

Phosphatase and Tensin Homolog (PTEN)-induced Putative Kinase 1 (PINK1)-dependent Ubiquitination of Endogenous Parkin Attenuates Mitophagy

STUDY IN HUMAN PRIMARY FIBROBLASTS AND INDUCED PLURIPOTENT STEM CELL-DERIVED NEURONS*

Received for publication, June 18, 2012, and in revised form, November 27, 2012. Published, JBC Papers in Press, December 4, 2012, DOI 10.1074/jbc.M112.391680

Aleksandar Rakovic[‡], Katharina Shurkewitsch[‡], Philip Seibler[‡], Anne Grünewald[‡], Alessandra Zanon^{‡§}, Johann Hagenah^{‡¶}, Dimitri Krainc^{||}, and Christine Klein^{‡¶1}

From the [‡]Section of Clinical and Molecular Neurogenetics at the Department of Neurology, University of Lübeck, 23562 Lübeck, Germany, the [¶]Department of Neurology, University of Lübeck, 23562 Lübeck, Germany, the [§]Center for Biomedicine, European Academy Bozen, 39100 Bolzano, Italy, and the ^{||}Department of Neurology, Massachusetts General Hospital, Harvard Medical School, MassGeneral Institute for Neurodegenerative Disease, Charlestown, Massachusetts 02129

Background: The Parkinson disease-related proteins PINK1 and Parkin initiate mitophagy of damaged mitochondria.

Results: Endogenous Parkin is not sufficient to induce mitophagy due to PINK1-dependent ubiquitination of Parkin.

Conclusion: Mitophagy is detectable only with supraphysiological levels of Parkin and differs between fibroblasts and iPS-derived neurons.

Significance: Stresses the importance of future studies in Parkinson disease-relevant tissue, *i.e.*, dopaminergic neurons.

Mutations in the E3 ubiquitin ligase Parkin and the mitochondrial PTEN-induced putative kinase 1 (PINK1) have been identified to cause autosomal recessive forms of familial Parkinson disease, with PINK1 functioning upstream of Parkin in a pathway important for the maintenance of mitochondrial function and morphology. Upon the loss of the mitochondrial membrane potential, Parkin translocates to mitochondria in a PINK1-dependent manner to ubiquitinate mitochondrial proteins. Parkin-mediated polyubiquitination of outer mitochondrial membrane (OMM) proteins recruits the ubiquitin- and LC3-binding adaptor protein p62 to mitochondria and induces mitophagy. Although previous studies examined mitophagy in established cell lines through overexpression approaches, there is an imperative to study the role of endogenous Parkin and PINK1 in human-derived and biologically relevant cell models. Here, we demonstrate in human primary fibroblasts and induced pluripotent stem-derived neurons from controls and PINK1 mutation carriers that endogenous levels of Parkin are not sufficient to initiate mitophagy upon loss of the mitochondrial membrane potential, caused by its (self-)ubiquitination, followed by degradation via the ubiquitin proteasome system. Next, we showed differential PINK1-dependent, Parkin-mediated ubiquitination of OMM proteins, which is Parkin dose-dependent and affects primarily OMM proteins of higher molecular mass. In contrast to the situation in fibroblasts, we did not detect mitophagy in induced pluripotent stem-derived neurons even upon overexpression of Parkin. Taken

together, our data demonstrate that mitophagy differs between human non-neuronal and neuronal cells and between “endogenous” and “Parkin-overexpressing” cellular models.

Parkinson disease (PD)² is the second most common neurodegenerative disorder. Although its etiology is mainly elusive, it has been shown that mitochondrial dysfunction caused by toxins such as 1-methyl-phenyl-1,2,3,6-tetrahydropyridine and rotenone can induce parkinsonism in humans and animal models (1, 2). Supporting an important role of mitochondria in the pathophysiology of PD, mutations in the E3 ubiquitin ligase Parkin (3) and the mitochondrial PTEN-induced putative kinase 1 (PINK1) (4) have been identified to cause autosomal recessive forms of familial PD, with PINK1 functioning upstream of Parkin in a pathway important for the maintenance of mitochondrial function and morphology (5–7). More recently, it has been shown that PINK1 recruits Parkin to depolarized mitochondria and subsequently promotes their degradation by autophagy (mitophagy) (8, 9). Following its mitochondrial translocation, Parkin induces polyubiquitination of the mitochondrial fusion proteins Mfn1 and Mfn2, followed by their degradation via the ubiquitin proteasome system (UPS) (10, 11). Additionally, it has been shown that overexpressed Parkin activates the UPS for widespread degradation of outer

* This work was supported by the Fritz Thyssen Foundation (to A. G.), National Genome Research Network-plus (Federal Ministry of Education and Research, to C. K.), and EU Grant European Project on Mendelian Forms of Parkinson's Disease (FP7).

¹ Recipient of a career development award from the Hermann and Lilly Schilling Foundation. To whom correspondence should be addressed: Section of Clinical and Molecular Neurogenetics at the Dept. of Neurology, Maria-Goeppert-Str. 1, 23562 Lübeck, Germany. Tel.: 494512903351; Fax: 494512903355; E-mail: christine.klein@neuro.uni-luebeck.de.

² The abbreviations used are: PD, Parkinson disease; PINK1, phosphatase and tensin homolog-induced putative kinase 1; FCCP, carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone; Grp, glucose-regulated protein; Hsp, heat shock protein; Mfn, mitofusin; MT-CO2, cytochrome c oxidase subunit II; iPS, induced pluripotent stem; MTG, Mitotracker Green; OMM, outer mitochondrial membrane; TH, tyrosine hydroxylase; Tom, translocase of outer membrane; VDAC, voltage-dependent anion channel; MTG, Mitotracker Green; UPS, ubiquitin proteasome system; LC3, microtubule-associated protein light chain 3; p62, sequestosome 1; PBS, phosphate-buffered saline; FL, full-length.

Ubiquitination of Endogenous Parkin Attenuates Mitophagy

mitochondrial membrane (OMM) proteins (12, 13). Although mitochondrial translocation to depolarized mitochondria and mitophagy has been clearly demonstrated in various non-neuronal cellular models, the results obtained from animal primary neurons are rather controversial (9, 14, 15). In the only published study on human neurons, *i.e.*, induced pluripotent stem (iPS)-derived neurons, mitochondrial translocation of Parkin has been observed within 12 h upon mitochondrial depolarization (16).

It is important to bear in mind that the majority of these studies have been performed in cells overexpressing Parkin, which may result in artifacts *per se*. This idea is further supported by the observation that selected overexpressed pathogenic Parkin mutants retain their ability to translocate to damaged mitochondria (8) and even to induce mitophagy at a level similar to that overexpressed wild type Parkin (18). This prompted us, first, to study the process of mitophagy in cells expressing endogenous (“physiological”) levels of Parkin with those overexpressing it. The second aim of the present study was to investigate mitophagy in iPS-generated neurons from PINK1 mutants and controls, a model closely resembling the most relevant disease-affected tissue in PD.

EXPERIMENTAL PROCEDURES

Cell Cultures and Generation of iPS-derived Neurons—Human dermal fibroblasts from controls and a PD patient harboring a homozygous *PINK1* missense mutation (V170G) and neuroblastoma (SH-SY5Y) cells were grown at 37 °C under a 5% CO₂ humidified atmosphere in DMEM (PAA laboratories) supplemented with 10% FBS (PAA laboratories) and 1% penicillin/streptomycin (PAA laboratories). The cells used in the present studies were between passages 4 and 12.

Generation of iPS cells and their differentiation into dopaminergic neurons was carried out as previously published (16). Immunofluorescence staining showed that ~70% of the total cells derived from iPS-Control and iPS-PINK1^{mut} expressed the neuron-specific marker neuronal class III-Tubulin (TUJ1) (control, 72 ± 15%; PINK1^{mut}, 76 ± 16%).

The percentage of neurons coexpressing TUJ1 and tyrosine hydroxylase (TH) was similar between controls (11 ± 3%) and PINK1^{mut} (10 ± 3%) iPS-derived neurons. Expression of TH was confirmed by Western blotting (see Fig. 9A).

Mitochondrial Preparation—Mitochondria were isolated from fibroblasts and SH-SY5Y cells as described previously (17). In brief, the cells were harvested and homogenized in buffer containing 250 mM sucrose, 10 mM Tris, and 1 mM EDTA, pH 7.4. Next, nuclei and intact cells were removed by centrifugation at 1500 × *g* for 20 min. The supernatant containing intact mitochondria was transferred into a new tube and centrifuged at 12,000 × *g* for 10 min. Supernatant (“cytosolic fraction”) was transferred into another new tube, and the mitochondria-enriched pellet (“mitochondrial fraction”) was dissolved in RIPA buffer containing a mixture of protease and phosphatase inhibitors (Roche Diagnostics). Cytoplasmic fractions were concentrated using Centricon YM-10 devices (Millipore) according to the manufacturer’s instructions. Proteins of the mitochondrial and cytoplasmic fractions were separated

by SDS-PAGE and detected by Western blot analysis using various antibodies.

Antibodies—In this study we used the following antibodies: anti- β -actin (Sigma), anti- β -tubulin (Sigma), anti-Complex II Fp subunit (Mitosciences), F1F0ATPase (α subunit) (Mitosciences), anti-Grp75 (Abcam), anti-MT-CO2 (Mitosciences), anti-Mitofusin 1 (Abcam), anti-Mitofusin 2 (Abcam), anti-Parkin (Cell Signaling), anti-Parkin (Abcam), anti-Tom20 (Santa Cruz), anti-Tom40 (Santa Cruz), anti-Tom70 (Abcam), anti-TUJ1 (Covance), anti-TH (Calbiochem), anti-ubiquitin (Cell Signaling), anti-V5 (Invitrogen), and anti-VDAC1 (Abcam). To quantify band intensities of immunoblots, the TotalLab TL100 v2006 one-dimensional gel analysis software (Nonlinear Dynamics) was used.

Immunofluorescence—Fibroblasts and differentiated neurons stably expressing Parkin or/and MitoDsRed were grown on glass coverslips, fixed in 4% formaldehyde for 15 min, permeabilized with 0.1% Triton X-100, and blocked in 4% normal goat serum in PBS for 1 h. Immunofluorescence staining was performed using primary antibodies against Parkin (1:200; Cell Signaling), TH (1:400; Calbiochem), and Grp75 (1:400; Abcam). Appropriate secondary antibodies were obtained from Invitrogen.

Immunoprecipitation—To immunoprecipitate Parkin, cells overexpressing WT Parkin were lysed using 25 mM Tris, 150 mM NaCl, and 1% Nonidet P-40. Next 5 μ l of an anti-Parkin antibody (Abcam) was bound with 50 μ l of Dynabeads protein A (Invitrogen) according to the manufacturer’s protocol. Finally, cell lysates were mixed with antibody-conjugated Dynabeads. The resulting immunoprecipitates were separated by SDS-PAGE and detected by Western blot analysis using anti-Parkin (Cell Signaling) or anti-ubiquitin (Cell Signaling) antibodies.

Measurement of MitoDsRed and Mitotracker Green Fluorescence—To measure MitoDsRed fluorescence, fibroblasts were grown in 96-well culture plates. Shortly before measurement, the cells were washed with PBS, and fluorescence was measured using a fluorescence plate reader (Biotek) with excitation/emission filters suitable for the red fluorescent MitoDsRed (530/590 nm).

For evaluation of the mitochondrial mass, the cells were incubated with 150 nM MitoTracker Green FM (Molecular Probes) 30 min before measurement. The cells were washed with 1× PBS, and fluorescence was measured using a plate reader (Biotek; 490/516 nm).

Plasmids for Expression of Mito-DsRed, PINK1-V5, and Parkin—To construct the Mito-DsRed lentiviral expression plasmid, whole Mito-DsRed2 fragment was cloned from pDsRed2-Mito (Clontech) into a lentiviral pER4 vector resulting in pER4-Mito-DsRed. Lentiviral plasmids expressing PINK1-V5 (pER4-PINK1-V5) and Parkin (pER4-Parkin) have been described previously (16). For transient expression of Parkin, a mammalian expression vector pcDNA3 (Invitrogen) containing WT Parkin cDNA was used.

RNA Extraction and Real Time PCR Analysis—Total RNA from fibroblasts was prepared by using the RNA easy protect kit (Qiagen) according to the manufacturer’s instructions and then reverse-transcribed into cDNA with the SuperScript first

strand synthesis system (Invitrogen). The resulting cDNAs were quantified by real time PCR using LightCycler DNA Master SYBR Green I on the Light Cycler 2.0 real time PCR system (Roche Diagnostics).

RESULTS

Overexpression of Parkin Is Required to Induce Detectable Loss of Mitochondrial Proteins—Previous studies analyzing PINK1/Parkin-dependent removal of depolarized mitochondria by mitophagy mainly used cells overexpressing Parkin (8, 9, 12, 18). To study this process in primary human cells in a similar setting, we generated control and PINK1^{mut} fibroblasts stably expressing C-terminally V5-tagged wild type PINK1 (PINK1-V5), wild type Parkin, or an empty expression vector using lentiviral particles. mRNA levels of *PINK1* and *Parkin* were measured by real time PCR using the mRNA levels of β -actin for normalization. mRNA levels of overexpressed PINK1 or Parkin were comparable between controls and PINK1 mutants ($\sim 250\times$ higher in comparison with cells expressing endogenous *PINK1* of *Parkin*; data not shown). In these cells, we first applied a routinely used method for mitochondrial labeling, *i.e.*, Mito-DsRed labeling (19, 20) and labeling with Mitotracker Green (MTG). MTG is thought to stain lipids of both mitochondrial membranes, *i.e.*, the outer and the inner membrane. On the other hand, Mito-DsRed is a fusion between red fluorescent protein (DsRed2) and the mitochondrial targeting sequence from subunit VIII of human cytochrome *c* oxidase localized exclusively in the inner mitochondrial membrane. Using both methods in parallel allowed us to monitor which one of the two mitochondrial membranes is affected by our treatments. In addition, it has been shown that Parkin modulates lipid metabolism (21), which further prompted us to include another method beside MTG. Mitochondria labeled with Mito-DsRed completely colocalized with mitochondria stained with MTG (Fig. 1A), which has previously been used to measure mitochondrial mass of mitochondria (8, 22). For Mito-DsRed labeling, control and PINK1^{mut} fibroblasts stably expressing empty vector, Parkin, or PINK1-V5 were transduced with lentiviral particles expressing Mito-DsRed to generate stable cell lines. Next, these cells were treated with valinomycin or with the commonly used mitochondrial depolarization agent carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) for 12 h, and DsRed fluorescence was measured. Here, we detected a decrease in DsRed-labeled mitochondria only in controls overexpressing Parkin but not in any other cells (Fig. 1B). To measure mitochondrial mass, control and PINK1^{mut} fibroblasts stably expressing empty vector, Parkin, or PINK1-V5 were treated with valinomycin or with FCCP for 12 h followed by MTG staining. Only in controls overexpressing Parkin did we detect a decrease in mitochondrial mass upon either treatment (Fig. 1C).

Next, we analyzed steady-state levels of proteins localized in different mitochondrial compartments, *i.e.*, the OMM protein VDAC1, the inner mitochondrial membrane protein MT-CO2, the mitochondrial matrix protein Grp75 in controls, and PINK1^{mut} stably expressing PINK1-V5, Parkin, or an empty vector. Note that Grp75 accumulates as a nonprocessed pre-protein (full-length, FL Grp75) (18, 19) upon the loss of the

mitochondrial membrane potential, which prevents its mitochondrial import and cleavage into a mature, mitochondrial form (Grp75). As in our previous experiment, we detected a significant decrease in levels of the mitochondrial proteins VDAC1, MT-CO2, and Grp75 only in control fibroblasts stably overexpressing Parkin when compared with levels of valinomycin- or FCCP-treated control cells expressing empty vector. In all other cells, no decrease in levels of these mitochondrial proteins was detected (Fig. 1D). In this experiment, endogenous Parkin is not detected because of a high dilution of the anti-Parkin antibody (2000 \times in comparison with the dilution necessary to detect endogenous Parkin). When using a less diluted anti-Parkin antibody, we detected both endogenous and overexpressed Parkin (data not shown).

To confirm our findings in another cell line, we analyzed SH-SY5Y cells stably expressing empty vector, Parkin, or PINK1-V5 under basal conditions and upon treatment with valinomycin or with FCCP for 12 h. As in fibroblasts, we detected a significant decrease in levels of the mitochondrial proteins VDAC1, MT-CO2, and Grp75 only in cells overexpressing Parkin but not in those expressing endogenous level of Parkin or overexpressing PINK1 (Fig. 1E).

Furthermore, we used immunostaining to show that only cells overexpressing Parkin are capable of removing mitochondria completely. For this, we transiently transfected fibroblasts from a control and a PINK1 mutant with empty vector or with vector expressing WT Parkin for 24 h. Upon transfection, the cells were treated with valinomycin for 16 h followed by immunostaining. In valinomycin-treated controls, we detected complete loss of the mitochondrial marker Grp75 (*red*) only in cells overexpressing Parkin (*green, outlined cell*), but not in those expressing endogenous levels of Parkin (Fig. 2A). In contrast, in PINK1^{mut} we detected no valinomycin-induced loss of Grp75 in cells with either endogenous or with overexpressed Parkin (Fig. 2B). We found that $79 \pm 2\%$ of control fibroblasts overexpressing Parkin displayed complete loss of mitochondria, whereas all other cells showed no loss of mitochondria (Fig. 2C). Note that all anti-Parkin antibodies we used were not sensitive enough to detect endogenous Parkin by immunostaining.

Because we detected no reduction in levels of mitochondrial proteins in cells expressing only endogenous levels of Parkin in the first 12 h upon valinomycin- or FCCP-induced mitochondrial depolarization in our previous experiment (Fig. 1), we sought to analyze cells upon prolonged mitochondrial depolarization. First, we verified that human fibroblasts and SH-SY5Y cells express relatively high levels of endogenous Parkin compared with other commonly used human cell lines (Fig. 3A). Next, fibroblasts from a control and a PINK1 mutant (PINK1^{mut}) were treated with valinomycin or FCCP for 12 and 24 h, respectively, followed by Western blot analysis of VDAC1, MT-CO2, Grp75, and Hsp60, another mitochondrial matrix protein. We detected comparable levels of all mitochondrial proteins tested upon 12 or 24 h of treatment with either valinomycin or FCCP in both controls and PINK1^{mut} (Fig. 3, B and C).

In addition, we measured steady-state levels of VDAC1, MT-CO2, and Grp75 in SH-SY5Y cells expressing endogenous levels of Parkin. Cells were analyzed under basal conditions and upon valinomycin or FCCP treatment for 12 and 24 h by West-

Ubiquitination of Endogenous Parkin Attenuates Mitophagy

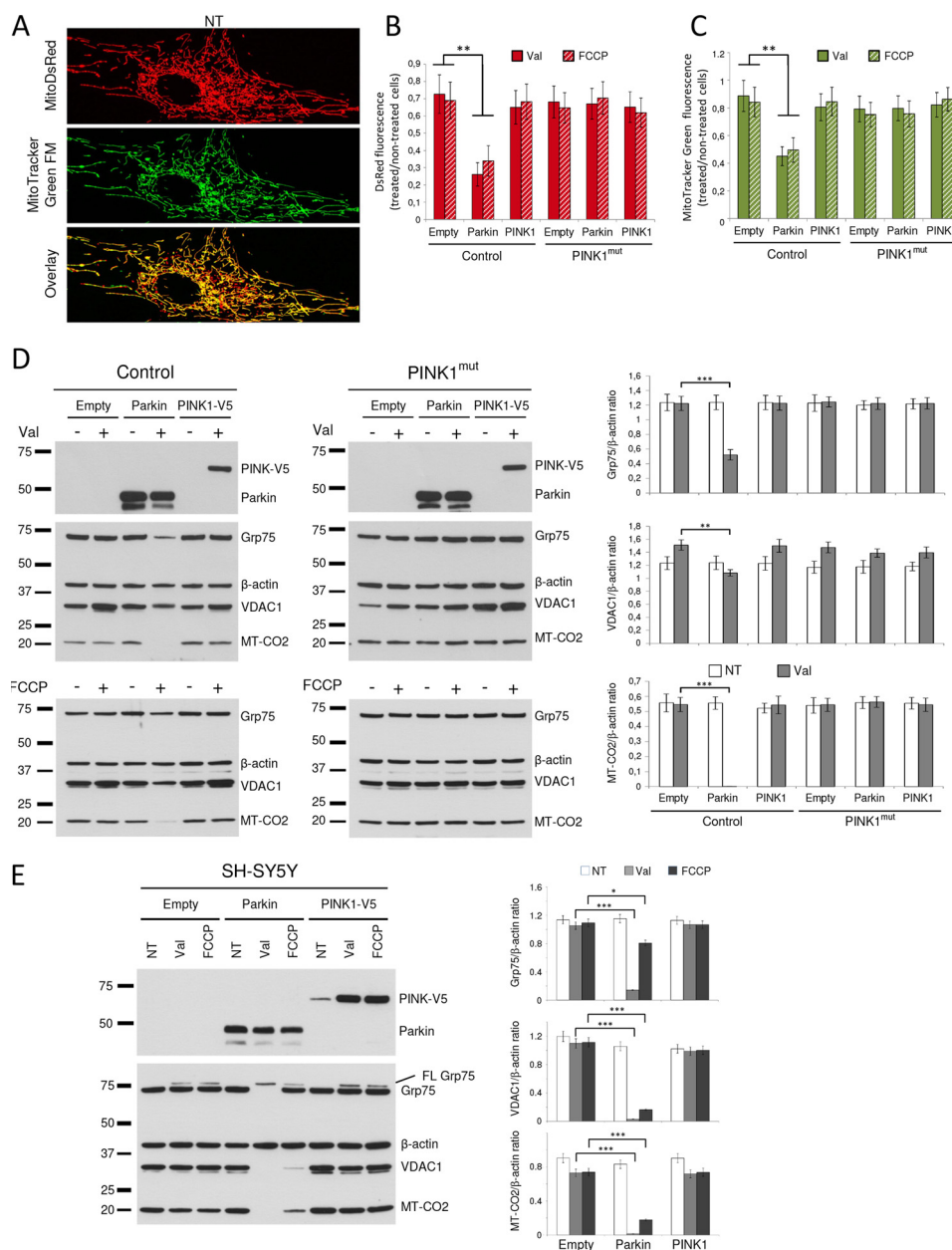


FIGURE 1. Overexpression of Parkin is required for a significant reduction in levels of mitochondrial proteins and mitochondrial mass. *A*, nontreated fibroblasts expressing MitoDsRed were stained using MTG. *B* and *C*, fibroblasts from a control and a PINK1 mutant stably expressing Parkin or PINK-V5 were transduced with lentiviral particles expressing MitoDsRed or stained using MTG followed by treatment with 1 μ M valinomycin or with 10 μ M FCCP for 12 h. DsRed (*B*) or MTG fluorescence (*C*) was measured using a plate reader. The results were normalized for cell number and expressed as a ratio between valinomycin- or FCCP-treated and nontreated cells. *D*, fibroblasts from a control and a PINK1 mutant were transduced with lentiviral particles expressing Parkin or C-terminally V5-tagged PINK1 (PINK1-V5) to generate stable cell lines. Next, cells were treated for 12 h with 1 μ M valinomycin or with 10 μ M FCCP. The proteins were extracted, separated on SDS gels, and immunoblotted using antibodies against Parkin and V5 or against mitochondrial proteins Grp75, VDAC1, and MT-CO2 with β -actin as a loading control. These proteins are reliable mitochondrial markers and mitochondrial loading controls because of their constitutive and ubiquitous expression. Note that endogenous Parkin is not detected in this experiment because of a high dilution of the anti-Parkin antibody (2000 \times in comparison with the dilution necessary to detect endogenous Parkin). Immunoblots were quantified densitometrically, and the intensity of each band was normalized to β -actin. *E*, SH-SY5Y cells stably expressing Parkin or PINK-V5 were treated with 1 μ M valinomycin or with 10 μ M FCCP for 12 h. Proteins were extracted, separated on SDS gels, and immunoblotted using antibodies against Parkin and V5 or against mitochondrial proteins Grp75, VDAC1, and MT-CO2 with β -actin as a loading control. Immunoblots were quantified densitometrically, and the intensity of each band was normalized to β -actin. The error bars represent the means \pm standard deviations from three independent experiments. NT, nontreated; Val, valinomycin.

ern blotting. Again, we detected no reduction in levels of any of the analyzed mitochondrial proteins (Fig. 3D). It is of note that FL Grp75 accumulates upon mitochondrial depolarization.

PINK1-dependent Ubiquitination of Endogenous Parkin Decreases Its Mitochondrial Translocation—Because endogenous Parkin is not sufficient to induce reduction in steady-state levels of mitochondrial proteins and mitochondrial mass meas-

ured by MTG, we next investigated whether protein levels of endogenous Parkin and its mitochondrial translocation might be affected in response to dissipation of the mitochondrial membrane potential. When we studied the effect of valinomycin or FCCP treatment on the total protein levels of endogenous Parkin using immunoblotting, we detected a loss of signal caused by either treatment only in controls but not in PINK1^{mut}

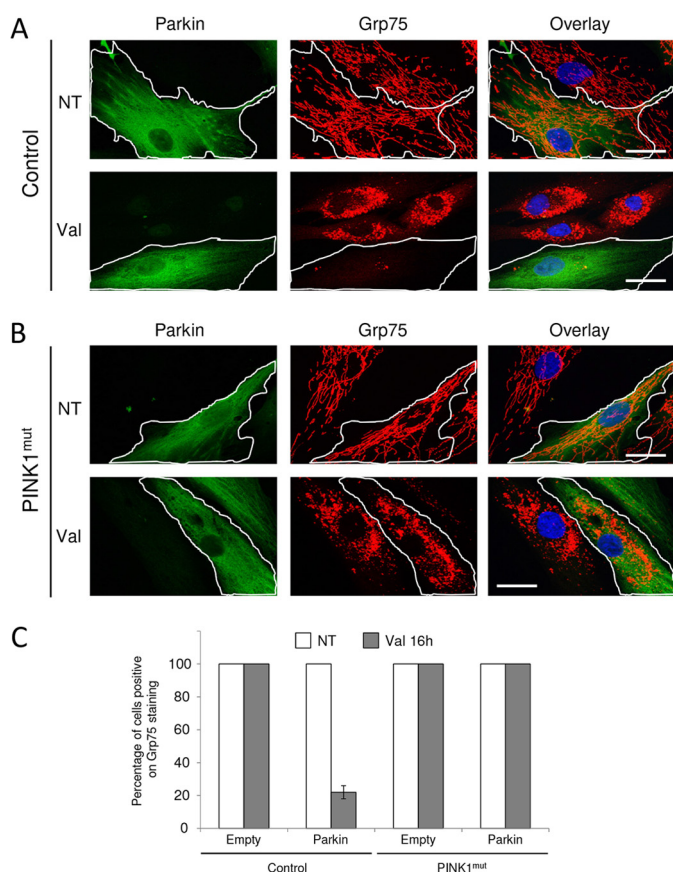


FIGURE 2. Overexpression of Parkin is required for mitophagy. *A* and *B*, fibroblasts from a control (*A*) and a PINK1 mutant (*B*) were transiently transfected with Parkin. Upon transfection, the cells were treated with 1 μ M valinomycin for 16 h followed by immunostaining using an antibody against Parkin (green, circled cells) and mitochondrial protein Grp75 (red). *C*, the percentage of cells with detectable mitochondria was determined for 100 cells/condition in three independent experiments. Scale bar, 25 μ m. NT, non-treated; Val, valinomycin.

(Fig. 4A). Next, we analyzed the kinetics of the valinomycin-induced translocation of endogenous Parkin from the cytosol to mitochondria and monitored its proteins levels in each of those fractions. For this, fibroblasts from a control and PINK1^{mut} were separated into mitochondrial and cytosolic fractions at different time points upon administration of valinomycin and analyzed by Western blotting (Fig. 4, B–E). In controls, Parkin translocated to mitochondria within 1 h of treatment followed by further mitochondrial accumulation until the ~3-h time point. After this time point, the levels of mitochondrial Parkin started to continuously decrease, whereas the levels of all mitochondrial markers, VDAC1, MT-CO2, and Grp75, did not change during the entire experiment (Fig. 4B). In contrast, in the cytosolic fraction, we detected a continuous decrease in the levels of endogenous Parkin. Additionally, proteins from both fractions were immunoblotted using anti-Parkin antibody only (Fig. 4C) to avoid any possibility of cross-reaction with other antibodies used in Fig. 4B. In the cytosolic fraction, we saw additional Parkin antibody-immunoreactive bands of higher molecular mass corresponding to possible mono- and diubiquitination of Parkin (Fig. 4C, right panel). In contrast, ubiquitinated Parkin was not detected in the mitochondrial fraction (Fig. 4C, left panel).

In PINK1^{mut}, endogenous Parkin remained exclusively in the cytosol upon mitochondrial depolarization (Fig. 4D). Furthermore, protein levels of endogenous Parkin remained unchanged for the entire duration of the experiment, and no ubiquitinated Parkin was detected in either fraction (Fig. 4E).

To confirm that valinomycin-induced reduction in levels of endogenous Parkin and its ubiquitination is PINK1-dependent, we used fibroblasts from PINK1 mutant stably expressing empty vector or PINK1 (PINK-V5). Here, we were able to rescue the WT phenotype in PINK1^{mut} by exogenous expression of WT PINK1 (Fig. 4, F and G). Taken together, these experiments demonstrate that endogenous Parkin is ubiquitinated in a PINK1-dependent manner in response to mitochondrial depolarization. Moreover, detection of the ubiquitinated species of “cytosolic” Parkin parallels a reduction in levels of mitochondrial Parkin, suggesting that ubiquitination of Parkin might be a reason for the reduction in its appearance in the mitochondrial fraction observed at a later time point.

Next, we aimed to test our findings from fibroblasts in SH-SY5Y cells expressing endogenous levels of Parkin. For this, we treated cells with valinomycin or FCCP for 12 and 24 h and analyzed whole cell lysate or separated mitochondrial and cytosolic fractions by Western blotting. In Fig. 5A, using whole cell lysate, we confirmed valinomycin- or FCCP-induced reduction in Parkin protein levels. Then we monitored the valinomycin-induced mitochondrial translocation of Parkin for 12 h and measured its proteins levels in each of the fractions at different time points (Fig. 5B). As in fibroblasts, we detected a peak in mitochondrial accumulation of Parkin between the 1- and 3-h time points, after which the levels of mitochondrial Parkin continuously decreased. In the cytosolic fraction, Parkin levels continuously decreased.

When we analyzed proteins from both fractions using anti-Parkin antibody only (Fig. 5C), we detected additional Parkin antibody-immunoreactive bands corresponding to possible mono- and diubiquitination of Parkin in the cytosolic but not in the mitochondrial fraction. In addition, we found ubiquitination of endogenous Parkin in SH-SY5Y cells treated with FCCP and immunoblotted at different time points (Fig. 5D).

Inhibition of the UPS Prevents Loss of Cytosolic Parkin and Increases Its Levels on Mitochondria—Next, we tested whether the ubiquitin proteasome system (UPS) is involved in the observed PINK1-dependent loss of endogenous Parkin. For this, control fibroblasts were treated for 12 h with valinomycin alone or with valinomycin in combination with an inhibitor of the UPS, *i.e.*, MG132 followed by Western blot analysis of the mitochondrial and cytosolic fractions (Fig. 6A). When compared with treatment with valinomycin alone, inhibition of the UPS significantly increased levels of endogenous Parkin in both fractions (Fig. 6B). Furthermore, when cellular fractions were analyzed using an anti-Parkin antibody alone, we detected ubiquitination of cytosolic Parkin upon valinomycin treatment. In contrast to our expectations, inhibition of the UPS did not preserve the ubiquitinated Parkin species, but conversely resulted in their loss and increase in levels of nonmodified Parkin (Fig. 6C). Likewise, we observed a reduction in ubiquitination levels upon inhibition of the UPS in case of the Mfn2 protein (Fig. 6, D and E), which was previously shown to be

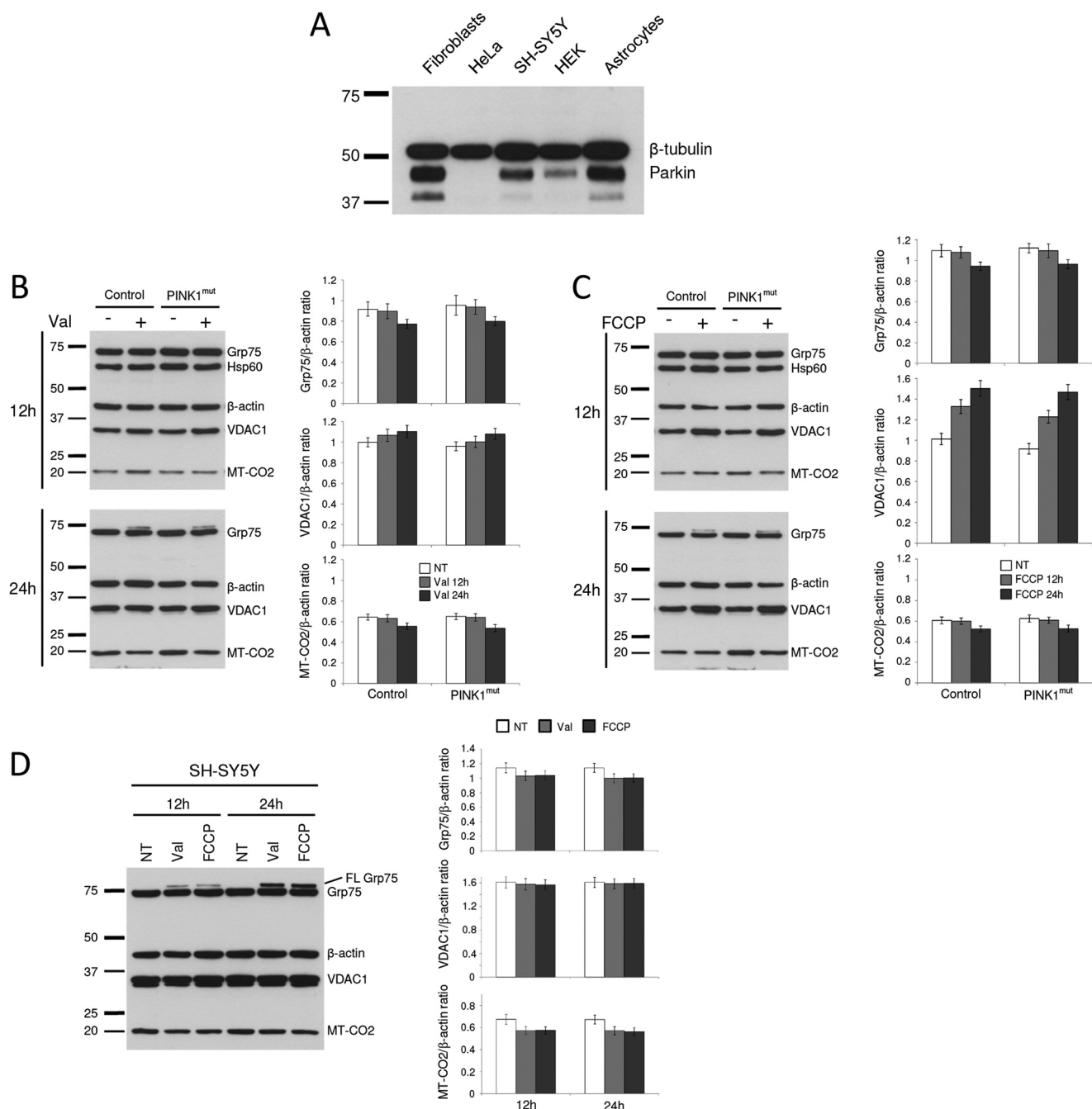


FIGURE 3. Endogenous Parkin is not sufficient to cause a detectable reduction in levels of mitochondrial proteins. *A*, whole cell lysate of fibroblasts and of HeLa, SH-SY5Y, HEK cells, and astrocytes was analyzed by Western blotting using antibodies against Parkin and β -tubulin as a loading control. Specificity of the anti-Parkin antibody used in this study was demonstrated previously (11). *B* and *C*, fibroblasts from control and PINK1 mutant were treated for 12 or 24 h with 1 μ M valinomycin (*B*) or with 10 μ M FCCP (*C*). Proteins were extracted, separated on SDS gels, and immunoblotted against mitochondrial proteins: Grp75, Hsp60, VDAC1, and MT-CO2. β -Actin served as a loading control. Note an additional band of Grp75 (FL Grp75) upon 24 h of treatment with either valinomycin or FCCP in both controls and PINK1^{mut}. This is due to the fact that loss of the mitochondrial membrane potential prevents mitochondrial import of Grp75 and its mitochondrial processing. Therefore, this protein remains in the cytosol as a nonprocessed pre-protein as shown before (26, 34). Next, we detected a PINK1-independent increase in levels of VDAC1 in both valinomycin- and FCCP-treated cells. This increase was more pronounced in cells treated with FCCP and is in accordance with previous findings that mitochondrial toxins increase the level of the VDAC1 protein but not its mRNA expression (35). Finally, protein levels of the two remaining mitochondrial markers Hsp60 and MT-CO2 were unaffected by treatment and also comparable between controls and PINK1^{mut}. The resulting immunoblots were quantified densitometrically. *D*, SH-SY5Y cells were treated for 12 or 24 h with 1 μ M valinomycin or with 10 μ M FCCP. The proteins were extracted, separated on SDS gels, and immunoblotted against mitochondrial proteins: Grp75, VDAC1, and MT-CO2. β -Actin served as a loading control. The intensity of each band was normalized to the intensity of β -actin. The error bars represent the means \pm standard deviations from three independent experiments. NT, nontreated; Val, valinomycin; FL, full length.

polyubiquitinated and degraded via the UPS (10–12). To confirm our findings from fibroblasts, we used SH-SY5Y cells expressing endogenous levels of Parkin or overexpressing it. In cells expressing endogenous Parkin, we detected moderate

ubiquitination of Mfn2 upon 12 h of valinomycin treatment (Fig. 6F). When valinomycin was combined with epoxomycin, we detected deubiquitination of Mfn2 but not preservation of its ubiquitinated forms, as one would expect. When we ana-

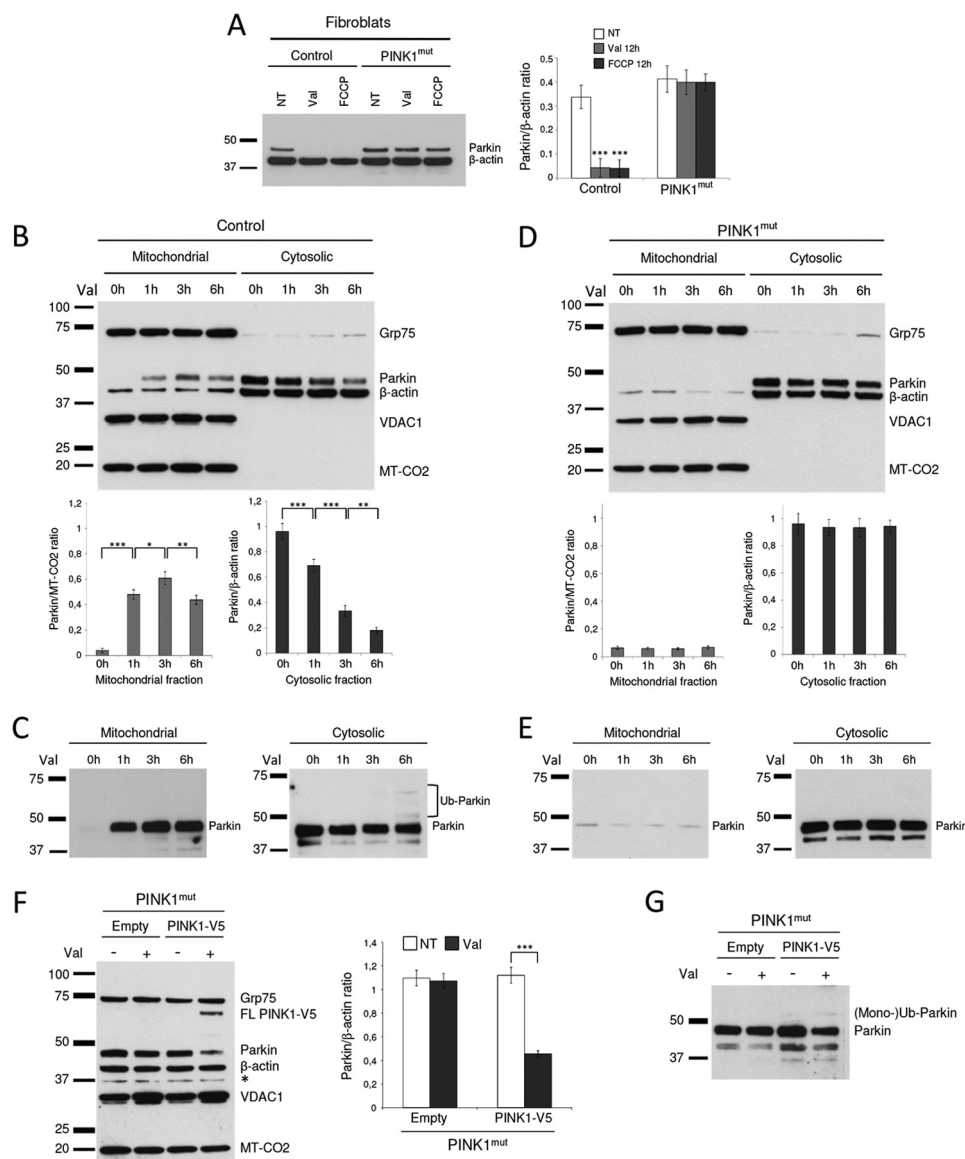


FIGURE 4. PINK1-dependent ubiquitination of endogenous Parkin prevents its mitochondrial translocation. *A*, fibroblasts from a control and a PINK1 mutant were treated for 12 h with 1 μ M valinomycin or 10 μ M FCCP. *B*, fibroblasts from a healthy control were treated with 1 μ M valinomycin. Cells were harvested at different time points to prepare mitochondrial and cytosolic fractions and analyzed by Western blotting using antibodies against Parkin and mitochondrial proteins Grp75, VDAC1, and MT-CO2 and β -actin as a loading control. After densitometric quantification, the intensity of each band was normalized to the intensity of either MT-CO2 or β -actin. The error bars represent the means \pm standard deviations from three independent experiments. *C*, proteins from each fraction were analyzed by Western blotting using an antibody against Parkin only. *D*, fibroblasts from a PINK1 mutant were treated with 1 μ M valinomycin. Cells were harvested at different time points to prepare mitochondrial and cytosolic fractions and analyzed by Western blotting using antibodies against Parkin and mitochondrial proteins Grp75, VDAC1, and MT-CO2. β -Actin served as a loading control. Densitometric quantification of the immunoblots from three independent experiments normalized to β -actin. *E*, proteins from each fraction were analyzed by Western blotting using an antibody against Parkin only. *F* and *G*, fibroblasts from PINK1 mutant stably expressing PINK1-V5 were treated with 1 μ M valinomycin for 12 h. The proteins were extracted, separated on SDS gels, and immunoblotted against Grp75, V5 tag, Parkin, VDAC1, and MT-CO2 (*F*) or against an anti-Parkin antibody only (*G*). NT, nontreated; Val, valinomycin.

lyzed Parkin in these cells, we observed moderate valinomycin-induced ubiquitination of endogenous Parkin but only in the cytosolic fraction (Fig. 6G). As in the case of Mfn2, combined treatment with valinomycin and epoxomycin resulted in a reduction of ubiquitination of Parkin in the cytosol and an increase in levels of its nonubiquitinated form in both the mitochondrial and the cytosolic fraction (Fig. 6G). On the other hand, in SH-SY5Y cells overexpressing Parkin, treatment with valinomycin resulted in a loss of Mfn2 (and VDAC1; Fig. 6H). Note that cells overexpressing Parkin were treated only for 6 h, because prolonged treatment would result in complete loss of

mitochondria. Combined treatment with epoxomycin and valinomycin resulted in preservation of ubiquitinated Mfn2. When we analyzed overexpressed Parkin, we could not detect its ubiquitination at this time point (6 h). Furthermore, treatment with valinomycin and epoxomycin together had no effect on the levels of overexpressed Parkin in either the mitochondrial or the cytosolic fraction (Fig. 6I), as observed with endogenous levels of Parkin (Fig. 6G).

To demonstrate that the observed Parkin antibody-immunoreactive bands of higher molecular mass were due to ubiquitination of Parkin, we used immunoprecipitation (Fig. 7). For

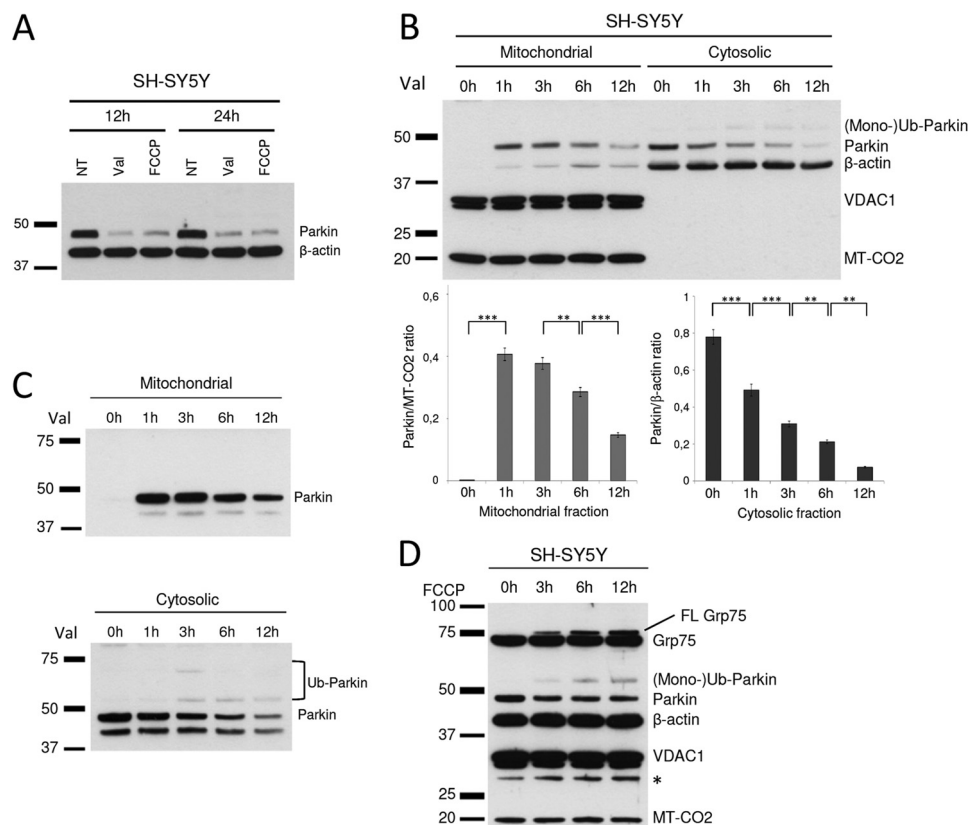


FIGURE 5. Ubiquitination of endogenous Parkin prevents its mitochondrial translocation in neuroblastoma cells. A, SH-SY5Y cells were treated for 12 and 24 h with 1 μ M valinomycin or 10 μ M FCCP. B, SH-SY5Y cells were treated with 1 μ M valinomycin. The cells were harvested at different time points to prepare mitochondrial and cytosolic fractions and analyzed by Western blotting using antibodies against Parkin and mitochondrial proteins Grp75, VDAC1, and MT-CO2 with β -actin as a loading control. After densitometric quantification, the intensity of each band was normalized to the intensity of either MT-CO2 or β -actin. The error bars represent the means \pm standard deviations from three independent experiments. C, proteins from each fraction were analyzed by Western blotting using an antibody against Parkin only. D, SH-SY5Y cells were treated with 1 μ M valinomycin. Proteins were extracted at different time points, separated on SDS gels, and immunoblotted against Grp75, Parkin, VDAC1, and MT-CO2. β -Actin served as a loading control. NT, nontreated; Val, valinomycin.

this, we employed SH-SY5Y cells overexpressing WT Parkin. Indeed, Western blot analysis of Parkin immunoprecipitates revealed highly ubiquitinated species in response to valinomycin using an anti Parkin antibody (Fig. 7A) or an anti-Ubiquitin antibody (Fig. 7B). These data are in keeping with the notion that endogenous Parkin is indeed polyubiquitinated and represents a substrate for the UPS.

Parkin Ubiquitinates Multiple OMM Proteins at Different Rates—Previously, we and others reported that Parkin ubiquitinates the mitochondrial GTPases Mfn1 and Mfn2 on depolarized mitochondria and promotes their degradation via the UPS (10, 11, 23). More recently, two groups found that overexpression of Parkin causes the UPS-mediated degradation of multiple OMM proteins; however, they did not determine whether these proteins are indeed substrates for Parkin, or whether they are degraded as nonubiquitinated proteins by the proteasome *in trans* (12, 13). To test whether Parkin directly ubiquitinates multiple OMM proteins in response to mitochondrial depolarization, we monitored the change in levels of several OMM proteins of different molecular mass ranging from \sim 15 to \sim 90 kDa, as well as the presence of specific immunoreactive bands of higher molecular mass corresponding to possible mono-, di-, or polyubiquitination in controls and PINK1 mutants containing different “doses” of Parkin, *i.e.*, endogenous (empty vector) *versus* overexpressed (Parkin) (Fig. 8). Because Parkin acts in

the same pathway together with PINK1, we used fibroblasts overexpressing WT PINK1 (PINK1-V5). The cells were analyzed at different time points within the first 6 h of valinomycin treatment to monitor changes in OMM proteins in early stages of mitophagy. In controls expressing endogenous levels of Parkin (empty vector) or overexpressing PINK1 (PINK1-V5), we detected rapid (mono-)ubiquitination of Mfn1 and Mfn2 and (mono-)ubiquitination of Tom70; however, this was observed only upon 3–6 h of treatment (Fig. 8A, *left panels*). The levels of all other OMM proteins studied were unaffected, and no higher molecular mass species were detected. In contrast, in controls overexpressing Parkin, we found rapid polyubiquitination of Mfn1, Mfn2, and Tom70 that led to almost complete degradation of these proteins within 6 h of valinomycin treatment. In addition, overexpression of Parkin induced ubiquitination of all other OMM proteins studied, but at slower rates (Fig. 8A, *right panels*).

In PINK1^{mut}, overexpression of wild type PINK1 (PINK1-V5) was sufficient to rescue (mono-)ubiquitination of Mfn1, Mfn2, and Tom70 but not of any other mitochondrial proteins analyzed in the present study (Fig. 8B, *left panels*). In contrast, in PINK1^{mut}, overexpression of Parkin had no effect on the levels of mitochondrial proteins, and no ubiquitination was detected (Fig. 8B, *right panels*).

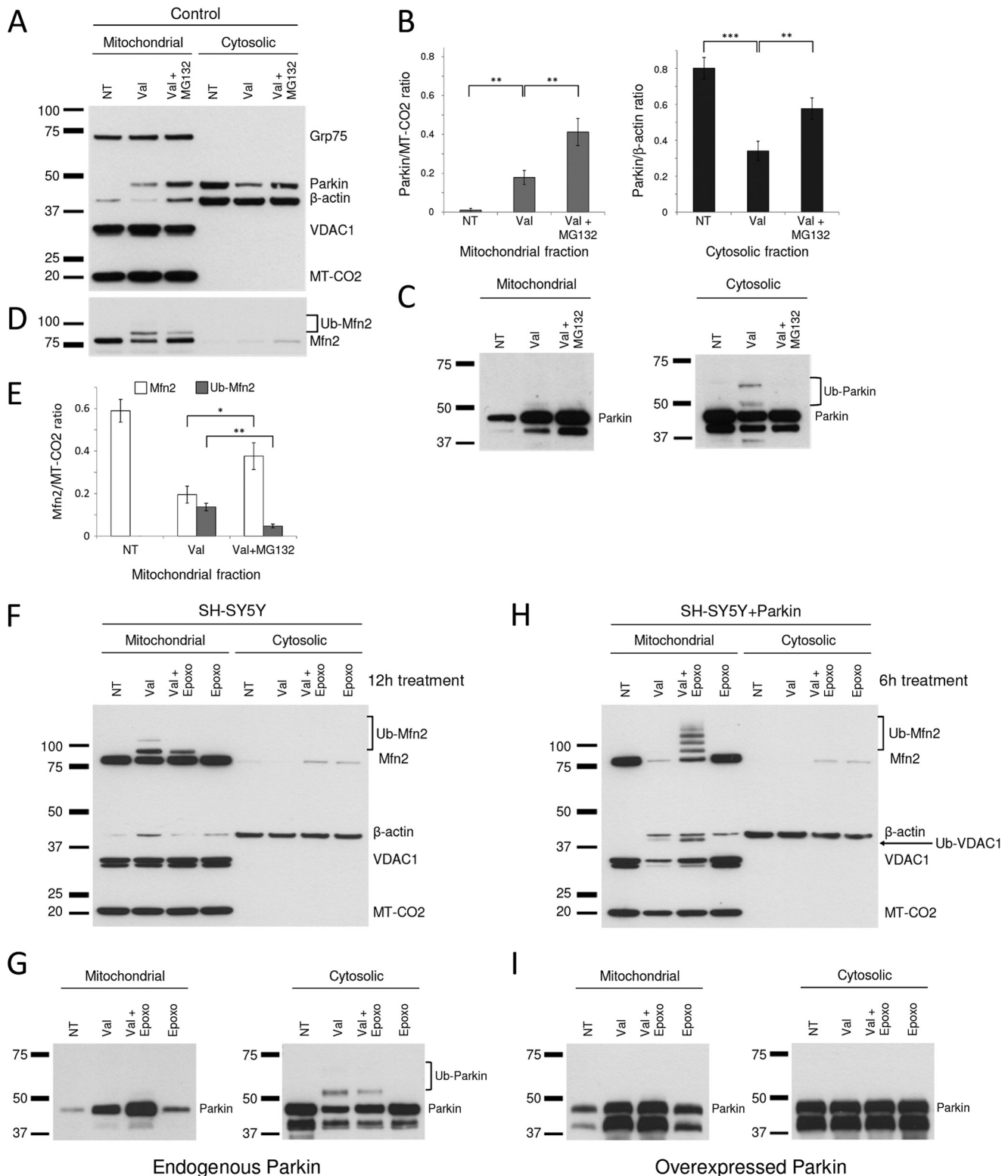


FIGURE 6. Inhibition of the UPS prevents ubiquitination of endogenous Parkin and increases its levels in mitochondria. *A*, fibroblasts from controls were treated with 1 μ M valinomycin alone or with 1 μ M valinomycin plus 10 μ M MG132 for 12 h. Cells were harvested to prepare mitochondrial and cytosolic fractions and analyzed by Western blotting using antibodies against Parkin, Grp75, VDAC1, MT-CO2, and β -actin. *B*, Densitometric analysis of immunoblots normalized to the intensity of either MT-CO2 or β -actin. Error bars represent means \pm standard deviations from three independent experiments. *C*, Proteins from each fraction were analyzed by Western blotting using an antibody against Parkin only. *D* and *E*, immunoblots from *A* were reprobed using an anti-Mfn2 antibody (*D*) and quantified densitometrically (*E*). *F–I*, SH-SY5Y cells expressing endogenous Parkin (*F* and *G*) and SH-SY5Y cells overexpressing Parkin (*H* and *I*) were treated with 1 μ M valinomycin alone or with 1 μ M valinomycin plus 4 μ M epoxomicin for 12 and 6 h. Cells were harvested to prepare mitochondrial and cytosolic fractions and analyzed by Western blotting using antibodies against Grp75, VDAC1, MT-CO2, and β -actin. *G* and *I*, proteins from each fraction were analyzed by Western blotting using an antibody against Parkin only. Val, valinomycin; Mfn2, mitofusin 2; Ub-Mfn2, ubiquitinated mitofusin 2.

Ubiquitination of Endogenous Parkin Attenuates Mitophagy

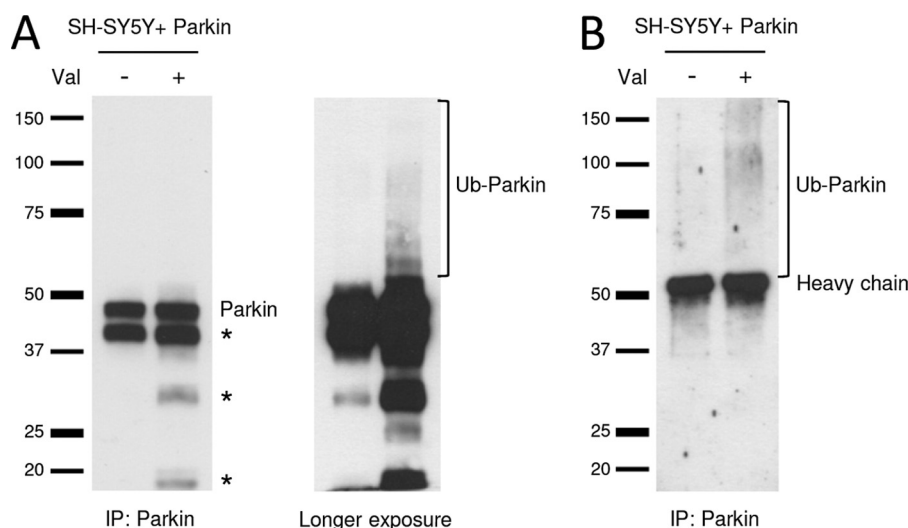


FIGURE 7. Immunoprecipitation confirmed ubiquitination of Parkin. Whole cell lysate from SH-SY5Y cells overexpressing WT Parkin was immunoprecipitated using an anti-Parkin antibody. The resulting immunoprecipitates (IP) were separated by SDS-PAGE and immunoblotted using antibodies against Parkin (A) or ubiquitin (B). Val, valinomycin.

These results collectively show that overexpressed Parkin ubiquitinates all OMM proteins analyzed in this study on a wild type PINK1 background in response to mitochondrial depolarization. The intensity of these observed ubiquitinations was more pronounced in those with higher molecular mass and was dependent on the Parkin dose.

Mitophagy Was Not Observed in iPS-derived Neurons—Because we showed that endogenous levels of Parkin are not sufficient to induce detectable mitophagy in fibroblasts and SH-SY5Y cells, we wanted to confirm our findings in a more disease-relevant tissue, *i.e.*, iPS-derived neurons. First, we compared the levels of the mitochondrial proteins Grp75, Mfn2, and Tom20 between fibroblasts and TH-positive iPS-derived neurons upon 12 h of mitochondrial depolarization. As in fibroblasts, we detected no differences in protein levels of Grp75 and Tom20 between neurons from controls and PINK1^{mut} (Fig. 9A). Similarly, valinomycin-induced ubiquitination of Mfn2 was observed in both fibroblasts and neurons from a control but not from PINK1^{mut} (Fig. 9A). We also detected higher protein levels of endogenous Parkin in neurons in comparison with fibroblasts (~4-fold) (Fig. 9B) and valinomycin-induced ubiquitination of endogenous Parkin in neurons (Fig. 9C).

To test whether overexpression of Parkin is sufficient to induce detectable mitophagy in iPS-derived neurons, we analyzed neurons derived from controls and PINK1 mutants stably expressing empty vector and Parkin under basal conditions and upon valinomycin treatment. Neurons expressing only endogenous levels of Parkin exhibited no decrease in levels of the mitochondrial proteins Grp75, VDAC1, and MTCO2 upon 16 h of mitochondrial depolarization (Fig. 9D, *left panel*). Although in neurons overexpressing Parkin, we detected a reduction in levels of VDAC1 and a band corresponding to its monoubiquitination, no reduction in levels of the matrix protein Grp75 and the inner mitochondrial membrane protein MT-CO2 was observed (Fig. 9D, *middle panel*). Overexpressed Parkin was detected using a high dilution of the anti-Parkin antibody (2500× in comparison with the dilution necessary to detect endogenous Parkin) (Fig. 9D, *right panel*).

A lack of valinomycin-induced mitophagy in neurons overexpressing Parkin was confirmed by immunostaining (Fig. 9E). These findings are consistent with previous work in iPS-derived dopaminergic neurons showing absence of mitophagy in those cells upon 12 h of valinomycin treatment (16).

Finally, we compared valinomycin-induced mitophagy between fibroblasts and iPS-derived neurons overexpressing Parkin by measuring steady-state levels of several proteins located in all three mitochondrial compartments (Fig. 10). Although in fibroblasts we detected PINK1-dependent valinomycin-induced loss of all mitochondrial proteins (Fig. 10A), in neurons, we detected a reduction only in OMM proteins of higher molecular mass, most likely because of their ubiquitination. Levels of all other mitochondrial proteins tested were unaffected (Fig. 10B). Taken together, our data indicate that neither endogenous nor overexpressed Parkin is sufficient to induce detectable mitophagy in iPS-derived neurons.

DISCUSSION

In this study, we analyzed the process of PTEN-induced putative kinase 1 (PINK1)-Parkin-dependent mitophagy by measuring levels of several proteins that are localized in different mitochondrial compartments and represent reliable mitochondrial markers and loading controls because of their constitutive and ubiquitous expression. In contrast to measuring mitochondrial mass using Mitotracker Green FM, which is thought to stain mitochondrial membranes only, our approach allowed us to simultaneously monitor changes in all mitochondrial compartments, *i.e.*, the outer mitochondrial membrane (OMM), inner mitochondrial membrane, and the mitochondrial matrix upon mitochondrial depolarization.

An important aspect of this study is the use of cells containing endogenous levels of both PINK1 and Parkin, which minimizes the occurrence of artifacts caused by protein overexpression. When we measured levels of mitochondrial proteins in response to dissipation of the mitochondrial membrane potential in primary fibroblasts expressing endog-

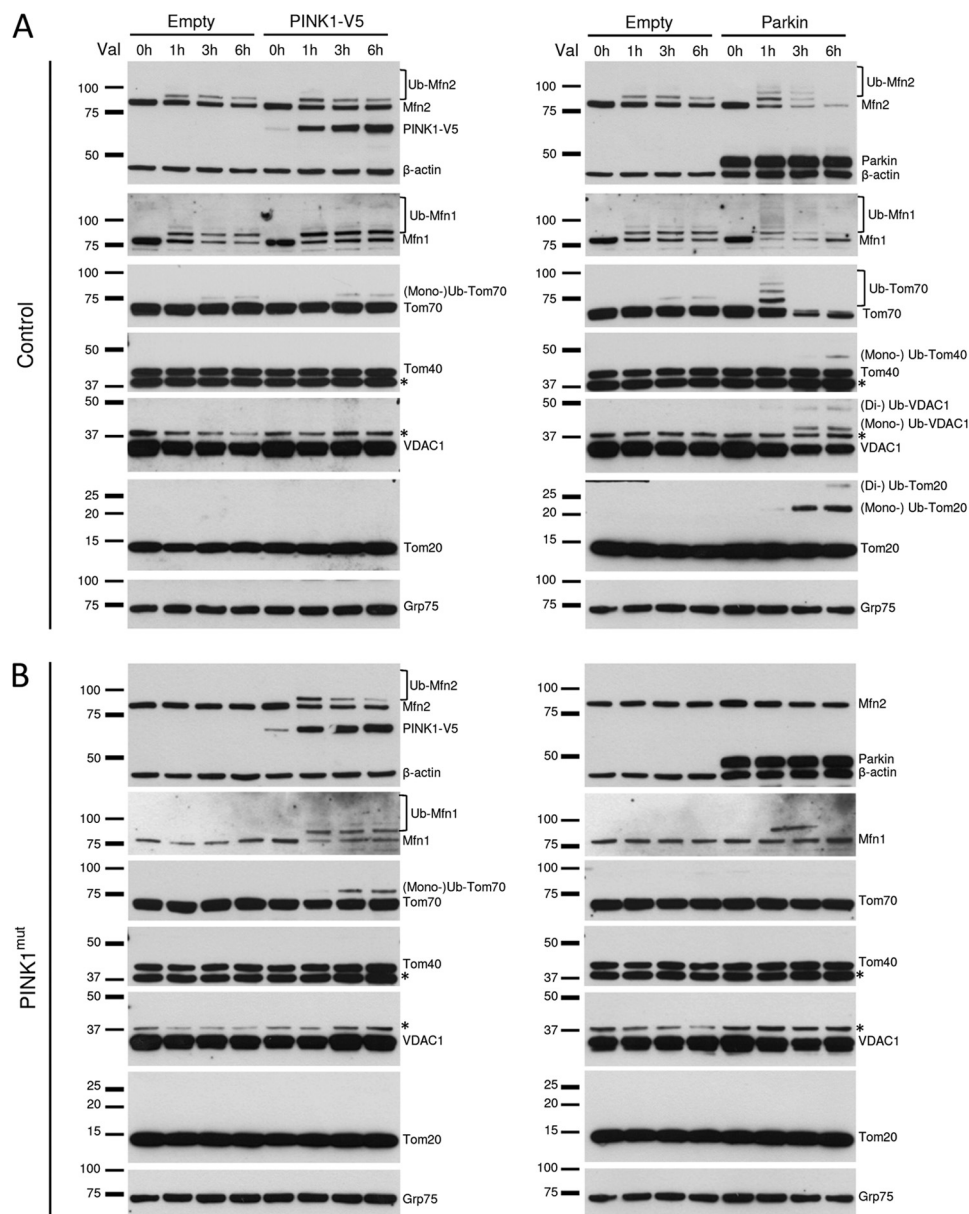


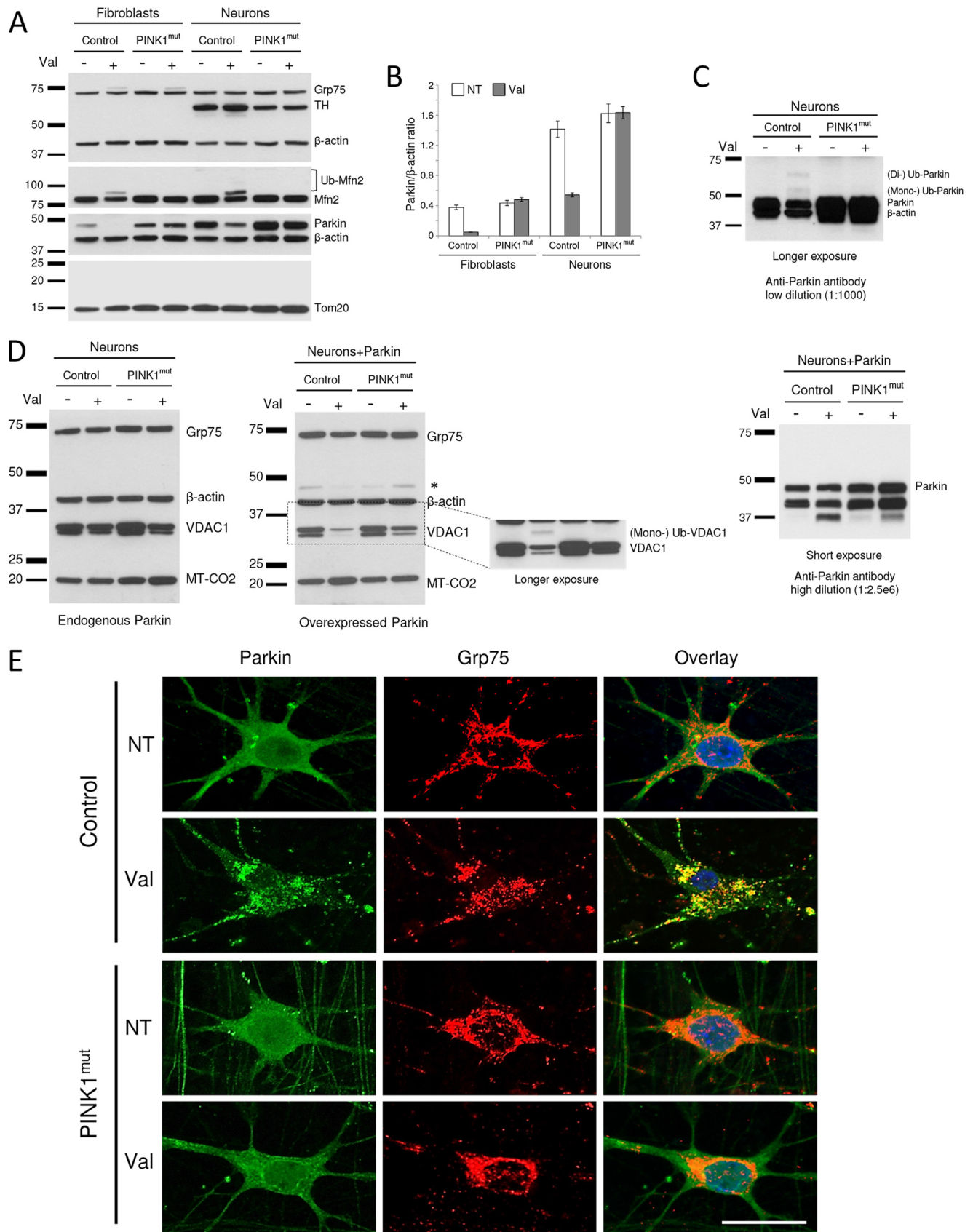
FIGURE 8. Overexpression of Parkin but not of PINK1 increases ubiquitination rates of outer mitochondrial membrane proteins. Fibroblasts from a control (A) or from a PINK1 mutant (B) stably expressing Parkin or PINK-V5 were treated with 1 μ M valinomycin. Cells were harvested at different time points, and total proteins were analyzed by Western blotting using antibodies against the following outer mitochondrial membrane proteins: Mfn1, Mfn2, Tom70, Tom40, VDAC1, and Tom20. Grp75 and β -actin served as a loading controls. Exogenous expression of Parkin and PINK1-V5 was confirmed using antibodies against Parkin and V5 tag. Val, valinomycin; *, nonspecific band; Ub, ubiquitin.

enous levels of Parkin, we detected no difference between controls and mitophagy-incompetent PINK1 mutants. Although a few studies reported mitophagy in cells expressing only endogenous levels of Parkin, mitophagy rates were at very low levels and dramatically differed from those in cells overexpressing Parkin (8, 24). Conversely, upon overexpression of Parkin, we detected a significant, PINK1-dependent decrease in levels of all mitochondrial proteins, possibly indicative of mitophagy. This was additionally confirmed using Mitotracker Green FM, an established and widely used method for measuring mitochondrial mass (8, 22, 25). In contrast, overexpression of PINK1, which acts in the same pathway as Parkin and is required for its mitochondrial translocation, had no effect on the levels of the mitochondrial proteins studied, suggesting that

levels of Parkin are the rate-limiting factor in this pathway. Of note, we cannot exclude that mitophagy takes place also under endogenous conditions, however, to a much lesser extent, which is difficult to detect. This could reflect the “endogenous” situation in human PD patients, in whom it takes decades to develop the disease.

We and others detected that Parkin is degraded upon stress in a PINK1-dependent manner and can be rescued upon inhibition of the ubiquitin proteasome system (UPS) (26, 27). Moreover, several groups reported ubiquitination of overexpressed YFP- and GFP-tagged Parkin coinciding with its mitochondrial translocation, however, suggesting that the GFP or YFP tags served as pseudo-substrates for the ubiquitination (10, 28). Here, we showed that endogenous (nontagged) Parkin

Ubiquitination of Endogenous Parkin Attenuates Mitophagy



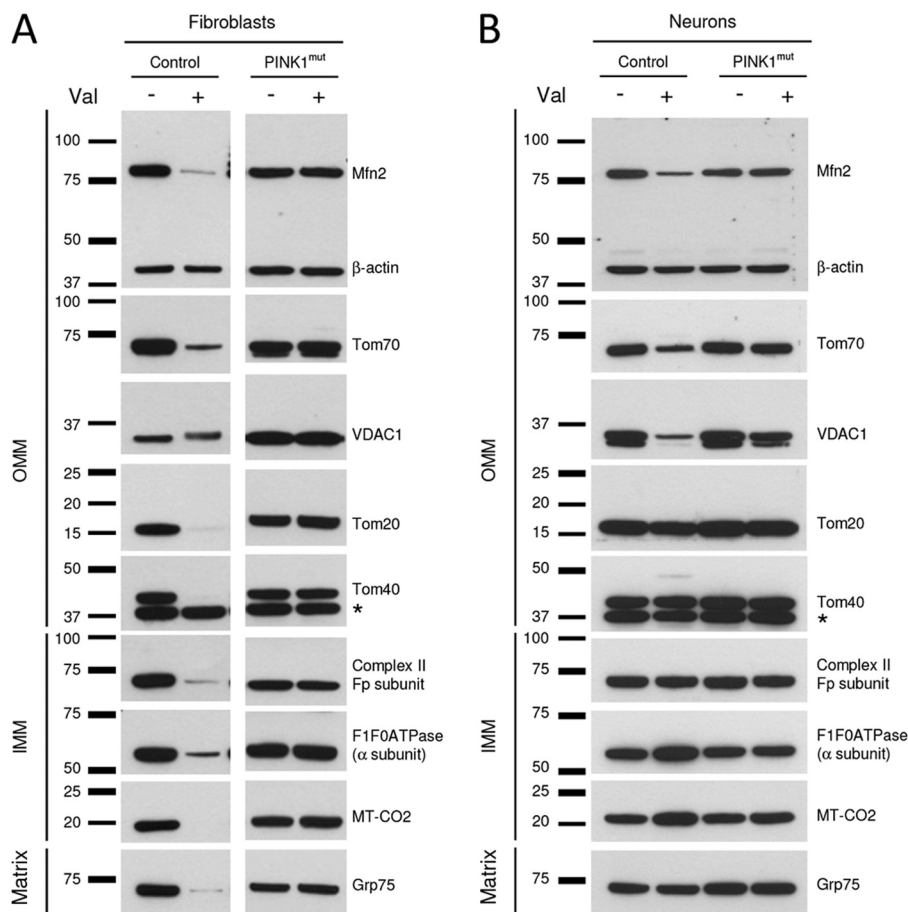


FIGURE 10. **PINK1-dependent valinomycin-induced degradation of mitochondrial proteins is observed in fibroblasts but not in iPS-derived neurons.** Fibroblasts (A) and iPS-derived neurons (B) from control and PINK1 mutant stably expressing Parkin were treated with valinomycin for 16 h. Extracted proteins were separated on SDS gels and immunoblotted against various mitochondrial proteins with β -actin serving as a loading control. IMM, inner mitochondrial membrane; Val, valinomycin; *, nonspecific band.

translocates to depolarized mitochondria but, at the same time, is getting (self-)ubiquitinated in a PINK1-dependent manner and likely degraded via the UPS. The observation that inhibition of the UPS caused deubiquitination but not preservation of ubiquitinated Parkin in fibroblasts was at first sight unexpected and may be explained as follows: Although ubiquitin chains consisting of at least four ubiquitin moieties are required for guiding a substrate to the proteasome, these conjugates eventually need to be removed by deubiquitinases associated with the 19 S subunit of the proteasome before degradation in the 20 S subunit (29). Epoxomycin is an inhibitor of the 20 S proteasome subunit but does not influence the deubiquitinase activity of the 19 S particle (30). Using neuroblastoma cells, we showed that Mfn2 and Parkin are ubiquitinated at a lower rate in cells expressing endogenous levels of Parkin. In these cells, inhibition of the UPS also results in deubiquitination rather than in preservation of ubiquitinated forms of these proteins. In con-

trast, when the ubiquitination rate of Mfn2 is increased upon overexpression of Parkin, inhibition of the UPS results in preservation of the ubiquitinated species of Mfn2, as one would have expected. In this context, it is again important to note that overexpressed Parkin differs in its response to valinomycin and/or epoxomycin treatment when compared with endogenous Parkin. A conceivable explanation of our observation in cells overexpressing Parkin could be as follows: Because of the high levels of Parkin in these cells, primarily nonubiquitinated forms of Parkin can be detected by Western blot, thus masking post-translational changes of Parkin observed at the endogenous level.

Based on our findings, we propose that ubiquitination of endogenous Parkin (and subsequent UPS-mediated degradation) decreases the “cytosolic” pool of Parkin and consequently results in lower levels of mitochondrially translocated Parkin, attenuating further mitophagy. Furthermore, loss of endoge-

FIGURE 9. **Valinomycin-induced mitophagy is not observed in iPS-generated neurons.** A, fibroblasts and iPS-derived neurons from a control and a PINK1 mutant were treated with 1 μ M valinomycin for 12 h. Proteins were extracted, separated on SDS gels, and immunoblotted using various antibodies. B, densitometric quantification of immunoblots probed with an antibody against Parkin. C, longer exposure of the immunoblot probed with an antibody against Parkin. D, iPS-derived neurons expressing endogenous levels of Parkin (left panel) or overexpressing Parkin (middle panel) were treated with 1 μ M valinomycin for 16 h. Cells were analyzed by Western blotting using antibodies against Grp75, VDAC1, and MT-CO2. β -Actin served as a loading control. Overexpressed Parkin was detected using high dilution of the anti-Parkin antibody (2500 \times in comparison with the dilution necessary to detect endogenous Parkin) (right panel). E, iPS-derived neurons from a control and a PINK1 mutant stably expressing Parkin were treated with 1 μ M valinomycin for 16 h. The cells were immunostained using antibodies against Parkin (green) and Grp75 (red). NT, nontreated; Val, valinomycin; Ub, ubiquitin. Scale bar, 25 μ m.

nous Parkin may play a broader role in PD pathology, because it has also been observed in cells from patients suffering from idiopathic PD (31).

Previously, we and others have shown that the large mitochondrial GTPases Mfn1 and Mfn2 are ubiquitinated in a PINK/Parkin-dependent manner and degraded via the UPS (10, 11, 32). Here, we extend these data, demonstrating Parkin-mediated ubiquitination of multiple OMM proteins on depolarized mitochondria. Interestingly, however, OMM proteins were ubiquitinated differentially with larger OMM proteins Mfn1 (~84 kDa), Mfn2 (~86 kDa), and Tom70 (~70 kDa) being rapidly ubiquitinated and intensities of nonubiquitinated bands severely reduced. Conversely, in the case of smaller OMM proteins Tom40 (~38 kDa), VDAC1 (~31 kDa), and Tom20 (~16 kDa), we detected only bands corresponding to mono- and di-ubiquitination of these proteins and no or only slight reduction in the intensities of the nonubiquitinated bands. Because Mfn1 and Mfn2 do not share any homology with Tom70, it is tempting to speculate that the degree of Parkin-mediated ubiquitination of OMM proteins may be size-dependent. However, this will have to be confirmed analyzing additional mitochondrial proteins of different size. In favor of our speculation are recent findings showing that two other large OMM proteins, Miro1/Rhot1 (~71 kDa) and Miro2/Rhot2 (~68 kDa), are rapidly degraded upon mitochondrial depolarization (12).

Analysis of mitophagy in iPS-derived neurons confirmed our findings from primary fibroblasts and neuroblastoma cells showing that endogenous Parkin is not sufficient to induce a detectable reduction in the levels of mitochondrial proteins because of its ubiquitination, although its protein levels were ~4-fold higher in neurons than in fibroblasts. Furthermore and in keeping with previous studies (14, 33), overexpression of Parkin failed to induce detectable mitophagy in iPS-derived neurons, suggesting a possible difference in this process between neurons and non-neuronal cells.

Taken together, our data have important implications for future directions of the study of Parkin-/PINK1-dependent mitophagy: (i) rates of mitophagy significantly differ in models using overexpressed compared with endogenous Parkin, warranting further studies at the endogenous level, and (ii) further study of mechanisms of mitophagy in iPS-generated neurons is needed to evaluate the utility of this recent PD-related human cellular model, which may serve as a basis to elucidate the selective vulnerability of the substantia nigra in PD.

REFERENCES

1. Langston, J. W., Ballard, P., Tetrud, J. W., and Irwin, I. (1983) Chronic Parkinsonism in humans due to a product of meperidine-analog synthesis. *Science* **219**, 979–980
2. Betarbet, R., Sherer, T. B., MacKenzie, G., Garcia-Osuna, M., Panov, A. V., and Greenamyre, J. T. (2000) Chronic systemic pesticide exposure reproduces features of Parkinson's disease. *Nat. Neurosci.* **3**, 1301–1306
3. Kitada, T., Asakawa, S., Hattori, N., Matsumine, H., Yamamura, Y., Minoshima, S., Yokochi, M., Mizuno, Y., and Shimizu, N. (1998) Mutations in the Parkin gene cause autosomal recessive juvenile parkinsonism. *Nature* **392**, 605–608
4. Valente, E. M., Abou-Sleiman, P. M., Caputo, V., Muqit, M. M., Harvey, K., Gispert, S., Ali, Z., Del Turco, D., Bentivoglio, A. R., Healy, D. G., Albanese, A., Nussbaum, R., González-Maldonado, R., Deller, T., Salvi, S., Cortelli,

- P., Gilks, W. P., Latchman, D. S., Harvey, R. J., Dallapiccola, B., Auburger, G., and Wood, N. W. (2004) Hereditary early-onset Parkinson's disease caused by mutations in PINK1. *Science* **304**, 1158–1160
5. Clark, I. E., Dodson, M. W., Jiang, C., Cao, J. H., Huh, J. R., Seol, J. H., Yoo, S. J., Hay, B. A., and Guo, M. (2006) *Drosophila* PINK1 is required for mitochondrial function and interacts genetically with Parkin. *Nature* **441**, 1162–1166
6. Exner, N., Treske, B., Paquet, D., Holmström, K., Schiesling, C., Gispert, S., Carballo-Carbajal, I., Berg, D., Hoepken, H. H., Gasser, T., Krüger, R., Winklhofer, K. F., Vogel, F., Reichert, A. S., Auburger, G., Kahle, P. J., Schmid, B., and Haass, C. (2007) Loss-of-function of human PINK1 results in mitochondrial pathology and can be rescued by Parkin. *J. Neurosci.* **27**, 12413–12418
7. Park, J., Lee, S. B., Lee, S., Kim, Y., Song, S., Kim, S., Bae, E., Kim, J., Shong, M., Kim, J. M., and Chung, J. (2006) Mitochondrial dysfunction in *Drosophila* PINK1 mutants is complemented by Parkin. *Nature* **441**, 1157–1161
8. Narendra, D. P., Jin, S. M., Tanaka, A., Suen, D. F., Gautier, C. A., Shen, J., Cookson, M. R., and Youle, R. J. (2010) PINK1 is selectively stabilized on impaired mitochondria to activate Parkin. *PLoS Biol.* **8**, e1000298
9. Vives-Bauza, C., Zhou, C., Huang, Y., Cui, M., de Vries, R. L., Kim, J., May, J., Tocilescu, M. A., Liu, W., Ko, H. S., Magrané, J., Moore, D. J., Dawson, V. L., Grailhe, R., Dawson, T. M., Li, C., Tieu, K., and Przedborski, S. (2010) PINK1-dependent recruitment of Parkin to mitochondria in mitophagy. *Proc. Natl. Acad. Sci. U.S.A.* **107**, 378–383
10. Tanaka, A., Cleland, M. M., Xu, S., Narendra, D. P., Suen, D. F., Karbowski, M., and Youle, R. J. (2010) Proteasome and p97 mediate mitophagy and degradation of mitofusins induced by Parkin. *J. Cell Biol.* **191**, 1367–1380
11. Rakovic, A., Grünwald, A., Kottwitz, J., Brüggemann, N., Pramstaller, P. P., Lohmann, K., and Klein, C. (2011) Mutations in PINK1 and Parkin impair ubiquitination of Mitofusins in human fibroblasts. *PLoS One* **6**, e16746
12. Chan, N. C., Salazar, A. M., Pham, A. H., Sweredoski, M. J., Kolawa, N. J., Graham, R. L., Hess, S., and Chan, D. C. (2011) Broad activation of the ubiquitin-proteasome system by Parkin is critical for mitophagy. *Hum. Mol. Genet.* **20**, 1726–1737
13. Yoshii, S. R., Kishi, C., Ishihara, N., and Mizushima, N. (2011) Parkin mediates proteasome-dependent protein degradation and rupture of the outer mitochondrial membrane. *J. Biol. Chem.* **286**, 19630–19640
14. Van Laar, V. S., Arnold, B., Cassady, S. J., Chu, C. T., Burton, E. A., and Berman, S. B. (2011) Bioenergetics of neurons inhibit the translocation response of Parkin following rapid mitochondrial depolarization. *Hum. Mol. Genet.* **20**, 927–940
15. Cai, Q., Zakaria, H. M., Simone, A., and Sheng, Z. H. (2012) Spatial Parkin translocation and degradation of damaged mitochondria via mitophagy in live cortical neurons. *Curr. Biol.* **22**, 545–552
16. Seibler, P., Graziotto, J., Jeong, H., Simunovic, F., Klein, C., and Krainc, D. (2011) Mitochondrial Parkin recruitment is impaired in neurons derived from mutant PINK1 induced pluripotent stem cells. *J. Neurosci.* **31**, 5970–5976
17. Almeida, A., and Medina, J. M. (1997) Isolation and characterization of tightly coupled mitochondria from neurons and astrocytes in primary culture. *Brain Res.* **764**, 167–172
18. Geisler, S., Holmström, K. M., Skujat, D., Fiesel, F. C., Rothfuss, O. C., Kahle, P. J., and Springer, W. (2010) PINK1/Parkin-mediated mitophagy is dependent on VDAC1 and p62/SQSTM1. *Nat. Cell Biol.* **12**, 119–131
19. Greenwood, S. M., Mizielinska, S. M., Frenguelli, B. G., Harvey, J., and Connolly, C. N. (2007) Mitochondrial dysfunction and dendritic beading during neuronal toxicity. *J. Biol. Chem.* **282**, 26235–26244
20. Wang, X., Winter, D., Ashrafi, G., Schlehe, J., Wong, Y. L., Selkoe, D., Rice, S., Steen, J., LaVoie, M. J., and Schwarz, T. L. (2011) PINK1 and Parkin target Miro for phosphorylation and degradation to arrest mitochondrial motility. *Cell* **147**, 893–906
21. Kim, K. Y., Stevens, M. V., Akter, M. H., Rusk, S. E., Huang, R. J., Cohen, A., Noguchi, A., Springer, D., Bocharov, A. V., Eggerman, T. L., Suen, D. F., Youle, R. J., Amar, M., Remaley, A. T., and Sack, M. N. (2011) Parkin is a lipid-responsive regulator of fat uptake in mice and mutant human cells. *J. Clin. Invest.* **121**, 3701–3712
22. Rodriguez-Enriquez, S., Kim, I., Currin, R. T., and Lemasters, J. J. (2006)

- Tracker dyes to probe mitochondrial autophagy (mitophagy) in rat hepatocytes. *Autophagy* **2**, 39–46
23. Ziviani, E., and Whitworth, A. J. (2010) How could Parkin-mediated ubiquitination of mitofusin promote mitophagy? *Autophagy* **6**, 660–662
 24. Gegg, M. E., Cooper, J. M., Chau, K. Y., Rojo, M., Schapira, A. H., and Taanman, J. W. (2010) Mitofusin 1 and mitofusin 2 are ubiquitinated in a PINK1/Parkin-dependent manner upon induction of mitophagy. *Hum. Mol. Genet.* **19**, 4861–4870
 25. Kundu, M., Lindsten, T., Yang, C. Y., Wu, J., Zhao, F., Zhang, J., Selak, M. A., Ney, P. A., and Thompson, C. B. (2008) Ulk1 plays a critical role in the autophagic clearance of mitochondria and ribosomes during reticulocyte maturation. *Blood* **112**, 1493–1502
 26. Rakovic, A., Grünwald, A., Seibler, P., Ramirez, A., Kock, N., Orolicki, S., Lohmann, K., and Klein, C. (2010) Effect of endogenous mutant and wild-type PINK1 on Parkin in fibroblasts from Parkinson disease patients. *Hum. Mol. Genet.* **19**, 3124–3137
 27. Xiong, H., Wang, D., Chen, L., Choo, Y. S., Ma, H., Tang, C., Xia, K., Jiang, W., Ronai, Z., Zhuang, X., and Zhang, Z. (2009) Parkin, PINK1, and DJ-1 form a ubiquitin E3 ligase complex promoting unfolded protein degradation. *J. Clin. Invest.* **119**, 650–660
 28. Matsuda, N., Sato, S., Shiba, K., Okatsu, K., Saisho, K., Gautier, C. A., Sou, Y. S., Saiki, S., Kawajiri, S., Sato, F., Kimura, M., Komatsu, M., Hattori, N., and Tanaka, K. (2010) PINK1 stabilized by mitochondrial depolarization recruits Parkin to damaged mitochondria and activates latent Parkin for mitophagy. *J. Cell Biol.* **189**, 211–221
 29. Verma, R., Aravind, L., Oania, R., McDonald, W. H., Yates, J. R., 3rd, Koonin, E. V., and Deshaies, R. J. (2002) Role of Rpn11 metalloprotease in deubiquitination and degradation by the 26S proteasome. *Science* **298**, 611–615
 30. Verma, R., and Deshaies, R. J. (2005) Assaying degradation and deubiquitination of a ubiquitinated substrate by purified 26S proteasomes. *Methods Enzymol.* **398**, 391–399
 31. Shin, J. H., Ko, H. S., Kang, H., Lee, Y., Lee, Y. I., Pletinkova, O., Troconso, J. C., Dawson, V. L., and Dawson, T. M. (2011) PARIS (ZNF746) repression of PGC-1 α contributes to neurodegeneration in Parkinson's disease. *Cell* **144**, 689–702
 32. Ziviani, E., Tao, R. N., and Whitworth, A. J. (2010) *Drosophila* Parkin requires PINK1 for mitochondrial translocation and ubiquitinates mitofusin. *Proc. Natl. Acad. Sci. U.S.A.* **107**, 5018–5023
 33. Sterky, F. H., Lee, S., Wibom, R., Olson, L., and Larsson, N. G. (2011) Impaired mitochondrial transport and Parkin-independent degeneration of respiratory chain-deficient dopamine neurons in vivo. *Proc. Natl. Acad. Sci. U.S.A.* **108**, 12937–12942
 34. Mizzen, L. A., Chang, C., Garrels, J. I., and Welch, W. J. (1989) Identification, characterization, and purification of two mammalian stress proteins present in mitochondria, grp 75, a member of the hsp 70 family and hsp 58, a homolog of the bacterial groEL protein. *J. Biol. Chem.* **264**, 20664–20675
 35. Burté, F., De Girolamo, L. A., Hargreaves, A. J., and Billett, E. E. (2011) Alterations in the mitochondrial proteome of neuroblastoma cells in response to complex 1 inhibition. *J. Proteome Res.* **10**, 1974–1986