LRRK2 regulates endoplasmic reticulum–mitochondrial tethering through the PERK-mediated ubiquitination pathway

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Abstract

Mutations in the leucine-rich-repeat kinase 2 (LRRK2) gene are the most common cause of familial Parkinson’s disease (PD). Impaired mitochondrial function is suspected to play a major role in PD. Nonetheless, the underlying mechanism by which impaired LRRK2 activity contributes to PD pathology remains unclear. Here, we identified the role of LRRK2 in endoplasmic reticulum (ER)–mitochondrial tethering, which is essential for mitochondrial bioenergetics. LRRK2 regulated the activities of E3 ubiquitin ligases MARCH5, MULAN, and Parkin via kinase-dependent protein–protein interactions. Kinase-active LRRK2(G2019S) dissociated from these ligases, leading to their PERK-mediated phosphorylation and activation, thereby increasing ubiquitin-mediated degradation of ER–mitochondrial tethering proteins. By contrast, kinase-dead LRRK2(D1994A)-bound ligases blocked PERK-mediated phosphorylation and activation of E3 ligases, thereby increasing the levels of ER–mitochondrial tethering proteins. Thus, the role of LRRK2 in the ER–mitochondrial interaction represents an important control point for cell fate and pathology in PD.

Keywords endoplasmic reticulum; LRRK2; mitochondria; PERK; ubiquitin ligase

Subject Categories Autophagy & Cell Death; Membranes & Trafficking; Metabolism

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Introduction

Parkinson’s disease (PD) is a neurodegenerative disorder with no cure. Genetic studies revealed that missense mutations in the protein LRRK2 are the most common cause of familial PD (Funayama et al., 2002; Paisan-Ruiz et al., 2004; Zimprich et al., 2004). In addition, genome-wide association studies have identified a common variation in the LRRK2 gene as a risk factor for sporadic PD (Satake et al., 2009; Simon-Sanchez et al., 2009). LRRK2 encodes a 2,527–amino acid protein consisting of an ankyrin-repeat (ANK) domain, a leucine-rich repeat (LRR), a Ras of complex proteins (ROC) domain, a C-terminal of Roc (COR) domain, and kinase and WD40 domains.

Mitochondrial dysfunction has been implicated in a range of neurodegenerative diseases and in PD in particular (Winklhofer & Haass, 2010). The molecular pathogenesis of sporadic PD and the basis of selective dopaminergic neuronal loss remain unclear. Mutations in several genes, including SNCA (encoding alpha-synuclein), DJ-1, LRRK2, PINK1, and PRKN (encoding Parkin), cause forms of familial PD that are clinically indistinguishable from sporadic PD (Klein & Westenberger, 2012). PINK1 and PRKN encode mitochondrial localized proteins that participate in mitochondrial quality control, further supporting the idea that mitochondrial dysfunction is sufficient to cause PD.

Mitochondria play major roles in multiple cellular processes, including energy metabolism, calcium homeostasis, and lipid metabolism. Mitochondria are associated with the endoplasmic reticulum (ER), with 5–20% of the mitochondrial surface apposed to ER membranes (Rizzuto et al., 1998; Csdorlas et al., 2006). The regions of the ER associated with mitochondria are termed mitochondria-associated ER membranes (MAMs), and these contacts facilitate a variety of signaling processes between the two organelles, including calcium (Gincel et al., 2001; Rizzuto et al., 2012) and phospholipid exchange (Rowland & Voeltz, 2012), and impact diverse physiological processes including ATP production, autophagy, protein folding, and apoptosis (Simmen et al., 2010; Rowland & Voeltz, 2012; Hamasaki et al., 2013; Kornmann, 2014). Despite the fundamental importance of these interactions to cell metabolism, the mechanisms that mediate recruitment of ER membranes to mitochondria are not fully understood. Several protein complexes have been proposed as ER–mitochondrial tethers, implying that different ER–mitochondrial tethering protein complexes may permit selective recruitment of different domains of the ER, causing the distances between physiological ER–mitochondrial contacts to vary 10–30 nm (Csdorlas et al., 2006; Rowland & Voeltz, 2012).
To maintain energy production and various cellular processes, mitochondrial protein quality control mechanisms are required to counteract the continuous accumulation of defective mitochondrial components. One such mechanism is the dynamic remodeling of mitochondrial membrane through fission and fusion (Karbowsk & Youle, 2011), and the other is the ubiquitin/proteasome system, which removes damaged proteins in mitochondria and ER (Christianson & Ye, 2014; Ruggiano et al, 2014). The covalent attachment of ubiquitin to target proteins (substrates) is mediated by the sequential action of an E1-activating enzyme, an E2 conjugase, and an E3 ubiquitin ligase (Pickart & Eddins, 2004). E3 ligases have the ability to bind both E2 proteins (via a RING domain, Ubox, or HECT domain) and substrates. Mitochondria localized E3 ubiquitin ligases such as MARCH5, MULAN, and Parkin ubiquitinate MAM components to regulate MAM formation and mitochondrial morphology (Harder et al, 2004; Braschi et al, 2009; Lokireddy et al, 2012; Nagashima et al, 2014; Gladkova et al, 2018).

On the other hand, recent reports have revealed the contribution of ER stress to the pathogenesis of PD (Mercado et al, 2013). ER stress activates the unfolded protein response (UPR), a complex signal-transduction pathway that mediates restoration of ER homeostasis (Doyle et al, 2011). Under chronic ER stress, the UPR triggers cell death by apoptosis, eliminating damaged cells. In mammalian cells, the UPR is initiated by activation of three distinct types of stress sensors located at the ER membrane: two transmembrane kinases, PERK and IRE1α, and transcription factor ATF6. Immunohistochemistry of post-mortem brain tissue from PD patients revealed that the phosphorylated forms of PERK and its substrate, eukaryotic initiation factor 2 (eIF2α), are present in dopaminergic neurons of the substantia nigra (Hoozemans et al, 2007). However, the mechanisms leading to ER stress in PD and the actual impact of the UPR on this disease remain unclear.

In this study, we investigated how LRRK2 is mechanistically involved in mitochondrial biogenesis. By analyzing metabolism and Ca2+ transport in MEFs genetically engineered using the CRISPR/Cas9 system, we identified the mitochondrial ubiquitination system as a key target in LRRK2-mediated mitochondrial biogenesis and showed that LRRK2 regulates ubiquitin ligase activity via PERK under ER stress. Thus, our findings reveal a new functional link between vulnerability to ER stress and mitochondrial biogenesis in the context of PD pathophysiology.

Results

Experiments were performed using genome-engineered mouse MEFs in which LRRK2 was deleted or replaced with either a kinase-active LRRK2 harboring the most common PD-related mutation (G2019S) or kinase-inactive LRRK2 with the D1994A mutation (Fig EV1A). Kinase assays using a synthetic substrate peptide (LRRK2-tide) revealed that LRRK2(D1994A) had lower activity, and LRRK2 (G2019S) had higher activity, than wild-type LRRK2 (Fig EV1B–D).

Mitochondrial morphology

In PD neurons, un-fragmented damaged materials accumulate, possibly due to impaired vesicular trafficking to the lysosome (Abeiovich & Rhinn, 2016). Phosphoproteomics has revealed that LRRK2 phosphorylates a subset of Rab GTPases thereby regulating intracellular endosome trafficking (Steger et al, 2016). Especially, the activity of Rab7 GTPase, a mediator for the late endosome–lysosome transport, is regulated by drosophila LRRK2 homolog (Dodson et al, 2012). Electron micrography revealed multiple large, electron-dense materials in the cytoplasm of both LRRK2+/−, LRRK2 (D1994A) and LRRK2(G2019S)-expressing MEFs (Fig 1A), suggesting that LRRK2 mutation impairs the lysosomal degradation of cytosolic debris through defects in trafficking of endosome to lysosome. Visualization of mitochondrial morphology using Mitotracker revealed that the proportion of fragmented mitochondria was elevated in LRRK2+/− and LRRK2(G2019S)-expressing MEFs (Fig EV1E). Consistent with the differences in mitochondrial morphology among MEFs, the activity of citrate synthase, the initial enzyme of the tricarboxylic acid (TCA) cycle and an exclusive marker of the mitochondrial matrix, was reduced in LRRK2+/− and LRRK2(G2019S)-expressing MEFs but elevated in LRRK2(D1994A)-expressing MEFs (Fig 1B). Thus, LRRK2 mutations disturbed mitochondrial biogenesis and/or proteasomal degradation processing.

Mitochondrial oxidative phosphorylation

To determine the role of LRRK2 in mitochondrial energetics, we measured basal and maximal (i.e., uncoupled with FCCP) oxygen consumption rate (OCR), an indicator of mitochondrial OXPHOS, and the extracellular acidification rate (ECAR), an indicator of aerobic glycolysis in living MEFs (Figs 1C and D, and EV1F and G). Maximal OCRs were significantly lower in LRRK2(G2019S)-expressing and LRRK2+/− MEFs, but higher in LRRK2(D1994A)-expressing MEFs, indicating that OXPHOS was enhanced by LRRK2(D1994A) but suppressed by loss of LRRK2 or expression of LRRK2(G2019S) (Fig 1C and D). By contrast, ECAR did not differ significantly between MEFs expressing LRRK2 mutants (Fig EV1F), indicating that aerobic glycolysis was not altered by LRRK2 mutation. Relative utilization of OXPHOS and glycolysis, as indicated by the OCR/ECAR ratio, was higher in LRRK2(D1994A)-expressing MEFs and lower in LRRK2+/− and LRRK2(D2019S)-expressing MEFs (Fig EV1G). These results suggested that OXPHOS is regulated by LRRK2 in a kinase-dependent manner.

The reduced rate of OXPHOS in LRRK2(G2019S)-expressing MEFs could be due to less active mitochondria, a lower density of mitochondria, or a combination of both. Because the rate of OXPHOS predicts ATP production, we next estimated the relative contribution of mitochondrial OXPHOS to ATP production (Fig 1E). To this end, we measured intracellular basal ATP content in MEFs in the absence and presence of oligomycin, a specific inhibitor of the mitochondrial F1δ-OAT synthase, to confirm the involvement of OXPHOS as the source of ATP production. MEFs expressing LRRK2+/− or LRRK2(G2019S) had significantly lower oligomycin-sensitive ATP content than wild-type MEFs. Thus, the reduction in OXPHOS activity due to LRRK2(G2019S) resulted in a decrease in ATP production.

Autophagy

To determine whether LRRK2 regulates autophagy, we measured the LC3-II level in MEFs (Fig 1F). Under basal conditions, as well as under ER stress induced by tunicamycin, the LC3-II level was higher...
in LRRK2−/− and LRRK2(S2019S)-expressing MEFs than in LRRK2+/+ MEFs, but lower in LRRK2(D1994A)-expressing MEFs. When LC3-II degradation was blocked with 200 nM bafilomycin A1 (BFA), a specific inhibitor of autophagic degradation, the higher LC3-II levels in LRRK2+/+ and LRRK2(S2019A)-expressing MEFs and the lower LC3-II levels in LRRK2(D1994A)-expressing MEFs than in that in LRRK2+/+ MEFs were also detected. Thus, the lower LC3-II levels in LRRK2(D1994A)-expressing MEFs indicated that autophagosome formation was suppressed. By contrast, the higher LC3-II levels in LRRK2+/+ and LRRK2(G2019S)-expressing MEFs indicated that autophagosome formation was enhanced. Consistent with the results for LC3-II, the levels of p62, another substrate of autophagy, were
reduced in LRRK2(D1994A)-expressing MEFs, but elevated in LRRK2(−/−) and LRRK2(S2019S)-expressing MEFs. Thus, LRRK2-mutant MEFs exhibited impaired autophagic flux, as previously demonstrated (Alegre-Abarrategui et al., 2009; MacLeod et al., 2013).

**Cell survival under ER stress**

To determine whether LRRK2 regulates cellular vulnerability to ER stress, we performed MTT assays to measure the viability response to ER stress inducers such as tunicamycin and thapsigargin, as well as oxidative stressors such as hydrogen peroxide (Fig 1G). Treatment with inducers of ER stress or oxidative stress decreased cell viability more strongly in LRRK2(−/−) and LRRK2(G2019S)-expressing MEFs than in MEFs expressing other LRRK2 mutants. Thus, LRRK2 (G2019S) exhibited greater vulnerability to ER and oxidative stresses. Together, these biochemical analyses indicated that LRRK2 regulates the viability response to ER stress, whereas kinase-active LRRK2(G2019S) enhances cellular vulnerability to this type of stress.

**Calcium homeostasis**

Key enzymes of OXPHOS, such as F1F0-ATPase and pyruvate dehydrogenase, are regulated by mitochondrial matrix Ca²⁺ (Territo et al., 2000; Balaban et al., 2005). Autophagy has been implicated in the IP3R-mediated mechanism (Sarkar et al., 2005; Criollo et al., 2007; Vicencio et al., 2009) and is activated by defects in IP3-induced Ca²⁺ release (Cardenas et al., 2010). Celluar vulnerability to stress is associated with mitochondrial mishandling of Ca²⁺ (Orrenius et al., 2003). These findings suggested that changes in mitochondrial biogenesis of MEFs expressing mutant LRRK2 could be due to a defect in a mitochondrial Ca²⁺-dependent mechanism. Accordingly, we examined the Ca²⁺ machinery on both the ER and mitochondrial sides. Specifically, we measured Ca²⁺ transfer from ER to mitochondria by monitoring bradykinin-stimulated calcium release from ER.

**Mitochondrial Ca²⁺ transfer**

To monitor mitochondrial Ca²⁺ concentration ([Ca²⁺]m), we targeted the protein-based Ca²⁺ indicator cameleon to mitochondria and then continuously visualized free Ca²⁺ in the mitochondrial matrix using fluorescence energy transfer (FRET; Miyawaki et al., 1997, 1999). Data in the figures are presented as absolute Ca²⁺ concentrations (Fig 2A–D). On average, basal [Ca²⁺]m in all MEFs was similar levels (2–3 μM). Treatment with 2.5 μM bradykinin significantly increased the [Ca²⁺]m transient in wild-type MEFs; the level was higher than that in LRRK2(−/−) and LRRK2(G2019S)-expressing MEFs, but lower than that in LRRK2(D1994A)-expressing MEFs (Fig 2B). Thus, mitochondrial Ca²⁺ transfer is inactivated by LRRK2(−/−) and LRRK2(G2019S), but activated by LRRK2(D1994A). By contrast, treatment with bradykinin significantly decreased ER Ca²⁺ concentration ([Ca²⁺]ER), as measured by the protein-based Ca²⁺ indicator ER-D1 targeted to the ER, in wild-type MEFs; the level was lower than that in LRRK2(−/−) and LRRK2(G2019S)-expressing MEFs (Fig EV2A and B). Thus, the magnitude of the change in [Ca²⁺]ER was reciprocal with that of the change in [Ca²⁺]m.

Close proximity between ER-localized IP3R and OMM-localized VDAC1 at the MAM potentiates rapid transfer of Ca²⁺ through the OMM. Mitochondrial Ca²⁺ accumulation is augmented by IP3-activated IP3R (Rizzuto et al., 1993) or over-expression of VDAC1 (Madesh & Hajnoczky, 2001; Rapizzì et al., 2002), but attenuated by down-regulation of either protein. To determine whether IP3R or VDAC1 is involved in disrupting mitochondrial Ca²⁺ accumulation in LRRK-modified MEFs, we measured [Ca²⁺]m in MEFs in which IP3R or VDAC1 was modified. shRNA-mediated down-regulation of IP3R or pretreatment with 20 μM 2-APB, a membrane-permeable blocker of IP3R, attenuated [Ca²⁺]m in all MEFs (Fig 2C), confirming the crucial role of IP3R in mitochondrial Ca²⁺ transfer. Over-expression of IP3R increased peak [Ca²⁺]m in LRRK2(D1994A)-expressing MEFs but not in LRRK2(−/−) or LRRK2(G2019S)-expressing MEFs. Down-regulation of VDAC1 decreased peak [Ca²⁺]m in all MEFs, whereas over-expression of VDAC1 increased peak [Ca²⁺]m in LRRK2(D1994A)-expressing MEFs but not in LRRK2(−/−) or LRRK2(G2019S)-expressing MEFs (Fig 2D). Thus, Ca²⁺ transfer through IP3R and VDAC1 was suppressed by LRRK2(G2019S), but enhanced by LRRK2(D1994A).

**Physical interaction and Ca²⁺ transfer between ER and mitochondria**

To obtain insight into the mechanism by which LRRK2 influences ER-mitochondrial Ca²⁺ transfer, we analyzed the relationship between the ER and mitochondria. Specifically, we performed ultrastructural analysis by electron microscopy to evaluate ER–mitochondrial contacts (Figs 2E and EV2C). Visual inspection of EM images acquired by facility personnel blinded to sample identity revealed a reduction in the number of ER–mitochondrial contact sites per unit of mitochondrial perimeter in LRRK2(−/−) and LRRK2(G2019S)-expressing MEFs. Thus, LRRK2 ablation and LRRK2(G2019S) block ER–mitochondrial contacts.

Next, we examined the physical interaction between the ER and mitochondria by in situ proximity ligation assay (PLA) using two organanelle-surface proteins involved in the calcium channeling complex: IP3R and VDAC1 at the MAM interface (Fig 2F; De Vos et al., 2012; Hedskog et al., 2013). IP3R and VDAC1 were in close proximity in wild-type MEFs, in which PLA intensity was higher than that in LRRK2(−/−) and LRRK2(G2019S)-expressing MEFs, but lower than that in LRRK2(D1994A)-expressing MEFs. Thus, LRRK2 might be involved in MAM formation in a kinase-dependent manner.

To determine whether reduced ER-mitochondrial Ca²⁺ transfer in LRRK2(−/−) and LRRK2(G2019S)-expressing MEFs was indeed due to a decrease in ER–mitochondrial contacts, we performed a rescue experiment using a synthetic ER–mitochondrial tethering protein, TOM-mRFP-ER) (Csordas et al., 2006; Kornmann, 2013), which restores changes in PLA intensity in LRRK2(−/−) and LRRK2(G2019S)-expressing MEFs (Figs 2G and EV2E). Over-expression of TOM-mRFP-ER significantly rescued mitochondrial Ca²⁺ transfer in LRRK2(−/−) and LRRK2(G2019S)-expressing MEFs (Fig 2G). These results indicated that ER–mitochondrial tethering was suppressed by loss of LRRK2 or expression of LRRK2(G2019S).

**ER–mitochondrial tethering proteins**

MAM integrity depends on the interaction of ER–mitochondrial tether proteins, whose levels are regulated by the ubiquitin/proteasome pathway (Karbowksi & Youle, 2011; Christianson & Ye, 2014;
Marchi et al., 2014; Ruggiano et al., 2014). It is plausible that the catalytic activities of E3 ubiquitin ligases in MEFs expressing kinase-active LRRK2(G2019S) could change the levels of MAM components in such a manner as to diminish ER–mitochondrial Ca^{2+} transfer. To explore this possibility, we analyzed the expression levels of each component of isolated MAM fractions by immunoblot (Fig 3A, Appendix Fig S1A). Among the components we analyzed, those involved in the Ca^{2+} transfer pathway including IP3R, VDAC1 and GRP75, and ER membrane proteins including Bap31, VAPB and Formin 2 were unchanged in all MEFs. Levels of ER and mitochondrial membrane proteins such as mitofusins 1 and 2, and mitochondrial membrane proteins including Fis1 and PTPIP51 were lower, and DRP1 was higher, in LRRK2−/− and LRRK2(G2019S)-expressing MEFs than in wild-type MEFs, whereas mitofusins 1 and 2 were present at higher levels in LRRK2(D1994A)-expressing MEFs. MAM provides a platform for mitochondrial dynamics, including DRP1-mediated fission and mitofusin 1/2-mediated fusion (Youle & van der Bliek, 2012). Thus, changes in the relative abundances of MAM components, specifically, reductions in the levels of mitofusins 1 and 2 and an increase in the level of DRP1, caused greater mitochondrial fragmentation in MEFs expressing LRRK2(G2019S) (Fig EV1E). Impaired ER–mitochondrial contact caused by changes
in MAM components could lead to changes in mitochondrial Ca\textsuperscript{2+} transfer.

**E3 ubiquitin ligases involved in the ER–mitochondrial interaction**

To determine which domain of LRRK2 was responsible for the ER–mitochondrial Ca\textsuperscript{2+} transfer, we introduced deletion constructs of LRRK2(G2019S) into LRRK2\textsuperscript{+/C0/C0} MEFs (Fig 3B and C, Appendix Fig S1B). A construct lacking the N-terminal region containing the ANK, LRR, and COR domains rescued the decreased Ca\textsuperscript{2+} transfer observed in LRRK2\textsuperscript{+/C0/C0} MEFs, suggesting that the N-terminal domain (a.a. 1–1,515) contains the regulatory site for LRRK2. Next, we searched the binding proteins with the yeast two-hybrid system, using the N-terminal region of LRRK2 as bait and a mouse brain cDNA library as prey. Among the potential binding proteins, we identified E3 ubiquitin ligases, including MARCH5, MULAN, and AC

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**Figure 3**. Physical and functional interactions between LRRK2 and E3 ubiquitin ligases.

A Representative immunoblots of MAM components in MEFs of indicated genotype. MAM fraction was extracted from MEFs by the Percoll gradient method and immunoblotted with antibodies indicated at the right.

B Diagram showing full-length and deletion constructs of LRRK2(G2019S). ANK, Ankyrin-repeat domain; LRR, leucine-rich domain; ROC, Ras complex domain; COR, C-terminus of Roc domain; Kinase, kinase domain.

C Peak values of Ca\textsuperscript{2+} transients in MEFs of the indicated genotypes transfected with deletion constructs of LRRK2(G2019S). Error bars represent ± SD from six independent experiments.

D Binding assays to detect interactions between deletion constructs of LRRK2(G2019S) and E3 ubiquitin ligases. HEK293 cells were transfected with deletion constructs of LRRK2(G2019S) and E3 ubiquitin ligases, and cell lysates were immunoprecipitated with antibody against the V5 or HA epitope. Precipitated proteins were subjected to SDS/PAGE, and blots were stained with antibody against the V5 or HA epitope, as indicated to the right of each panel.

E, F Peak values of Ca\textsuperscript{2+} transients in MEFs of the indicated genotypes transfected with ligase-active MARCH5(WT), MULAN(WT), and Parkin(WA) [Parkin(WA)] or dominant-negative forms of MARCH5(H43W) [MARCH5(HW)], MULAN(C339A) [MULAN(CA)], and Parkin(C431A) [Parkin(CA)] (E) or in MEFs transfected with active USP30 or inactive USP30(C77S) [USP30(CS)] (F). Error bars represent ± SD from six independent experiments.

Data information: For graphs (C, E and F), the P values were determined by a Mann–Whitney U-test. ns = not significant, *P < 0.05.

Source data are available online for this figure.
Parkin. All of these molecules belong to the really interesting new genes (RING) domain E3 ubiquitin ligase family, which is characterized by the presence of the RING domain (Deshaies & Joazeiro, 2009). During the ubiquitination process, E3 ubiquitin ligase binds to the E2-co-enzyme via its RING domain and it physically receives the ubiquitin moiety on its active center (Caulfield et al., 2015). Immunoprecipitation/immunoblot analysis confirmed the binding of each of these molecules to the N-terminal domain of LRRK2 (Fig 3D).

Several lines of evidence indicate the importance of ubiquitination and proteasomal degradation in MAM formation (Karbowiak & Youle, 2011; Christianson & Ye, 2014; Marchi et al., 2014; Ruggiano et al., 2014). Mitofusin 2, a critical component in MAM formation, is ubiquitinated and degraded by ligase-active E3 ubiquitin ligases such as MARCH5(WT), MULAN(WT), or Parkin(W403A), but not by ligase-negative E3 ubiquitin ligases such as MARCH5(H43W), MULAN(C339A), or Parkin(C431A) (Gegg et al, 2010; Sugliera et al., 2013; Yun et al., 2014). Therefore, we considered it likely that LRRK2 regulates ER–mitochondrial interaction through bound E3 ubiquitin ligases. To determine whether bound E3 ubiquitin ligases are involved in LRRK2-mediated ER–mitochondrial Ca\(^{2+}\) transfer, we introduced the ligase-active or ligase-dead forms of each molecule into LRRK2\(^{-/-}\) and LRRK2(G2019S)-expressing MEFs, and analyzed ER–mitochondrial Ca\(^{2+}\) transfer in the transfected cells (Fig 3E, Appendix Fig S1C). The combination of ligase-active MARCH5(WT), MULAN(WT), and Parkin(W403A) decreased Ca\(^{2+}\) transfer in LRRK2(D1994A)-expressing MEFs, but not in LRRK2\(^{-/-}\) or LRRK2(G2019S)-expressing MEFs, whereas the combination of ligase-dead MARCH5(H43W), MULAN(C339A), and Parkin(C431A) increased Ca\(^{2+}\) transfer in LRRK2\(^{-/-}\) and LRRK2(G2019S)-expressing MEFs, but not in LRRK2(D1994A)-expressing MEFs. Thus, ligase-negative E3 ubiquitin ligases suppressed the endogenous E3 ubiquitin ligases in the dominant-negative manner, by which over-expressed mutant E3 ubiquitin ligases may compete with endogenous E3 ubiquitin ligases for endogenous E2-co-enzyme (Caulfield et al., 2015). These results indicated that ligases were more active in LRRK2\(^{-/-}\) and LRRK2(G2019S)-expressing MEFs than in LRRK2(D1994A)-expressing MEFs. Collectively, these results suggested that the ER–mitochondrial interaction is regulated by LRRK2 through a MARCH5-, MULAN-, and Parkin-mediated mechanism involving the ubiquitin/proteasome system, in which the activities of these E3 ubiquitin ligases are promoted by loss of LRRK2 or kinase-active LRRK2(G2019S), but suppressed by kinase-dead LRRK2(D1994A).

The role of ubiquitin deubiquitylase

Ubiquitination of mitochondrial proteins is a reversible process in which ubiquitin is not only conjugated to substrates via the ubiquitin pathway, but also removed from substrates by deubiquitylating enzyme (Livnat-Levanon & Glickman, 2011). USP30, a mitochondrially tethered deubiquitylase, antagonizes MULAN as well as Parkin (Bingol et al, 2014; Cunningham et al, 2015). If E3 ubiquitin ligase-mediated ubiquitination is enhanced in LRRK2(G2019S)-expressing MEFs, active USP30, but not catalytically inactive USP30 (USP30(C77S)), should antagonize the high-ubiquitination state. To confirm the observation that the perturbation of ER–mitochondrial Ca\(^{2+}\) transfer in LRRK2(G2019S)-expressing MEFs resulted from enhanced E3 ubiquitin ligase activity, we over-expressed wild-type USP30 or the C77S mutant (Fig 3F). Wild-type USP, but not USP30(C77S), partially rescued ER–mitochondrial Ca\(^{2+}\) transfer in LRRK2\(^{-/-}\) and LRRK2(G2019S)-expressing MEFs, but not in LRRK2(D1994A)-expressing MEFs. These results indicated that ubiquitination activity was higher in LRRK2\(^{-/-}\) and LRRK2(G2019S)-expressing MEFs than in LRRK2(D1994A)-expressing MEFs. This finding supported the idea that the activities of E3 ubiquitin ligases such as MARCH5, MULAN, and Parkin are promoted by loss of LRRK2 and kinase-active LRRK2(G2019S).

The role of LRRK2 kinase activity in E3 ubiquitin ligase

Does LRRK2 regulate E3 ubiquitin ligase-mediated ubiquitination and degradation of MAM components via its kinase activity? Multiple reports have shown that the activities of E3 ubiquitin ligases are regulated by phosphorylation (Gallagher et al., 2006; Smith et al., 2009; Lewandowski & Piwnica-Worms, 2014). Immunoblots of MEFs using anti-phosphoserine antibody detected higher levels of the phosphorylated forms of endogenous E3 ubiquitin ligases in LRRK2(G2019S)-expressing MEFs in the presence of ER stressors such as tunicamycin (Fig 4A). This implied that phosphorylation of E3 ubiquitin ligases is involved in their activation. To explore this possibility, we focused on ubiquitination of mitofusin 2, which was present at reduced levels in LRRK2\(^{-/-}\) and LRRK2(G2019S)-expressing MEFs (Fig 3A, Appendix Fig S1A). We performed immunoblots of LRRK2-expressing MEFs transfected with each of the endogenous E3 ubiquitin ligases, cultured in the presence or absence of tunicamycin (Fig 4B). In wild-type MEFs, tunicamycin significantly increased the levels of phosphorylated E3 ubiquitin ligases, decreased the level of mitofusin 2, and reciprocally increased the level of ubiquitinated mitofusin 2. These results indicated that phosphorylation of E3 ubiquitin ligase increases its activity toward mitofusin 2. By contrast, under tunicamycin treatment, LRRK2(D1994A)-expressing MEFs contained less phosphorylated E3 ubiquitin ligase and ubiquitin-conjugated mitofusin 2, and reciprocally more mitofusin 2, than wild-type MEFs. These results indicated that LRRK2(D1994A) suppressed phosphorylation of E3 ubiquitin ligase, and the subsequent ubiquitination and degradation of mitofusin 2, under ER stress. On the other hands, LRRK2\(^{-/-}\) and LRRK2(G2019S)-expressing MEFs had more phosphorylated E3 ubiquitin ligase and ubiquitin-conjugated mitofusin 2, and reciprocally less mitofusin 2, under both control conditions and tunicamycin treatment. These results indicated that loss of LRRK2 or LRRK2(G2019S) promoted phosphorylation of E3 ubiquitin ligase and subsequent ubiquitination and degradation of mitofusin 2.

To confirm this finding, we pretreated LRRK2 mutant-expressing MEFs with the LRRK2 kinase inhibitor LRRK2-IN-1 (Deng et al., 2011; Figs 4B and EV3B). In LRRK2(G2019S)-expressing MEFs pretreated with LRRK2-IN-1, E3 ubiquitin ligase phosphorylation, and the level of mitofusin 2 in the MAM fraction recovered to the levels in wild-type MEFs. In line with this finding, ER–mitochondrial Ca\(^{2+}\) transfer in LRRK2-IN-1-treated LRRK2(G2019S)-expressing MEFs increased to the level in wild-type MEFs (Fig 5A). Collectively, these results indicated that LRRK2 regulates E3 ubiquitin ligase-mediated ubiquitination and proteasomal degradation in a kinase-dependent manner.
PERK phosphorylates E3 ubiquitin ligase

Kinase activity of LRRK2 was required for phosphorylation and activation of E3 ubiquitin ligase. However, in vitro phosphorylation assays did not show direct phosphorylation of E3 ubiquitin ligase by LRRK2 (data not shown), indicating that kinases other than LRRK2 phosphorylate these ligases.

To identify the kinase responsible for phosphorylation of E3 ubiquitin kinase, we introduced an siRNA library targeting the expression of 628 kinases into LRRK2(G2019S)-expressing MEFs under tunicamycin treatment. If the responsible kinase is knocked down, un-phosphorylated and inactive E3 ubiquitin ligases might rescue the cell viability of LRRK2(G2019S)-expressing MEFs treated with tunicamycin. The cell viability of MEFs in each well was measured using the Vybrant MTT Cell Proliferation Assay. Using a statistical Z-score to quantify the deviation of cell viability from the mean of all measurements, we selected four siRNAs that induced significantly higher viability of LRRK2(G2019S)-expressing MEFs under tunicamycin treatment. Next, we performed immunoblots with anti-phospho-serine antibody to examine the phosphorylation of E3 ubiquitin ligases in LRRK2(G2019S)-expressing MEFs harboring each of the selected siRNAs in the presence of tunicamycin. Among siRNA specific for candidate kinases, we found that siRNA specific for PERK decreased phosphorylated E3 ubiquitin ligases in LRRK2(G2019S)-expressing MEFs.

PERK and kinase-active PERK-ΔN increased the levels of phosphorylated E3 ubiquitin ligases, whereas kinase-dead PERK(K618R) and siRNA targeting PERK had the opposite effect (Fig EV4A).
is mainly localized at the MAM (Verfaillie et al., 2012). Considering the close proximity between PERK and E3 ubiquitin ligase at the MAM, it is possible that PERK directly phosphorylates E3 ubiquitin ligases under ER stress. Alternatively, PERK may be cleaved by an ER-localized protease (Ye et al., 2000; Lichtenthaler et al., 2018), giving the soluble cytoplasmic domain of PERK access to E3 ubiquitin ligases.§

Next, we performed in vitro kinase assays using isolated PERK-DN or PERK(K618R) (Verfaillie et al., 2012), E3 ubiquitin ligase, and $^{32}$P-ATP (Fig 5C). PERK directly phosphorylated E3 ubiquitin ligase, whereas PERK(K618R) did not phosphorylate them. Previous reports identified S65 of Parkin as the phosphorylation site by PINK1 at the damaged mitochondrial membrane (Kondapalli et al., 2012; Durcan & Fon, 2015). Immunoprecipitation/immunoblotting of transfected cells co-expressing Parkin or phosphorylation-defective Parkin(S65A) with PERK showed less serine-phosphorylated Parkin (S65A) than Parkin (Fig EV4B). These results implied PERK as the kinase for MARCH5, MULAN, and Parkin under ER stress and

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5 Correcrions added on 15 September 2020, after first online publication: panel B in Fig 5 has been corrected, and the main text has been updated; see the associated Corrigendum at https://doi.org/10.15252/embj.2020105826
strongly suggested that PERK-mediated phosphorylation affects the activity of E3 ubiquitin ligase. Hence, we performed in vitro ubiquitination assays with recombinant HA-tagged Ub, E1, and E2 (UbC17) enzymes, His6-tagged mitofusin 2, and Myc-tagged E3 ubiquitin ligases, which were first in vitro phosphorylated by PERK or PERK(K618R), in the presence of ATP. Mitofusin 2 ubiquitinated with Ubl-HA was detected in nickel bead pull-downs in the presence of PERK, but not PERK(K618R) (Fig 5D). Thus, PERK phosphorylation increased E3 ubiquitin ligase activity.

**LRRK2 blocks PERK-mediated phosphorylation of E3 ubiquitin ligases**

The final question was how LRRK2 regulates PERK-mediated phosphorylation of E3 ubiquitin ligases through its kinase activity. Immunoblotting of MEFs to detect endogenous proteins revealed similar amounts of phosphorylated PERK, but larger amounts of phosphorylated E3 ubiquitin ligases, in LRRK2(G2019S)-expressing MEFs under tunicamycin than LRRK2+/+ or LRRK2(G1994A)-expressing cells transfected with LRRK2(G2019S) (Fig 6A and B). In contrast–mitochondrial Ca2+ transfer in LRRK2(D2019S)-expressing MEFs, the level of which was the highest, was not increased any more by two maneuvers (Figs 4C and 5A), where a fraction of LRRK2, not auto-phosphorylated, constitutively bound to E3 ubiquitin ligase (Fig 6A and B). In comparison with mutant LRRK2-expressing MEFs, the ER–mitochondrial Ca2+ transfer in LRRK2(G2019S)-expressing MEFs, the level of which was the highest, was significantly increased by over-expression of LRRK2-D1 as well as LRRK2-IN-1 (Fig 5A, Appendix Fig S1B), where LRRK2 (D2019S), fully auto-phosphorylated, lacked the binding to E3 ubiquitin ligase (Fig 6A and B). In contrast–mitochondrial Ca2+ transfer in LRRK2(D1994A)-expressing MEFs, the level of which was the lowest, was significantly increased by over-expression of LRRK2-D1 as well as LRRK2-IN-1 (Fig 5A, Appendix Fig S1B), where the majority of LRRK2(D1994A), not auto-phosphorylated, constitutively bound to E3 ubiquitin ligase (Fig 6A and B). In comparison with mutant LRRK2-expressing MEFs, the ER–mitochondrial Ca2+ transfer in LRRK2(G2019S)-expressing MEFs, the level of which was the lowest, was significantly increased by over-expression of LRRK2-D1 as well as LRRK2-IN-1 (Fig 5A, Appendix Fig S1B), where the majority of LRRK2(D1994A), not auto-phosphorylated, constitutively bound to E3 ubiquitin ligase (Fig 6A and B). Thus, the regulatory model of E3 ubiquitin ligases by LRRK2 could explain changes in the ER–mitochondrial Ca2+ transfer in mutant LRRK2-expressing MEFs.

In order to overcome excess activation of E3 ubiquitin ligases and ubiquitination/degradation of MAM proteins in LRRK2 (G2019S)-expressing MEFs, it is necessary to suppress PERK activity. To this end, we treated LRRK2(G2019S)-expressing MEFs with shRNA targeting PERK (Fig 7A and B). Knockdown of PERK rescued the increased autophagic flux and increased ER–mitochondrial Ca2+ transfer in LRRK2(G2019S)-expressing MEFs. By contrast, PERK-AN augmented autophagic flux and further decreased ER–mitochondrial Ca2+ transfer in these cells. Thus, suppression of PERK kinase activity, which is required for the UPR, overcame ER–mitochondrial dysfunction in LRRK2(G2019S)-expressing MEFs.

In summary, our findings show that in addition to clearing damaged mitochondrial components as part of the canonical UPR pathway, PERK contributes to MAM formation by phosphorylating E3 ubiquitin ligase, thereby promoting the ubiquitination and degradation of substrates such as mitofusin 2. We conclude that LRRK2 is involved in the ER–mitochondrial interaction through the ubiquitination/proteasome system.

**Discussion**

In this study, we demonstrated that LRRK2 binds to E3 ubiquitin ligase and blocks PERK phosphorylation and E3 ligase activity toward MAM components. This interaction between LRRK2 and E3 ubiquitin ligases depends on the kinase activity of LRRK2; kinase-active LRRK2(G2019S) auto-phosphorylates at S1292, releasing it from E3 ubiquitin ligase, which then promotes PERK phosphorylation and ligase activation, thereby promoting ubiquitination/proteasomal degradation of MAM components. Subsequently, the reduction in the ER–mitochondrial interaction decreases IP3R/
VDAC1-mediated ER–mitochondrial Ca\textsuperscript{2+} transfer and inhibits mitochondrial energy production (Fig 7C). Of note, loss of LRRK2 causes similar phenotypic changes in the ER–mitochondrial interaction, further supporting a model in which E3 ubiquitin ligase is activated following its detachment from LRRK2.

Mitochondrial Ca\textsuperscript{2+} is a positive effector of the tricarboxylic acid (TCA) cycle and ATP generation (McCormack et al., 1990), and also plays a major role in the regulation of autophagy and apoptosis (Cardenas et al., 2010). Efficient mitochondrial Ca\textsuperscript{2+} uptake is supported by the close apposition of the ER membrane and the OMM, i.e., the MAM (Gincel et al., 2001; Rapizzi et al., 2002). In LRRK2(D2019S)-expressing MEFs, mitochondrial Ca\textsuperscript{2+} measurements using targeted recombinant Ca\textsuperscript{2+} probes revealed reduced mitochondrial Ca\textsuperscript{2+} uptake in response to bradykinin-mediated IP3R activation. In accordance with the Ca\textsuperscript{2+} measurements, mitochondrial O\textsubscript{2} consumption was reduced by loss of LRRK2 or LRRK2 (G2019S), but was enhanced by LRRK2(D1994A). Therefore, kinase-active LRRK2 regulates the ER–mitochondrial interaction; kinase-dead LRRK2 activates this interaction, whereas kinase-active LRRK2 inactivates it.

We considered the possibility that LRRK2 controls the interactions between ER and mitochondrial proteins at existing sites of organelle contact. Electron microscopy and in situ PLA revealed that ER–mitochondrial contact sites were more abundant in LRRK2(D1994A)-expressing MEFs, but less abundant in LRRK2 (G2019S)-expressing MEFs. Furthermore, over-expression of the synthetic tethering protein TOM-mRFP-ER rescued the decrease in mitochondrial contacts in LRRK2(D2019S)-expressing MEFs. Several different protein complexes have been proposed as ER–mitochondrial tethers, including interactions between ER-anchored IP3Rs and the mitochondrial voltage-dependent anion channel (VDAC1) mediated by GRP75 (Rapizzi et al., 2002; Szabadkai et al., 2006), homo-,
heterotypic interactions between mitochondrial mitofusin 1/2 and ER-localized mitofusin 2 (de Brito & Scorrano, 2008; Cosson et al., 2012; Filadi et al., 2015; Wang et al., 2015), interactions between the integral ER protein VAPB and mitochondrial tyrosine phosphatase-interacting protein 51 (PTPIP51) (De Vos et al., 2012; Stoica et al., 2014), interactions between Fission 1 homologue (Fis1) and ER-

$^{a}$Correction added on 15 September 2020, after first online publication: panel C in Fig 7 has been corrected; see the associated Corrigendum at https://doi.org/10.15252/embj.2020105826
located Bap31 (Iwasawa et al., 2011), and interactions between protein phosphofurin acidic cluster sorting protein 2 (PACS-2) and Bap31 (Simmen et al., 2005). Among the MAM components, the levels of mitofusins 1 and 2, Fos1, and PTPP51 were decreased by LRRK2(G2019S). These results indicated that kinase-active LRRK2 directly down-regulates MAM formation by decreasing the abundance of MAM components.

Screening for the binding partners of LRRK2 revealed that this kinase functionally interacts with the mitochondrial membrane-bound E3 ubiquitin ligases MARCH5, MULAN, and Parkin through its N-terminal region. LRRK2-bound E3 ubiquitin ligases belong to the RING family (Vittal et al., 2015). Many E3 ubiquitin ligases exist in an auto-inhibitory state in which a region of the protein outside the catalytic domain prevents access to the active site (Deshaies & Joazeiro, 2009; Vittal et al., 2015). Alternatively, phosphorylation induces dimer or multi-complex formation through the RING domain, thereby activating RING E3 ubiquitin ligase (Metzger et al., 2014). Indeed, in the inactive state of Parkin, the RING domain is involved in the auto-inhibitory mechanism. PINK1-mediated phosphorylation of Parkin releases this autoinhibition through a conformational change (Chaugule et al., 2011; Shiba-Fukushima et al., 2012; Dove et al., 2015). Based on these findings, along with many reports showing that the activities of E3 ubiquitin ligases are regulated by phosphorylation (Gallagher et al., 2006; Smith et al., 2009; Lewandowski & Piwnica-Worms, 2014), we consider it likely that phosphorylated MARCH5, MULAN, and Parkin undergo conformational changes from the E2 binding–incompetent state to the E2 binding–competent state.

siRNA screening for the kinases responsible for phosphorylation of E3 ubiquitin ligases identified PERK, an ER stress sensor, as a candidate. Biochemical studies and measurements of mitochondrial Ca2+ transfer revealed that PERK phosphorylates E3 ubiquitin ligases, thereby inducing their ligase activities toward MAM components. However, it remains unclear how ER-localized PERK can phosphorylate mitochondrially localized ligases through the gap between ER and mitochondria at the MAM (10–30 nm) (Csordás et al., 2006; Rowland & Voeltz, 2012). One possibility is that the distance between the ER and mitochondria at the MAM is short enough for PERK to directly phosphorylate its target ligases. Alternatively, the cytoplasmic domain of PERK may be released from the ER membrane in order to gain access to its targets. Several proteases localized at the ER membrane cleave ER-localized proteins (Espenshade et al., 1999; Ye et al., 2000; Lichtenhaler et al., 2018). Notably in this regard, ER membrane-bound ATF6, another ER stress sensor, is cleaved by S1P under ER stress, resulting in release of its cytoplasmic domain, which subsequently enters the nucleus (Ye et al., 2000). However, it remains unclear whether the cytoplasmic domain of PERK is cleaved by ER stress and whether cleaved PERK released from ER phosphorylates mitochondria-bound E3 ubiquitin ligases.8

Beyond its canonical role as ER stress sensor, PERK is a novel mediator of ER–mitochondrial contact sites, where it phosphorylates and activates the E3 ubiquitin ligases, thereby decreasing MAM formation. Among MAM components, mitofusin 2 can tether two mitochondria together, as well as tethering mitochondria to the ER (Rowland & Voeltz, 2012). Ablation of mitofusin 2 in mouse proopiомelanocorticotropin neurons decreases the number of ER–mitochondrial contacts, leading to ER stress (Schneeberger et al., 2013). Consistent with this, loss of PINK1 or Parkin concomitant with an increase in the level of mitofusin 2 causes accumulation of misfolded proteins, leading to excess ER stress (Doyle et al., 2011). Thus, one interesting possibility would involve cross-talk between the ER and mitochondria in which PERK sensitizes the mitochondria to ER stress through the ubiquitination/proteasome pathway.

The final question was how LRRK2 regulates PERK-mediated phosphorylation in dependent of its kinase activity. Phosphorylation and activation of E3 ubiquitin ligases were augmented in LRRK2(G2019S)-expressing MEFs, and these effects were suppressed by the LRRK2 kinase inhibitor LRRK2-IN-1. In line with this finding, ER–mitochondrial Ca2+ transfer in LRRK2(G2019S)-expressing MEFs was rescued by treatment with LRRK2-IN-1. Thus, LRRK2 kinase activity was crucial for the regulation of E3 ubiquitin ligase activity. As noted above, LRRK2 auto-phosphorylates at S1292 (Sheng et al., 2012). Phosphorylation-defective LRRK2(S1292A) bound E3 ubiquitin ligase more strongly than phosphomimetic LRRK2(S1292D). Furthermore, LRRK2(S1292A) blocked the PERK-mediated phosphorylation of E3 ubiquitin ligases, whereas LRRK2(S1292D) did not. Together, these results indicate that LRRK2 not phosphorylated at S1292 interacts with E3 ubiquitin ligases, thereby blocking PERK-mediated phosphorylation of E3 ubiquitin ligases. The E3 ubiquitin ligase–interacting region of LRRK2 (a.a. 1–1,515) covers an ankyrin-repeat domain, a leucine-rich repeat, a Ras complex domain, and a C-terminal Roc domain, all of which are involved in the protein–protein interaction (Gilisbach & Kortholt, 2014). S1292 is localized within the leucine-rich repeat. This finding implies that phosphorylation of S1292 induces a conformational change in the interacting sites, thereby decreasing affinity for E3 ubiquitin ligases.

In conclusion, our results show that LRRK2 regulates the phosphorylation of the mitochondrial E3 ubiquitin ligases via ER-localized PERK, thereby determining ER–mitochondrial tethering. These findings provide insight into the mechanism by which two major processes involved in PD, mitochondrial dysfunction, and ER stress, converge in modulating the PD phenotype.

Materials and Methods

Generation of genome-engineering MEFs

Specific targeted alterations in the LRRK2 gene of MEFs were generated using CRISPR-Cas9 genome-engineering system (Moyer & Holland, 2015). Briefly: To create LRRK2 knock-out MEFs, cells were electrophoretically transduced with Cas9 vector (SBI, Polo Alto, CA, USA) annealed with sgRNA; to create LRRK2-mutant MEFs, cells were transduced with Cas9 vector (SBI) annealed to sgRNA conjugated with homology-directed repair (HDR) dsDNA (Appendix Fig S2).

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Generation of expression vectors and site-directed mutagenesis
cDNAs encoding mouse IP3R, LRRK2, MARCH5, mitofusin 2, MULAN, MCU, MCUB, Parkin, PERK, ubiquitin, USP30, and VDAC1 were synthesized by PCR. IP3R, LRRK2, and mitofusin 2 were ligated into pcDNA3.1/V5-His (Invitrogen/Life Technologies); MCU, MCUB, ubiquitin, USP30, and VDAC1 were ligated into pcMV-HA (Clontech Laboratories, Polo Alto, CA, USA); MARCH5, MULAN, and Parkin were ligated into pCMV-Myc (Clontech Laboratories); and PERK was ligated into 3XFLAG-CMV-13 (Sigma-Aldrich, St Louis, MO, USA). Deletion constructs of LRRK2 [LRRK2-d1 (a.a. 1–1,114), LRRK1-d2 (a.a. 1,516–2,527)] were synthesized by PCR and ligated into pcDNA3.1/V5-His. A deletion construct of PERK [PERKΔN (a.a. 10–1,114)] was synthesized by PCR and ligated into 3XFLAG-CMV-13. Mutations of D1994 to A and G2019 to S in LRRK2 were introduced to create kinase-dead and kinase-active forms of the protein, respectively. Mutations of S1292 to A and S1252 to D in LRRK2 were introduced to create phosphorylation-defective and phosphomimetic LRRK2, respectively. Mutations of H65 to W in MARCH5, C659 to A in MULAN, and C431 to A in Parkin were introduced to create ligase-inactive MARCH5(H63W), MULAN(C339A), and Parkin(C431A), respectively. Mutation of W403 to A in Parkin was introduced to create ligase-active Parkin(W403A). Mutation of C77 to S in USP30 was introduced to create deubiquitinase-inactive USP30(C77S). Mutation of K618 to R in PERK was introduced to create kinase-dead and kinase-active forms of the protein, respectively. Mutations of R33 to A was introduced to create an S1P-resistant form. Mutations were confirmed by immunoblotting of E3 ubiquitin ligases extracted from transfected cells treated with or without phosphatase (Appendix Fig S3A).

RNAi
shRNA oligonucleotides specific for the target sequence of mouse IP3R, PERK, or VDAC1 were designed (Appendix Fig S3A) and ligated into expression vector pcDNA6.2-GW-miR (Clontech Laboratories). Cultured cells were transfected with shRNA vectors using Lipofectamine 2000 (Thermo Fisher, Waltham, MA USA), LRRK2-IN-1 (Cayman Chemical, Ann Arbor, MI, USA), MISSION siRNA mouse kinase panel library (Sigma-Aldrich, Mitotracker (Molecular Probe), Ni-NTA beads (Qiagen, Germantown, MD, USA), Ru360 (Sigma-Aldrich), and tunicamycin (Sigma-Aldrich), Ubch7 (Abcam), and Vybrant MTT Cell Proliferation Assay Kit (Thermo Fisher).

Antibodies, immunoprecipitation, and immunoblotting
Antibodies were obtained from the indicated suppliers (Appendix Fig S4): anti-actin (Abcam), anti-Bap31 (Abcam), anti-calnexin (Abcam), anti-DRP1 (Abcam), anti-Fis1 (Merck Millipore), anti-GRP75 (Abcam), anti-IP3R (Abcam), anti-LC3 (MBL, Nagoya, Aichi 460-0008, Japan, Merck Millipore, Burlington, MA, USA), anti-LRRK2 (Abcam), anti-MARCH5 (Abcam), anti-mitofusin 1 (Abcam), anti-mitofusin 2 (Abcam), anti-MULAN (United State Biological), anti-Parkin (Abcam), anti-Ptp1P51 (Abcam), anti-p62 (MBL), anti-PERK (Abcam), anti-VAPB (Abcam), anti-VDAC1(Abcam), anti-FLAG (Sigma-Aldrich), anti-HA (MBL), anti-Myc (MBL), anti-phosphoserine (Abcam), and anti-V5 (Thermo Fisher). Transfected cells were incubated for 2 days, harvested, and then lysed in lysis buffer (50 mM Tris–HCl [pH 8.0], 150 mM NaCl, and 0.1% SDS) for immunoblotting or in TNE buffer (50 mM Tris–HCl, pH 7.4 150 mM NaCl, 5 mM EDTA, 1% NP-40, 0.25% Na-deoxycholate, and 1 mM NaF) for immunoprecipitation/immunoblotting. Immunoprecipitation/immunoblot analyses were performed using standard protocols. The specificity of anti-phospho-serine antibody to phosphorylated serine was confirmed by immunoblotting of E3 ubiquitin ligases extracted from transfected cells treated with or without phosphatase (Appendix Fig S3A).

Subcellular fractionation
MAM, mitochondria, and microsomes were isolated from cells using Percoll gradient fractionation (Bozidis et al, 2007). In brief, cells were harvested and lysed in Sucrose Homogenization Medium (0.25 M sucrose, 10 mM HEPES, pH 7.4). Differential centrifugation was used to isolate the post-nuclear supernatant from nuclei and cellular debris. The total microsomal fraction and crude mitochondrial fraction were isolated by centrifugation at 10,000 g. Following this step, the microsomal fraction was isolated following centrifugation at 100,000 g. The resulting supernatant from this spin is the “cytosol” fraction, and it was concentrated by using Amicon Ultra 15-mL filters. The crude mitochondrial fraction was purified through a self-generating Percoll gradient, and the collected mitochondrial and MAM fractions were further purified by centrifugation at 6,300 g. The MAM was then collected following centrifugation at 100,000 g. Equivalent amounts of protein from each fraction were analyzed by SDS–PAGE and immunoblot.

Oxygen consumption
Oxygen consumption rate (OCR) was measured at 37°C using an XF96 Extracellular Flux Analyzer (Seahorse Bioscience, North Billerica, MA). Cells seeded into 96-well plates (1 × 10⁶ per well)
were loaded into the machine for determination of oxygen concentration. Cells were exposed to oligomycin (1 μM), carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP; 300 nM), and rotenone (100 nM) plus actinomycin (100 nM). After each injection, OCR was measured for 5 min. Representative traces are shown in Fig 1C. Every point represents the average of four different wells. Basal OCR was calculated as the difference between OCR measurements taken before and after oligomycin. Maximum OCR was calculated as the difference over the OCR measurements taken after FCCP and after exposure to rotenone plus actinomycin.

### ATP production

ATP content in MEFs was measured using the ATP Determination Assay kit (Molecular Probes). ATP concentration was calculated using an ATP standard curve.

### Calcium imaging

Plasmids for expression of the mitochondrial Ca$^{2+}$ sensor (pcDNA3-0.2mt-cameleon) and the ER Ca$^{2+}$ sensor (D1ER pcDNA3) were kindly provided by Dr. Roger Tsien (University of California; Palmer et al., 2006).

To measure the Ca$^{2+}$ concentration in mitochondria and ER, MEFs plated on 3.5-cm confocal dishes were transfected with pcDNA3-0.2mt-cameleon and D1ER pcDNA3 using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Along with the Ca$^{2+}$ sensor expression plasmids, an expression vector containing the target construct was transfected (1:10 molar ratio of Ca$^{2+}$ sensor expression plasmid to target expression vector). After 2 days, cells were rinsed twice and then maintained in Hanks’ balanced salt solution (HBSS: 142 mM NaCl, 5.6 mM KCl, 1 mM MgCl$_2$, 2 mM CaCl$_2$, 0.34 mM Na$_2$HPO$_4$, 0.44 mM KH$_2$PO$_4$, 4.2 mM NaHCO$_3$, 10 mM HEPES, and 5.6 mM glucose [pH 7.4]). Calcium imaging experiments were performed using an LSM 700 microscope (Carl Zeiss, Oberkochen, Germany). Dual-emission ratio imaging of cameleon was accomplished using the BP420/10 excitation filter, a 440/320 dichroic mirror, and two emission filters (BP472/30 for cyan fluorescent protein and BP542/27 for YFP), which were alternated using a filter changer. Exposure time was 100 ms, and images were collected every 3 s. Baseline (50-s) measurements were acquired before the first pulse of bradykinin (BK). BK was dissolved in HBSS, and the working concentration was 2.5 μM. In some experiments, MEFs were preincubated with 2-amino-ethoxydiphenyl borate (2-APB, 20 μM) for 30 min at room temperature prior to stimulation with BK. The free Ca$^{2+}$ concentration in mitochondria or ER was determined as previously described (Palmer et al., 2006). Peak values of Ca$^{2+}$ transients in MEFs transfected with constructs or treated with agents were compared with those in MEFs transfected with empty vectors or vehicles, which had no significant effects on these values (Appendix Fig S5B).

### In situ proximity ligation assay

Quantification of protein interactions (< 40 nm) as individual fluorescent dots was performed using the Duolink in situ assay (Sigma-Aldrich). MEFs on slides were fixed and permeabilized. The samples were probed with rabbit anti-IP3R antibody and mouse anti-VDAC1 antibody, and then with anti-mouse IgG and anti-rabbit IgG conjugated to oligonucleotide extensions. In this system, if the oligonucleotides are within a distance of 40 nm, they hybridize with subsequently add connector oligonucleotides to form a circular DNA template, which is ligated and subsequently amplified to create a single-stranded DNA product. In MEFs, the size of ER–mitochondrial junctions (10–25 nm) enabled proximity ligation and subsequent detection by hybridization of Texas red-labeled oligonucleotide probes. Fluorescence was analyzed on a Zeiss inverted fluorescence microscope. Each fluorescent dot represents the formation of one IP3R–VDAC1 interaction.

### Cell viability assay

Cell viability was measured using the Vybrant MTT Cell Proliferation Assay Kit (Thermo Fisher). MEFs were incubated with MTT solution in phenol red-free DMEM for 4 h at 37°C. The MTT assay involves the conversion of water-soluble MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) to insoluble formazan. The formazan is then solubilized by DMSO, and the concentration is determined by measuring optical density at 570 nm.

### Citrate synthase assay

Citrate synthase, the initial enzyme of the tricarboxylic acid (TCA) cycle and an exclusive marker of the mitochondrial matrix, was measured using the Citrate Synthase Assay kit (Sigma-Aldrich). Whole-cell lysates of MEFs were incubated with acetyl coenzyme A (acytely CoA) and oxaloacetic acid, yielding CoA with a free thiol group. The CoA then reacted with 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB) to form yellow TNB, which was spectrophotometrically measured at 412 nm.

### In vitro kinase assay

Reactions were performed at 30°C for 30 min in 40 μl of kinase buffer (20 mM HEPES, pH 7.5, 50 mM KCl, 1.5 mM DTT, 2 mM MgCl$_2$, 0.1 mM ATP) containing 6 μCi of $[^{32}P]ATP$ (1 Ci = 37 GBq), 2 μg of E3 ubiquitin ligase isolated from cell lysates with anti-Myc Sepharose, and 4 μg of PERK isolated from cell lysates with anti-FLAG Sepharose and FLAG peptide (Sigma-Aldrich). Reaction mixtures were subjected to 12% SDS/PAGE and visualized by autoradiography on a phosphorimager.

### In vitro ubiquitination assay

Reactions were carried out in a total volume of 100 μl of ubiquitination buffer (50 mM Tris–HCl, pH 7.5, 5 mM MgCl$_2$, 1 mM DTT, 100 mM NaCl) containing 90 nM E1 enzyme, 4 mM ATP, 0.4 mM HA-tagged Ub, 4 μg UbcH7, 4 μg His-tagged mitofusin 2, and 2 μg of MARCH5, MULAN, or Parkin. The latter proteins were first phosphorylated by PERK or PERK(K618R) in kinase buffer at 37°C for 30 min and then isolated with anti-Myc Sepharose. The reaction was incubated for 2 h at 37°C, followed by centrifugation at 3,000 g. The pellet containing His-tagged mitofusin 2 was washed with ubiquitination buffer and subjected
to SDS/PAGE. The supernatant was incubated in Ni-NTA binding buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole, pH 8.0) containing 20 ml of Ni-NTA beads. The Ni-NTA beads were washed with Ni-NTA binding buffer and then subjected to SDS/PAGE.

Kinase activity assay
LRRK2 kinase activity was measured using the ADP-Glo Assay kit. The kinase reaction was performed with LRRK2 isolated from MEFs with anti-LRRK2 antibody conjugated to Protein G Sepharose, LRRKside (RLGRDKYKTLRQIRQ) as a substrate, and ATP. ADP formed from the kinase reaction was converted into ATP, and the newly synthesized ATP was measured using a coupled luciferase/adenylyl cyclase reaction.

Yeast two-hybrid library screening
The BD Matchmaker Pretransformed cDNA Library was used in this study. Yeast strain AH109 was transformed with the bait plasmid pGBDU-C1 encoding the N-terminus (aa. 1–1,515) of LRRK2 and then screened against a mouse brain cDNA Matchmaker library (BD Biosciences, San Jose, CA, USA). Interacting proteins were identified by plasmid sequencing and BLAST searching. To confirm the interaction with the identified prey DNA, the indicated regions of LRRK2 and prey DNA were cloned into the GAL4-DNA binding-domain and GAL4-DNA activation-domain plasmids, respectively. The resultant plasmids were transformed into yeast strain PJ69-4A, and the interaction between the two proteins was tested in the yeast two-hybrid system. Interactions between binding- and activation-domain fusion proteins were scored based on yeast growth.

Electron microscopy
MEFs were incubated in 0.1 M phosphate buffer containing 2.5% glutaraldehyde and then post-fixed in 1% osmium tetroxide. The cells were embedded in Spurr’s resin. Embedded samples were cut into ultrathin sections. Sections were counterstained with uranyl acetate and lead citrate. Micrographs were obtained at 5,000× or 12,000× magnification. To obtain the interface percentage, mitochondrial perimeter and area, and the lengths of ER interfaces, were measured using Metamorph. Distances of ER–mitochondrial interfaces were determined by measuring the shortest distance between the ER membrane and the mitochondrial OMM at two sites for each contact. Eight images, each of which contained 2–4 mitochondria, were obtained from 5 MEFs of indicated genotypes.

Statistical analysis
All statistical analyses were performed using GraphPad Prism. All samples were first subjected to a D’Agostino–Pearson omnibus normality test. If values were distributed in a Gaussian manner, t-test was used for paired comparisons, and one-way ANOVA followed by Bonferroni’s multiple comparison tests for multiple comparisons. For non-Gaussian distributions, a Mann–Whitney U-test was used for paired comparisons, and a Kruskal–Wallis nonparametric ANOVA test was used for multiple comparisons.
Author contributions
TT, YO, TI, and SS carried out most of the experiments. TT wrote the manuscript. AK provided advice on project planning and data interpretation.

Conflict of interest
The authors declare that they have no conflict of interest.

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