (SNCA) gene encodes for a 140 amino acid protein that is abundant in the human brain and primarily localized in the presynaptic terminals of the neurons. The

protein consists of three domains: the N-terminal lipid-binding α-helix, which contains several repeat composed of 11 amino acids, the central hydrophobic domain (NAC), and a C-terminal acid domain (Giasson et al., 2001). The structure of α-Syn allows the molecule to exist in either a natively unfolder conformation or as an α-helix in the presence of phospholipids. This variability suggest that α-synuclein function may vary depending on its structure or cellular localization (Ahn et al., 2002).

Four mutations, genomic duplication, and triplications have been found in families with PD. The first and most common mutation identified is A53T. A53T is an autosomal dominant mutation with a high penetrance (about 85%) and an aggressive clinical course that causes an early onset in comparison to other missense mutations (Kasten et al., 2013; Polymeropoulos et al., 1997). The other three mutations are A30P, E46K and H50Q (Kasten et al., 2013; Zarranz et al., 2004; Krüger et al., 1998). All the missense mutations impair the amino acid terminal domain, generating protein more flexible and able to sample a large range of conformations. In detail the mutations simplify the β-sheet formation and exacerbate the formation of toxic oligomers, protofibrils and fibrils and likely cause PD through a toxic gain of function (Bertoncini et al., 2005).

4.2.2. LRRK2

The LRRK2 gene codifies for a large multidomain protein of 2527 amino acids, a member of the Roco family of proteins. The protein LRRK2 has a GTPase Ras-like G-domain (Roc), COR domain (C-terminal Roc), WD40 repeat domain and a kinase domain leucine-rich repeat (LRR) (Gandhi et al., 2009; Mata et al., 2006). The protein is mainly localized in the cytoplasm, but can be also associate with the OMM. LRRK2 mutations are the most frequent causes of autosomal dominant late-onset PD. Several of these mutations induce a reduction in the GTPase activity and increase in the kinase activity, probably inducing a gain of function mechanism (Greggio et al., 2006; West et al., 2005). The most widespread mutation is G2019S, and is found in 1% of sporadic forms and 4% of familial PD. It has a very variable penetrance of between 25 and 100% by 80 years of age (Marder et al., 2015; Healy et al., 2008).

4.2.3. DJ-1

DJ-1 gene encodes for a 20 KDa protein with a predicted secondary structure of seven β-strands and nine α-helices. It is present as a dimer and it is ubiquitously expressed (Tao and Tong, 2003; Wilson et al., 2003). DJ-1 protein functions include response to oxidative stress, RNA binding, androgen-receptor signaling, spermatogenesis, and fertilization (Lev et al., 2006). DJ-1 is localized in the cytoplasm and translocates to the nucleus upon oxidation of cysteine 106 (Kim et al., 2012), but it is also found in mitochondria in neurons where it can mitigate oxidative stresses; interestingly, its mitochondrial localization increases during oxidative stress (Lev et al., 2008; Li et al., 2005). DJ-1 has been associated with recessive autosomal cases of PD with early onset and constitutes about 1-2% of the recessive forms (Pankratz et al, 2006). Large deletions and missense mutation (L166P) impair the folding of the protein, causing a structure unable to form a dimer, which undergoes rapid degradation (Olzmann et al., 2004).

4.2.4. PINK1

PTEN-induced putative kinase (PINK1) gene encodes for a protein of 581 amino acids with an N-terminal mitochondrial targeting motif, a highly conserved serine-threonine kinase domain and a C-terminal autoregulatory domain (Beilina et al., 2005) (Fig.9). Full-length PINK1 (66 KDa) is ubiquitously expressed and is localized to the outer mitochondrial membrane (Gandhi et al., 2006). PINK1 is constitutively imported through the outer and the inner mitochondrial membrane where it is cleaved by the mitochondrial processing peptide (MPP) and PARL (Greene et al., 2012; Deas et al., 2011; Meissner et al., 2011; Jin et al., 2010). The cleaved 52 KDa fragment is subsequently rapidly degraded by the proteasome.

When mitochondria depolarize, PINK1 import is impaired, thus resulting in its accumulation on the outer mitochondria membrane to activate selective recruitment of Parkin and promote mitophagy (Lin and Kang, 2008). Studies in *Drosophila* demonstrated the PINK1 induces the phosphorylation of Parkin at serine 65 (Shiba-Fukushima et al., 2012) and the phosphorylation of ubiquitin at the same serine (Ser 65). Parkin, which exists in an autoinhibited conformational structure that resembles a coiled snake, is activated by PINK1 in a two-steps process, which consists of both phosphorylation of Parkin in its UBL domain

and ubiquitin. Following PINK1 dependent phosphorylation, Parkin inactive state is released and it allows its translocation to mitochondria to promote mitophagy (Koyano et al., 2014).

PINK1 can also phosphorylates the HtrA serine peptidase 2 (HTRA2), a mitochondria protease which plays a role in mitochondrial homeostasis. PINK1-dependent phosphorylation of HTRA2 modulate HTRA2 proteolytic activity, contributing to an increased resistance of cells to mitochondrial stress (Plun-Favreau et al., 2007). It has also been reported that PINK1 interacts with Miro a well studied protein that is implicated in mitochondrial trafficking (Weihofen et al., 2009; John et al., 2005). PINK1 phosphorylates Miro, which activates Parkin-dependent proteasomal degradation of Miro, impairing mitochondrial movement along the microtubules (Wang et al., 2011).

Mutations in PINK1 are the second most common cause of autosomal recessive PD, after Parkin mutations, and constitute about 1–7% of early onset PD (Tan et al., 2007). Most of the mutations are found in the kinase domain and induce a complete loss of the kinase activity that is known to be responsible for the neuroprotective role of PINK1 (Petit et al., 2005). The impairment in PINK1 function induces an alteration in mitochondrial Ca^{2+} buffering capacity and a reduction in the mitochondrial membrane potential. PINK1 impairment also results in complex I and complex IV activity loss, decreases ATP production, dysfunctional mitochondrial import and alteration of mtDNA levels (Gehrke et al., 2015; Gegg et al., 2009; Morais et al., 2009). In parallel, this causes an increase in the ROS production and increased sensitivity to apoptotic stress, as well as an alteration in the mitochondrial ultrastructure and synaptic transmission (Winklhofer and Haass, 2010).

Figure 9. Domains of PINK1. Pink1 is a serine-threonine kinase ubiquitously expressed. It possesses an N-terminal mitochondrial targeting sequence (MTS), follow by a transmembrane helix (TM) and serine/threonine kinase domain (Kinase).

4.2.5. Parkin

Parkin gene encodes a 465 amino acid protein with an N-terminal ubiquitin-like domain (UBL), two RING finger motifs, a RING-between-RING (RBR) domain with E3 ubiquitin ligase activity, and another RING domain at the C-terminus (Fig.10). The E3 ubiquitin ligase

catalyzes the transfer of ubiquitin (76 aa protein) from E2 enzyme to target proteins (Moore, 2006).

Figure 10. Characteristic Parkin domains. Parkin is a member of a family of RING-In Between-RING (RBR) E3 ubiquitin ligase, which contains a N-terminal ubiquitin-like domain (UBL), a RING 0, a RING 1 and an in-between-RING (IBR) linker domain, followed by a repressor element of Parkin (REP) and at C-terminal the RING2 domain. REP acts to suppress Parkin activity through the binding with RING1 domain.

The conjugation of ubiquitin to the substrate involves a three steps process: activation step catalyzed by E1, covalent binding of UBL to a conjugating enzyme (E2) and the final step where ubiquitin binds specifically its substrate, catalyzed by a ligase enzyme (E3). In the ubiquitination process, following addition of a single ubiquitin molecule to the target protein (monoubiquitination), further ubiquitin molecules can be added to the first ubiquitin via linkage on lysine residue, producing an ubiquitinated chain (polyubiquitination). Multiple lysine residues on the target protein can also be ubiquitinated to produce multi-monoubiquitination. Alternatively, an ubiquitin chain can form upon linear ubiquitination, in which the carboxy-terminal glycine of one ubiquitin molecule form a peptide bond with the amino-terminal methionine of another (linear or M1-linked ubiquitination) (Pickart and Eddins, 2004). Ubiquitin contains seven lysine residues (Lys 6, Lys 11, Lys 27, Lys 29, Lys 33, Lys 48 and Lys 63), which allow the generation of different ubiquitin chains. Although some chain-types, K48 and K63-chains, are more common than others (Tenno et al., 2004). The type of ubiquitin chain attached affects the physiological outcome. For example, the polyubiquitin K48 chains most commonly induce proteasome-dependent degradation of target proteins, while the K63 chain is associated with non-degradative ubiquitination and control signal transduction, DNA repair, membrane protein trafficking, endocytosis and autophagy. Monoubiquitylation and multi-monoubiquitylation are associated with the regulation of several physiological processes, including definition of subcellular localization as well as protein-protein interaction (Park and Ryu, 2014; Schnell and Hicke, 2003). Monoubiquitylation is also involved in at least three distinct cellular functions: histone regulation, endocytosis and the budding of retroviruses from the plasma membrane (Hicke, 2001).

The fate of Parkin substrates depends on the type of ubiquitinatin chain that is generated (Tab.1). Indeed recent studies suggest that Parkin acts as a dual-function ubiquitin protein ligase capable of both mediating degradative ubiquitination (proteosomal or autophagy-dependent degradation) as well as functional ubiquitination (non degradative ubiquitination). Two different *in vitro* works demonstrated that Parkin E3 ubiquitin ligase activity induces multiple monoubiquitination of several substrates and this function seems to depend on its RING2 domain (Hampe et al., 2006; Matsuda et al., 2006). In the same years, it was reported that Parkin is able to conjugate monoubiquitination of protein PICK, a synaptic scaffolding protein (Joch et al., 2007), Bcl-2 (Chen et al., 2010) and chaperones Hsp70/Hsc70 (Moore et al., 2008). Interestingly, it was also shown that Parkin could induce the formation of K6, K27, K29, K48 and K63 polyubiquitin chains (Durcan et al., 2012; Durcan et al. 2011). In the 2010 was demonstrated by Tanaka and colleagues that Parkin-mediated ubiquitination of mitofusin induces its degradation in a proteasome- and p97-dependent manner after mitochondria depolarization (Tanaka et al., 2010). Recently it was reported that Parkin can promote linear polyubiquitination of NEMO, NF-κB essential modulator. In response to cellular stress, Parkin works as supplementary component of the Linear Ubiquitin Assembly Complex LUBAC, increasing linear ubiquitination of NEMO resulting in the activation of NF-KB prosurvival pathway (Müller-Rischart et al., 2013). Moreover Parkin mediates its own ubiquitination via K48 proteasome dependent ubiquitin chain formation, thus impinging on its own protein turnover (Imai et al., 2000). All considered, ubiquitination by Parkin has the potential of controlling a variety of cellular processes and it does not come as a surprise that the protein exists in an autoinhibited state under normal conditions. In the last years thanks to high resolution determination of Parkin crystal structure, the mechanisms of Parkin autoinhibition was better elucidated. Parkin is basally auto-inhibited under normal conditions: the RING1 domain, which contains the binding site for E2 ubiquitin-conjugating enzyme is blocked by the REP and UBL domains. Also the RING2 domain, which is required for Parkin E3 ubiquitin activity, is suppressed by RING0. This close structure, which resembles that of a coiled snake, basically results in the repression of Parkin activity (Wauer et al., 2013; Dove et al., 2013).

Parkin is subjected to several post translational modifications (PTMs), such as ubiquitination, phosphorylation, acetylation and others, which can regulate its activation,

subcellular localization, conformation, E2 choice, substrate affinity, as well as specificity (Chakraborty et al., 2017). One of the most characterized Parkin post translational modification is phosphorylation. In the 2008 Kim and coworkers demonstrate that Parkin activity and localization is PINK1 kinase activity dependent (Kim et al., 2008). As previously mentioned, it was observed that PINK1 promotes Parkin phosphorylation within the UBL domain at Ser65 (Kondapalli et al., 2012) as well as phosphorylation of ubiquitin at the same residue, allowing ubiquitin binding to Parkin and to Parkin substrates (Kazlauskaite et al., 2015). PTMs are therefore essential for Parkin activity and translocation to damaged mitochondria.

Parkin mutations are the most frequent cause of autosomal recessive PD; sixty associated PD mutations were found with a frequency of 15,5% in familial cases and 4,3% among sporadic forms, beyond deletions and duplications (Kilarski et al., 2012). Initially, it was thought that the pathological mutations caused a loss of Parkin function, which results in accumulation of Parkin substrates and neurotoxicity as result of impaired proteasome dependent degradation. However later works showed that Parkin can also promote proteasome-independent ubiquitination, and therefore promote “functional” ubiquitination which dysregulation can also be linked to the degeneration of dopaminergic neurons (Henn et al., 2007; Fallon et al., 2006; Hampe et al., 2006; Matsuda et al.,2006; Doss-Pepe et al., 2005; Lim et al., 2005).

Substrates	Ub Type	Elevated			Reference
		KO mice	ARJP brain	PD brain	
Ataxin-2	–	Yes	–	–	Huynh et al. (2007)
Ataxin3 polyQ79	–	–	–	–	Tsai et al. (2003)
Bcl-2	Mono	–	–	–	Chen et al. (2010)
CDCrel-1	–	Yes/No	Yes/No	–	Ko et al. (2005)
CDCrel-2a	–	–	Yes	–	Choi et al. (2003)
Cyclin E	–	No	Yes/No	Yes	Staropoli et al. (2003) and Ko et al. (2005)
DJ-1 L166P	K63	–	–	–	Olzmann et al. (2007)
Dopamine Transporter	–	–	–	–	Jiang et al. (2004)
Drp1	K48	–	–	–	Wang et al. (2011a)
ps15	Mono	–	–	–	Fallon et al. (2006)
FBP1	–	Yes	Yes	Yes	Ko et al. (2006)
Fbw7β	K48	Yes	Yes	–	Ekholm-Reed et al. (2013)
Hsp70	Multiple Mono	No	No	Yes	Moore et al. (2008)
LIM Kinase	–	–	–	–	Lim et al. (2007)
Mitofusin	–	–	–	–	Poole et al. (2010) and Ziviani et al. (2010)
O-glycosylated α-synuclein	–	–	Yes	–	Shimura et al. (2001)
P38/AIMP2	Multiple Mono	Yes	Yes	Yes	Corti et al. (2003), Ko et al. (2005), Periquet et al. (2005) and Hampe et al. (2006)
Pael-R	–	No	Yes/No	–	Ko et al. (2005)
PARIS (ZNF746)	K48	Yes	Yes	Yes	Shin et al. (2011)
PDCP2–1	–	–	Yes	Yes	Fukae et al. (2009)
Phospholipase Cγ1	–	Yes	–	–	Dehvari et al. (2009)
PICK1	Mono	No	–	–	Joch et al. (2007)
RanB2	–	–	–	–	Um et al. (2006)
Synaptotagmin XI	–	Yes/No	–	–	Periquet et al. (2005)
Synphilin-1	K63	No	No	–	Chung et al. (2001), Ko et al. (2005) and Lim et al. (2005)
VDAC1	K27, Mono	Yes	–	–	Periquet et al. (2005), Geisler et al. (2010) and Narendra et al. (2010b)
α/β tubulin	–	Yes/No	No	–	Ren et al. (2003) and Ko et al. (2005)

Table 1. Parkin substrates (Zhang et al., 2015**).**

5. Mitophagy

PINK1 and Parkin have emerged as key players in the mitochondria quality control pathway. In healthy mitochondrial, PINK1 undergoes rapid turnover and proteasome degradation, as previously explained (Matsuda et al., 2010). The loss of mitochondria membrane potential causes the inhibition of PINK1 proteolysis, and subsequent accumulation of PINK1 on the OMM. This induces the phosphorylation of Parkin at the Ser65 and the PINK1-dependent phosphorylation of ubiquitin at the same residue (Kazlauskaite et al., 2015; Kim et al., 2008). These two steps are essential for Parkin recruitment on damaged mitochondria (Narendra et al., 2010). Following recruitment to mitochondria, Parkin provokes the ubiquitination of several substrates inducing rapid degradation of MFN1, MFN2 (and Marf in *Drosophila* cells), of mitochondria import receptor (Tom70) and a degradation with slower kinetics of solute transporter (VDAC1), regulator of apoptosis (Bak), mitochondrial fission protein (Fis1) and mitochondrial import protein (Tom20) (Sarraf et al., 2013; Glauser et al.2011; Chan et al., 2011; Ziviani et al., 2010; Tanaka et al., 2010; Poole et al., 2010; Gegg et al., 2010). In 2010 Tanaka and co-authors demonstrated that mitofusin degradation not only works as signal for mitophagy activation, but it is also required to

inhibit mitochondrial fusion and isolate damaged mitochondria from the mitochondrial network (Tanaka et al., 2010). Upon depolarization, OPA1 undergoes proteolytic dependent inactivation, which leads to a fusion-inactive form. In parallel, mitofusin is degraded in a Parkin-, p97-, and proteasome-dependent manner. These events both inhibit mitochondrial fusion and isolate damage mitochondria from the mitochondrial network (Tanaka et al., 2010.)

In addition recent work demonstrates the involvement of AMBRA1, an upstream autophagy regulator and a Parkin interactor, in mitophagy. In physiological condition AMBRA1 is localized at mitochondria, where its pro-autophagy activity is inhibited by Bcl-2. After mitophagy activation AMBRA1 interacts with LC3, an autophagosomal marker. This interaction is an essential step both for Parkin-dependent and –indipendent mitochondrial clearance (Strappazzon et al., 2015).

6. Animal model of Parkinson's disease

The pathology hallmarks of PD are loss of dopaminergic neurons in the substantia nigra pars compacta and the intracytoplasmic accumulation of aggregated proteins called Lewy bodies (Dauer and Przedborski, 2003). The degeneration of dopaminergic neurons induces the reduction of dopamine levels and the onset of motor symptoms such as tremor, rigidity, bradykinesia and postural instability (Braak et al., 2004; Forno, 1996). Dopaminergic neurons loss is also associated with mitochondrial dysfunction, oxidative stress, neuroinflammation, reduction of autophagy and impaired proteasome-dependent protein degradation (Martin et al., 2010; Hirsch and Hunot, 2009; Dauer and Przedborski 2003). PD affects not only the striatum, but also other areas of the central neuron system (CNS) and outside the CNS, such as the myenteric plexus where Lewy Bodies have been detected (Braak et al., 2004; Hornykiewicz and Kish, 1987; Kupsky et al., 1987). These effects in central and peripheral nervous system are the cause of onset of no motor symptoms which include depression, sleep disturbances, olfactory deficit, constipation and rapid-eye movement (Chaudhuri et al., 2006).

Unfortunately, no mammalian model has been established that can recapitulate all the pathological features that are present in human patients (Chaudhuri et al., 2007; Langston, 2006; Jain, 2011). Nevertheless a number of *in vivo* animal models have been established

which allowed tackling the disease onset. In particular in the following paragraphs we will give a detailed overviews of the animal models that are available so far, mainly divided in synthetic neurotoxin and genetic models.

6.1. Synthetic neurotoxins models

6-Hydroxydopamine (6-OHDA) is a selective catecholaminergic neurotoxin with a structure similar to dopamine (Ungerstedt, 1968; Senoh et al., 1959). Administration of this compound induces the selective degeneration of dopaminergic and noradrenergic neurons in the brain caused by its high affinity for catecholaminergic transporters (Sachs and Jonsson, 1975), dopamine depletion, motor imbalance and behaviour deficit, without presence of Lewy bodies inclusions (Ungerstedt, 1968) (Tab.2). This molecule, isolated for the first time in 1950, does not cross the brain-blood barrier and requires direct injection in the SNC to induce neuronal cell death in 12h/2-3 days (Blandini et al., 2008; Jeon et al. 1995; Faull and Laverty, 1969; Senoh et al., 1959). 6-OHDA is accumulated in the cytosol of neurons where it is oxidized causing the production of reactive oxygen species, oxidative stress, eventually leading to cell death (Blum et al., 2001; Graham, 1978; Saner and Thoenen, 1971). This model was mainly used to assess the neuroprotective effects of pharmacological therapies. Its greatest advantage is the possibility to do a unilateral injection, leaving one hemisphere as an internal control (Ilijic et al., 2011; Chan et al., 2010; Jiang et al., 1993). Several animals were found to be sensitive to 6-OHDA, but the most frequently treated model is the rat (Luthman et al., 1989).

MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) sold as a new “synthetic heroin” in California was identify in 1983 (Langston et al, 1983). It is a lipophilic molecule able to cross the blood-brain barrier. In the astrocytes it is metabolized into 1-methyl-4-phenylpyridinium (MPP+) by MAO-B, released form these cells and subsequently imported into neurons by the dopamine transporter (DAT) (Dauer and Przedborski, 2003). In neurons, MPP+ induces neurotoxicity by blocking complex I activity, thereby causing a reduction in ATP production, and increasing oxidative stress (Mizuno et al., 1987; Nicklas et al., 1985). MPTP is toxic for many species, but the most treated models are mouse and monkey, where causes dopaminergic neurons loss in the striatum without Lewy body formation (Tab.2). In

these models MPTP administration leads to motor symptoms that resemble the motor deficits of PD patient, as rigidity, tremor gait and posture disturbance (Tieu, 2011; Halliday et al., 2009; Shimoji et al., 2005; Dauer and Przedborski, 2003). Of note rats are resistant to MPTP toxicity (Chiueh et al., 1984). Mouse models are used to investigate the roles of genetic mutations in response to MPTP toxicity. Whereas monkey model is the best model to test the therapeutic agents for PD and to analyze the non motor preclinical symptoms that characterize PD, as well as to examine the motor symptoms considering that mouse models do not develop symptoms that truly recapitulates those seen in human patients (Vezoli et al., 2011; Bezard and Przedborski, 2011). Administration of low doses of MPTP for long periods in monkey causes motor deficits, which more closely represents the human PD phenotype (Bezard and Przedborski, 2011; Bezard et al., 1997).

Paraquat (N,N'-dimethyl-4-4-4'-bypiridinium) (PQ) is a herbicide that is able to cross the blood–brain barrier. It has a structure comparable to MPP+, but a different transport property and mechanism of action to induces toxicity (Berry et al., 2010; Shimizu et al., 2001). PQ exhibits toxicity by generating superoxide radical, hydrogen peroxide and hydroxyl radicals, which cause damage to proteins, lipids, RNA and DNA (Przedborski and Ischiropoulos, 2005; Day et al, 1999). The advantage of this model is that paraquat induces the formation of α-synuclein aggregates, similar to Lewy bodies (Mak et al., 2010; Fernagut et al., 2007; Manning-Bog et al., 2002). The administration of PQ in mice leads to dopaminergic neurons loss, which causes motor deficits in dose- and age- dependent manner but do not affect the striatum dopamine levels (Thiruchelvam et al., 2003; McCormack et al., 2002; Thiruchelvam et al., 2000b; Brooks et al., 1999) (Tab.2).

Rotenone is an herbicide and an insecticide with a half-life of 3–5 days that is naturally present in tropical plants. It is a lipophilic molecule that easily crosses the blood-brain barrier without a specific transporter. Rotenone causes toxicity in rat brain by inhibiting complex I activity. Initial work shows that administration of high doses of rotenone induces diffuse lesions in several brain regions. In contrast, low continuous dose via subcutaneous infusion leads to selective nigrostriatal degeneration with cytoplasmic inclusions similar to Lewy bodies (Höglinger et al., 2003; Betarbet et al., 2000; Ferrante et al., 1997; Heikkila et al.,

1985) (Tab.2). The advantage of this model is that reproduces all of the pathology hallmarks of human PD including bradykinesia, postural instability and rigidity; its limitation is the variability of the percentage of animals that develop clear nigrostriatal lesions (Cannon et al., 2009).

6.2. Genetic mouse models

The discovery of familiar forms of PD caused by mutation in specific genes led to the identification of common pathways in the pathogenesis of PD, and the development of several genetic animal models. As previously mentioned, genetic and sporadic forms manifest similar clinical symptoms and indistinguishable PD hallmarks (dopaminergic neuron loss, intracellular inclusions, mitochondrial abnormalities). Therefore, sporadic PD will likely benefit from studies of the molecular pathways which are impaired in familiar cases.

6.2.1. α-synuclein model

The mutants A53T and A30P of α-synuclein, which can cause autosomal dominant forms of PD, have been used to generate transgenic mice. These models show a reduction of dopamine level in the striatum, but most of them do not present nigrostriatal degeneration. The phenotype in the mouse model with α-syn overexpression depends primarily on the promoter and on the level of transgene expression. With particular adeno-associated virus vectors and lentiviral vectors, rat and mouse model of α-syn overexpression were established. Unlike the previously described transgene model, α-syn overexpression models display cytoplasmic α-syn aggregates, dopaminergic neuron loss age-dependent and movement dysfunction (Decressac et al., 2012; Kirik et al., 2002; Klein et al., 2002; Lo Bianco et al., 2002) (Tab.2).

6.2.2. LRRK2 model

Mutations in *LRRK2* gene cause autosomal dominant late-onset forms of PD. Mice with the knock in mutations G2019S or R1441C, which cause aberrant kinase activity, show a mild phenotype with α-syn accumulation and no impairment in the dopaminergic neurons (Hinkle et al., 2012; Tong et al., 2010; Lin et al., 2009). The overexpression of the mutation G2019S

induces mild degeneration of DA neurons in the substantia nigra in mice up 2 years of age (Ramonet et al., 2011) (Tab.2).

6.2.3. PINK1, Parkin and DJ-1 models

Mutations in PINK1, Parkin, and DJ-1 cause autosomal recessive forms of PD. *Pink1* knockout and knockdown mice exhibit a mild reduction of dopamine in the striatum accompanied by mild alterations in respiratory capacity and electrochemical potential, with no dopaminergic neuronal degeneration or Lewy bodies (Gispert et al., 2009; Gautier et al., 2008). The only exception is the PINK1 KO model generated by the deletion of exons 4-5, which shows a progressive reduction of DA in the striatum, but no degeneration in the SNc (Akundi et al., 2011) (Tab.2).

Parkin null mice have a phenotype comparable to that of PINK1 KO and exhibit progressive loss of DA in the striatum without neurodegeneration (Goldberg et al., 2003). Bacterial artificial chromosome (BAC) transgenic mice expressing the Q311X Parkin mutant in DA neurons under the control of a dopaminergic promoter displays DA reduction accompanied by an age-dependent dopaminergic neuronal loss in the striatum and progressive hypokinetic motor deficits (Lu et al., 2009) (Tab.2). Recently another Parkin model was generated in rat via overexpression of T240R Parkin mutant using the adeno-associated viral vector in dopaminergic neurons. This animal model shows, both with mutant and wild type gene overexpression, dose-dependent dopaminergic neuron degeneration (Van Rompuy et al., 2014).

Similar to PINK1 KO and Parkin KO, the DJ-1 KO mouse model presents mild dopamine reduction and mitochondrial dysfunction and no dopaminergic neuron loss. Unlike the DJ1-C57 mouse, DJ-1 KO mouse in C57/BL6 background shows an early-onset unilateral dopaminergic neurons loss in SNc and a late onset of bilateral degeneration of the nigrostriatal axis (Rousseaux et al., 2012) (Tab. 2).

	Animal model	Motor behavior	SNc neuron loss	Striatal DA loss	Lewy body/Syn pathology
Toxin-based	MPTP Mice	Reduced locomotion, bradykinesia	↑ ↑ ↑	↑ ↑ ↑	NO
	MPTP Monkeys	Reduced locomotion, altered behavior, tremor, and rigidity	↑ ↑ ↑	↑ ↑ ↑	NO
	6-OHDA rat	Reduced locomotion, altered behavior	↑ ↑ ↑	↑ ↑ ↑	NO
	Rotenone	Reduced locomotion	↑ ↑	↑ ↑ ↑	YES
	Paraquat/maneb	Reduced locomotion	↑ ↑	↑ ↑ ↑	YES
	MET/MDMA	Reduced locomotion	↑ ↑	↑ ↑ ↑	NO
Genetic mutations*	α-Synuclein	Altered behavior, reduced or increased motor activity	↑ Not consistent	↑	↑ (in old animals)
	LRKK2	Mild behavioral alteration	NO	NO	NO
	PINK1	No obvious alterations or reduced locomotion	NO	NO	NO
	PARKIN	No obvious locomotion or reduced locomotion	NO	↑	NO
	DJ-1	Decreased locomotor activity	NO	NO	NO
	ATP13A2	Late onset sensorimotor deficits	NO	NO	NO
Others	SHH	Reduced locomotion	↑ ↑	↑ ↑	NO
	Nurr1	Reduced locomotion	↑ ↑	↑ ↑	NO
	Engrailed 1	Reduced locomotion	↑ ↑	↑	NO
	Pitx3	Reduced locomotion	↑ ↑ ↑	↑ ↑ ↑	NO
	C-Rel-NFKB	Gait, bradykinesia, rigidity	↑ ↑	↑ ↑	YES
	MitoPark	Reduced locomotion, tremor, and rigidity	↑ ↑	↑ ↑	YES
	Atg7	Late onset locomotor deficits	↑ ↑	↑ ↑	YES
	VMAT2	Reduced locomotion and altered behavior	↑ ↑	↑ ↑	YES

↑ ↑ ↑, *Severe loss;* ↑ ↑, *Moderate loss;* ↑, *Mild loss.*
**This table summarizes general observations for each model. See the main text for full and specific description of different animal models for each genetic mutation.*

Table 2. Animal model of Parkinson disease (Blesa e Przedborski, 2014).

7. *Drosophila* model

As previously mentioned no single PD animal model can completely recapitulate the complexity of human Parkinson's disease. However, enormous achievements toward the understanding of the etiology of PD were made thanks to clever genetic interaction experiments that were done in the fruitfly *Drosophila melanogaster*. Very rapidly, *Drosophila* model became a powerful tool to study PD because most mechanisms involved in the pathophysiology are common to human and flies are extremely easy to genetically manipulate. The advantages of this model are numerous: easy maintenance of large numbers of stocks, short life-cycle (10 days) and large progeny (St Johnston, 2002), easy genetic manipulation thanks to the use of P-element transposons to induce downregulation or overexpression of proteins by the bipartite transcription activation system UAS-GAL4 or site specific integration. Moreover *Drosophila* is also a good model for genetic or chemical screening. Its genome is quite small (4 pairs chromosomes) with quite small genetic redundancy (Venken et al., 2011; Chang et al., 2008; Adams and Sekelsky, 2002; Brand and

Perrimon, 1993; Rubin and Spradling, 1982). Not last, most (77%) human disease genes have fly homologues including PINK1 and Parkin (for which mutant KO lines have been generated and have been extensively characterized) and several works, that uses pharmacological treatment, generated different flies model of sporadic PD. For example, chronical exposure to rotenone causes locomotor impairments that increased with the dose of rotenone and determines loss of dopaminergic neurons in all of the brain clusters (Coulom and Birman, 2004). Also, paraquat exposure replicate a broad spectrum of parkinsonian features including movement disorder, resting tremor, bradykinesia, rotational behaviors and postural instability and loss of specific DA neuron clusters (Chaudhuri et al., 2007). All together these works highlight the huge impact of *Drosophila* model to study the mechanisms of DA neurodegeneration.

The first genetic fly PD model was obtain through the overexpression of wild type or mutant forms of human α-syn in the *Drosophila* neurons because fly doesn't have a homolog of this protein. This model has a clear phenotype characterized by selective loss of DA neurons and Lewy bodies formation and locomotor defects. The fact that both wild type and mutant α-syn cause PD phenotype supports the hypothesis that abnormal protein accumulation in the form of α-synuclein aggregates formation can contribute to neurodegeneration (Feany and Bender, 2000).

Two orthologs of DJ-1 exist in *Drosophila*, DJ-1α and DJ-1β. While *DJ-1α* expression is restricted to the male germline, *DJ-1β* is ubiquitously expressed as its human counterpart (Meulener et al., 2005; Menzies et al., 2005). Both are similar to human DJ-1, but DJ-1α has higher homology than DJ-1 β. The DJ-1 β KO fly has a reduction in lifespan and higher sensibility to paraquat and rotenone, as well as locomotor defects and reduction in ATP levels. However these mutations do not lead to DA neuron loss (Lavara-Culebras et al., 2007; Park et al., 2005).

Parkin gene is highly conserved in *Drosophila*, for this reason several groups generated Parkin KO flies. The flies are viable and characterized by mitochondria defects, degeneration of flight muscle, hypersensitivity to environmental and oxidative stress, which is likely

caused by impairment in mitochondrial functions. They also have reduced lifespan and locomotor defects in flight and climbing abilities, as well as DA neurons loss of protocerebral posterior lateral 1 (PPL1) cluster and males are sterile. Parkin fly model summarized many features of autosomal recessive juvenile PD (Cha et al., 2005; Pesah et al, 2004; Greene et al., 2003).

Drosophila PINK1 gene contains the same domain of homolog human gene. Interestingly the PINK1 KO fly generated by transposon-mediated mutagenesis and RNAi presents a phenotype similar to Parkin KO fly. PINK1 KO model displays male sterility, muscle degeneration, hypersensitivity to oxidative stress, mitochondrial dysfunction, reduction in lifespan, locomotor defects and DA neuron loss (Clark et al., 2006; Park et al. 2006; Yang et al., 2006; Wang et al., 2006). Parkin and PINK1 double mutants do not show exacerbation of the phenotype. Also, overexpression of Parkin in PINK1 KO flies ameliorates the phenotypes that are associated to PINK1 loss of function, but not *vice versa* (Park et al., 2006; Yang et al., 2006; Clark et al., 2006). These observations support the hypothesis that PINK1 and Parkin operate in the same signaling pathway with PINK1 working upstream of Parkin (Park et al., 2006; Clark et al., 2006; Yang et al., 2006).

PINK1 KO flies also present reduction in complex I activity, as observed in PD patients (Morais et al., 2009). Overexpression of ND42, a subunit of complex I, in PINK1 KO background, but not in Parkin KO flies, partially rescue the locomotor defect, supporting the hypothesis that PINK1 modulates complex I activity in Parkin independent fashion and independently to its role in mitophagy (Pogson et al., 2014).

RESULTS

Regulation of ER-Mitochondria tethering in an *in vivo* animal model of Parkinson's disease.

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Abstract

Parkin, an E3 ubiquitin ligase and a Parkinson's disease (PD) related gene, translocates to impaired mitochondria and drives their elimination via autophagy, a process known as mitophagy. Mitochondrial pro-fusion protein Mitofusins (MFN1 and MFN2) were found to be a target for Parkin mediated ubiquitination. MFNs are transmembrane GTPase embedded in the outer membrane of mitochondria, which are required on adjacent mitochondria to mediate fusion. In mammals, MFN2 also forms complexes that are capable of tethering mitochondria to endoplasmic reticulum (ER), a structural feature essential for mitochondrial energy metabolism, calcium (Ca^{2+}) transfer between the organelles and Ca^{2+} dependent cell death. Despite its fundamental physiological role, the molecular mechanisms that control ER-mitochondria cross talk are obscure. For example, is ER-mitochondria physical connection decreased upon stimuli that promote Ca^{2+} release from the ER and how is this achieved? Does ER-mitochondria phospholipid exchange require a more intimate ER-mitochondria connection? Ubiquitination has recently emerged as a powerful tool to modulate protein function, via regulation of protein subcellular localization and protein ability to interact with other proteins. Ubiquitination is also a reversible mechanism, which can be actively controlled by opposing ubiquitination-deubiquitination events. In this work we found that in Parkin deficient cells, the tether between ER and mitochondria is decreased. We identified the site of Parkin dependent ubiquitination and showed that the non-ubiquitinatable Mfn2 mutant fails to restore ER-mitochondria physical and functional interaction. Finally, we took advantage of an established *in vivo* model of PD to demonstrate that manipulation of ER-mitochondria tethering by expressing an ER-mitochondria synthetic linker is sufficient to rescue the locomotor deficit associated to an *in vivo Drosophila* model of PD.

Introduction

Parkinson's disease is the second most common neurodegenerative disease, for which there is no cure. Although the exact cause of most cases is unknown, both environmental and genetic factors are implicated in the onset of the disease. In the last few years, a number of genes have been identified, which cause inherited PD and account for ~10% of PD cases (Thomas and Beal, 2007). Some of these genes encodes for proteins that are either expressed in the mitochondria or targeted to the mitochondria upon stressful condition. Indeed, mitochondrial dysfunction is strongly implicated in the aetiology of the disease and impaired mitochondria are found in animal and cell models of PD (Bogaerts et al., 2008).

Mitochondria form a tubular, reticulated network which shape is controlled by opposing fusion and fission events (Bereiter-Hahn and Voth, 1994). The mitofusins 1 and 2 (Mfn1 and Mfn2) are conserved dynamin-like GTPases, composed of a large cytosolic GTPase domain, an HR1 domain, a double transmembrane domain embedded in the outer mitochondrial membrane (OMM) and a second HR2 domain (HR2) (Rojo et al., 2002). Mfn1 mediate mitochondrial fusion of OMM in coordination with OPA1, which mediates the fusion of IMM (Wong et al., 2000). Mitochondrial shaping proteins have pleiotropic functions. While Mfn1 seems primarily involved in organellar docking and fusion, Mfn2 is enriched at contact sites between ER and mitochondria called MAMs (mitochondria-associated ER-membranes) where it is implicated in the formation of molecular linkers that are capable of organelles tethering (Chen et al., 2012; de Brito and Scorrano, 2008). This model has been recently challenged by two works (Filadi et al., 2015; Cosson et al., 2012) which, by using electron and confocal based microscopy techniques, produced the opposite result that Mfn2 removal results in increased ER-mitochondria interaction. Despite the controversy, both schools and a number of previous works attribute to these points of close contact between the OMM and the nearby ER, a fundamental role in the propagation of cellular signals, including those that control lipid metabolism, calcium (Ca^{2+}) homeostasis and cell death (Rowland et al., 2012; Rizzuto et al., 1998; Vance, 1990). Indeed, aberrations in ER-mitochondria juxtaposition have been described in cellular models of a number of neurodegenerative diseases, including Alzheimer's, Huntington's and Parkinson's disease (Krols et al., 2016; Calì et al., 2013; Ottolini et al., 2013; Area-Gomez et al., 2012; Calì et al., 2012; Panov et al.,

2001). Although the exact cause for neuronal loss is not clear. Of note, in mice disruption of Mfn2 is lethal at embryonic stage, while in human MFN2 mutants caused Charcot Marie Tooth type 2A (CMT2A), a peripheral motor and sensory neuropathy (Züchner et al., 2004; Chen et al., 2003).

Parkin, an E3-ubiquitin ligase mutated in familiar Parkinson's Disease (PD) is selectively recruited to dysfunctional mitochondria and promotes their elimination via autophagy, a process known as mitophagy (Narendra et al., 2008). PINK1, a protein kinase, also a PD related gene, is required for Parkin recruitment and stress induced mitophagy (Ziviani et al., 2010). In 2008 Poole et al. showed a strong genetic interaction between PINK1/Parkin pathway and mitochondrial fission and fusion machinery. By using the fruitfly *Drosophila*, they found that PINK1 or Parkin mutant (knock out) phenotypes, including locomotor defects, loss of dopaminergic neurons and degeneration of the muscle of the thorax, is suppressed by inhibiting mitochondrial fusion or by promoting mitochondrial fission (Poole et al., 2008). This genetic interaction was explained by the subsequent discovery that Parkin physically interacts with and ubiquitinates Marf (MFN), fly homologue of Mfn, to control its steady state levels (Ziviani et al., 2010). This work was followed by others that showed that in several model systems, including HeLa cells (Tanaka et al., 2010) and human neuroplastoma cells SH-SY5Y (Gegg et al., 2010), Parkin selectively ubiquitinates mammalian Mfn1 and Mfn2. Accordingly, lack of Parkin (or PINK1 which operates upstream Parkin in the same pathway) (Exner et al., 2007; Clark et al., 2006; Park et al., 2006; Yang et al., 2006) results in increased amount of the steady state levels of MFN and a reduction of MFN ubiquitination forms (Ziviani et al., 2010).

The area of ER-mitochondria contacts has emerged as one of the most novel and controversial fields of research in cell biology. Despite its fundamental physiological role, the molecular mechanisms that control ER-mitochondria physical and functional interaction are yet to be deciphered. In this work we show that a post-translational modification (PTM), in this case ubiquitination, plays an indispensable role in the formation of ER-mitochondria contacts sites. Moreover, we demonstrate for the first time that *in vivo* manipulation of ER-mitochondria tethering by expressing an ER-mitochondria synthetic linker is sufficient to rescue the locomotor deficits associated to an *in vivo Drosophila* model of PD.

Results

Parkin downregulation results in decreased Mfn2 ubiquitination

In S2R+ *Drosophila* cells overexpression of Flag tagged MFN allows the identification of three to four specific bands by western blotting (WB). As previously shown, the smaller molecular weight band (~90KDa) corresponds to unmodified MFN whereas the upper bands (~100KDa, 130KDa and 140KDa) correspond to monoubiquitinated and multiubiquitinated forms of MFN, respectively (Ziviani et al., 2010). A similar pattern of ubiquitination was also previously described for Hela cells (Tanaka et al., 2010), for human neuroblastoma cells SH-SY5Y (Gegg et al., 2010) and for the yeast Mfn homologue, fuzzy onions (Fzo1) (Anton et al., 2013). This pattern of ubiquitination is PINK1/Parkin dependent (Ziviani et al., 2010). Accordingly, in the absence of PINK1 or Parkin, steady state levels of MFN are increased and the pattern of ubiquitination is abolished (Ziviani et al., 2010). We thought to extend these data and investigate the pattern of Mfn2 ubiquitination in MEFs coming from conditional Parkin KO mice in which Parkin expression can be deleted upon expression of Cre recombinase by recombining loxP sites *ad hoc* inserted in exon 7 ($Parkin^{Flx/Flx}$ mice) (Von Coelln et al., 2004). Transfection of MEFs with pcDNA 3.1-Cre leads to an obvious decrease in Parkin mRNA as compared to pcDNA 3.1 transfected cells (Figure 1A). We then investigated the pattern of Mfn2 ubiquitination in MEFs upon Parkin deletion. To do that, we transfected $Parkin^{Flx/Flx}$ MEFs (Von Coelln et al., 2004) with empty vector, pcDNA 3.1 (CTRL), or pcDNA 3.1-Cre (CRE) and in combination with Myc-tagged form of Mfn2 (MFN2) and hemagglutinin (HA)-tagged ubiquitin (HA-Ub). Western blotting analysis of Mfn2 by using anti Mfn2 antibody revealed a single band of the predicted size of Mfn2 (~86 KDa) (Figure 1B, upper panel). Western blotting analysis with antibody against HA revealed the expected smear of ubiquitinated forms, which was partially decreased upon Parkin deletion (Figure 1B, lower panel). To assess whether Mfn2 is ubiquitin modified, we performed coimmunoprecipitation analysis against HA-Ub. Immunoprecipitates of Mfn2 were prepared by using anti Mfn2 antibody (Figure 1C, upper panel) and WB analysis was performed with antibody against HA on the pulled down samples (Figure 1C, lower panel). Following enrichment by immuniprecipitation, we observed a clear reduction of Mfn2-Ub. Interestingly, Parkin

downregulation appeared to be also accompanied by accumulation of steady state Mfn2 (Figure 1C).

Thus, Parkin downregulation in MEFs results in decreased ubiquitinated forms of Mfn2.

Parkin downregulation affects ER-mitochondria tethering

Considering the role of Mfn2 in ER-mitochondria tethering (Filadi et al., 2015; Cosson et al., 2012; Chen et al., 2012; de Brito and Scorrano, 2008), we next investigated the degree of tethering between ER and mitochondria upon Parkin downregulation. Volume rendered 3D reconstruction of *z*-axis stacks of confocal images of fluorescent-tagged mitochondria and ER has been previously used to measure contact sites between the two organelles (Rizzuto et al., 1998). We therefore transfected MEFs with expression vectors encoding mitochondrial and endoplasmic reticulum fluorescent marker (mRFP and ER-YFP respectively) and upon treatment with scramble (Scrb) or Parkin siRNA (Figure 2A). We then measured the degree of tethering by using Mander's coefficient of co-localization upon volume-rendered 3D reconstruction of *z*-axis stacks of confocal images. In such 3D reconstruction, the overlap between red and green fluorescence (yellow spots) represents area of organelle tethering. We found a clear decrease in ER-mitochondria interaction in Parkin downregulating cells (Figure 2B)

To consolidate these data, we also measured ER-mitochondria proximity by using a modified FRET-based indicator of ER-mitochondria proximity named FEMP (Naon et al., 2016). This sensor is targeted to the mitochondrial outer membrane (OMM) (targeting sequence mAKAP1 connected to YPF fluorescent protein) and ER (targeting sequence Sac1 connected to CFP fluorescent protein) and contains a self-leaving Tav2A peptide, which undergoes autocleavage releasing YFP and CFP. FRET intensity is inversely proportional to the distance between the two fluorophores that are appropriately targeted to the two compartments. In the FEMP probe, OMM and ER targeting sequence are coupled with the two components of the FKBP-FRP heterodimerization system that allows covalent linkage between ER and mitochondria upon rapamycin administration. ER-mitochondria juxtaposition can therefore be correlated to FRET intensity and rapamycin treatment allows heterodimerization between adjacent FKBP and FRB domains to maximize FRET intensity. We transfected $Parkin^{Flx/Flx}$ MEFs with pcDNA 3.1 (CTRL) or pcDNA 3.1-Cre (CRE) and in combination with the

FEMP probe. We measured a decrease in the FEMP FRET ratio in Parkin downregulating cells further sustaining the knowledge that ER-mitochondria tethering is reduced in Parkin downregulating conditions (Figure 2C-D).

To extend these observations we addressed whether PINK1, which acts upstream Parkin in the same pathway, may affect ER-mitochondria tethering. To do this, we took advantage of a previously established MEF cell line coming from embryonic PINK1 KO mice. Unexpectedly we observed no differences in the FEMP FRET ratio in PINK1 deficient MEFs (Figure supplementary 1A-B). Since embryonic PINK1 KO mice lack PINK1 from the point of conception, it is possible that compensatory mechanisms are in place, which account for the lack of effect. Of note, PINK1 KO mice show a very mild phenotype (Gispert et al., 2009; Gautier et al., 2008). Also, western blotting analysis of protein lysates coming from PINK1 KO MEFs revealed no changes in Mfn2 steady state levels as compared to control cells (Figure supplementary 1C) in contrast with what was previously observed *in vitro* upon transient PINK1 downregulation (Rojas-Charry et al., 2014)

Altogether these results indicate that Parkin downregulation decreases ER-mitochondria interaction.

CMT type 2A disease-associated MFN mutants are not ubiquitinated and are incompetent in promoting ER-mitochondria interaction

Ablation of *Mfn2* in mice is embryonically lethal and *MFN2* mutations in humans are associated with the onset of Charcot-Marie-Tooth neuropathy type 2A (CMT2A), an hereditary axonal peripheral neuropathy with patients developing early and severe motor disabilities that in the most severe cases constrict them to wheelchair (Züchner et al., 2004; Chen et al., 2003). Interestingly, the most frequent mutation in Mfn2 found in CMT2A patients, R94Q, cannot restore ER-mitochondria contacts in *Mfn2* KO MEFs (de Brito and Scorrano, 2008). We looked at the pattern of ubiquitination in S2R+ *Drosophila* cells upon expression of Flag-tagged form of MFN^{R94Q} mutant and two additional CMT type 2A disease-associated MFN mutations (P251A and R280H, respectively) and we found a complete loss of the ubiquitinated MFN forms in S2R+ *Drosophila* cells (Figure 3A). To consolidate these data, we also investigated Mfn2 ubiquitination in *Mfn2* KO MEFs reconstituted with either wild type Mfn2 or mutants $Mfn2^{P251A}$ and $Mfn2^{R280H}$. Western blotting analysis of Mfn2 by using

anti Mfn2 antibody revealed a single band of the predicted size of Mfn2 (~86 KDa) (Figure 3B). Immunoprecipitates of wt Mfn2, Mfn2^{P251A} and Mfn2^{R280H} were prepared by using anti Mfn2 antibody and WB analysis was performed with antibody against Ubiquitin on the pulled down samples to identify ubiquitinated forms of Mfn2. We observed a clear reduction of Mfn2-Ub in all CMT type 2A disease-associated Mfn2 mutants (Figure 3C). Remarkably, we observed a reduction in ER-mitochondria contacts in *Mfn2* KO MEFs reconstituted with Mfn2^{P251A} or Mfn2^{R280H} as compared to wt Mfn2 (Figure 3D-E).

These results led us to the intriguing hypothesis that ubiquitination of Mfn2, rather than its steady state levels, is an essential prerequisite for ER-mitochondria tethering.

Lysine 416 in the HR1 Mfn2 domain is a genuine site of ubiquitination

Starting from the rationale that ubiquitination may be required for proper ER-mitochondria interaction, we conducted a bioinformatics study to identify the potential MFN site that could be ubiquitinated by Parkin. Mfn1 and Mfn2 are anchored at the outer mitochondrial membrane and have N- and C-terminal domains that protrude into the cytosol. At the N-terminus, there is a GTP binding domain, followed by a hydrophobic heptad repeat region (HR1) and a transmembrane domain (TM), which allows anchorage to the outer membrane. At the C-terminal, there is a second hydrophobic heptad repeat (HR2) (Rojo et al., 2002). This protein structure is highly conserved between different species. Also, a number of publications reported an almost identical pattern of ubiquitination for yeast homologue *fuzzy onion* (fzo1) (Anton et al., 2013; Cohen et al., 2008), fly homologue Marf (Ziviani et al., 2010) and human Mfn2 (Rakovic et al., 2011; Glauser et al., 2011; Tanaka et al., 2010). We therefore thought it was reasonable to hypothesis that the site of ubiquitination may be evolutionary conserved among species. Indeed, multiple sequence alignments using MultAlin revealed a high degree of homology between human, mouse and fly MFN protein sequence (Figure 4A). We identified twenty Lysine residues (K) that are conserved between species (Figure 4A, arrowheads and Table 4B). Interestingly, by using a mass spectrometry approach, Bingol et al. also identified a number of Lysine residues that are ubiquitinated upon Parkin overexpression (Bingol et al., 2014 and Table 4B). We matched the residues identified by Bingol with ours, and we identified six Lysine residues that are likely to represent good candidates for Parkin-dependent ubiquitination of MFN. As illustrated in

Figure 4C, K36 and K84 are located at the N-terminal of the protein, before the GTPase domain, K355 is positioned between the GTPase and HR1 domain, K416 is within the HR1 domain and K732 and K737 are in the HR2 domain. We proceeded by generating non-ubiquitinatable mutants of K36, K416 and K737 by substituting Lysine with Arginine (R), a common procedure to impair ubiquitination (Xu and Jaffrey, 2013). We expressed Flag-tagged mutants MFN^{K36R}, MFN^{K416R} and MFN^{K737R} in control and MFN RNAi treated S2R+ *Drosophila* cells. Western blotting analysis of MFN by using anti Flag antibody revealed a single band of the predicted size of unmodified MFN (~92 KDa) and a number of higher molecular weight bands which corresponds to mono and poly ubiquitinated forms of MFN, as previously described (Ziviani et al., 2010). Interestingly, expression of non-ubiquitinatable mutant MFN^{K416R} failed to reproduce the predicted pattern of ubiquitination (Figure 5A). Confocal microscopy analysis confirmed the mitochondrial subcellular localization of mutant MFN^{K416R} (Figure 5B).

To corroborate these findings, we next analysed the pattern of ubiquitination of non-uniquitinatable mutant $Mfn2^{K416R}$ in MEFs. To do that, we transfected *Mfn2* KO MEFs with Myc-tagged form of wild type Mfn2 (Mfn2) or the non-ubiquitinatable mutant ($Mfn2^{K416R}$) and HA-Ub. Western blotting analysis of Mfn2 by using anti Mfn2 antibody revealed a single band of the predicted size of Mfn2 (~86 KDa). Of note, no changes in the steady state levels of mutant $Mfn2^{K416R}$ was observed (Figure 5C). To assess whether Mfn2 was ubiquitin modified, immunoprecipitates of Mfn2 were prepared by using anti Mfn2 antibody and WB analysis was performed with antibody against HA on the pulled down samples. We observed a clear reduction of ubiquitinated forms in the non-ubiquitinatable mutant $Mfn2^{K416R}$ (Figure 5D), further supporting the notion that K416 is a genuine Mfn2 ubiquitination site.

Ubiquitination of Lysine 416 in the HR1 Mfn2 domain control physical and functional ER-mitochondria interaction.

Our results indirectly suggest that lack of MFN ubiquitination correlates to impaired ER-mitochondria interaction. To directly demonstrate the physiological significance of impaired MFN ubiquitination and ER-mitochondria miscommunication, we next evaluated whether non-ubiquitinatable mutant $Mfn2^{K416R}$ is incompetent in promoting ER-mitochondria physical and functional interaction. To this aim, we expressed $Mfn2^{K416R}$ or wild type Mfn2 in *Mfn2*

KO MEFs and measured ER-mitochondria physical interaction as previously described. Remarkably, we observed a reduction in ER-mitochondria contacts in *Mfn2* KO MEFs reconstituted with non-ubiquitinatable mutant $Mfn2^{K416R}$ (Figure 6A-B).

To evaluate the functional counterpart of ER-mitochondria physical interaction, we next measured ER-mitochondria Ca^{2+} transfer in *Mfn2* KO MEFs reconstituted with non-ubiquitinatable mutant $Mfn2^{K416R}$. Remarkably, in *Mfn2* KO cells expressing mutant $Mfn2^{K416R}$, mitochondrial Ca^{2+} uptake following ATP-generated $InsP_3$ signaling is significantly diminished (Figure 6C). This is accompanied by a decrease in mitochondrial Ca^{2+} uptake speed in µM/s calculated at the half of peak upon treatment with 200 µM ATP (Figure 6D).

Thus, ubiquitination of Lysine 416 in the HR1 Mfn2 domain controls physical and functional ER-mitochondria interaction.

Expression of ER-mitochondria synthetic tether partially rescues the locomotor defect of a *Drosophila* model of PD

Our results strongly indicate a causal link between impaired ubiquitination of MFN and physical and functional ER-mitochondria miscommunication, specifically in the context of Parkin deficiency. One of the most important functions of ER-mitochondria cross talk includes coordinating Ca^{2+} transfer and elegant studies have shown that artificial tether between the ER and mitochondria can be used to modulate Ca^{2+} transfer (Csordás et al., 2010; Csordás et al., 2006). A number of studies have also shown impaired Ca^{2+} homeostasis in cellular models lacking PINK1 or Parkin (Heeman et al., 2011; Sandebring et al., 2009). With that in mind, we investigated the effect of expressing an ER-mitochondria synthetic linker in a well-established *in vivo Drosophila* model of PINK1 loss of function. We generated two *Drosophila* transgenic lines that allow visualising *in vivo* in the adult wing ER-mitochondria contacts through expression of a synthetic tether construct driven by a neuron-specific driver. The construct was created by Csordás et al. (Csordás et al., 2006) and encodes monomeric red fluorescent protein (mRFP) fused to the OMM-targeting sequence of mAKAP1 at the N-terminus and fused to the ER-targeting sequence of yUBC6 at the C-terminus. We cloned the construct for the synthetic linker into a fly vector and generated two "tethering" lines by random insertion in a wild type *(white1118)*$^{-}$ background. These lines expressed mild (TM, Tether Mild) to high (TH, Tether High) levels of the tethering

construct. In order to activate neuronal expression of the UAS tether lines we crossed these lines with nSyb-Gal4 (neuronal Synaptobrevin) expressing flies. As a read out for visualizing *in vivo* ER-mitochondrial contacts in the fly, we took advantage of a recently described model, which allows imaging of sensory neurons in the translucent fly wing (Vagnoni and Bullock, 2016). We fixed flies of the desired age in PFA, detached the wing and measured ER-mitochondria contact sites by imaging at confocal microscope of the red fluorescent signal in the L1 vein neuron bundle. We observed well-defined and easily quantifiable red fluorescent spots throughout the L1 vein that perfectly matched the pattern seen when expressing only mito-GFP or ER-GFP in the wing neurons (Figure supplementary 2A).

We expressed the TM (tether mild) "tethering" line in wild type (*white1118)*$^{-}$ and PINK1 mutant (KO) background (PINK1^{B9}). Of note, we observed a clear reduction in the RFP signal in PINK1 mutant (KO) background as compared to wild type (Figure 7A-B). Defects in the mitochondrial protein import machinery of the PINK1 deficient flies (Gehrke et al., 2015) might account for the reduced expression of the tethering probe. This hypothesis is also supported by the observation that expression of the TH (tether high) synthetic tethering construct is lethal in wild type but not in PINK1 mutant (KO) background (Figure 7C). Interestingly, TH expression does not impact eggs to pupae viability (Figure supplementary 2B), but impaired adults/pupae ratio suggesting that lethality occurs at later stage when pupae become adults (Figure supplementary 2C).

Remarkably, climbing performance of PINK1 KO flies was significantly improved upon expression of the synthetic ER-mitochondria linker *in vivo* (Figure7D).

Thus, *in vivo* genetic manipulation of ER-mitochondria tethering rescues the locomotor impairment associated to a *Drosophila* animal model of PD.

Discussion

Parkin, a protein which mutations have been linked to the onset of a rare autosomal recessive form of familiar PD, is an E3 ubiquitin ligase that belongs to the RBR (Ring-between-RING) type of E3 ubiquitin ligases, also known as RING/HECT hybrids (Müller-Rischart et al., 2013). By mediating the covalent attachment of the highly conserved 76-amino acid protein ubiquitin on target proteins, Parkin controls a fundamental post-

translational modification (PTM) that is required for proteasome-dependent protein degradation. However, recent studies suggest that Parkin acts as a dual-function ubiquitin protein ligase capable of both mediating degradative ubiquitination (proteosomal or autophagy-dependent degradation) (Glauser et al.2011; Ziviani et al., 2010; Tanaka et al., 2010; Poole et al., 2010; Gegg et al., 2010), as well as functional ubiquitination (non degradative ubiquitination) (Müller-Rischart et al., 2013; Henn et al., 2007; Fallon et al., 2006; Hampe et al., 2006; Matsuda et al.,2006; Doss-Pepe et al., 2005; Lim et al., 2005), which may act as a regulative mechanism to modulate protein activity (Salmena and Pandolfi, 2007). In the ubiquitination process, following addition of a single ubiquitin molecule to the target protein (monoubiquitination), further ubiquitin molecules can be added to the first ubiquitin molecule via linkage on each lysine (K) residue, producing an ubiquitinated chain (poliubiquitination). Multiple K residues on the target protein can also be ubiquitinated to produce multi-monoubiquitination. Alternatively, an ubiquitin chain can form upon linear ubiquitination, in which the carboxy-terminal glycine of one ubiquitin molecule form a peptide bond with the amino-terminal methionine of another (linear or M1-linked ubiquitination). Different ubiquitin chains can generate different physiological outcomes. For example, the K48-linked ubiquitin chain of the target induces the proteasome degradation of the substrate (Grice and Nathan, 2016). Differently the conjugation of monoubiquitination or K63- linked ubiquitin chain regulates ribosome function, endocytosis of the proteins, protein sorting, trafficking and recycling or degradation by lysosome (Park and Ryu, 2014; Schnell and Hicke, 2003). In that respect, ubiquitination by Parkin has the potential of controlling almost every cellular process. Not surprisingly, Parkin exists in an inactive state that resembles that of a coiled snake (Wauer et al., 2013; Dove et al., 2013) and its activity is normally repressed by mechanisms of autoinhibition.

Parkin is predominantly localized in the cytosol, but it can translocate to depolarized mitochondria where it promotes polyubiquitination of outer mitochondrial membrane resident proteins, which is a prerequisite for mitochondria quality control or mitophagy, a well characterized cellular process that leads to the elimination of a selective subset of damaged mitochondria via autophagy (Narendra et al., 2008). In a number of cellular models, Parkin promotes the ubiquitination of Marf (fly homologue of mammalian Mitofusins) (Ziviani et al., 2010) and both mitofusin 1 (Mfn1) and mitofusin 2 (Mfn2) (Tanaka

et al., 2010; Gegg et al., 2010). Ubiquitination of Mitofusins leads to proteasome- and p97-dependent degradation of the proteins (Tanaka et al., 2010). However, a number of studies reported proteosome-independent-mediated ubiquitinations by Parkin, which do not result in degradative ubiquitination. For example, in 2006 two groups independently observed that Parkin catalyzes the formation of multiple monoubiquitination *in vitro* (Matsuda et al., 2006; Hampe et al., 2006). Additional study propounded that Parkin activation results in the formation of K63-linked ubiquitin chains, which does not results in decreased levels of the target protein (Lim et al., 2005). Similarly it was demonstrated that Parkin can form linear ubiquitination of NF-κB essential modulator (NEMO) causing the activation of NF-KB pro-survival pathway (Müller-Rischart et al., 2013). All these observations suggest that Parkin may act like a multi-functions ubiquitin protein ligase capable of mediating both degradative or non degradative ubiquitination.

Mitofusins are large GTPase proteins that are required on adjacent mitochondria to promote tethering and fusion of the outer mitochondria membrane. In mammals Mfn2 was also demonstrated to regulate ER-mitochondrial tethering (Filadi et al., 2015; Cosson et al., 2012; de Brito and Scorrano, 2008). ER-mitochondria physical interaction is a structural feature that is indispensable for Ca^{2+} cross talk, phospholipid biosynthesis, autophagosome formation, DRP1 mediated mitochondrial fission and a number of additional physiological signaling pathways that are essential for cell survival (Paillusson et al., 2016; Hamasaki et al., 2013; De Stefani et al, 2011; Friedman et al., 2011; Kirichok et al., 2004; Rizzuto et al., 1998; Vance, 1990). Starting from the observation that Parkin downregulation in MEFs results in decreased ubiquitination forms of Mfn2 (Figure 1), as also observed in *Drosophila* cells (Ziviani et al., 2010), HeLa and human neuroblastoma cells (Tanaka et al., 2010; Gegg et al., 2010), we addressed the effect of Parkin downregulation on ER-mitochondria physical and functional communication in MEFs. By using two different approaches, we indeed showed that Parkin downregulation induced a clear decrease in ER-mitochondria interaction (Figure 2).

Ablation of *Mfn2* in mice is embryonically lethal and *MFN2* mutations in humans are associated with the onset of Charcot-Marie-Tooth neuropathy type 2A (CMT2A), an hereditary axonal peripheral neuropathy with patients developing early and severe motor disabilities (Züchner et al., 2004; Chen et al., 2003). Interestingly, the most frequent

mutation in Mfn2 found in CMT2A patients, R94Q, cannot restore ER-mitochondria contacts in *Mfn2* KO MEFs (de Brito and Scorrano, 2008). With that in mind, we analyzed the ubiquitination pattern of R94Q as well as two additional Charcot Marie Tooth type 2A disease-associate MFN2 mutants (P251A and P280H). Of note, these MFN mutants failed to be ubiquitinated both in S2R fly cells and in MEFs (Figure 3A-C). Moreover when expressed in *Mfn*2 KO background, they were unable to restore the ER-mitochondria contacts (Figure 3D-E).

These observations indirectly suggest that ubiquitination of MFN might operate as a functional ubiquitination that is required on adjacent organelles to promote physical interaction. To prove this hypothesis, we engaged on a bioinformatics as well as mass spectrometry-based analysis to identify the specific site of Parkin dependent ubiquitinaition on Mfn2 molecule. We identified lysine 416 localized in the HR1 domain of Mfn2 as a potential very promising site for Parkin dependent ubiquitination (Figure 4). We generated a non-ubiquitinatable K416 mutant by substituting Lysine with Arginine (R), a common procedure to impair ubiquitination (Xu and Jaffrey, 2013). Of note, mutant K416R failed to be ubiquitinated and was incompetent in promoting ER-mitochondria physical interaction (Figure 5-6). The non ubiquitinatable mutant not only altered physical contacts formation, but also impaired the functionality of ER-mitochondria communication, reducing mitochondrial Ca^{2+} uptake and mitochondrial Ca^{2+} uptake speed upon treatment with 200 µM ATP (Figure 6).

Aberrations in ER-mitochondria juxtaposition have been described in cellular models of a number of neurodegenerative diseases, including Parkinson's disease (Ottolini et al., 2013; Calì et al., 2013; Calì et al., 2012). One of the most important functions of ER-mitochondria cross talk includes coordinating Ca^{2+} transfer and a number of studies have also shown impaired Ca^{2+} homeostasis in cellular models lacking PINK1 or Parkin (Heeman et al., 2011; Sandebring et al., 2009). Although it is not clear why dopaminergic neurons specifically degenerate in PD, it is tempting to hypothesis that this could be due to impaired Ca^{2+} cross talk at ER-mitochondria interface.

Elegant studies have shown that artificial tether between the ER and mitochondria can be used to modulate Ca^{2+} transfer *in vitro* (Csordás et al., 2010; Csordás et al., 2006). Starting from the rational that normalization of ER-mitochondria Ca^{2+} transfer could also be

beneficial *in vivo*, we investigated the effect of expressing an ER-mitochondria synthetic linker in a well-established *in vivo Drosophila* model of PINK1 loss of function. PINK1 mutant (KO) flies have a very obvious phenotype characterized by male sterility, degeneration of thorax muscle, degeneration of dopaminergic neurons, locomotor defects, reduction in lifespan and mitochondrial dysfunction (Clark et al., 2006; Park et al. 2006; Yang et al., 2006). We crossed PINK1 KO or control (WT) flies with two different fly lines expressing mild (TM) and high (TH) levels of the synthetic tethering construct (Csordás et al., 2006). We demonstrated that expression of the mild tether (TM) induced a significantly improvement in the climbing performance of PINK1 KO flies (Figure 7).

Altogether, these results led us to the intriguing hypothesis that Parkin dependent ubiquitination of K416 is required on healthy mitochondria for proper ER-mitochondria interaction under physiological conditions. This model does not exclude the existence of a parallel stress induced pathway (like that generated by intoxication of mitochondria with CCCP), which promotes Parkin-dependent ubiquitination of Mfn1 and Mfn2 and additional outer mitochondrial membrane resident proteins, to promote mitophagy (Figure 8).

Materials and methods

Cell culture

Drosophila S2R+ cells grow at 25°C without CO_2, as semi-adherent monolayer in tissue culture flasks. The complete medium for SR2+ cells is Schneider's *Drosophila* Medium (Gibco) containing 10% heat-inactivated fetal bovine serum (FBS). Transfection was performed using Effectene Transfection Reagent (QIAGEN) following manufacturer instruction. 5 hours after the transfection medium was removed and change with fresh medium and after 72 hours the cells were used to the indicated experiments.

The mouse embryonic fibroblasts (MEFs) grow in Dulbecco's modified Eagle medium (DMEM) (Gibco) with addition of 1% penicillin/streptomycin, 1% non-essential amino acids solution, L-glutammine and 10% FBS at 37°C with 5% CO_2 atmosphere.

Mfn2 KO MEFs were provided by Dr. Luca Scorrano, Department of Biology, University of Padova. *PINK1* KO MEFs were a kind gift of the lab of Prof. Brini, Department of Biology, University of Padova (Requejo-Aguila et al., 2014). *Parkin*$^{Flx/Flx}$ MEF were obtained from Dr. Ted Dawson, Johns Hopkins University (Von Coelln et al., 2004). TransFectin™ Lipid Reagent (Bio-Rad) or GenJet™ In Vitro DNA Transfection Reagent (SignaGen laboratories) were used for the transfection following the manufacturer's instruction. The medium was changed after 5h and the experiments performed after 24h/48h from the transfection.

Molecular Biology

pEYFP-ER (ER YFP), mRFP, pCB6-Myc-Mfn2 (Mfn2 WT) were previously described (de Brito and Scorrano, 2008). pcDNA 3.1 CRE (CRE), pcDNA 3.1 (Empty vector) and pcDNA 3.1 mitoKate were available in our lab. Site directed mutagenesis was performed from Myc-Mfn2 using QuikChange II XL Site-Directed Mutagenesis Kit (Agilent) to obtain Myc-Mfn2^{P251A} (Mfn2^{P251A}), Myc-Mfn2^{R280H} (Mfn2^{R280H}) and Myc-Mfn2^{K416R} (Mfn2^{K416R}) (primers in Table.1). pAct-PPA Marf-Flag (MFN) and pAct-PPA Marf GFP (MFN GFP) constructs were previously generated (Ziviani et al., 2010) and were available in the lab. Site directed mutagenesis was performed to generate MFN^{P251A} Flag, MFN^{R280H} Flag, MFN^{K36R} Flag, MFN^{K416R} Flag, MFN^{K737R}

Flag, MFN^{K416R} GFP (primers in Table.1) using QuikChange II XL Site-Directed Mutagenesis Kit (Agilent). HA-Ubiquitin (HA-Ub) was obtained from Addgene. FRET based Mito-ER Linker probe (FEMP) and mitochondrial low-affinity aequorin (mtAEQ mut) were previously described (Naon et al., 2016 and Kendall et al., 1992 respectively).

Constructs	Primers
Myc-Mfn2^{P251A}	Forward: 5'-GCGTCTCTCCCGGGCAAACATCTTCATC-3' Reverse: 5'-GATGAAGATGTTTGCCCGGGAGAGACGC-3'
Myc-Mfn2^{R280H}	Forward: 5'-CAGCACATGGAGCATTGTACCAGCTTCC-3' Reverse: 5'- GGAAGCTGGTACAATGCTCCATGTGCTG -3'
Myc-Mfn2^{K416R}	Forward: 5'-GAGCTCTTGGCTCAAGACTATAGGCTCCGAATTAAGCAGATTACG-3' Reverse: 5'-CGTAATCTGCTTAATTCGGAGCCTATAGTCTTGAGCCAAGAGCTC-3'
MFN^{P251A} Flag	Forward: 5'-TCGCAGAAGCTAAGCAAGGCCAACATCTTCATCCTGAAC-3' Reverse: 5'-GTTCAGGATGAAGATGTTGGCCTTGCTTAGCTTCTGCGA-3'
MFN^{R280H} Flag	Forward: 5'-AAGTCTCAGCACACGGAACACTGCATCGACTTCCTCACC-3' Reverse: 5'-GGTGAGGAAGTCGATGCAGTGTTCCGTGTGCTGAGACTT-3'
MFN^{K36R} Flag	Forward: 5'-TTTGTGCGCGCCAGGAGGAGGATCAACGATATC-3' Reverse: 5'-GATATCGTTGATCCTCCTCCTGGCGCGCACAAA-3'
MFN^{K416R} Flag/ MFN^{K416R} GFP	Forward: 5'-GGTTACGCGGGAAATGAGGATGAGGATCCACAACATGGTCG-3' Reverse: 5'-CGACCATGTTGTGGATCCTCATCCTCATTTCCCGCGTAACC-3'
MFN^{K737R} Flag	Forward: 5'-AAGCTGCTCAGGAATAGGGCCGGTTGGTTGGAC-3' Reverse: 5'-GTCCAACCAACCGGCCCTATTCCTGAGCAGCTT-3'

Table.1: Primers used for Site directed mutagenesis. Underscores indicate mismatch.

Parkin siRNA and MFN RNAi Treatment

Parkin siRNA (Invitrogen) was transfected using Oligofectamine^{TM} reagent (Invitrogen) and 100nM of siRNA for each treatment. The same amount of scrambled siRNA was used as control. For Parkin knockdown, cells were incubated with siRNA for at least 48h (Müller-Rischart et al., 2013).

Double-stranded RNAs (dsRNAs) were prepared using the MEGAscript kit (Ambion) according to the manufacturer's instructions. Primers used to generate dsRNAs contained a T7 promoter sequence at the 5' end (MFN forward primer 5'-GGAACCTCTTTATTCTCTAT-3' and reverse primer 5'-GGTTTGCTTTGCCCCAACAT-3')**.** A total of 1 million S2R+ cells were

plated on a six-well plate and treated with 15 µg dsRNA probe in serum-free medium. One hour after probe treatment, complete medium was added to the wells, and cells were cultured for 2 days before being transfected.

RNA isolation and Real time PCR

Parkin$^{Flx/Flx}$ MEF cells were transfected with the pcDNA 3.1-CRE (CRE) or empty vector (CTRL) and after 48h total RNA was extracted from the cells with TRIzol™ Reagent (Invitrogen™) according to the manufacturer's instructions. Two hundred and fifty ng of RNA was reverse transcribed with SensiFAST™ cDNA Synthesis Kit (Bioline). Briefly, GoTaq qPCR Master mix containing SYBR green fluorescent dye (Promega) was mixed with 1 µl cDNA and 0.2 µM primers: *Parkin* forward primer 5'-AGGAATGCGTGCTGCAAATG-3', reverse primer 5'-CTGTAGGCCTGAGAAGTGGC-3'; *mRPL13A* forward primer 5'-TGAAGCCTACCAGAAAGTTTGC-3', reverse primer 5'-CCAGGAGTCCGTTGGTCTTG-3' in a final volume of 10 µl. Each cycle consisted of denaturation at 95°C for 15 secs, annealing at 60°C for 60 secs and extension at 95°C for 15 secs. Reactions were carried out on the 7900 HT Fast Real-time PCR System (Applied Biosystems). Quantification of gene expression (Relative level) was performed according to the $2^{-\Delta CT}$ method using *mRPL13A* gene as internal control. Reactions were run in triplicate (technical replicates) and three independent experiments were performed.

Immunoblotting

MEFs were washed with phosphate-buffered saline (PBS) and scraped off using plastic cell scraper in PBS. The cells were centrifuged at 3000 rpm at 4°C for 5 minutes. The supernatant was discarded and the pellet resuspended in appropriate volume of modified RIPA buffer (50 mM Tris-HCl, pH 7.4; 1% TritonX; 0.5% Na-deoxycholate; 0.1% SDS; 150 mM NaCl; 2 mM EDTA; 50 mM NaF), upon addition of fresh protease Inhibitor Cocktails (PIC), 50µM MG132 and 10mM *N*-Ethylmaleimide (NEM; a deubiquitinase inhibition). The cells were incubated in ice for 30 mins and vigorously mixed every 10 mins and were centrifuged at 4000 rpm for 10 mins at 4˚C and the supernatant was transferred to new tube.

SR2+ cells protein lysate was obtained following incubation with a buffer composed of: 50 mM Tris-HCl, 150 mM NaCl, 1% Triton X, 2 mM EGTA, 1 mM MgCl2 with in addition 10% glycerol, PIC (100X), 10 mM NEM and 50 µM MG132. Cells were centrifuged at 4000 rpm for 10 mins at 4˚C and the supernatant was transferred to new tube.

Protein content was quantified using the Pierce™ BCA Protein Assay Kit (Thermo Scientific™). NuPAGE™ LDS Sample Buffer (4X) (Invitrogen™) and 2-Mercaptoethanol (SIGMA) were added to the samples and proteins were boiled at 95°C for 10 mins. Proteins were loaded in ExpressPlus™ PAGE Gel,10×8, 8% (GenScript) or NuPAGE™ 3-8% Tris-Acetate Protein Gels, 1.0 mm (Invitrogen™) and separated using a constant voltage of 100 mV for the appropriate time. After electrophoresis run, proteins were transferred from the gel matrix to Polyvinylidene difluoride (PVDF) membrane (Thermo Fisher Scientific) applying a constant voltage of 100 mV for 2 hours at 4°C. The membrane was saturated upon incubation with 5 % milk in T-BST (mixture of tris-buffered saline and Tween 20) for 1 hours. Proteins were probed using the following antibodies: α-Flag (1:1000; Cell Signaling Technology: 2368S), α-Mfn2 (1:1000; Abnova: H00009927-M03), α-PINK1 (1:500; Novus Biologicals: BC100-494), α-HA (1:1000; Cell Signaling Technology: 3724).

Immunoprecipitation (IP)

MEFs (about 500 000 cells) were plated in a 100 mm dish and the day after were transfected with the Mfn2 WT or the Mfn2 mutants and HA-Ub. 12 or 24 hours later, cells were lysed with a buffer composed of: 50 mM Tris-HCl, 150mM NaCl, 1% Triton X, 2 mM EGTA, 1 mM MgCl2 with in addition 10% glycerol, PIC (100X), 10mM NEM and 50µM MG132. Protein extract (250µg-1mg) was incubated with 10 µl of protein A agarose beads (Roche), previously balanced in lysis buffer, for 30 mins at 4°C on the wheel (pre-cleaning). In parallel 30 µl of balanced beads were incubated with 1 µg of α-Mfn2 (Abcam: ab56889) or without the antibody (negative control) for 1 hour and half at 4°C on wheel in 70 µl of lysis buffer. Pre-cleaning beads were centrifugated at 4000 rpm for 5 mins and the supernatant was incubated overnight at 4°C with antibody-conjugated beads. The day after the mix was centrifugated and the supernatant was discarded. Beads were washed 3 times for 10 mins at 4°C with lysis buffer and boiled for 10 mins at 95°C in 30-50 µl Laemmli loading buffer 2X

(Laemmli 4X: Tris HCL 300 µM, pH 6.8; SDS 300 µM; Sucrose 1,4 M; Beta mercaptoethanol 8%;+ Bromophenol blue). Supernatant was recovered upon maximum speed centrifugation and analyzed by western blotting in NuPAGE™ 3-8% Tris-Acetate Protein Gels.

Imaging

MEFs or S2R+ cells were plated on 24 mm round glass coverslips and co-transfected with mitoKate or mito-RFP and ER-YFP and the indicated plasmids for 48-72 hours before imaging. Images were acquired using an UPlanSApo 60X/1.35 objective (iMIC Adromeda) upon excitation with 561 and 488 lasers. The percentage of ER that colocalize with mitochondria was measured with Mander's coefficient of colocalization (JACoP), following 3D volume rendered reconstruction of 60 *z*-axis images separated by 0.2 µm (software: imageJ, plug in: volumeJ).

For FRET imaging about 1200-1800 MEFs cells were seeded on 384 well plate (Perkin Elmer). After 12 h cells were transfected with FEMP probe and the indicated plasmids (ratio 1:3) using Genjet in Vitro DNA Transfection Reagent and analyzed using Perkin Elmer Operetta High-Content Imaging System objective 20X after 12 or 24 hours. FEMP probe is targeted to the mitochondrial outer membrane (OMM) (targeting sequence mAKAP1 connected to YPF fluorescent protein) and ER (targeting sequence Sac1 connected to CFP fluorescent protein) and contains a self-leaving Tav2A peptide, which undergoes autocleavage releasing YFP and CFP. FRET intensity is inversely proportional to the distance between the two fluorophores that are appropriately targeted to the two compartments. In the FEMP probe, OMM and ER targeting sequence are coupled with the two components of the FKBP-FRP heterodimerization system that allows covalent linkage between ER and mitochondria upon rapamycin administration. ER-mitochondria juxtaposition can therefore be correlated to FRET intensity and rapamycin treatment allows heterodimerization between adjacent FKBP and FRB domains to maximize FRET intensity. The basal FRET level (FRET basal) was obtained using ex 410-430 and em 460-500 for CFP and ex 490-510 and em 520-560 for YFP while YFP_{FRET} was obtained using ex 410-430 and em 520-560. The maximum FRET intensity (FRETmax) was measured after treatment with 100 µM Rapamycin for 15 mins, on fixed cells in PBS (1% Formaldehyde for 10 mins). The images were analyzed using Perkin Elmer

Harmony 3.5 image software. The YFP channel was used to mark the ROI (Fcell) and a second bonder, around each ROI, was plotted to measure and subtract the background intensity (Fbg). FRETbasal and FRETmax were calculated as: FYFPFRETcell-FYFPFRETbg)/(FCFPcell-FCFPbg). The FRET Ratio (ΔR/R) is (FRETmax-FRETbasal)/FRETbasal.

Bioinformatic analysis

Protein sequences of MFN2_HUMAN (O95140), MFN2_MOUSE (Q80U63) and MARF_DROME (Q7YU24) were obtained from database Protein (UniProt). The alignment was performed using MultAlin software.

Aequorin measurement

MEFs were grown on 13 mm round glass coverslips at 50-60% of confluence and co-transfected with the indicated constructs and mitochondrial low affinity aequorin (mtAEQ mut). Cells were incubated for 1.5 h at 37°C in DMEM 1% FBS after being reconstituted with 5 µM coelenterazine wt (invitrogene). To monitor mitochondrial Ca^{2+} transients, cells were perfused with Krebs Ringer buffer (KRB: 125 mM NaCl, 5 mM KCl, 1 mM Na_3PO_4, 1 mM $MgSO_4$, 5.5 mM glucose, 20 mM HEPES, pH 7.4, 37 °C, 1 Mm $CaCl_2$) for 20–30 s with or without 200 µM ATP. At the end of each experiment, the cells are perfused with milliQ H_2O_2 containing 10 mM $CaCl_2$ and 100 mM digitonin to calibrate the luminescence signal in Ca^{2+} values. The rate of Ca^{2+} uptake is calculated as Ca^{2+} uptake speed in µM/s at the half of peak upon treatment with 200 µM ATP.

Fly stocks and breeding condition

Fly lines were grown on standard cornmeal medium and were maintained at 23°C, 70% relative humidity, on a 12-h light:12-h dark cycle. The UAS mitoGFP/TM6B and UAS KDELGFP/TM6B were obtained from the lab of Prof. Daga, Department of Pharmaceutical and Pharmacological Sciences, University of Padova. The mito-ER syntetic linker, created by Csordás, consists of a monomeric fluorescent protein (mRFP) fused to the OMM targeting

sequence of mAKAP1 at the N terminus and fused to the ER targeting sequence of yUBC6 at the C terminus (Csordás et al., 2006). The construct was cloned into a fly vector (pUAST) and two fly lines expressing different levels of the tethering construct were generated by random insertion into a *white1118*$^{-}$ background or PINK1 mutant (KO) background (PINK1^{B9}/FM7): UAS-mito-mRFP-ERTH/TM6B (TH, high expression of tether) and UAS-mito-mRFP-ER^{TM}/TM6B (TM, mild expression of tether). In order to activate neuronal expression of the tethering construct, flies were crossed with nSyb-Gal4/TM6B, neuronal Synaptobrevin-expressing flies obtained from the Bloomington stock center. PINK1 mutant (KO) flies (PINK1B9/FM7) (Park et al., 2006) were a kind gift of Dr. Alexander Whitworth.

Mounting and imaging of fly wing

Flies at 3-5 days of age were fixed with 4% of paraformaldehyde (PFA) for 2 hours at room temperature on a rotating wheel and then washed 3 times for 10 minutes with PBS. The whole wings were cut with Castro-Viejo Scissors, mounted on 24 mm round glass coverslips with a small amount of ProLong™ Gold Antifade Mountant and subsequently covered with another 12 mm round glass coverslip which was fixed with nail polish. The images were acquired using an UPlanSApo 20X/0.75 objective (iMIC Adromeda) after the excitation with a 561 laser. The quantification of the RFP signal was performed counting the spots using ImageJ along 150 µm of length of the L1 vein neural bundle (Vagnoni and Bullock, 2016) from the intersection of the L2 vein to proximal side of the wing flies.

Climbing assay

The climbing test was performed at 3 days of age at the same time of the day with male flies of the indicated genotypes. 10 flies for each genotype were transferred in a plastic cylinder of 12 cm of length and 5 cm of diameter, marked with a line at 6 cm from the bottom of the tube. Flies were tapped to the bottom of the tube and the number of flies that successfully climbed across the 6 cm line in 10 seconds was counted. Fifteen separate and consecutive trials were performed for each experiment, and the results were averaged. 50 flies for each genotype were analyzed. The results were expressed as percentage of climbing flies.

Eggs-to-adults viability

The assay was performed to analyze eggs-to-adults viability of the tethering TM and TH flies compared to WT flies. For each lines (WT, WT TM and WT TH) 10 virgin females were crossed with 10 males nSyb-Gal4/TM6B on a plate with sugar yeast fruit medium. The day after, adults were removed and after 3 days eggs were counted. Next the medium with eggs was added to the tube with standard cornmeal medium. After 5 and 10 days from egg laying, the number of pupae and the number of male adults were counted. To analyze the eggs-to-pupae viability the number of pupae was divided by the number of eggs for each genotype and the resulting value was normalized to control (WT). In order to address the larvae-to-adults viability, the number of male adults was divided by the total number of pupae and expressed as percentage.

Statistics

Data were presents as mean ±SEM from at least three independent experiments. Statistical significance was determined using Unpaired t-test and p values are indicated (GraphPad software).

Figure legends

FIGURE 1: Parkin downregulation results in decreased Mfn2 ubiquitination.

(A) Total RNA was extracted from Parkin$^{Flx/Flx}$ MEF cells transfected with pcDNA 3.1 (CTRL) or pcDNA 3.1-Cre (CRE) for 48 h and retrotranscribed into cDNA. Specific Parkin and endogenous control oligonucleotides primers were used to perform quantitative RT-PCR. Graph bar indicates mean ±SEM of Parkin mRNA levels relatively to endogenous control in treated cells as indicated. Student's t test, ****p<0.0001, n=3.

(B) Parkin$^{Flx/Flx}$ MEFs were transfected with pcDNA 3.1 (CTRL) or pcDNA 3.1-Cre (CRE) and in combination with Myc-tagged form of Mfn2 (Mfn2) and hemagglutinin (HA)-tagged ubiquitin (HA-Ub). Equal amounts of protein (50 μg) were separated by SDS-PAGE and immunoblotted using the indicated antibodies. Western blotting analysis of Mfn2 by using α-Mfn2 antibody revealed a single band of the predicted size of Mfn2 (~86 KDa).

(C) Parkin$^{Flx/Flx}$ MEF cells were transfected with pcDNA 3.1-CRE or empty vector, Mfn2 (Myc-Mfn2) and HA-Ub and subjected to immunoprecipitation (IP) of Mfn2 using α-Mfn2 antibody. WB analysis was performed with antibodies α-Mfn2 or α-HA on the pulled down samples. NEG identify samples incubated without antibody (negative control).

FIGURE 2: Parkin downregulation decreases ER-mitochondria tethering.

(A) MEFs were transfected with either Parkin siRNA or Scrb siRNA. Mitochondria and ER were probed using organelles targeted fluorescence probes (mRFP and ER-YFP respectively). The overlap between red and green fluorescence (yellow spots) represents area of organelle tethering. Panels on the right show enlarged views of the boxed areas. Scale bar, 20μm.

(B) Graph bar indicates mean ±SEM of percentage of ER co-localizing with mitochondria (degree of tethering) by using Mander's coefficient of co-localization upon volume-rendered 3D reconstruction of *z*-axis stacks of confocal images (n = 3, 15 cells per experiment). ** 0.001<p< 0.01 (Student's t test).

(C) Representative images of Parkin$^{Flx/Flx}$ MEFs transfected with pcDNA 3.1 (CTRL) or pcDNA 3.1-Cre (CRE) and in combination with the FEMP probe. The first and second panel on the left part represents the YFP (mito) and the CFP (ER) signals of the probe, respectively. The third panel represents the YFP_{FRET} in basal condition.

(D) Parkin$^{Flx/Flx}$ MEFs were transfected with pcDNA 3.1 (CTRL) or pcDNA 3.1-Cre (CRE) and in combination with the FEMP probe. FRET ratio (ΔR/R) was calculated as described in materials and methods. Graph bar indicates mean ±SEM. n=4; * 0.01<p<0.05 (Student's t test).

FIGURE 3: CMT type 2A disease-associated MFN mutants are not ubiquitinated and are incompetent in promoting ER-mitochondria interaction.

(A) S2R+ were treated with MFN RNAi or CTRL RNAi and were transfected with the indicated plasmids and lysed after 3 days. Equal amounts of protein (50 μg) were separated by SDS-PAGE and immunoblotted using the indicated antibodies. Asterisk (*) indicated ubiquitinated forms of MFN. Arrow (►) indicated unmodified MFN.

(B) *Mfn2* KO MEFs were transfected with the indicated plasmids and lysed after 24 hours. Equal amounts of protein (50 μg) were separated by SDS-PAGE and immunoblotted using the indicated antibodies. Western blotting analysis of Mfn2 by using α-Mfn2 antibody revealed a single band of the predicted size of Mfn2 (~86 KDa).

(C) *Mfn2* KO MEFs were transfected with the indicated plasmids and in combination with HA-Ub. After 24 hours, cells were lysed and subjected to immunoprecipitation (IP) by α-Mfn2 antibody. Western blotting analysis was performed by using α-HA antibody on the pulled down samples. NEG identify samples incubated without antibody (negative control).

(D) *Mfn2* KO MEFs were transfected the indicated plasmids. Mitochondria and ER were probed using organelles targeted fluorescence probes (mitoKate and ER-YFP respectively). Yellow spots represent area of organelle tethering. Panels on the right show enlarged views of the boxed areas. The scale bar is 20μm.

(E) Graph bar indicates mean ±SEM of percentage of ER co-localizing with mitochondria (degree of tethering) by using Mander's coefficient of co-localization upon volume-rendered 3D reconstruction of z-axis stacks of confocal images of cells transfected as indicated. (n=4, 15 cells per experiment). ** 0.001<p<0.01 and *** 0.0001<p<0.001 (Student's t test). The scale, 20μm.

FIGURE 4: Lysine 416 in the HR1 Mfn2 domain is a genuine site of ubiquitination.

(A) Multiple sequence alignments using MultAlin of the Mitofusin protein sequences from *Homo sapiens, Mus musculus and Drosophila melanogaster*. Arrowheads identify conserved lysines residues.

(B) Table shows Lysine residues (K) that are conserved between human, mouse and flies (upper row) and those identified by Bingol et al. 2014, following mass spectrometry analysis (middle row).

(C) Schematic representation of Mfn2 protein. Residues K36 and K84 are located at the N-terminal of the protein, before the GTPase domain. Residue K355 is positioned between the GTPase and HR1 domain. Residue K416 is within the HR1 domain. Residues K732 and K737 are in the HR2 domain.

FIGURE 5: Lysine 416 in the HR1 Mfn2 domain is a genuine site of ubiquitination.

(A) S2R+ cells were transfected with the indicated plasmids and lysed after 3 days. Equal amounts of protein (50 μg) were separated by SDS-PAGE and immunoblotted using the indicated antibodies. Asterisk (*) indicates ubiquitinated forms of MFN. Arrow (►) indicates unmodified MFN.

(B) Representative confocal image of SR2+ cells treated with Mito Tracker Red and transfected with the indicated plasmids. Scale bars, 40μm.

(C) *Mfn2* KO MEFs were transfected with the indicated plasmids and in combination with HA-Ub and lysed after 24 hours. Equal amounts of protein (50 μg) were separated by SDS-PAGE and immunoblotted using the indicated antibodies. Western blotting by using α-Mfn2 antibody revealed a single band of the predicted size (~86 KDa).

(D) *Mfn2* KO MEFs were transfected with the indicated plasmids and in combination with HA-Ub and lysed after 24 hours. Protein lysates were subjected to IP by using α-Mfn2 antibody. WB analysis on the pulled down sample was performed by using α-Mfn2 or α-HA antibody. NEG identify samples incubated without antibody (negative control).

FIGURE 6: Ubiquitination of Lysine 416 in the HR1 Mfn2 domain control physical and functional ER-mitochondria interaction.

(A) *Mfn2* KO MEFs were transfected with the indicated plasmids. Mitochondria and ER were probed using organelles targeted fluorescence probes (mitoKate and ER-YFP respectively). Yellow spots represents area of organelle tethering. Panels on the right show enlarged views of the boxed areas. The scale bar is 20 μm.

(B) Graph bar indicates mean ±SEM of percentage of ER co-localizing with mitochondria (degree of tethering) by using Mander's coefficient of co-localization upon volume-rendered 3D reconstruction of z-axis stacks of confocal images. n=4, 15 cells per experiment. ** 0.001<p< 0.01 (Student's t test).

(C) *Mfn2* KO MEFs were transfected with the indicated plasmids and in combination with Ca^{2+} probe aequorin targeted to the mitochondria matrix (mtAEQ). Graph bar show mean ±SEM of mitochondrial Ca^{2+} uptake upon ATP generated Ca^{2+} release (ATP 200 μM). n=3. ** 0.001<p<0.01 (Student's t test).

(D) *Mfn2* KO MEFs were transfected with the indicated plasmids and in combination with Ca^{2+} probe mtAEQ. Graph bar show mean ±SEM of mitochondrial Ca^{2+} uptake speed expressed in μM/s calculated at the half of peak upon treatment with 200 μM ATP. n=3. * 0.01<p<0.05 (Student's t test).

FIGURE 7: Expression of ER-mitochondria synthetic tether partially rescues the locomotor defect of a *Drosophila* model of PD.

(A) Representative images of TM (tether mild) "tethering" line in wild type (*white1118*)$^{-}$ and PINK1 mutant (KO) background ($PINK1^{B9}$). The down panels shown enlarge views of the 150 nm length along the L1 vein neural bundle from the intersection of the L2 vein to proximal side of the wing flies. Arrow (►) indicates the RFP signal.

(B) RFP signal in the wing of flies of the indicated genotype was quantified as described in materials and methods. Graph bar indicates mean ±SEM of the number of RFP spots. n=3. * 0.01<p<0.05 (Student's t test).

(C) Graph bar represents means ±SEM of percentage (%) of climbing of flies of the indicated genotype. TH (tether high) "tethering" line in wild type (*white1118*)$^{-}$ and PINK1 mutant (KO) background ($PINK1^{B9}$) n=5, 10 flies per experiment. NS p> 0.05. (Student's t test).

(D) Graph bars represents means±SEM of percentage (%) of climbing of flies of the indicated genotype. TM (tether mild) "tethering" line in wild type (*white1118*)$^{-}$ and PINK1 mutant (KO) backgound (PINK1B9). n=5, 10 flies per experiment. NS p> 0.05, ** 0.001<p<0.01 (Student's t test).

FIGURE 8: Regulation of ER-mitochondria interaction by Parkin via MFN2: a schematic representation.

Parkin ubiquitinates Mfn2 on lysine K416. This event is a prerequisite for ER-mitochondria physical and functional interaction.

SUPPL. FIGURE 1: In PINK1 KO MEFs the percentage of ER interacting with mitochondria is comparable to WT cells.

(A) Representative confocal images of PINK1 WT and PINK1 KO MEFs transfected with FEMP probe. The first and second panel on the left part represents the YFP (mito) and the CFP (ER) signals of the probe, respectively. The third panel represents the YFP_{FRET} in basal condition.

(B) PINK WT and PINK1 KO MEFs were transfected with FEMP probe and FRET Ratio (ΔR/R) was calculated as described. Graph bar indicates mean ±SEM. (n=5). NS p> 0.05 (Student's t test).

(C) Equal amount of protein (50 µg) lysates from WT and PINK1 KO MEFs were separated by SDS-PAGE and immunoblotted using the indicated antibodies.

SUPPL. FIGURE 2: Expression of synthetic tether TH (Tether High) is toxic.

(A) Representative image of a small portion of the L1 vein neural bundle of the wing of flies either expressing mitochondria targeted green fluorescent probe (upper panel), ER targeted green fluorescent probe (middle panel) or the red synthetic tether (TM, tether mild) (lower panel) in the wing neurons. Bright-field images of the wings and overlapping images of fluorescent and bright-field are also shown.

(B) Graph bar indicates means ±SEM of egg to pupae ratio (% of WT) calculated as described in materials and methods. n=3. NS p> 0.05 (Student's t test).

(C) Graph bar indicates means ±SEM of adults to pupae ratio (%). n=3. NS p> 0.05, *** 0.0001<p<0.001 (Student's t test).

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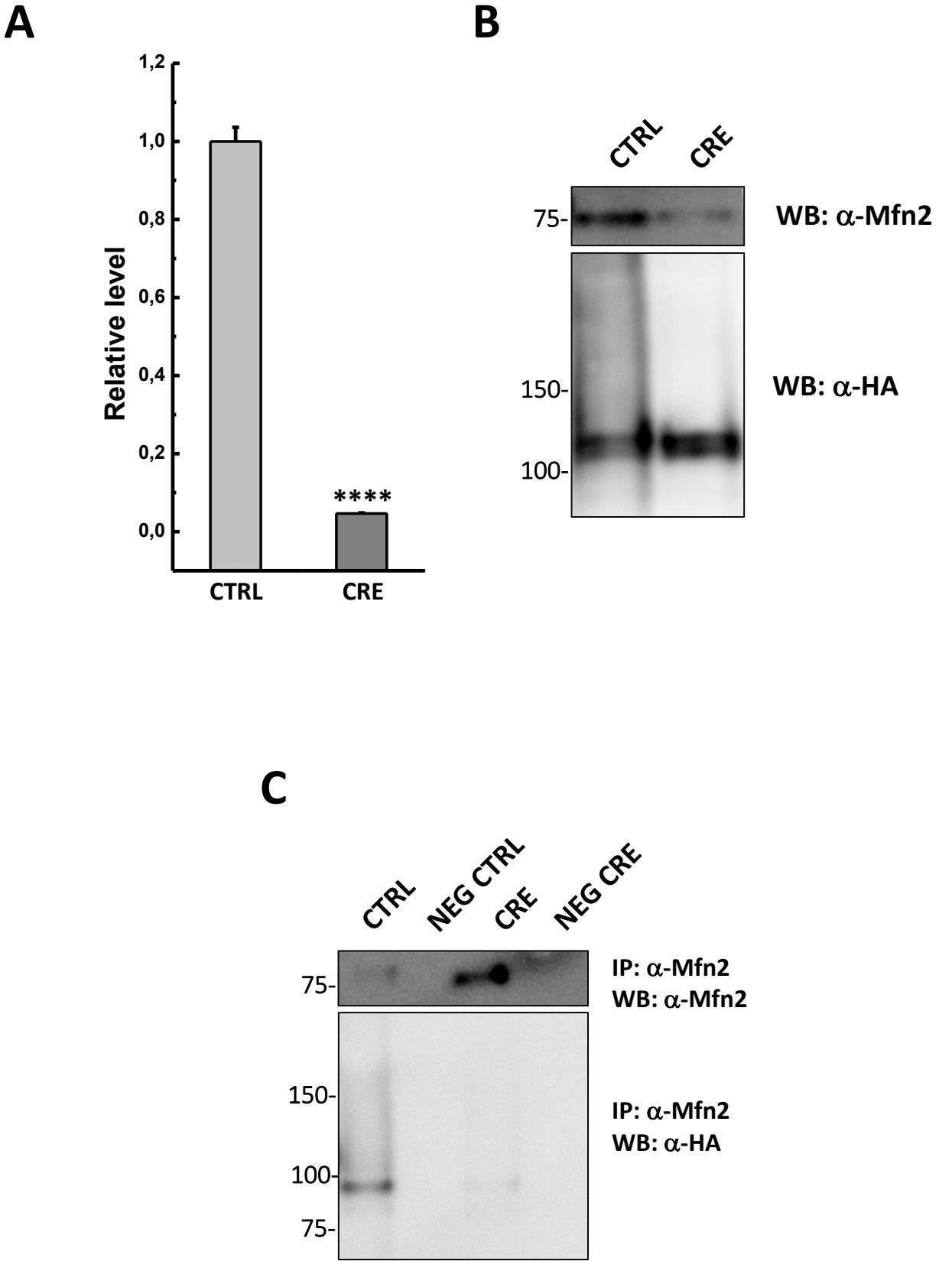

A
B
C
Relative level
1,2
1,0
0,8
0,6
0,4
0,2
0,0
****
CTRL
CRE
CTRL
CRE
75-
150-
100-
WB: α-Mfn2
WB: α-HA
CTRL
NEG CTRL
CRE
NEG CRE
75-
150-
100-
75-
IP: α-Mfn2
WB: α-Mfn2
IP: α-Mfn2
WB: α-HA

FIGURE 1

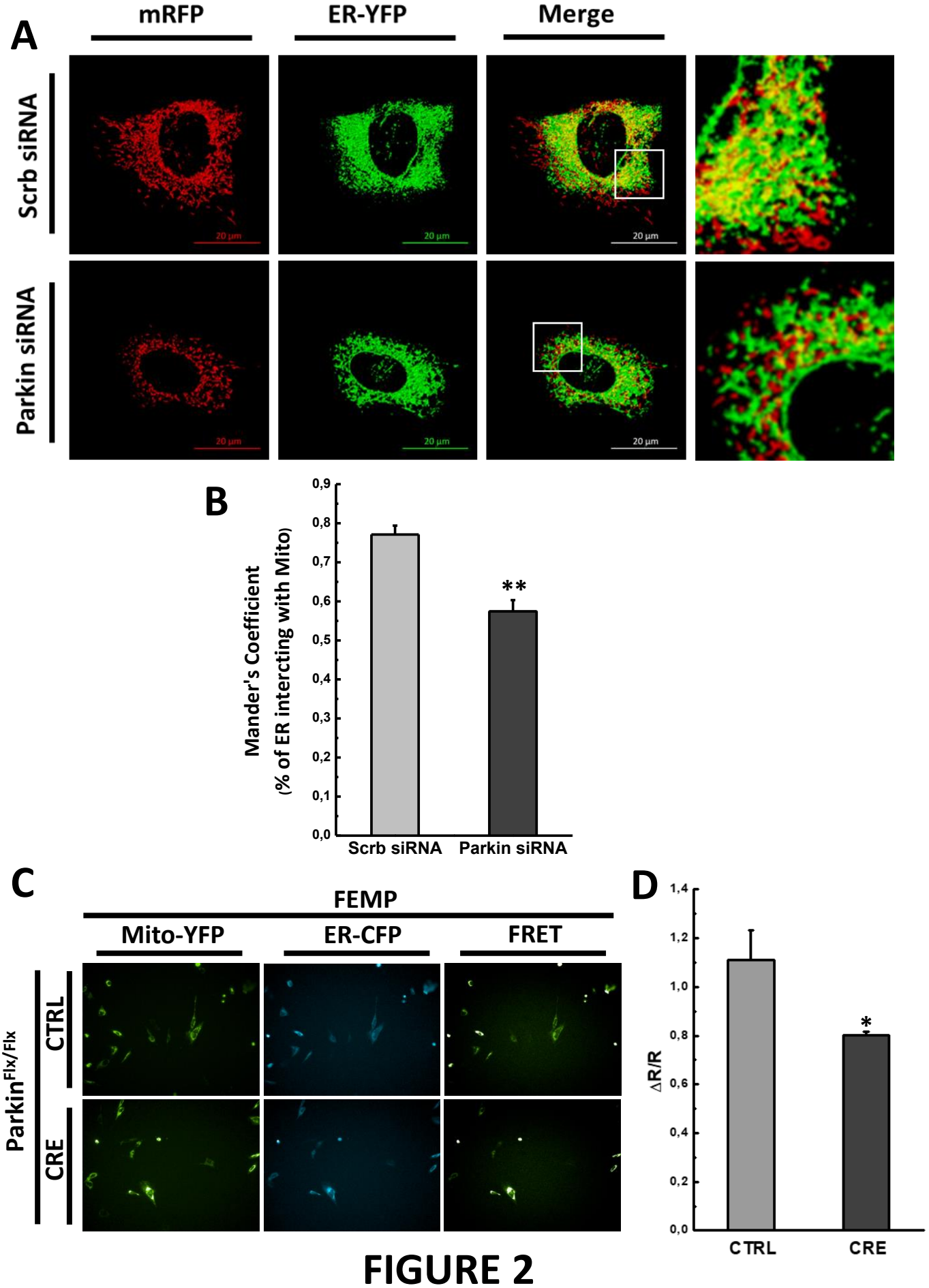

A
mRFP
ER-YFP
Merge
Scrb siRNA
Parkin siRNA
20 µm
B
Mander's Coefficient
(% of ER intercting with Mito)
0,9
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0,0
**
Scrb siRNA
Parkin siRNA
C
FEMP
Mito-YFP
ER-CFP
FRET
Parkin Flx/Flx
CTRL
CRE
D
ΔR/R
1,4
1,2
1,0
0,8
0,6
0,4
0,2
0,0
*
CTRL
CRE

FIGURE 2

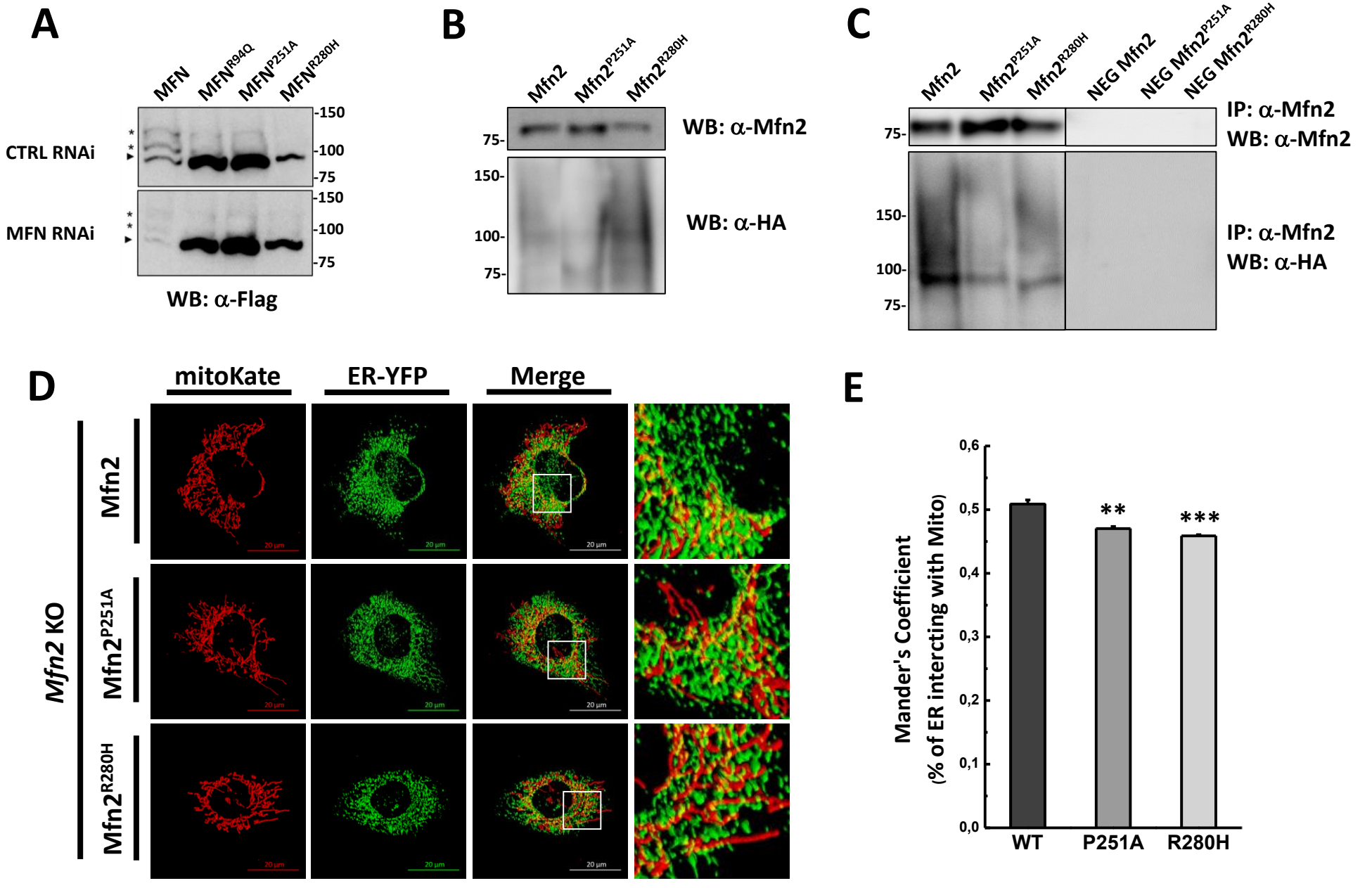
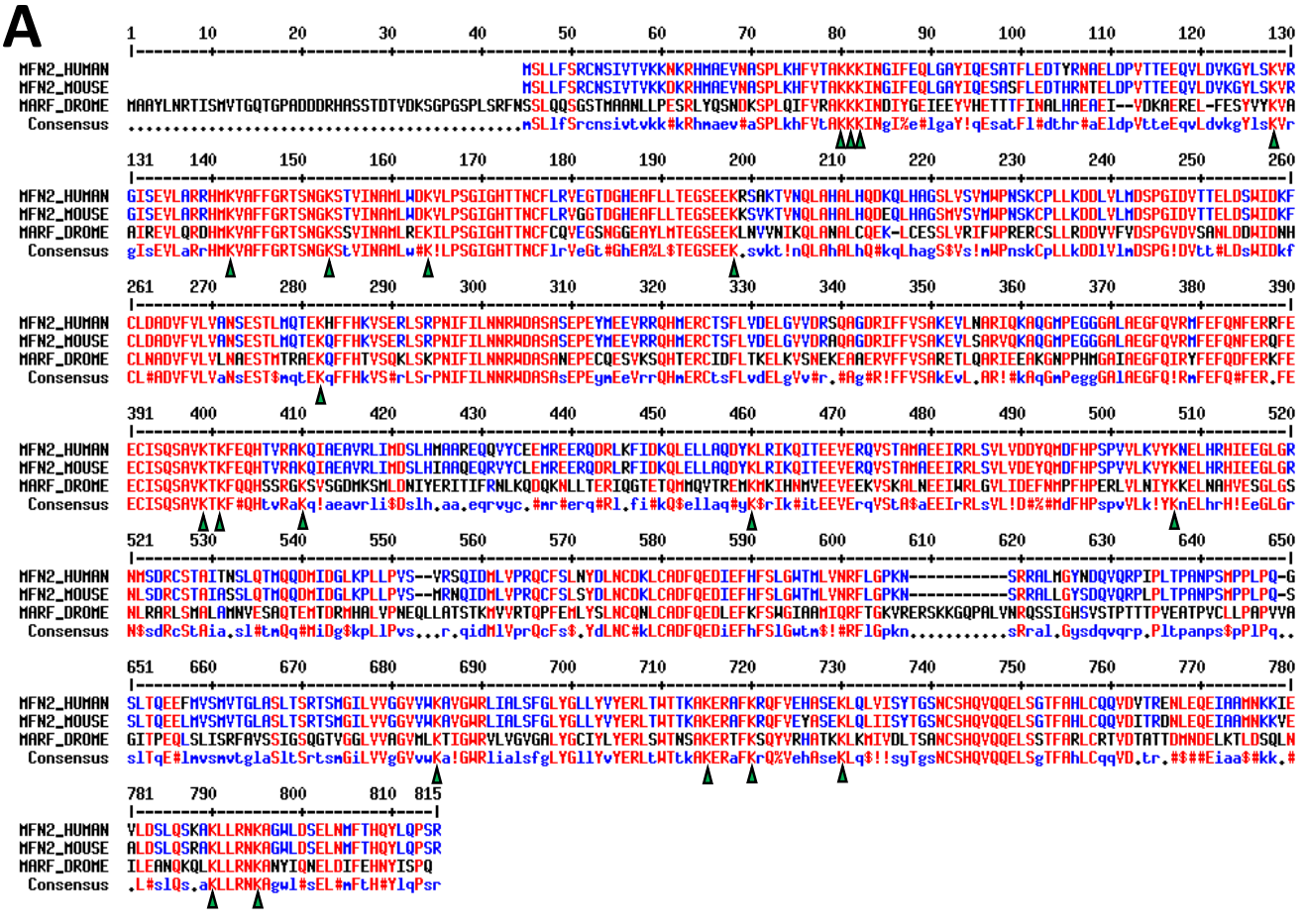

A
MFN
MFN^R94Q
MFN^P251A
MFN^R280H
CTRL RNAi
MFN RNAi
150
100
75
WB: α-Flag
B
Mfn2
Mfn2^P251A
Mfn2^R280H
75-
150-
100-
75-
WB: α-Mfn2
WB: α-HA
C
Mfn2
Mfn2^P251A
Mfn2^R280H
NEG Mfn2
NEG Mfn2^P251A
NEG Mfn2^R280H
75-
150-
100-
75-
IP: α-Mfn2
WB: α-Mfn2
IP: α-Mfn2
WB: α-HA
D
mitoKate
ER-YFP
Merge
Mfn2 KO
Mfn2
Mfn2^P251A
Mfn2^R280H
20 µm
E
Mander's Coefficient
(% of ER intercting with Mito)
0,6
0,5
0,4
0,3
0,2
0,1
0,0
**
***
WT
P251A
R280H

FIGURE 3

A



B

MultAlin Human Mfn2, mouse Mfn2, fly MFN	36-37-38-84-98-109-120-154-238-355-357-366-416-463-627-657-662-672-732-737
Ubiquitination sites identified by mass spectrometry analysis (Bingol et al., 2014)	30-36-79-84-307-355-406-416-420-460-560-719-720-730-732-737
MATCH	36-84-355-416-732-737

C

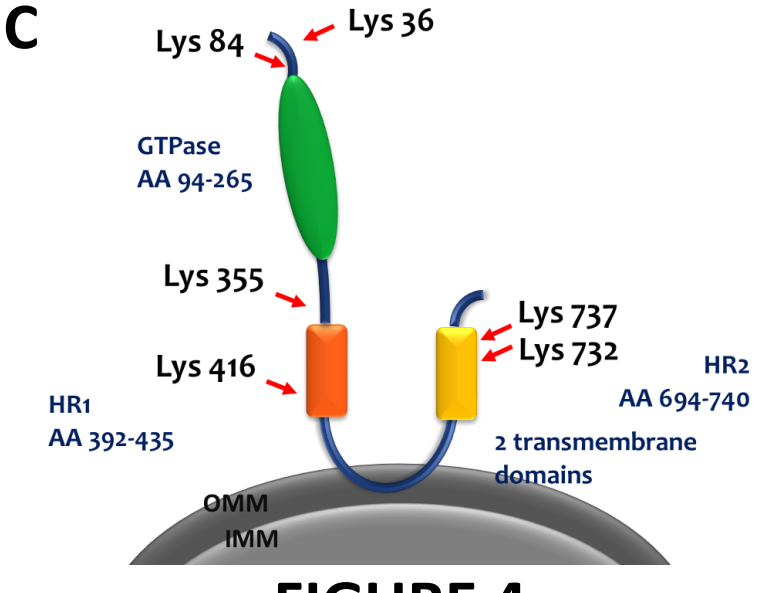
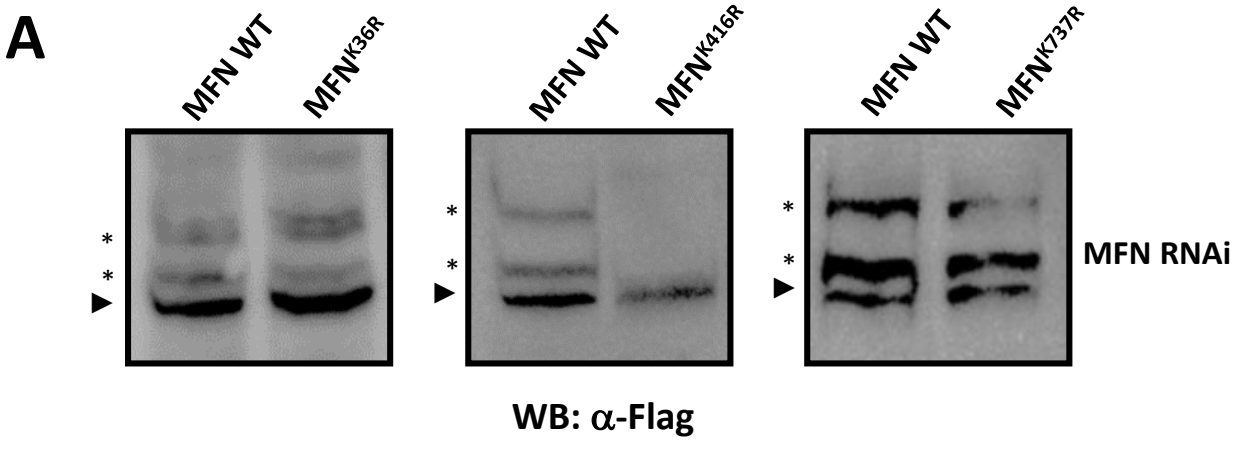
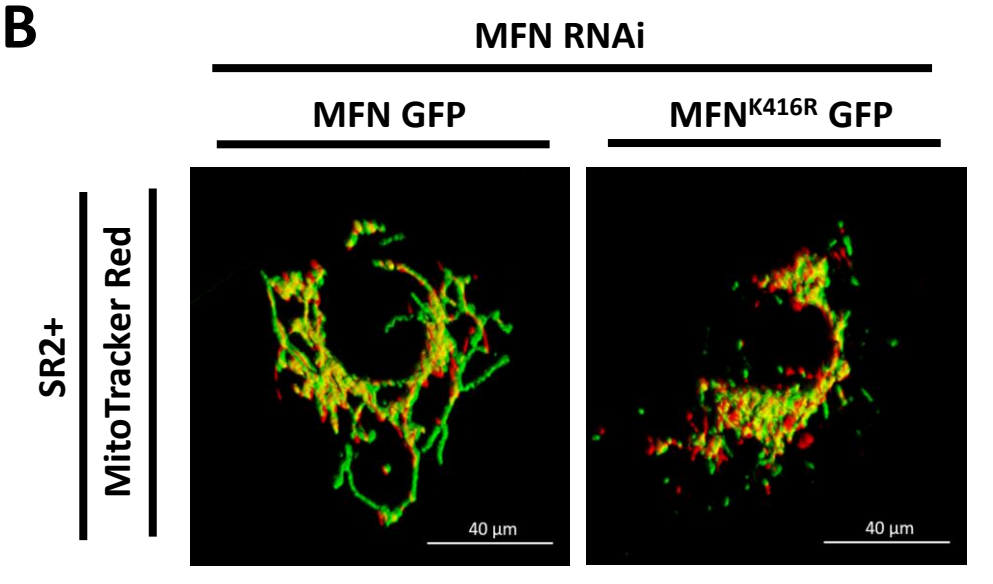
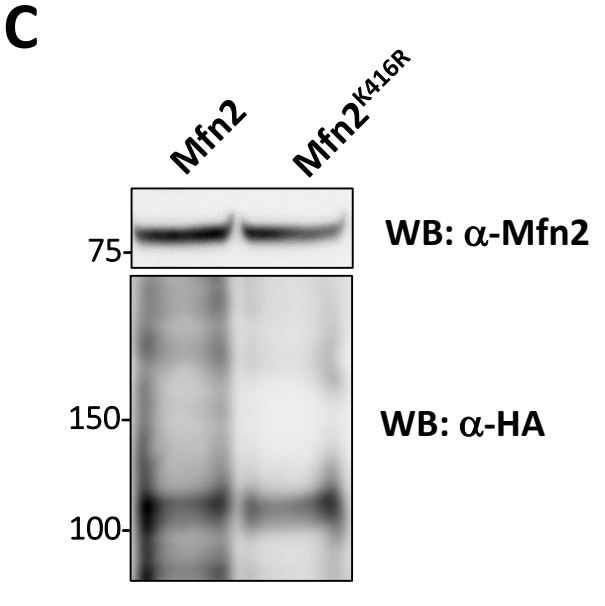


FIGURE 4


A
MFN WT
MFN^K36R
MFN WT
MFN^K416R
MFN WT
MFN^K737R
MFN RNAi
WB: α-Flag


B
MFN RNAi
MFN GFP
MFN^K416R GFP
SR2+
MitoTracker Red
40 µm
40 µm


C
Mfn2
Mfn2^K416R
75-
150-
100-
WB: α-Mfn2
WB: α-HA

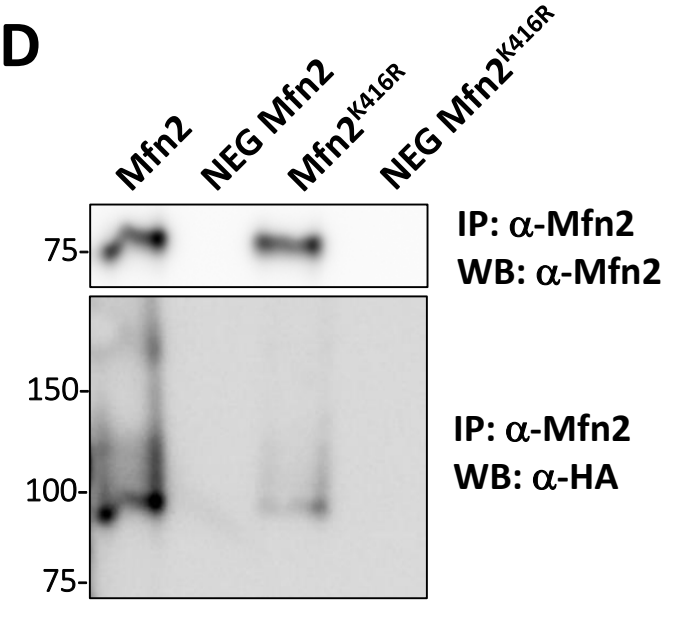
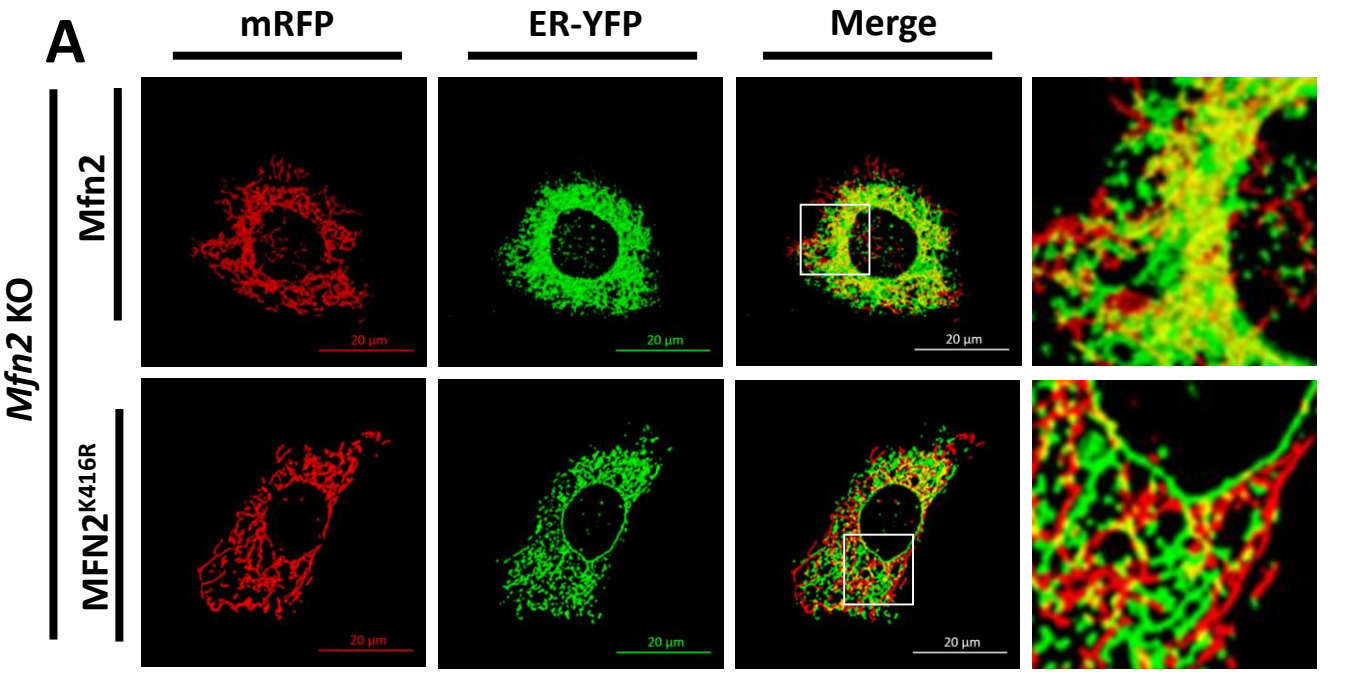
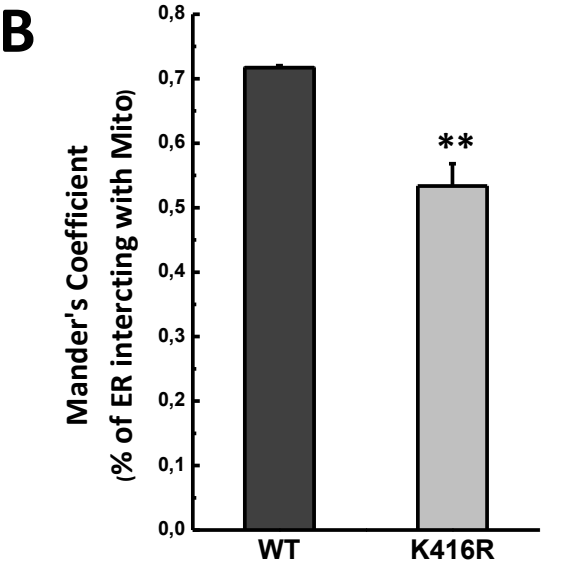
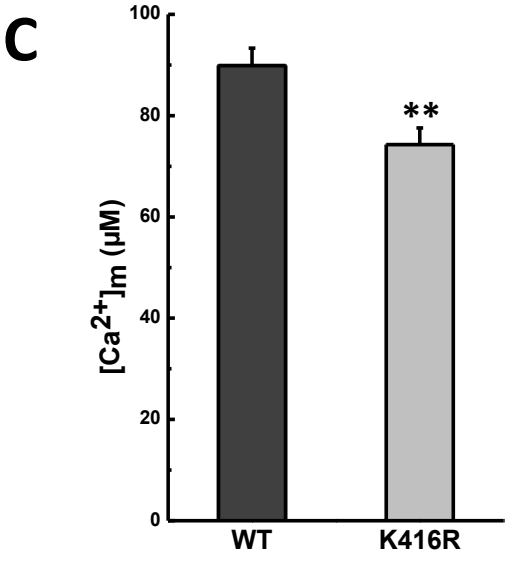

D
Mfn2
NEG Mfn2
Mfn2^K416R
NEG Mfn2^K416R
75-
150-
100-
75-
IP: α-Mfn2
WB: α-Mfn2
IP: α-Mfn2
WB: α-HA

FIGURE 5


A
mRFP
ER-YFP
Merge
Mfn2 KO
Mfn2
MFN2K416R
20 µm


B
Mander's Coefficient
(% of ER intercting with Mito)
0,0
0,1
0,2
0,3
0,4
0,5
0,6
0,7
0,8
**
WT
K416R


C
[Ca2+]m (µM)
0
20
40
60
80
100
**
WT
K416R

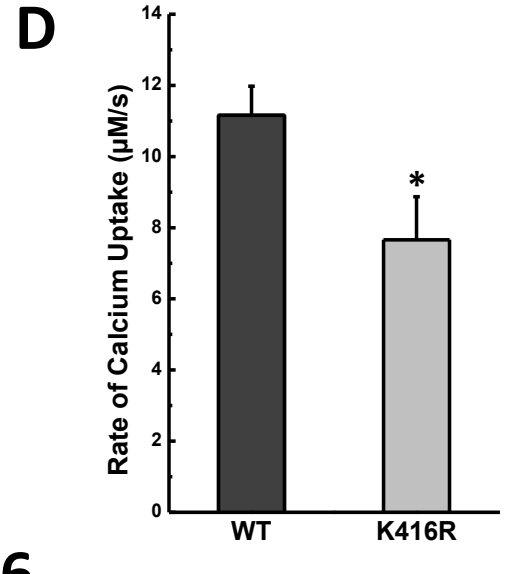

D
Rate of Calcium Uptake (µM/s)
0
2
4
6
8
10
12
14
*
WT
K416R

FIGURE 6

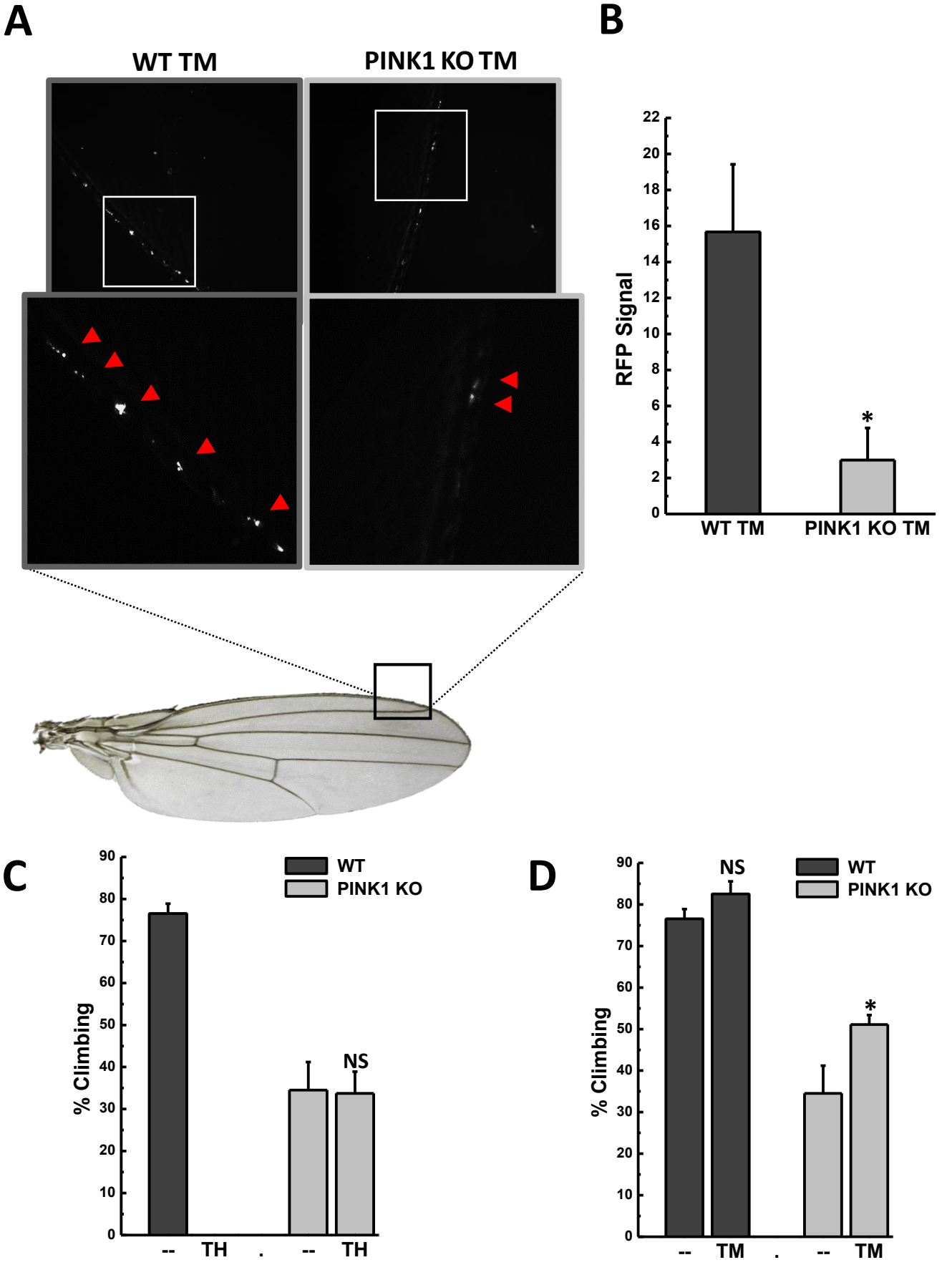

A
WT TM
PINK1 KO TM
B
RFP Signal
22
20
18
16
14
12
10
8
6
4
2
0
*
WT TM
PINK1 KO TM
C
% Climbing
90
80
70
60
50
40
30
20
10
0
WT
PINK1 KO
NS
-- TH . -- TH
D
% Climbing
NS
*
WT
PINK1 KO
-- TM . -- TM

FIGURE 7

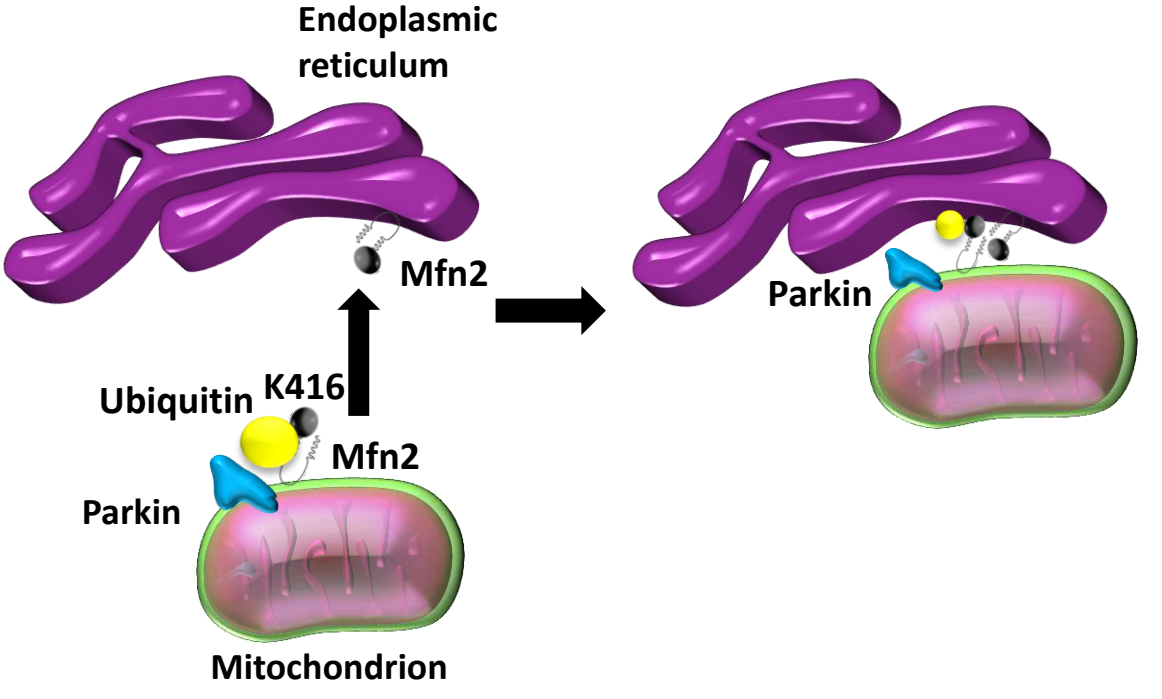

Endoplasmic reticulum
Mfn2
Parkin
Ubiquitin
K416
Mfn2
Parkin
Mitochondrion

FIGURE 8

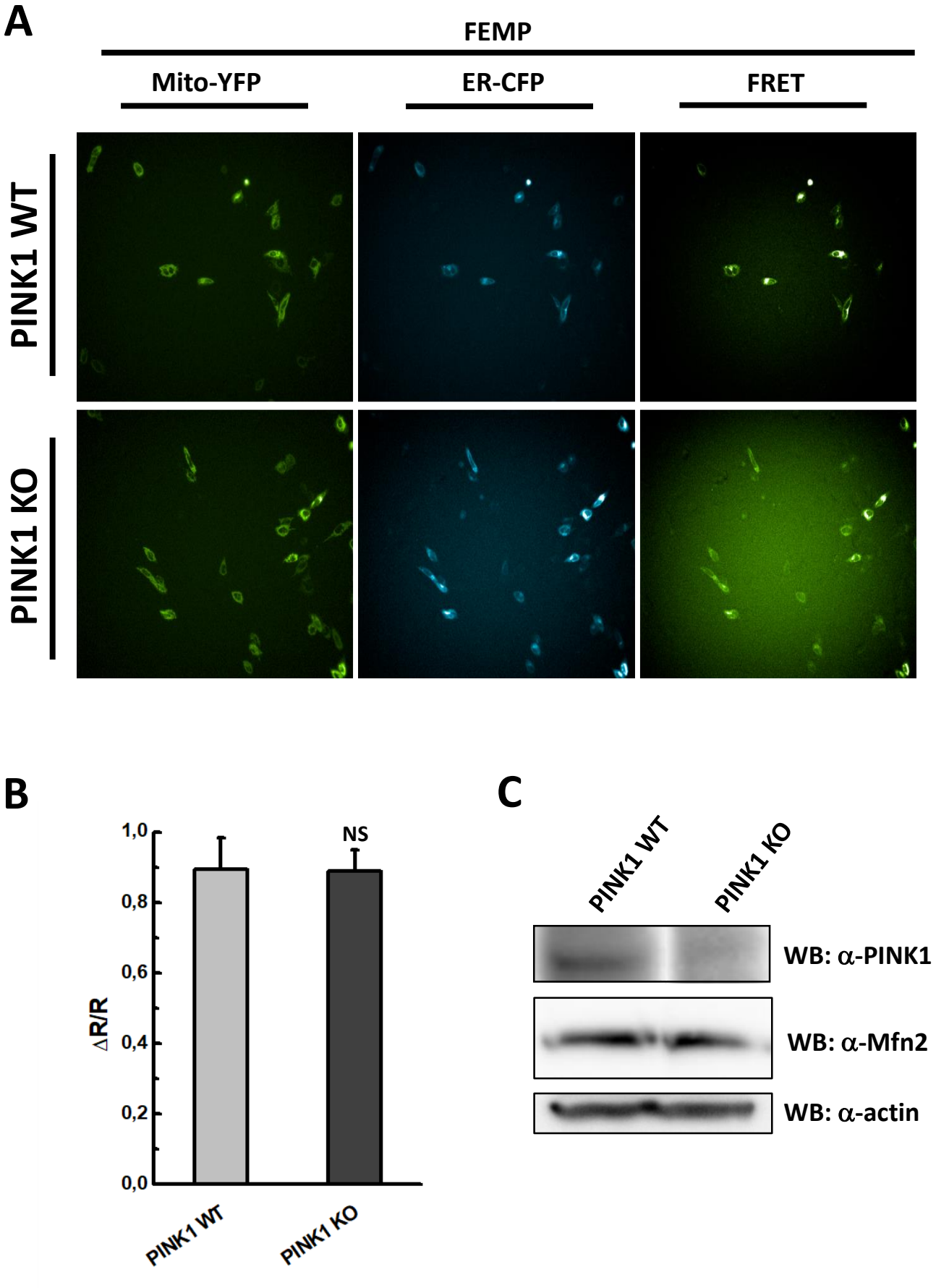

A
FEMP
Mito-YFP
ER-CFP
FRET
PINK1 WT
PINK1 KO
B
1,0
0,8
0,6
0,4
0,2
0,0
ΔR/R
NS
PINK1 WT
PINK1 KO
C
PINK1 WT
PINK1 KO
WB: α-PINK1
WB: α-Mfn2
WB: α-actin

FIG. SUPPL. 1

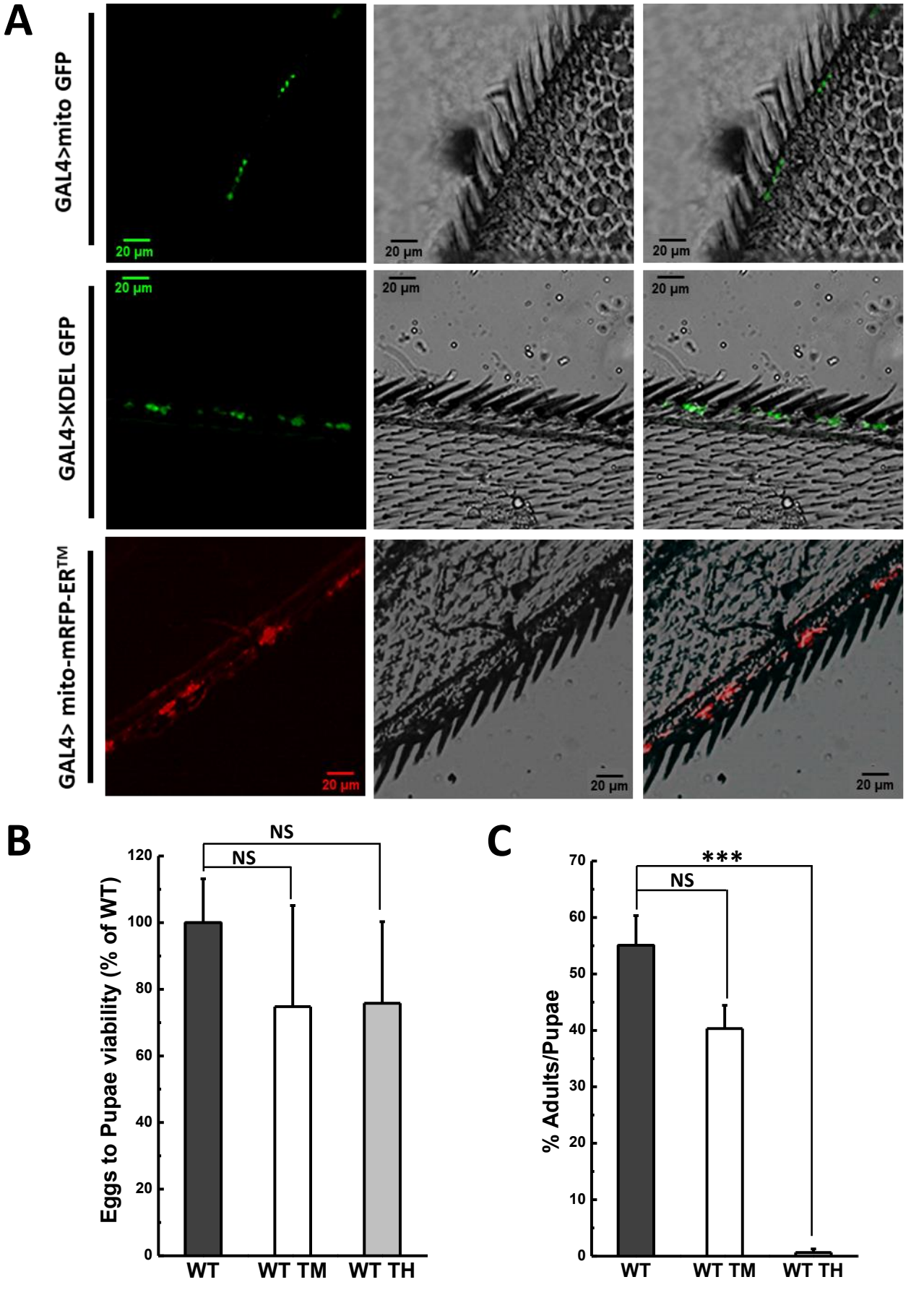

A
GAL4>mito GFP
GAL4>KDEL GFP
GAL4> mito-mRFP-ER™
20 µm
B
Eggs to Pupae viability (% of WT)
NS
NS
120
100
80
60
40
20
0
WT
WT TM
WT TH
C
% Adults/Pupae
***
NS
70
60
50
40
30
20
10
0
WT
WT TM
WT TH

FIG. SUPPL. 2

Conclusions

The purpose of this PhD thesis was to investigate the role of Parkin-dependent ubiquitination of Mfn2 in the regulation of ER-mitochondria tethering and its relevance in the neurodegenerative pathways leading to the onset of PD.

We hypothesized that Parkin, the *bona fide* E3 ubiquitin ligase that ubiquitinates MFN, regulates ER-mitochondria tethering, a structural feature that is required for a number of physiological processes including lipids biosynthesis and Ca^{2+} transfer between the two compartments (Rizzuto et al., 1998; Vance, 1990).

To address the role of Parkin and Parkin-dependent Mfn2 ubiquitination in the regulation of ER-mitochondria interaction, we took advantage of an animal model that was previously generated in the lab of Dr Ted Dawson, the conditional Parkin KO mouse (Von Coelln et al., 2004). We obtained mouse embryonic fibroblasts (MEFs) from these mice and we investigated the ubiquitination pattern of Mfn2 upon ablation of Parkin. Interestingly we observed that Parkin downregulation in MEFs resulted in decreased Mfn2 ubiquitination, which was accompanied by a significant reduction in ER-mitochondria interaction. We used two independent approaches to measure ER-mitochondria tethering: we first measured the percentage of ER co-localizing with mitochondria by using Mander's coefficient of co-localization upon volume-rendered 3D reconstruction of *z*-axis stacks of confocal images of cells expressing organelles targeted fluorescence probes (mito-RFP and ER-YFP, respectively) (Rizzuto et al., 1998). Secondly, we took advantage of a FRET based probe that was generated in the lab of Dr Luca Scorrano (Naon et al., 2016) to measure ER-mitochondria proximity. In this sensor, called FEMP, FRET intensity is inversely proportional to the distance between the two fluorophores (mito-YFP and ER-CFP) that are appropriately targeted to the two compartments. Both approaches indicated that in Parkin downregulating cells, ER-mitochondria physical interaction was impaired.

Starting from the observation that one of the most frequent Mfn2 mutation in CMT2A patients, R94Q, cannot restore ER-mitochondria contacts in *Mfn2* KO MEFs (de Brito and Scorrano, 2008), we analysed the pattern of MFN ubiquitination upon expression of mutant MFN^{R94Q} as well as two additional Charcot Marie Tooth type 2A disease-associate Mfn2 mutants (P251A and P280H). Of note, these MFN mutants failed to be ubiquitinated both in

S2R+ fly cells and in MEFs. Moreover when expressed in *Mfn2* KO background, Mfn2 mutants were unable to restore ER-mitochondria contacts.

These results led us to the intriguing hypothesis that ubiquitination of Mfn2, rather than its steady state levels, is an essential prerequisite for ER-mitochondria tethering.

To prove this hypothesis, we worked really hard to identify the site of Parkin dependent ubiquitination on the Mfn2 molecule. We found that the highly conserved lysine 416 in the HR1 domain is a genuine Parkin-dependent Mfn2 ubiquitination site. Non-ubiquitinatable mutant $Mfn2^{K416R}$ failed to reproduce the predicted pattern of ubiquitination and was incompetent in promoting ER-mitochondria physical and functional interaction (i.e. Ca^{2+} transfer).

Our results strongly indicated a causal link between impaired ubiquitination of Mfn2 and physical and functional ER-mitochondria miscommunication.

A number of studies have shown impaired Ca^{2+} homeostasis in cellular models lacking PINK1 or Parkin (Heeman et al., 2011; Sandebring et al., 2009). Although it is not clear why dopaminergic neurons specifically degenerate in PD, it is tempting to hypothesis that impaired Ca^{2+} homeostasis could be due to impaired Ca^{2+} cross talk at ER-mitochondria interface. Elegant studies have shown that artificial tether between ER and mitochondria can be used to modulate Ca^{2+} transfer. With that in mind, we addressed whether expressing an ER-mitochondria synthetic linker in a well-established *in vivo Drosophila* model of PINK1 loss of function could ameliorate PINK1 KO phenotypes by impinging on ER-mitochondria cross talk. Indeed our *in vivo* results showed that PINK1 KO flies climbing performance was significantly improved upon expression of a synthetic ER-mitochondria linker, demonstrating for the first time that the manipulation of ER-mitochondria tethering *in vivo* could represent a valid therapeutic target for medical intervention.

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REVIEW Open Access

Post translational modification of Parkin

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Abstract: Mutations in the gene encoding for the E3 ubiquitin ligase Parkin are associated to a rare form of familiar autosomal recessive Parkinsonism. Despite decades of research on the Parkin protein, whose structure has been recently solved, little is known about the specific signalling pathways that lead to Parkin activation. Parkin activity spans from mitochondria quality control to tumor suppression and stress protection; it is thus tempting to hypothesize that the broad impact of Parkin on cellular physiology might be the result of different post translational modifications that can be controlled by balanced opposing events. Sequence alignment of Parkin from different species indicates high homology between domains across Parkin orthologs and identifies highly conserved amino acid residues that, if modified, impinge on Parkin functions. In this review, we summarize findings on post translational modifications that have been shown to affect Parkin activity and stability.

Reviewers: This article was reviewed by Prof. Dr. Konstanze F. Winklhofer and by Prof. Thomas Simmen. Both reviewers have been nominated by Professor Luca Pellegrini.

Keywords: Parkinson's disease, Parkin, Post translational modifications, Ubiquitination, Phosphorylation

Background

Parkinson's Disease (PD) is the second most common neurodegenerative disorder affecting primarily the survival of a specific subset of dopaminergic neurons residing in the *Substantia Nigra Pars Compacta* of the midbrain [1]. Most PD cases are sporadic in origin. However, a small proportion of PD cases derive from mutations in PD associated genes, which have been mainly identified by characterizing familiar Mendelian inherited PD forms [2, 3]. The discovery of these genes (S, PINK1, Parkin, DJ1, LRRK2, VPS35, FBXO7, PLA2G6 and ATP13A2) (see [4] for a review), has greatly enhanced our understanding of the neurodegenerative pathways leading to dopaminergic neurons loss. Although there seem to be various causes of PD, genetic and sporadic forms are almost undistinguishable in terms of specific hallmarks, which at the cellular level includes formation of intracellular inclusions named Lewy Bodies, mitochondria abnormalities and selective loss of DA neurons, leading to the well characterized locomotor impairments at the systemic level [1]. The reason for studying genetic mutations of PD is the belief that the similarities between the sporadic and the inherited forms share a common mechanism of neurodegeneration, which can be more easily dissected at the molecular level in the genetic forms.

The pleiotropic protein Parkin

Although there are no unequivocally accepted scientific data that explain the selective neurodegeneration of dopaminergic neurons, the pathogenesis of PD appear to converge on three common features: mitochondria dysfunction, oxidative stress and proteins misfolding and aggregation [5]. Indeed studies on Parkin (PARK2), an E3 ubiquitin ligase, which mutations have been associated to the early onset of autosomal recessive Parkinsonism [6], have provided evidences for a direct role of mitochondrial dysfunction in the onset of the disease by regulating the mitochondria quality control via mitophagy. In the mitophagy process, Parkin is selectively recruited to depolarised mitochondria by PINK1 [7], a mitochondrial serine/threonine kinase, also a PD related protein [8]. In healthy mitochondria PINK1 is imported into mitochondria by the TOM/TIM translocase complex, cleaved and rapidly degraded by the proteasome [9–11]. However, on depolarised mitochondria PINK1 remains stable on the surface of mitochondria where it mediates the phosphorylation of Parkin, Parkin substrates and ubiquitin [7, 12–15]. Primed phospho-ubiquitin is specifically used by Parkin to ubiquitinate its targets on the outer mitochondrial membrane (OMM) [16], leading to the recruitment of downstream cytosolic

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receptors that are required for the activation of autophagy, including p62/SQSTM1, HDAC6, NDP52 and Optineurin [17–20].

Parkin leads to the ubiquitination of a broad number of targets that are expressed on the OMM, among others TOM20, Mitofusins, VDAC and Fis1 [16, 20–22]. Moreover, by targeting proteins with ubiquitin molecules, Parkin plays a crucial role in the degradative pathways mediated by the ubiquitin–proteasome system (UPS), which is required for both clearance of misfolded proteins and stress-induced mitophagy [19]. The available literature suggests that Parkin mediated mitochondrial outer membrane protein ubiquitination recruits proteasome complex to mitochondria, in turn causing rupture of the outer membrane, thus exposing inner membrane proteins which can interact with LC3 and guides mitochondria to mitophagy [23, 24]. It is possible that widespread ubiquitination of mitochondrial surface proteins by Parkin, acts as a general signal for mitochondrial quality control. In that respect, Parkin substrate specificity is debatable because no single Parkin substrate is essential for mitophagy and mitochondrial localization of a number of deubiquitinating enzymes (DUBs), including USP30 and USP2 [25], is sufficient to inhibit mitophagy, where DUBs like USP30 or USP15 knock down [26, 27] rescues defective mitophagy even in the absence of Parkin.

In addition to its established role in mitophagy and UPS, Parkin impacts other neuroprotective cellular pathways [28], including TNFα signaling [29, 30], and Wnt/β catenin signaling [31]. Parkin is also a putative tumor suppressor [32–34]. Interestingly, many of these pleiotropic functions of Parkin, which are dependent on its E3 ubiquitin ligase activity, do not result in ubiquitin dependent degradation of Parkin targets [35–38].

The power of ubiquitination

How does Parkin fulfill its many biological functions? Recent studies proposed ubiquitination as a modulator of protein activity, via regulating its subcellular localization and its ability to interact with other proteins [39, 40]. In the ubiquitination process, multiple lysine residues on the target protein can be ubiquitinated to produce multi-monoubiquitination [41]. Alternatively, an ubiquitin chain can form upon linear ubiquitination, in which the carboxy-terminal glycine of one ubiquitin molecule form a peptide bond with the amino-terminal methionine of another (linear or M1-linked ubiquitination) [42, 43]. Also, following addition of a single ubiquitin molecule to the target protein, further ubiquitin molecules can be added to the first ubiquitin molecule via linkage on lysine residue, producing an ubiquitinated chain (polyubiquitination). Ubiquitin itself contains seven lysine residues (Lys 6, Lys 11, Lys 27, Lys 29, Lys 33, Lys 48 and Lys 63), which allows the generation of ubiquitin chains with different orientations. Although some chain-types (specifically, K48 and K63-chains [44]) are more common than others, all possible linkages have been detected in cells [45]. Interestingly, the type of ubiquitin chain attached impinges on chain conformation and impacts the physiological outcome. For example, Lys 48 and Lys 11-linked chains adopt compact conformation and target proteins to the 26S proteasome [46–50]. Lys 63-linked chains or monoubiquitination, on the other hand, are the post-translational modifications that regulate lysosome dependent degradation [51, 52]. Monoubiquitination, Lys 63-linked chains and linear ubiquitination have also been described as non-degradative ubiquitination, which can control protein-protein interaction, protein subcellular localization and protein activity [53–59]. Very little is known regarding the physiological relevance of Lys 6, Lys 27, Lys 29 and Lys 33-linked chains and which biological outcomes these modifications would lead to. In that respect, ubiquitin ligases are extremely versatile enzymes that can potentially control almost every cellular process.

It is known that Parkin can promote both degradative Lys 48-mediated ubiquitination and non classical, proteosome-independent ubiquitination, including Lys 6, Lys 11, Lys 63, mono ubiquitination and linear ubiquitination [30, 60, 61]. Although Parkin dependent "regulative" ubiquitination has only recently started to be specifically addressed, emerging evidences suggest that Parkin activity necessarily includes "functional" ubiquitination and has the potential of controlling a broad subset of cellular processes depending on the activating stimuli. Not surprisingly, Parkin exists in an inactive state and it is normally repressed under basal conditions by several mechanisms of autoinhibition.

Mechanisms of Parkin autoinhibition

High resolution Parkin crystal structure gave insight into the mechanisms of Parkin autoinhibition. Parkin belongs to the RBR (RING-between-RING) type of E3 ubiquitin ligases, also known as RING/HECT hybrids, consisting of an ubiquitin like domain (Ubl), followed by two RING fingers domains (RING0 and RING1), an in between RING finger domain (IBR), a linker domain called Repressor Element of Parkin (REP) and a third RING finger domain called RING2 [62–65] (Fig. 1a). In the ubiquitin process, ubiquitin-activating enzymes (E1s) activate the C-terminus of the ubiquitin molecule and pass it to E2 conjugating enzymes that accept the activated ubiquitin and coordinate with E3 ubiquitin ligases to finally transfer ubiquitin to the amino group of a substrate protein [66–68]. Ubiquitin coordination and transfer is allowed by forming a thioester bond between catalytic cysteine residues on the E ubiquitin enzymes

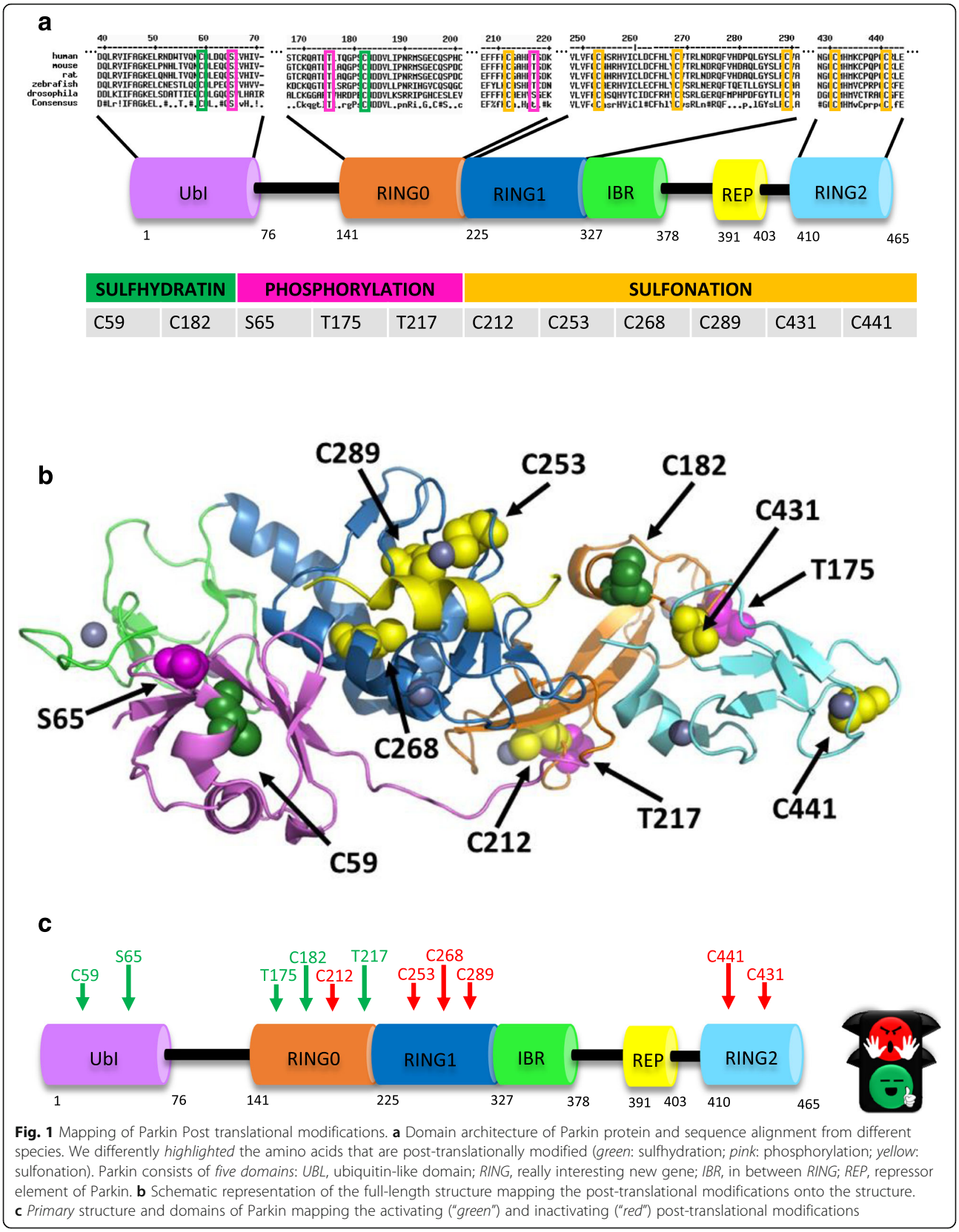

Fig. 1 Mapping of Parkin Post translational modifications. **a** Domain architecture of Parkin protein and sequence alignment from different species. We differently *highlighted* the amino acids that are post-translationally modified (*green*: sulfhydration; *pink*: phosphorylation; *yellow*: sulfonation). Parkin consists of *five domains*: *UBL*, ubiquitin-like domain; *RING*, really interesting new gene; *IBR*, in between *RING*; *REP*, repressor element of Parkin. **b** Schematic representation of the full-length structure mapping the post-translational modifications onto the structure. **c** *Primary* structure and domains of Parkin mapping the activating ("*green*") and inactivating ("*red*") post-translational modifications

and the C-terminal carboxyl group of ubiquitin. Parkin can selectively interact and coordinate with different E2 ubiquitin ligase to ubiquitinate its substrates. Interestingly, these E2s are selectively expressed in specific subcellular compartments. For example, Ubc6 and Ubc7 are endoplasmic reticulum-associated E2s that specifically interact with Parkin. Parkin can also interact with E2 enzymes UbcH7, UbcH8 and UbcH13/Uev1. Depending on which E2 enzyme Parkin couples with, different types of ubiquitin modification can arise, resulting in different biological outcomes. For example, Parkin can employ UbcH7 E2 ligase for K48-linked proteasome dependent polyubiquitination, and UbcH13/Uev1a for K63-linked autophagy dependent polyubiquitination [69–73]. How Parkin can choose to couple with a specific E2 ubiquitin ligase is largely unknown.

Two mechanisms of auto inhibition control Parkin activity. Fist, the RING1 domain that contains the binding site for the E2 ubiquitin-conjugating enzyme is occluded by the Ubl and the REP domains. Second, the catalytic site in the RING2 domain is blocked by the RING0 domain. Notably, Parkin catalytic Cys 431 in the RING2 domain is physically distant from the E2 conjugating site, which further suggest that Parkin needs to undergo a conformational change in order to function [62–65]. Therefore, under basal conditions, Parkin maintains a close structure that resembles that of a coiled snake [63] and its ubiquitin ligase activity is repressed (Fig. 1b). Not surprisingly, disease-associated mutations disrupt these interactions.

Post translational modifications of Parkin

Post translational modifications have 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. Most Post translational modifications, such as phosphorylation, acetylation, ubiquitination, are reversible modifications, mediated by large families of opposing enzymes. Balanced opposing events mediated by antagonistic enzymes might provide a potential molecular switch to modulate Parkin activity upon specific stimulation. Accordingly, Post translational modifications are required to trigger Parkin activity or to keep it repressed.

In the following sections, we summarize the so far reported Post translational modifications affecting Parkin activity and stability. Sequence alignment of Parkin indicates high homology between domains across Parkin orthologs and identifies highly conserved amino acid residues, most of which are post translationally modified from mammals to insects to impinge on Parkin functions. This analysis highlights evolutionarily conserved posttranslational processes, in relation to Parkin activation, which is not characterized yet.

Phosphorylation

There are multiple proteins, which are involved in Parkin phosphorylation, PINK1 being the most studied [74–79]. It was first reported by Kim et al. [78] that Parkin activity and mitochondrial localization is PINK1 kinase activity dependant. Authors further described that RING1 and REP domains are indispensable for PINK1 mediated mitochondrial translocation of Parkin, as well as PINK1 dependent phosphorylation of Thr 175/Thr 217 is crucial for its translocation. In a separate study [79] the same was confirmed, and it was further shown that phosphorylation of Parkin is required for Parkin to interact with E2 ubiquitin ligase UbcH13/Uev1a to mediate K63-linked polyubiquitination of IKKγ in NFκB stress response pathway.

Later, it was reported by two simultaneous studies [80, 81] that the PINK1-dependent phosphorylation of Ser 65 of the Ubl domain is required for Parkin translocation as well as stress-induced mitophagy. These findings were confirmed in a subsequent study that used as read out the degradation of Miro1, a *bona fide* Parkin substrate, upon expression of full-length wild type or PD disease-associated Parkin mutants [82]. In vivo data from *Drosophila* also confirmed these findings and additionally showed that phosphorylation of Parkin regulates spontaneous dopamine release from the neuron terminals, flight activity as well as survival of the flies [83].

One recent study deciphered the sequence of Parkin activation, and demonstrates that upon mitochondrial depolarization PINK1 phosphorylates ubiquitin at Ser 65, which goes to bind Parkin and Parkin substrates. Primed phospho-ubiquitin makes Parkin more accessible for PINK1 mediated Ser 65 phosphorylation [84]. So, in a nutshell, it has become evident that phosphorylation by PINK1 is the central point of Parkin activation and target recognition [81, 84, 85].

Phosphorylation is not only required for Parkin activation. Quite intriguingly, phosphorylation of Parkin by cyclin dependent kinase 5 (Cdk-5), casein kinase 1 (CK-1) and c-Abl modulates Parkin folding and/or activity [74–76, 86]. Phosphorylation by both Cdk-5 on Ser 131 and CK-1 on Ser 101, Ser 127 and Ser 378 influence the solubility of the protein, leading to increased Parkin aggregation [75]. Quite surprisingly, in both cases Parkin phosphorylation does not seem to particularly affect Parkin E3 ubiquitin activity. The role of c-Abl in regulating Parkin activity was reported by two different studies where the authors found that phosphorylation of Parkin by c-Abl at Tyr 143 can inactivate its E3 ligaes activity [74, 86] in human cell lines and MPTP treated mice. Consistent with this, increased protein levels of c-Abl and tyrosine phosphorylation of Parkin was reported in human post mortem brains from PD patients.

Ubiquitination

As previously mentioned, poly ubiquitination of proteins in general is the signal for proteasomal degradation. Parkin, though an E3 ubiquitin ligase itself, faces the same fate when multiple ubiquitin chains are attached to it. It has been reported that Parkin mediates its own ubiquitination via K48 proteosome dependent ubiquitin chain formation [87], thus impinging on its own protein turnover. Co-localization of ubiquitinated Parkin and Lewy body in PD patients brain might ignite the idea that ubiquitinated Parkin is a inactive form of the enzyme. However, in reality Parkin mono-ubiquitination at different sites can activate the enzyme. Mutations causing ubiquitination of the Ubl domain of Parkin makes the enzyme constitutively active and thus supports the idea of 'regulative" Parkin autoubiquitination [88]. One intriguing possibility is that Parkin dependent non-degradative self-ubiquitination might be required to regulate Parkin subcellular localization and its interaction with specific E2 ubiquitin ligases and/or Parkin substrates. In that context, further studies are required to identify the precise site of Parkin ubiquitination and dissect the functional role of specific site ubiquitination.

Consistent with the hypothesis that Parkin self-ubiquitination is a functional ubiquitination, Parkin autoubiquitination is subjected to tight regulation by other factors. Deubiquitinating enzymes (DUBs), for example, antagonize Parkin autoubiquitination [89]. Durcan and colleagues identified DUB Ataxin-3 as a binding partner of Parkin, which interacts with both the Ubl and IBR-RING2 domain of Parkin and promotes Parkin de-biquitination. Mutant Ataxin-3, which polyglutamine expansion is associated with the onset of Machado-Joseph neurodegenerative disease, promotes the degradation of Parkin via autophagy and leads to decreased Parkin levels in vivo [90, 91]. In a subsequent study, the same group showed that Ataxin-3 in fact binds to and coordinate with E2 ubiquitin ligase Ubc7 rather than Parkin, and promotes Parkin de-ubiquitination only upon Parkin autoubiquitination [92].

Recently, the same group has reported that DUB USP8 preferentially remove K6 linked Ub conjugates from Parkin and USP8 silencing hindered Parkin recruitment to depolarised mitochondria, suggesting that USP8 is required for active mitophagy [93].

Overall, these works highlighted an intricate regulation of Parkin ubiquitination that involves the coordinated activities of Parkin, DUBs and E2 ubiquitin ligases. It is tempting to hypothesis that such complex interplay is required to prime Parkin for further Post translational modifications that affect Parkin activity via regulation of its subcellular localization and/or interaction with specific E2 and/or substrates.

Sumoylation and Neddylation

Post-translational modification of proteins by small ubiquitin like modifiers (SUMO) or in general SUMOylation is still not fully unravelled, though holds the indications that like ubiquitination or phosphorylation, it might have far reaching implications as well. In a very simplistic way, SUMO gets matured, activated and attached to target proteins by a series of specific enzyme complexes, much like the ubiquitination process [94]. Interestingly, from the point of view of PD, three of the prominent proteins that are mutated in familiar Parkinsonism, SNCA, DJ-1 and Parkin, fall under the targets of SUMOylation [95–98]. At first sight, it appears that reports connecting SUMOylation of SNCA and aggregate formation followed by cell death are contradictory [97, 99], but the precise site of SUMOylation might be the deciding factor for aggregate formation and cell death by α-synucleinopathy [100]. As far as Parkin is concerned, reports are significantly less which properly deciphers the physiological role of SUMO attachment to Parkin. One solitary report by Um and Chung [98] demonstrates that covalent attachment of SUMO-1 (but not SUMO-2, which possible due to differential preference of subtrates by SUMO-1, 2 and 3) to Parkin, both in vitro and in vivo, increases its nuclear localization and autoubiquitination. In the nucleus, Parkin transcriptionally represses p53 by interacting with p53 promoter. Interestingly, this activity of Parkin is independent of its ligase function.

NEDD8 is another protein that shows similarity with ubiquitin, in terms of structural homology and the way of getting attached to other proteins as post-translational modification [101]. Like ubiquitin, NEDD8 is also expressed in most tissue types and strikingly concentrated in different types of protein aggregates, which includes Mallory bodies in liver, Rosenthal fibres in astrocytoma, neurofibrillary plaques of Alzheimer's disease and Lewy bodies in PD [101]. Um et al. [102] showed that attachment of NEDD8 to Parkin increase the E3 ligase activity by increasing affinity towards E2 ubiquitin ligase UbcH8 and the putative substrate the p38 subunit of aminoacyl trasferase. Choo et al. [103] also reported increased Parkin E3 ligase activity upon neddylation. The also found that PINK1 undergoes neddylation, which results in increased stability of PINK1 55KDa proteolytic fragment. Interestingly, genetic enhancement of neddylation was shown to rescue the phenotypes associated with a *Drosophila* in vivo model of PINK1 deficiency. Moreover, PD neurotoxin MPP^+ treatment showed decreased neddylation of both PINK1 and Parkin, clearly indicating a causal link between NEDD8 modification of PINK1/Parkin and PD pathogenesis.

Nitrosylation, sulfhydration and sulfonation

Among many other contributing factors, nitric oxide (NO), hydrogen sulphide (H_2S) and oxidative stress have been found to influence the progression of PD [104].

Increased attachment of NO to thiol groups (S-nitrosylation) of Parkin in PD was first reported by Ted Dawson's group [105] where the authors showed increased nitosylation of Parkin in MPTP treated mice and human patient's brains. They also demonstrated that nitrosylation decreased the protective effect of Parkin by inhibiting its E3 ligase activity. A concurrent study by Stuart Lipton's group also reported the same while showing a steep increase of the Parkin E3 ubiquitin ligase activity, which autoubiquitinates the enzyme, followed by decreased activity [106].

In contrast to these works, one solitary study demonstrates that S-nitrosylation of Parkin, more specifically at Cys 323, increases the Parkin E3 ligase activity, and it is required for efficient removal of depolarised mitochondria [107]. The authors suggest that Cys 323 is not involved in zinc ion coordination and therefore its modification can regulate Parkin activity without disrupting the ability of the protein to coordinate ion zinc that is required for Parkin activity. Furthermore, the authors give evidences that PINK1 dependent phosphorylation and nitrosylation of Parkin act independently and that Parkin nitrosylation is mostly cytosolic.

These conflicting studies identified sites of potential S-nitrosylation within the RING1, RING2 and the IBR domain [106, 107]. Most of those that were identified as potential sites of nitrosylation are highly conserved among different species (except for Cys 323 that is only conserved in vertebrates), although further studies are required to understand whether these nitrosylated sites are responsible for altered Parkin E3 activity. Cysteine sites in these domains are important for zinc coordination and their nitrosylation is likely to distrupt the conformation of these domains that are both important for E2 coordination and Parkin catalytic activity. It is therefore not surprisingly that nitrosylation at those sites affect Parkin E3 ligase activity.

Interestingly, a recent work demonstrated that nitrosylation of Parkin increased p53 level [108]. Authors suggested that cell death due to nitrosative stress occurs via increase of pro apoptotic factor p53 and correlated the increased nitrosylation of Parkin to p53 levels in human post mortem PD brains. It was reported that nitrosylated Parkin preferentially accumulates in the cytoplasm and does not translocate to the nucleus, where Parkin operates as repressor of p53 promoter. This finding might explain the correlation between increased p53 levels and nitrosylated Parkin in PD brains, and potentially p53 dependent-cell death due to nitrosative stress, the later being the causative factor for the increase of p53 levels.

Modification of Parkin by H_2S, termed sulfhydration, was found to be protective in nature. Three independent studies documented the protective effect of systemic administration of NaHS as H_2S donor in preventing the progression of Parkinsonism in toxin induced animal models of PD [109–111]. In a subsequent study, Vaniver et al. [112], specifically discovered the sulfhydrated cysteine residues which enhanced Parkin protective activity. The authors systematically mutated the various Parkin cysteines and measured Parkin activity. Mutation C95S completely abolished Parkin enhanced ubiquitination activity upon administration of GYY4137, a H_2S donor. Mutants C59S and C182S also result in substantial diminution of the enhancement of ubiquitination upon sulfhydration. Though the protective effect of sulfhydration against neurotoxin-induced Parkinsonism has been widely documented, the molecular mechanism is largely known.

Interestingly, the level of Parkin sulfhydration was found to be reduced in PD patient's brain, while nitrosylation showed a steep increase. These findings suggest that nitosylation and sulfhydration are two reciprocal, opposing events that both impinge on cysteine residues and provide them with chemical groups that opposingly impact Parkin E3 ligase activity [112].

In a slightly different context, it was shown that heat shock, oxidative stress induced by H_2O_2 or deletion of 13 amino acids from the C terminal end of Parkin can lead to its aggregation, which is inhibited by the overexpression of heat shock protein chaperones [113]. More recently, a study by Meng et al. [114] showed that oxidative stress induced by either MPP+ or H_2O_2 treatment result in oxidation of a specific subset of cysteine residues of Parkin. The process of cysteine oxidation, also known as sulfonation, alters Parkin solubility and leads to Parkin inactivation. Upon mass spectrometry analysis of Parkin oxidation, authors also showed that several of the Parkin cysteine that are sulfonated upon oxidative stress (Cys 212, Cys 253, Cys 268, Cys 289, Cys 431 and Cys 441) were previously reported to be mutated in familial PD cases, supporting the hypothesis that rare hereditary mutations and environmentally linked PD cases might share a common mechanism of inactivation of Parkin.

Post translational modifications of Parkin: a protein analysis between orthologs

In order to keep their function, proteins need to preserve their three-dimensional structure, meaning that they have to keep the same or similar amino acid sequence. If there are conserved amino acids in some regions of orthologous proteins, it can be concluded that these amino acids are important for the function of the protein. This is especially relevant when the conservation occurs at the protein rather than at the DNA level. Interestingly, amino acids sequence alignment between Parkin orthologs revealed that the Parkin residues that are post translationally modified are highly conserved from mammals to insects (Fig. 1a). These include sites

of phosphorylation (Ubl and RING0 resident residues Ser 65, Tyr 175 and Tyr 217), sulfhydration (Ubl and RING0 resident residues Cys 59 and Cys 182) and sulfonation (RING1 and RING2 resident residues Cys 212, Cys 253, Cys 268, Cys 289, Cys 431 and Cys 441) (Fig. 1a).

It is interesting to note that phosphorylation sites leading to Parkin activation are highly conserved, whereas those which impairs Parkin activity are only conserved in mammals. It is intriguing to hypothesis that evolution might have been more stringent when it comes to mechanisms of Parkin activation.

This analysis also led us to the interesting observation that the residues that are post translationally modified to activate Parkin are either in the Ubl or the RING0 domain, whereas the inactivating modifications mainly affect residues of the RING1 or RING2 domain, that contain the E2 binding site and the catalytic site, respectively (Fig. 1c).

Conclusions

Parkin is an E3 ubiquitin ligase with various pleiotropic functions. Elucidating the molecular mechanisms that control its function can have important implications not only in the regulation of mitochondria quality control and proteosomal dependent degradation of abnormal proteins, but also in the context of various Parkin cytoprotective functions, that include inhibition of the activity of pro apoptotic proteins p53 and Bax, and enhancement of expression of pro survival protein OPA1 via NF-kB signalling [29, 30].

Under basal conditions, Parkin adopts a coiled inhibited conformation and its activity is repressed [62–65, 115, 116]. Post translational modifications can control Parkin activity, subcellular localization, conformation, solubility, E2 choice and interaction with cofactors that are required for Parkin activation, substrate affinity as well as specificity. Post translational modifications can occur rapidly to respond to changes in metabolism or when cell experience environmental stress. More importantly, Post translational modifications are reversible and are controlled by the counteracting activities of opposing enzymes, which can be timely and rapidly regulated. Protein phosphatases oppose protein kinases, de-ubiquitinating enzymes oppose ubiquitin ligases, protein deacetylases counteract acetyltransferases, denitrosylation opposes S-nitrosylation and so on. Balanced regulation of opposing events can result in complex biological outcomes, particularly when the targets of this regulation are proteins with pleiotropic functions, like Parkin.

Potentially each PTM can be targeted for therapeutic intervention as long as its physiological outcomes is known and specific synthetic molecules are available to either inhibit or enhance such modification depending on its outcome. In this context, much effort has been recently put towards the identification of specific deubiquitinating enzymes that directly or indirectly oppose Parkin activity. Along the same line, it will be important to evaluate whether specific protein phosphatases are in place to oppose PINK1 in the phosphorylation of Parkin.

Future works will also clarify whether other modifications, such as acetylation (second most common PTM after phosphorylation) or glycosylation (third most common PTM) might play a role in the control of Parkin activation.

The next challenge will be to identify appropriate in vitro system that allows rapid and specific read out of Parkin activation. In that respect, in a very recent report Pao et al. [117] developed a newly engineered probe to monitor the thansthiolation activity of E3 ligases to decipher mechanisms of Parkin activation. Interestingly, this report demonstrates that initial phosphorylation of ubiquitin is upstream Parkin phosphorylation and subsequent activation, allowing the precise dissection of a rather complex multi step process.

The compatibility of newly generated probes to study Parkin activity from cell extracts and the potential reproducibility of the assay in samples extracted from human patients, will pave the way for the development of rapid methods to address how different post-translational modifications affect Parkin activity in vitro as well as in vivo.

Reviewers' comments

This article was reviewed by Prof. Dr. Konstanze F. Winklhofer, Faculty of Medicine, Biochemistry and Pathobiochemistry department, University of Ruhr-Bochumand (reviewer 1) and by Prof. Thomas Simmen, Department of Cell Biology, University of Alberta, Canada (reviewer 2).

Reviewer 1 summary: In their manuscript, the authors provide a comprehensive and timely review on the regulation of the E3 ubiquitin ligase Parkin by posttranslational modifications. This overview is well-balanced and includes a wide spectrum of Parkin functions. Therefore it will be interesting and helpful for a broad readership.

Reviewer 2 summary: The review article by Chakraborty et al. provides a useful, up-to-date resource for Parkin researchers by describing the complete set of known Parkin post-translational modifications. There is a three-part figure that accompanies the manuscript, which provides a helpful illustration of what is described within the manuscript. The sequence of chapters is logical, starting with phosphorylation. Ultimately, my suggestions are minor changes that would hopefully make the manuscript even more useful that it already is.

Response to reviewers: *First of all we would like to thank both reviewers for their valuable and relevant comments. As specified in the following chapters, reviewers concerns have been carefully addressed in the revised version of the manuscript.*

Reviewer 1

Comment: Some suggestions to increase linguistic clarity: *Page 10, line 15 to 18: "Quite intriguingly, phosphorylation of Parkin by cyclin dependent kinase 5 (Cdk-5), casein kinase 1 (CK-1) and c-Abl leads to either increased Parkin aggregates properties or Parkin inhibition." … modulates Parkin folding and/or activity.*

Response: *we have modified the sentence according to the suggestion.*

Comment: *Page 11, line 15: The statement that "mutation in the Ubl domain leads to multi ubiquitinated Parkin that is constitutively active" should be substantiated by a reference.*

Response: *We have modified the sentence and supported the statement by reference.*

Minor points: *Check correct spelling, for example:-Page 3, line 3: "familiar"-Page 4, line 15: "rational"-Page 8, line 15: "autoinhibitions"-Page 14, line 11: "S-nitrosylayion" - Fig. 1a: "Sulfhydatin" Check wording, for example:-Page 3, line 4 : "brought to"-Page 5, line 21: "whether" - Page 14, line 10: "imposes" - Page 17, line 4: "whether"*

Response: *we have modified the specified sentences.*

Reviewer 2

Comment: *what is the mechanism that causes proteasome degradation of mitochondrial proteins? Are these proteasomes mitochondria-localized? This is not well explained.*

Response: *we have explained this in the updated manuscript.*

Comment: *the description of highly conserved residues within Parkin on page 9 is confusing. Are all of these post-translationally modified? Are only these post-translationally modified?*

Response: *we have mentioned in the manuscript that most of these are post translationally modified. This list might not be exclusive and we have focused on the so far reported ones.*

Comment: *I would reconsider using the PTM abbreviation. I had some trouble getting used to it.*

Response: *to our knowledge, PTM is quite frequently used abbreviation for posttranslational modification. We however reconsidered using the abbreviation in the manuscript, as suggested by the reviewer.*

Comment: *on page 5, it is stated that PINK1 is a serine/threonine kinase, but on page 9, PINK1 is said to phosphorylate tyrosines on Parkin. It is later mentioned that Parkin serines are also phosphorylated. What is the relationship between tyrosine and serine phosphorylation? This needs to be better explained. What phosphorylation occurs in D. melanogaster? What is the relationship between phosphorylation by PINK1 and other kinases? What is known about Parkin serine phosphorylation in PD patient brains?*

Response: *We would like to thank the reviewer for actually spotting this. We realised that the abbreviation for Threonine was misspelled (Tyr instead of Thr): PINK1 indeed is a serine/threonine kinase that phosphorylates substrates on threonine (Thr) residues. We apologise for the confusion the misspelled abbreviation might have caused.*

To our knowledge, nothing is known about Parkin serine phosphorylation in PD patients brains.

Comment: *What is the functional difference between SUMO-1 and SUMO-2?*

Response: *The basic difference between SUMO-1 and 2 is their substrate preference. We have mentioned this in the manuscript.*

Comment: *Where could NO originate that modifies Parkin and why does it increase in PD brain? It is unclear whether there is a correlative or causative link between Parkin nitrosylation and p53.*

Response: *NO is synthesized by nitric oxide synthase (NOS), which comes in three forms: endothelial, neuronal and inducible NOS. So, in the brain the origin of NO could be due to the functionality of neuronal NOS. The increase in NO in PD brain and its relevance is quite controversial as it can be marked as both causative and/or after effect of neuro-inflammation. It is beyond the scope of this review to discuss that in details.*

In the stated report the authors discovered that nitrosylation of Parkin decreases its activity as a repressor of P53. Later increase in both nitrosylated Parkin and P53 was found in human patients brain and the conclusion was drawn stating nitrosylated Parkin is the causal factor for P53 increase. We have mentioned this in the updated MS.

Comment: *The authors may consider swapping panels a and b in Fig. 1.*

Response: *We don't understand the reason for this request. Panel a is described before panel b in the text.*

Comment: *English grammar and spelling needs to be improved in some spots. This is especially the case in the abstract, which I suggest to rewrite from scratch, but also in some other instances (e.g., lines 19–23 p. 5; line 18 p. 6; lines 11–13 p.9; lines 8–14 p. 11; lines 7, 13 p.12). Some issues with singular/plural confusion and wrong usage of articles.*

Response: *We have modified the manuscript according to the suggestions.*

Abbreviations

Cdk-5: Cyclin dependent kinase 5; Ck-1: Casein kinase 1; DUBs: Deubiquitination enzymes; H_2S: Hydrogen sulphide; IBR: In between RING finger domain; NO: Nitric oxide; OMM: Outer mitochondrial membrane; PD: Parkinson's Disease; REP: Repressive element of Parkin; SUMO: Small ubiquitin like modifiers; UPS: Ubiquitin-proteasome system

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Availability of data and materials
Not applicable.

Authors' contributions
JC, VB and EZ wrote the manuscript. VB drew the figure. All authors read and approved the final manuscript.

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Dr Elena Ziviani accomplished her PhD in Neuroscience at the MRC toxicology Unit in Leicester, UK, under the supervision of Professor P. Nicotera where she investigated the effect of nicotine in primary neurons on neuroprotection and synaptic plasticity. In 2007, she joined Dr. Alex Whitworth's group in Sheffield, UK, where she studied the role of Parkinson's Disease (PD)-related proteins PINK1 and Parkin in mitochondrial dynamic and mitophagy. In 2010 Dr Ziviani was awarded with both EMBO long term and Marie Curie Action fellowships to investigate the role of Parkin-dependent ubiquitination of Mitofusin-2 in regulating mitochondria-endoplasmic reticulum tethering in the onset of PD at the Department of cellular physiology and metabolism, Geneva medical school, Switzerland. Since 2014, she became assistant professor at the Department of biology, University of Padova.

Competing interests
The authors declare that they have no competing interests.

Consent for publication
Not applicable.

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Not applicable.

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