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The E3 Ligase Parkin Maintains Mitochondrial Integrity by Increasing Linear Ubiquitination of NEMO

Anne Kathrin Müller-Rischart,[1,3] Anna Pilsl,[1] Patrick Beaudette,[4] Maria Patra,[1] Kamyar Hadian,[5] Maria Funke,[1] Regina Peis,[6] Alexandra Deinlein,[1] Carolin Schweimer,[1] Peer-Hendrik Kuhn,[3] Stefan F. Lichtenthaler,[2,3] Elisa Motori,[1,8] Silvana Hrelia,[8] Wolfgang Wurst,[2,3,6,9] Dietrich Trümbach,[6] Thomas Langer,[10] Daniel Krappmann,[7] Gunnar Dittmar,[4] Jörg Tatzelt,[1,3] and Konstanze F. Winklhofer[1,2,3,*]

[1]Neurobiochemistry, Adolf Butenandt Institute, Ludwig Maximilians University, 80336 Munich, Germany
[2]Munich Cluster for Systems Neurology (SyNergy), 80336 Munich, Germany
[3]German Center for Neurodegenerative Diseases, 80336 Munich, Germany
[4]Max Delbrück Center for Molecular Medicine, 13125 Berlin, Germany
[5]Assay Development and Screening Platform, Institute of Molecular Toxicology and Pharmacology
[6]Institute of Developmental Genetics
[7]Research Unit Cellular Signal Integration, Institute of Molecular Toxicology and Pharmacology
Helmholtz Zentrum München—German Research Center for Environmental Health, 85764 Munich/Neuherberg, Germany
[8]Department of Life Quality Studies, Alma Mater Studiorum—University of Bologna, 40126 Bologna, Italy
[9]Max Planck Institute of Psychiatry, 80804 Munich, Germany
[10]Cologne Cluster of Excellence in Cellular Stress Responses in Aging-Associated Diseases, Center for Molecular Medicine, Institute for Genetics, University of Cologne, 50674 Cologne, Germany
*Correspondence: konstanze.winklhofer@med.uni-muenchen.de
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SUMMARY

Parkin, a RING-between-RING-type E3 ubiquitin ligase associated with Parkinson's disease, has a wide neuroprotective activity, preventing cell death in various stress paradigms. We identified a stress-protective pathway regulated by parkin that links NF-κB signaling and mitochondrial integrity via linear ubiquitination. Under cellular stress, parkin is recruited to the linear ubiquitin assembly complex and increases linear ubiquitination of NF-κB essential modulator (NEMO), which is essential for canonical NF-κB signaling. As a result, the mitochondrial guanosine triphosphatase OPA1 is transcriptionally upregulated via NF-κB-responsive promoter elements for maintenance of mitochondrial integrity and protection from stress-induced cell death. Parkin-induced stress protection is lost in the absence of either NEMO or OPA1, but not in cells defective for the mitophagy pathway. Notably, in parkin-deficient cells linear ubiquitination of NEMO, activation of NF-κB, and upregulation of OPA1 are significantly reduced in response to TNF-α stimulation, supporting the physiological relevance of parkin in regulating this antiapoptotic pathway.

INTRODUCTION

The modification of target proteins with ubiquitin has emerged as a highly versatile mechanism for regulating a plethora of biological functions. The functional outcome of ubiquitination depends on the type of ubiquitin linkage, determined by E2 ubiquitin-conjugating enzymes and/or E3 ligases. The E3 ubiquitin ligase parkin belongs to the RING-between-RING (RBR) family and can apparently catalyze different modes of ubiquitination in a context-specific manner (reviewed in Exner et al., 2012). RBR ligases were initially considered a subtype of RING ligases, which facilitate direct ubiquitin transfer from the ubiquitin-charged E2 to a lysine of the substrate protein. In contrast, HECT ligases form a thioester with ubiquitin before ligation onto a substrate. Two members of the RBR family, HHARI and HOIP, have recently been shown to function as RING/HECT hybrids in that they bind ubiquitin-charged E2s via RING1 and then form a ubiquitin thioester intermediate via a conserved cysteine residue in RING2 (Smit et al., 2012; Stieglitz et al., 2012; Wenzel et al., 2011). Although a parkin thioester intermediate initially could not be trapped, it can catalyze ubiquitination with lysine-unreactive but cysteine-reactive E2s, suggesting that the RING/HECT hybrid mechanism is a general feature of RBR ligases (Wenzel et al., 2011). Indeed, an oxyester intermediate was recently detected for the C431S parkin mutant under conditions of mitochondrial depolarization (Lazarou et al., 2013).

About 30 putative parkin substrates have been reported to date (reviewed in Dawson and Dawson, 2010). These putative substrates do not allow integration of the function of parkin into a unifying pathway, and the physiological and pathological relevance has been demonstrated for only a few of these substrates. Nonetheless, loss-of-function mutations in the *parkin* gene cause autosomal-recessive parkinsonism, indicating that parkin plays a role in maintaining neuronal integrity and function (Kitada et al., 1998). In line with this notion, parkin shows a wide neuroprotective activity in vitro and in vivo, preventing cell death

in various stress paradigms, such as mitochondrial stress, endoplasmic reticulum (ER) stress, excitotoxicity, and proteotoxic stress (reviewed in Dawson and Dawson, 2010; Exner et al., 2012).

Parkin has recently been implicated in a mitochondrial quality-control pathway to induce the removal of damaged mitochondria via mitophagy (Narendra et al., 2008). Upon dissipation of the mitochondrial membrane potential, parkin translocates to mitochondria, and remodeling of the outer mitochondrial membrane then recruits the autophagic machinery for mitochondrial clearance. It has been speculated that this mitochondrial quality-control pathway underlies the wide protective activity of parkin. We therefore experimentally addressed the question of whether parkin-induced mitophagy is sufficient to explain its prosurvival function. Our study revealed that the stress-protective activity of parkin is not compromised in the absence of mitophagy, but depends on NEMO (NF-κB essential modulator), the core regulatory component of the IκB kinase complex essential for canonical NF-κB signaling (Makris et al., 2000; Schmidt-Supprian et al., 2000). Parkin can enhance linear ubiquitination of NEMO via the linear ubiquitin chain assembly complex (LUBAC), resulting in increased expression of the mitochondrial guanosine triphosphatase (GTPase) OPA1, which we identified as an NF-κB target gene. OPA1 is a key regulator of cristae integrity and promotes mitochondrial fusion, which explains why parkin can mediate both cell survival and prevention of mitochondrial fragmentation under cellular stress.

RESULTS

The Stress-Protective Activity of Parkin Is Dependent on NEMO

To study the stress-protective activity of parkin, we employed three stress paradigms in human neuroblastoma SH-SY5Y cells. Cells moderately overexpressing wild-type (WT) parkin were treated with thapsigargin (TG), carbonyl cyanide 3-chlorophenylhydrazone (CCCP), or staurosporine (STS) for inducing cellular stress. TG acts as an inhibitor of the ER Ca^{2+} ATPase, causing an imbalance in calcium homeostasis and ER stress. CCCP renders the mitochondrial inner membrane permeable for protons and leads to dissipation of the proton gradient. STS induces apoptotic cell death by inhibiting several kinases. In all paradigms, parkin was able to prevent stress-induced cell death (Figure 1A). Supporting a protective activity of endogenous parkin, loss of parkin sensitized parkin-deficient SH-SY5Y cells to stress-induced cell death, and this phenomenon could be rescued by small interfering RNA (siRNA)-resistant WT parkin (Figure 1B; see Figure S1A, available online, for a different parkin siRNA). Moreover, a significant increase in apoptotic cell death upon stress treatment was also observed in primary cortical neurons and mouse embryonic fibroblasts (MEFs) from parkin knockout (KO) mice (Figures 1C–1F).

Parkin-induced mitophagy results in the elimination of damaged mitochondria via the autophagic machinery. To test the impact of mitophagy on the stress-protective activity of parkin, we made use of MEFs lacking essential components of the mitophagy pathway. The mitochondrial kinase PINK1 is required for recruitment of parkin to uncoupled mitochondria, ATG5 is a core component of the autophagic machinery, and the adaptor protein p62 links ubiquitinated cargo to the phagophore by binding to both ubiquitin and LC3 (Geisler et al., 2010; Narendra et al., 2008; Vives-Bauza et al., 2010). Surprisingly, the activity of parkin to prevent stress-induced cell death was not compromised in the absence of PINK1, ATG5, or p62 (Figures 2A–2D). In addition, recruitment of parkin to mitochondria is not required for its stress-protective activity, because only CCCP, but not TG or STS, induced mitochondrial translocation of parkin (Figure S1B). Moreover, a parkin mutant that lacks the N-terminal ubiquitin-like (UBL) domain (ΔUBL parkin) was not impaired in inducing mitophagy but had no prosurvival activity and could not rescue parkin deficiency (Figures S2A–S2C; Figure 1B), corroborating that mitophagy and stress protection are mediated by parkin via separate pathways.

Because parkin can stimulate canonical NF-κB signaling (Henn et al., 2007), we addressed the question of whether the prosurvival activity of parkin is dependent on this pathway by using MEFs from NEMO KO mice. NEMO, the core regulatory component of the IκB kinase complex, is essential for canonical NF-κB activation in response to all known stimuli (Makris et al., 2000; Schmidt-Supprian et al., 2000). Indeed, parkin was not able to prevent stress-induced cell death in NEMO KO MEFs (Figure 2E). Importantly, the protective effect of parkin was fully restored in NEMO KO MEFs upon reintroduction of NEMO by transient transfection (Figure 2F). In line with the existence of two independent pathways for mitophagy and stress protection, parkin-induced mitophagy was not impaired in NEMO-deficient cells (Figures S2D and S2E).

Parkin Induces Increased Expression of OPA1 in a NEMO-Dependent Manner

Parkin can reduce cytochrome *c* release from mitochondria in response to proapoptotic stimuli (Figure 3A) (Darios et al., 2003), indicating that the stress-protective activity of parkin could be linked to a mitochondrial pathway different from mitophagy. Cytochrome *c* release is controlled by OPA1, a dynamin-related GTPase located in the mitochondrial intermembrane space that maintains cristae structure and is implicated in the regulation of inner membrane fusion and apoptosis (Frezza et al., 2006; Olichon et al., 2003). To test whether parkin may have an effect on OPA1, we expressed parkin in WT and NEMO KO MEFs and analyzed OPA1 expression and processing. We did not observe an obvious effect of parkin on the processing of OPA1 induced by CCCP, but we noticed striking differences in the protein levels of OPA1 (Figure 3B). The increased expression of parkin induced a significant upregulation specifically of OPA1 protein and messenger RNA (mRNA) levels in WT MEFs both under basal and stress conditions, but not in NEMO KO MEFs (Figures 3C and 3D; Figure S3), indicating that parkin-induced upregulation of OPA1 requires NEMO. Supporting this idea, parkin was not able to prevent STS-induced cytochrome *c* release in the absence of NEMO (Figure 3A). A bioinformatical analysis revealed that the human OPA1 promoter contains three putative binding sites for NF-κB family members in proximity to the transcriptional start site (TSS) that are highly conserved among species, supporting

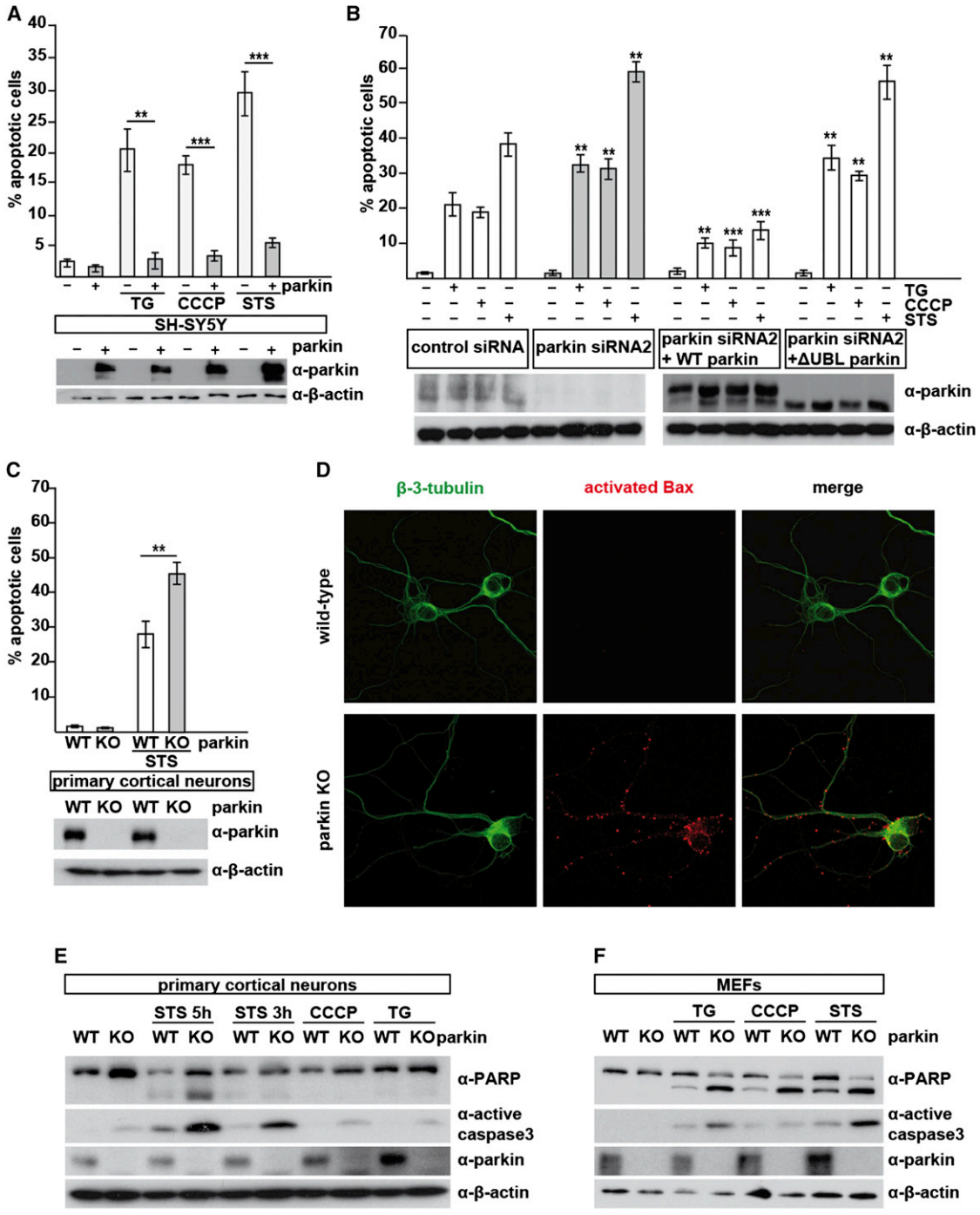

Figure 1. Parkin Prevents Stress-Induced Cell Death

(A) Increased expression of parkin confers stress protection in SH-SY5Y cells. Cells transiently expressing parkin (parkin +) or EYFP as a control (parkin −) were subjected to different cellular stressors (TG, CCCP, or STS). Apoptotic cell death was visualized by indirect immunofluorescence and quantified by counting transfected cells positive for activated caspase-3.

(B–E) Parkin-deficient cells are vulnerable to stress-induced cell death.

(B) SH-SY5Y cells transfected with parkin siRNA2 were treated with TG, CCCP, or STS. For rescue experiments, siRNA-resistant WT or ΔUBL parkin was cotransfected.

(C and D) Primary cortical neurons from parkin KO or WT mice were treated with STS. Apoptotic cell death was visualized by indirect immunofluorescence and quantified by counting neurons positive for activated Bax. Representative images are shown in (D).

(E) Apoptosis of primary cortical neurons from WT or parkin KO mice in response to TG, CCCP, or STS was monitored by western blotting using an antibody for PARP and active caspase-3.

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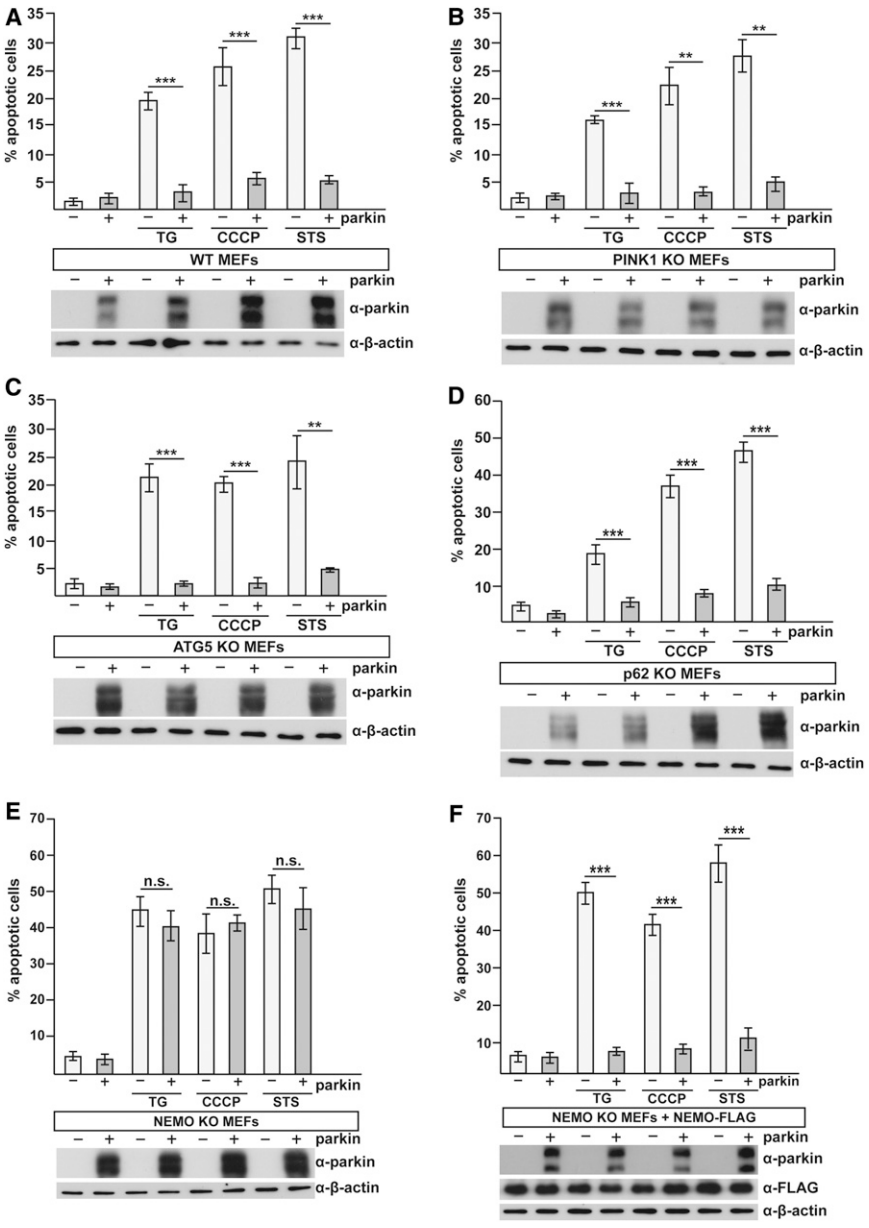

Figure 2. The Stress-Protective Activity of Parkin Is Not Compromised in Mitophagy-Deficient Models but Depends on NEMO

(A–F) MEFs from WT mice (A), PINK1 KO mice (B), ATG5 KO mice (C), p62 KO mice (D), or NEMO KO mice (E and F) transiently transfected with parkin (parkin +) or vector as a control (parkin −) were subjected to different cellular stressors (TG, CCCP, or STS). In (F), NEMO KO MEFs were reconstituted with NEMO by transient transfection. Apoptosis was quantified as described in Figure 1. Data represent the mean ± SEM of three to six independent experiments, each performed in triplicate. n ≥ 2,500 transfected cells. **p ≤ 0.01; ***p ≤ 0.001. See also Figure S2.

functional relevance (Figure 3E). To test a possible transcriptional regulation of OPA1 by NF-κB, we generated luciferase reporter constructs comprising either a longer part of the OPA1 promoter with three putative NF-κB binding elements (OPA1-Luc3) or a shorter part with two putative NF-κB binding elements (OPA1-Luc2) (Figure 3E). These reporter constructs were not only induced by activators of NF-κB (tumor necrosis factor α [TNF-α] treatment and coexpression of IKK-β) but also by the coexpression of WT parkin, whereas the ΔUBL parkin mutant, which lacks stress-protective activity, was not effective (Figures 3F and 3G). Mutation of the NF-κB binding motifs abrogated sensitivity of the reporter construct to TNF-α, IKK-β, or parkin (Figure 3G, mutOPA1-Luc2). These experiments confirmed that the binding elements identified in the OPA1 promoter are functional NF-κB-responsive elements.

OPA1 Acts Downstream of NEMO to Mediate the Stress-Protective Activity of Parkin

For validation of our observations in models of parkin deficiency, we first analyzed OPA1 levels in SH-SY5Y cells transiently depleted of parkin by RNA interference. Decreased OPA1 levels were observed upon parkin silencing, accompanied by an increase in mitochondrial fragmentation (Figures 4A, S4A, and S4B). Similarly, primary cortical neurons from parkin KO mice displayed a moderate but significant decrease in OPA1 levels, which were more pronounced under stress conditions (Figure 4B). Next, we wondered whether OPA1 might be a downstream effector of the stress-protective activity of parkin and tested whether parkin can protect from stress-induced cell death in the absence of OPA1. Strikingly, parkin was not able to prevent apoptotic cell death in STS-treated MEFs from OPA1 KO mice (Figure 4C). Reintroduction of OPA1 into OPA1 KO MEFs fully restored the activity of parkin to prevent STS-induced cell death, whereas the increased expression of NEMO was not effective, indicating that NEMO is acting upstream of OPA1 (Figure 4C). The impaired activity

(F) Apoptosis of WT or parkin KO MEFs treated with TG, CCCP, or STS was determined by western blotting using antibodies for PARP or active caspase-3.

Data represent the mean ± SEM of at least three independent experiments, each performed in triplicate. At least 2,500 transfected cells or 1,000 neurons were assessed per condition. **p ≤ 0.01; ***p ≤ 0.001. See also Figure S1.

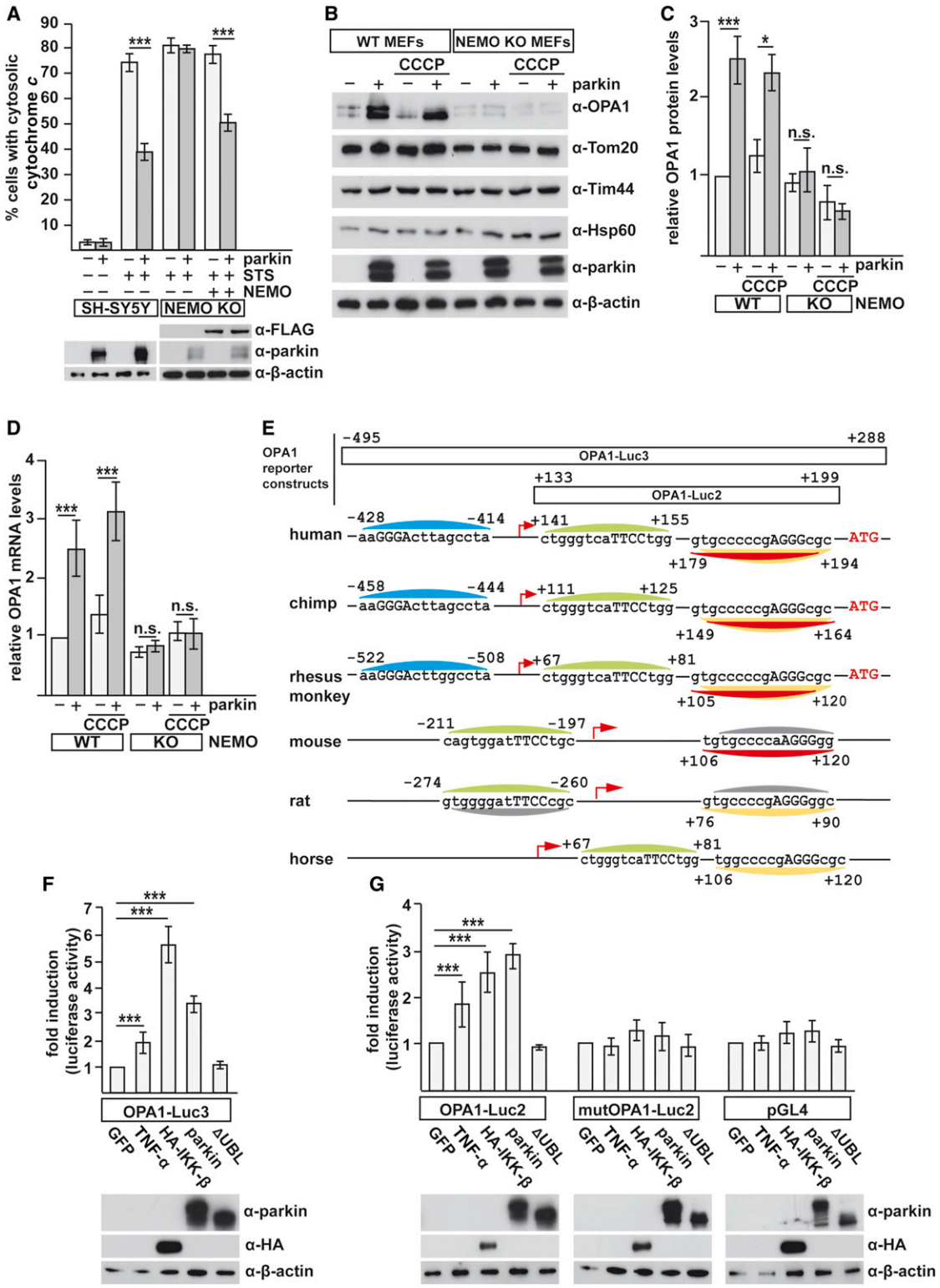

Figure 3. Parkin Increases Expression of OPA1 in a NEMO-Dependent Manner

(A) Parkin reduces cytochrome *c* release. SH-SY5Y cells or NEMO KO MEFs expressing parkin were treated with STS, and translocation of cytochrome *c* was detected by indirect immunofluorescence. In a subset of NEMO KO MEFs, NEMO was reintroduced by transient transfection. Quantification is based on triplicates of three independent experiments. n ≥ 1,000 transduced or transfected cells.

(B–D) Parkin increases OPA1 expression.

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of parkin to prevent STS-induced cell death in NEMO KO MEFs could be restored by NEMO, as already shown in Figure 2F for various stressors, but also by OPA1, confirming that OPA1 is downstream of NEMO (Figure 4D). In support of OPA1 being an essential downstream mediator of parkin, the increased expression of OPA1 was sufficient to prevent stress-induced cell death similarly to parkin in both MEFs and SH-SY5Y cells in all stress paradigms tested (Figures 4C–4E; see Figures S4C and S4D for other stressors). The activity of parkin to induce mitophagy was not impaired in OPA1 KO MEFs (Figure S4E), confirming the concept that parkin-induced mitophagy is independent from the stress-protective activity of parkin.

Parkin Increases Linear Ubiquitination of NEMO by LUBAC

Based on these findings, the question emerged of how the E3 ligase activity of parkin is linked to its stress-protective function. The NF-κB activation cascade is a paradigm for the role of ubiquitination in regulating signaling pathways, and both degradative and nondegradative ubiquitination is essential for inducing NF-κB signaling (reviewed in Iwai, 2012). An alternative mode of ubiquitin linkage involves the formation of linear head-to-tail polyubiquitin chains in which the C-terminal glycine of ubiquitin is conjugated to the α-amino group of the N-terminal methionine (M1) of another ubiquitin molecule (Kirisako et al., 2006). Formation of linear or M1-linked ubiquitin chains is catalyzed by LUBAC composed of two RBR proteins, HOIL-1L and HOIP, and SHARPIN as an adaptor subunit (Gerlach et al., 2011; Ikeda et al., 2011; Tokunaga et al., 2011). Linear ubiquitination of NEMO by LUBAC is required for efficient activation of the canonical NF-κB pathway by TNF-α and other inducers (Haas et al., 2009; Niu et al., 2011; Rahighi et al., 2009; Tokunaga et al., 2009). Remarkably, NEMO is not only modified by linear ubiquitination but also binds to linear ubiquitin chains via its UBAN (ubiquitin binding in ABIN and NEMO) domain. Therefore, the recombinant UBAN domain of NEMO can be employed to pull down proteins with linear ubiquitin chains (Figure S5A) (Hadian et al., 2011; Komander et al., 2009; Rahighi et al., 2009). We noticed a striking similarity of the modular structure between parkin and HOIL-1L, which prompted us to investigate a possible effect of parkin on linear ubiquitination. Lysates of HEK293T cells expressing either HOIL-1L and HOIP; parkin and HOIP; parkin and HOIL-1L; or parkin, HOIL-1L, and HOIP were incubated with the recombinant UBAN domain carrying an N-terminal Strep-Tag II, which allows affinity purification by Strep-Tactin resins. Proteins bound to the UBAN domain were then analyzed by western blotting using a ubiquitin antibody. Coexpression of parkin significantly enhanced linear ubiquitination in HOIL-1L- and HOIP-expressing cells, indicating that parkin can increase the activity of LUBAC (Figures 5A and S5B, left blots). Increased linear ubiquitination of NEMO in the presence of parkin was quantified using a selected reaction monitoring (SRM)-based mass spectrometry approach. Relative quantification of samples was achieved using isotope-labeled peptides (SpikeTides, JPT) and normalizing to a NEMO-derived peptide, which acted as a control for pull-down efficiency. Formation of linear ubiquitin chains was increased 3-fold by WT parkin, but not by the ΔUBL parkin mutant (Figure 5B). We also tested the pathogenic parkin mutants W453X and G430D, which show impaired E3 ligase activity in vivo (Chung et al., 2001; Geisler et al., 2010). Similarly to the ΔUBL mutant, these parkin mutants did not prevent stress-induced cell death (Figure 5C) and could not enhance LUBAC activity (Figures 5A and S5B, middle blots). Pull-down experiments with D311N-UBAN, a mutant defective in the binding of linear ubiquitin chains (Hadian et al., 2011; Rahighi et al., 2009) (Figure S5A), did not enrich ubiquitinated proteins in either of the samples (Figures 5A and S5B, right blots). To test a possible impact of endogenous parkin on linear ubiquitination, we performed linear ubiquitination assays in parkin-deficient cells. Human embryonic kidney (HEK) 293T cells silenced for parkin expression by RNA interference were transfected with HOIP and HOIL-1L for inducing linear ubiquitination of NEMO. In parkin-deficient cells, linear ubiquitination was reduced in comparison to control siRNA-treated cells, and this observation could be confirmed by quantitative mass spectrometry (Figures 5D, 5E, and S5C). Differences in the efficiency of linear ubiquitination between control and parkin knockdown cells were also obvious upon TNF-α treatment (Figure 5D). Because TNF-α signaling is critically dependent on LUBAC-mediated linear ubiquitination of NEMO, we chose this paradigm to establish a linear ubiquitination assay exclusively based on endogenous components. In comparison to WT MEFs, the ubiquitin signal originating from M1-linked chains was clearly weaker in MEFs

(B) WT and NEMO KO MEFs stably expressing parkin or GFP as a control were treated with CCCP for 3 hr, and OPA1 levels and processing were analyzed by western blotting 8 hr after treatment. Tom20, Tim44, Hsp60, and β-actin were immunoblotted as controls.
(C) The densitometric analysis of protein levels is based on western blots from at least five independent experiments. OPA1 levels in untreated WT MEFs were set as 1.
(D) OPA1-specifc mRNA of the experiment described in (B) was quantified by RT-PCR based on four independent experiments.
(E) The OPA1 promoter harbors functional NF-κB-responsive elements. Schematic representation of OPA1 promoter sequences from different mammalian species. The red arrow indicates the TSS, and positions are denoted relative to the TSS. The translation start site is indicated by a red ATG. NF-κB binding sites are denoted by semicircles. Blue (V$NFKAPPAB.02), green (V$NFKAPPAB65.01), red (V$NFKAPPAB.01), and yellow (V$NFKAPPAB50.01) binding sites are predicted by four different binding motifs that correspond to a Genomatix-defined family of seven matrices describing the NF-κB binding site. Blue and green NF-κB binding sites are located on the plus strand; red and yellow sites are located on the minus strand. Gray semicircles (not conserved) represent additional NF-κB binding sites. Regions cloned into the OPA1-Luc3 and OPA1-Luc2 reporter constructs are indicated.
(F and G) HEK 293T cells were transfected with the OPA1-Luc3 (F) or OPA1-Luc2 reporter (G). In mutOPA1-Luc2 the two NF-κB binding motifs are mutated. Cells were treated with TNF-α or cotransfected with hemagglutinin (HA)-IKK-β, parkin, or ΔUBL parkin. Luciferase activity in cell lysates was determined 16 hr after TNF-α treatment or 1 day after cotransfection. Luciferase activity in control GFP-expressing cells was set as 1. Quantifications are based on at least four independent experiments performed in triplicate.
Data represent the mean ± SEM. *$p \leq 0.05$; ***$p \leq 0.001$. See also Figure S3.

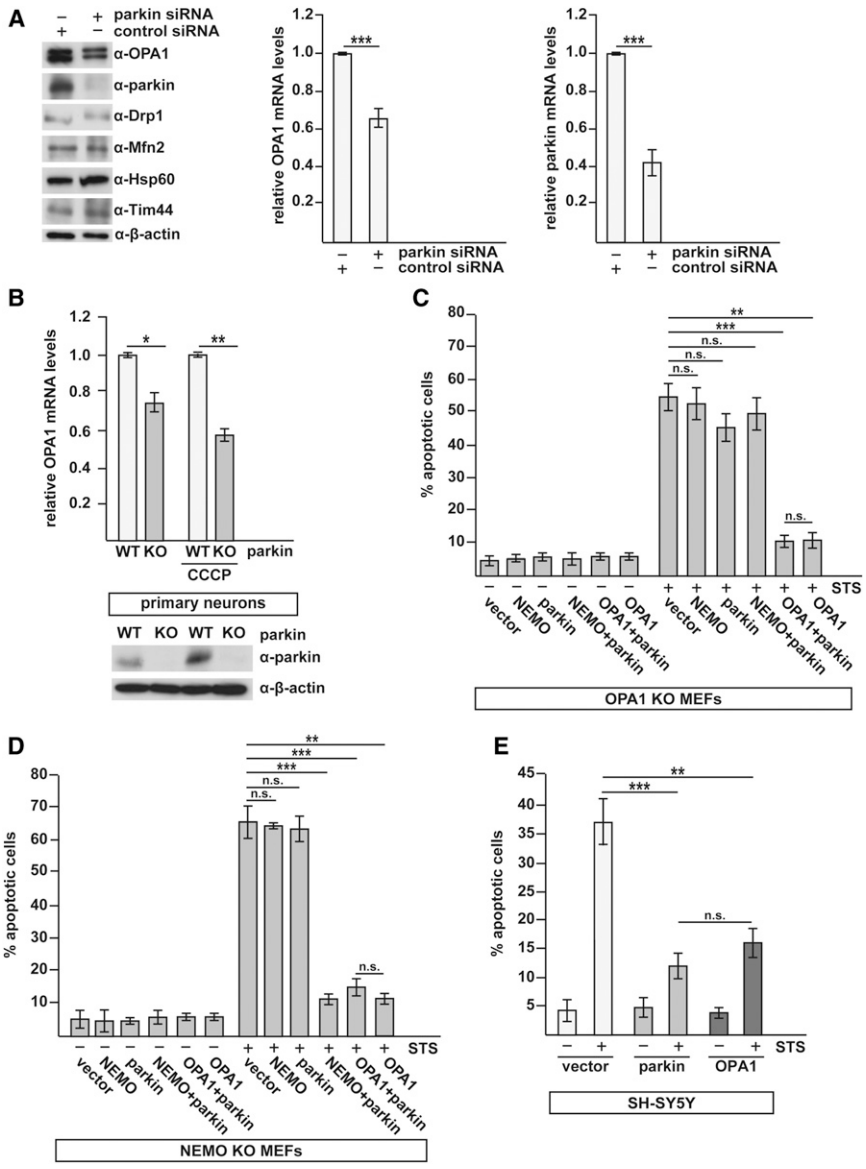

Figure 4. OPA1 Is an Essential Downstream Mediator of the Stress-Protective Activity of Parkin

(A) Transient silencing of parkin decreases OPA1 levels. SH-SY5Y cells were analyzed for OPA1 levels by immunoblotting 3 days after transfection with control or parkin siRNA. Drp1, Mfn2, Hsp60, Tim44, and β-actin were immunoblotted as controls (left panel). Quantifications of OPA1 (middle panel) or parkin (right panel) mRNA levels are based on three independent experiments with triplicate samples.

(B) OPA1 levels are significantly decreased in stressed primary neurons from parkin KO mice. Primary cortical neurons from parkin KO or WT mice were exposed to CCCP for 3 hr, and OPA1-specific transcripts were quantified 16 hr after treatment by RT-PCR (four independent experiments with triplicate samples). OPA1 mRNA levels in neurons from WT mice were set as 1.

(C and D) OPA1 is downstream of NEMO in mediating the stress-protective activity of parkin. OPA1 KO MEFs (C) or NEMO KO MEFs (D) were transfected with the plasmids indicated and stressed by STS. Apoptosis was quantified as described in Figure 1.

(E) OPA1 can reduce stress-induced cell death similarly to parkin. SH-SY5Y cells transiently expressing parkin or OPA1 were stressed by STS, and apoptotic cell death was analyzed.

Data represent the mean ± SEM. n ≥ 2,500 transfected cells per condition. *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001; n.s., not significant. See also Figure S4.

from parkin KO mice, both under basal conditions and upon TNF-α treatment (Figures 5F and S5D).

In line with a role of parkin in increasing the activity of LUBAC, parkin is physically recruited to LUBAC. A mass-spectrometry-based screen for interactors of HOIP and HOIL-1L revealed that parkin preferentially binds to HOIP with even higher efficiency than SHARPIN, a known LUBAC component (Figure S5E). Moreover, endogenous HOIP could be coimmunoprecipitated with endogenous parkin and vice versa (Figures 5G, S6A, and S6B). Notably, the interaction of parkin and HOIP was augmented under cellular stress (Figure 5H). The parkin mutants ΔUBL, G430D, and W453X interacted with HOIP similarly to WT parkin (Figure 5I) but did not increase linear ubiquitination (Figure 5A, middle blot), supporting the idea that the E3 ligase activity of parkin is required for enhancing LUBAC activity.

So far, only NEMO and RIP1, a component of the TNF receptor signaling complex, have been identified as targets for linear ubiquitination (Gerlach et al., 2011; Tokunaga et al., 2009). Endogenous NEMO coimmunoprecipitated with endogenous parkin, and this interaction was also strongly increased under cellular stress conditions (Figures 5J, S6C, and S6D). To add evidence for a role of parkin in ubiquitinating NEMO by an independent approach, we performed conventional ubiquitination assays under normal and stress conditions with endogenous levels of LUBAC and ubiquitin. In contrast to the ΔUBL mutant, WT parkin increased ubiquitination of NEMO (Figure S6E), and parkin-mediated NEMO ubiquitination was enhanced under cellular stress (Figure S6F). Notably, parkin was not able to increase ubiquitination of a NEMO mutant harboring two mutations at K285 and K309 (K285R/K309R NEMO), two residues required for NEMO linear ubiquitination (Tokunaga et al., 2009), adding further evidence for a role of parkin in mediating M1-linked ubiquitination of NEMO (Figure 5K).

To address the functional relevance of linear ubiquitination for the stress-protective activity of parkin, we followed various approaches. First, we tested two NEMO mutants defective in linear ubiquitination. The D311N NEMO mutant, which is unable

to bind to linear ubiquitin chains (Hadian et al., 2011; Rahighi et al., 2009), could neither restore the activity of parkin to prevent stress-induced cell death (Figure 6A) nor rescue the activity of parkin to reduce Drp1-induced mitochondrial fragmentation in NEMO KO MEFs (Figures S7A and S7B). Similarly, the K285R/K309R NEMO mutant, which lacks two critical sites for linear ubiquitin chain attachment, was also not able to rescue the stress-protective activity of parkin in NEMO KO MEFs (Figure 6A). Both NEMO mutants were significantly impaired to restore TNF-α- and parkin-induced NF-κB activation in NEMO KO MEFs (Figure 6B). Second, in cells depleted of HOIP, the main catalytically active component of LUBAC, the prosurvival activity of parkin was significantly impaired (Figure 6C). Further supporting a role of NF-κB in mediating this effect, the increased degradation of IκBα induced by parkin is markedly reduced in HOIP knockdown cells (Figure 6D, lanes 7–9 versus 10–12). Finally, the downregulation of HOIP impaired the activity of parkin to increase OPA1 levels (Figure 6E). Moreover, the increased expression of HOIP and HOIL-1L activated the reporter containing a fragment of the OPA1 promoter (OPA1-Luc3), and this effect was significantly increased by WT parkin, but not by the ΔUBL parkin mutant (Figure 6F).

TNF-α Signaling Is Impaired in the Absence of Parkin

The previous data indicated that the protective activity of parkin is causally related to its ability to increase linear ubiquitin chain assembly in the NF-κB pathway. Activation of the NF-κB pathway by TNF-α has been shown to be critically dependent on linear ubiquitination of NEMO (Haas et al., 2009; Rahighi et al., 2009; Tokunaga et al., 2009). We therefore addressed the question of whether NF-κB activation by TNF-α might be compromised in the absence of parkin. Indeed, nuclear translocation of p65 upon TNF-α treatment was significantly impaired in SH-SY5Y cells silenced for parkin expression and in primary neurons from parkin KO mice (Figures 7A–7D). We also observed reduced degradation of IκBα after TNF-α stimulation in these models of parkin deficiency (Figures S7C and S7D). Notably, impaired NF-κB activation upon TNF-α treatment was paralleled by an increase in apoptotic cell death in parkin KO primary neurons (Figure S7E). In addition, NF-κB activation by either TNF-α treatment or HOIP and HOIL-1L overexpression was markedly reduced in parkin KO MEFs, as determined by NF-κB reporter assays (Figure 7E). Thus, deficient NF-κB activation upon TNF-α stimulation was observed in several models of parkin deficiency via different approaches, supporting the functional relevance of endogenous parkin in this pathway.

DISCUSSION

Here we demonstrate that NF-κB signaling and mitochondrial integrity are linked through linear ubiquitination of NEMO in a stress-protective pathway that is regulated by the E3 ubiquitin ligase parkin. Parkin is recruited to LUBAC under cellular stress to increase linear ubiquitination of NEMO, activating canonical NF-κB signaling. As a result, the mitochondrial GTPase OPA1 is transcriptionally upregulated via NF-κB-responsive binding sites in its promoter (Figure 7F). OPA1 maintains cristae structure, prevents apoptosis, and mediates mitochondrial inner membrane fusion (Frezza et al., 2006; Olichon et al., 2003). Thus, the increased expression of OPA1 can explain not only the antiapoptotic effect of parkin but also its activity to prevent Drp1-induced mitochondrial fragmentation. In support of this notion, transient silencing of parkin increases mitochondrial fragmentation, which can be rescued by OPA1 (Lutz et al., 2009).

Several lines of evidence confirmed the functional relevance of the stress-protective pathway signaling via parkin, LUBAC, NEMO, NF-κB, and OPA1. First, parkin cannot prevent stress-induced cell death in the absence of NEMO. Second, the protective activity of parkin in NEMO-deficient cells can be rescued by WT NEMO, but not by NEMO mutants impaired in linear ubiquitination. Third, silencing of HOIP, the main catalytic component of LUBAC, impairs the activity of parkin both to prevent cell death and to increase OPA1 expression. OPA1 is probably not the only beneficial target regulated via this pathway; however, it is an essential target, given that parkin has no prosurvival activity in OPA1-deficient cells. In this context it is interesting to note that the pathway underlying the acute stress-protective activity of parkin we identified in this study implicates a transcriptional program which also applies to the effect of parkin in stimulating mitochondrial biogenesis (Shin et al., 2011).

Our study identifies parkin as an additional component of LUBAC, which can significantly increase linear ubiquitination of NEMO. The recruitment of parkin to both HOIP and NEMO is strongly enhanced under cellular stress, indicating a role of parkin in efficient stress management. This finding explains our previous observation that parkin can lower the threshold to efficiently induce NF-κB signaling in response to stress stimuli (Henn et al., 2007). Notably, we observed an unambiguous correlation between the capacity of parkin to prevent stress-induced cell death and to increase LUBAC activity. All pathogenic parkin mutants tested were neither able to mediate stress protection nor able to increase linear ubiquitination, although they can be recruited to HOIP. This finding strongly supports the concept that the E3 ligase activity of parkin is required to increase LUBAC activity and argues against the possibility that parkin just releases autoinhibition of HOIP by binding to the HOIP UBA domain via its UBL domain.

SHARPIN has recently been described as another LUBAC component associated with CD40 and TNF receptor complexes (Gerlach et al., 2011; Ikeda et al., 2011; Tokunaga et al., 2011). It can form a dimeric LUBAC together with HOIP, or a tripartite LUBAC consisting of SHARPIN, HOIP, and HOIL-1L. SHARPIN is required for TNF-α, CD40, and interleukin-1β (IL-1β) signaling, and mice carrying a spontaneous mutation in the *Sharpin* gene exhibit severe chronic dermatitis and immune deficiency (Gerlach et al., 2011; Seymour et al., 2007). In contrast to SHARPIN, parkin obviously cannot replace HOIL-1L within LUBAC. This may at least partially explain why parkin KO mice do not present a striking phenotype. Parkin functions instead as a modulator of a HOIL-1L- and HOIP-containing complex to transiently enhance NF-κB signaling under cellular stress. As a consequence, parkin deficiency may become obvious only after cellular stress or during aging, when the fidelity of quality-control systems

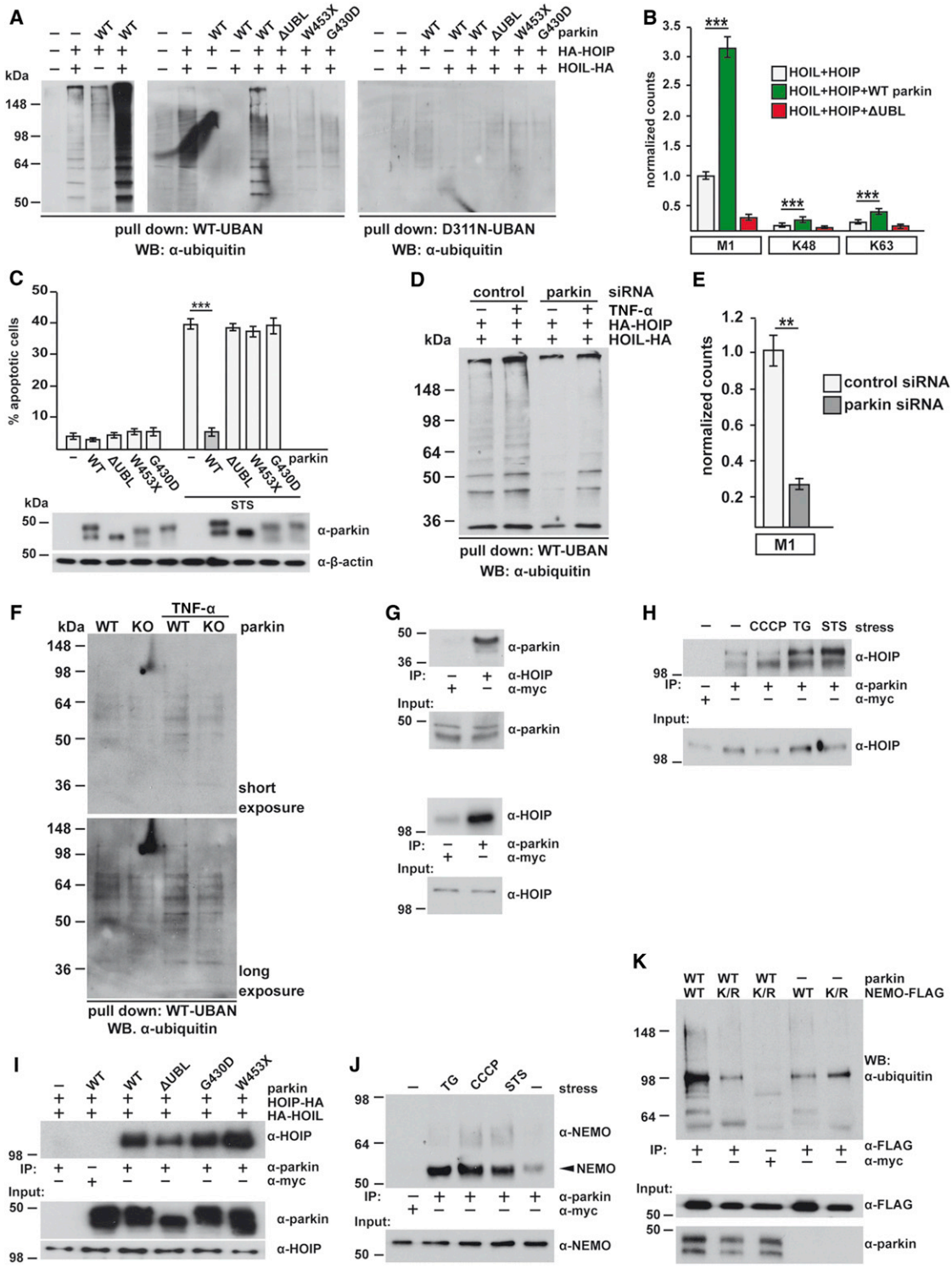

Figure 5. Parkin Increases LUBAC Activity

(A) Parkin increases the activity of HOIP and HOIL-1L to mediate linear ubiquitination. HEK 293T cells were transiently transfected with the plasmids indicated. Cells were lysed and a pull-down was performed with either the recombinant WT-UBAN (left and middle blot) or D311N-UBAN (right blot) domain of NEMO carrying an N-terminal Strep-Tag II. Proteins affinity-purified by Strep-Tactin beads were analyzed by immunoblotting using a ubiquitin antibody.

(B) Quantification of M1-, K48-, and K63-linked ubiquitin chains by SRM-based mass spectrometry. Normalized counts in the HOIP and HOIL sample were set as 1. Data represent the mean ± SEM of two independent experiments, with three technical replicates each.

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declines. Intriguingly, parkin expression is most abundant in postmitotic neurons and muscle cells, which are critically dependent on mitochondrial integrity. Strategies to compensate for a loss of parkin function may be efficient under normal conditions, but not under prolonged stress, which explains why patients usually present with symptoms only after some decades. In support of the functional relevance of our findings, linear ubiquitination and NF-κB activation is significantly impaired upon TNF-α stimulation in the absence of parkin. Therefore, it is tempting to speculate that parkin deficiency may also affect inflammatory and/or immune responses. In agreement with such a scenario, parkin KO mice display loss of nigral dopaminergic neurons upon systemic inflammation induced by lipopolysaccharide (Frank-Cannon et al., 2008).

We also demonstrate in this study that the mitophagy pathway is dispensable for the prosurvival activity of parkin, at least under acute stress. Parkin can prevent stress-induced cell death in the absence of PINK1 or components of the autophagic machinery required for mitophagy. In addition, the ΔUBL parkin mutant has no prosurvival activity and cannot mediate ubiquitination of NEMO but can promote mitophagy to the same extent as WT parkin. In line with the existence of two independent pathways for stress protection and mitophagy, parkin-induced mitophagy is not impaired in NEMO- or OPA1-deficient cells. Moreover, a recent study employing fluorescent-based cellular sensors for ubiquitin chains indicated that parkin-induced mitophagy predominantly involves K63-linked ubiquitin, whereas binding of a sensor for M1-linked chains to depolarized mitochondria was not observed (van Wijk et al., 2012).

Our data are consistent with the notion that parkin has adaptive effects on mitochondria depending on the severity of mitochondrial damage. Parkin prevents stress-induced cell death under moderate stress conditions with only minor mitochondrial defects by activating the NF-κB prosurvival pathway, resulting in increased OPA1 expression for the maintenance of mitochondrial integrity. However, when mitochondria are irreversibly damaged in response to severe stress, parkin can promote their elimination via mitophagy involving mainly K63-linked ubiquitination.

In conclusion, our findings establish a role for linear ubiquitination in the maintenance of mitochondrial integrity and the regulation of cell death. We propose that the composition of LUBAC is regulated in a context- and cell-type-specific manner, with parkin acting as a LUBAC modulator to increase neuronal viability under stress.

EXPERIMENTAL PROCEDURES

Apoptosis Assays

Activation of caspase-3 or Bax was determined by indirect immunofluorescence based on a single-cell analysis. In brief, cells were grown on glass coverslips. Cells were incubated 24 hr after transfection with CCCP (10 μM, 6 hr), TG (10 μM, 8 hr), or STS (1 μM, 5 hr). Fixed and permeabilized cells were incubated with an activated caspase-3 antibody or an activated Bax antibody overnight at 4°C, followed by incubation with Alexa 555-conjugated secondary antibody for 2 hr at room temperature. Cells were mounted onto glass slides and examined by fluorescence microscopy using a Leica DMRB microscope. Activation of caspase-3 and cleavage of PARP were also analyzed via western blotting.

Ubiquitination Assays

HEK 293T cells or MEFs transfected with the indicated constructs were lysed in denaturing lysis buffer and incubated at 95°C for 10 min. Protein extracts were diluted 1:10 with nondenaturing lysis buffer and cleared by centrifugation. To pull down proteins modified by linear ubiquitination, the recombinant UBAN domain of NEMO was added, which carries an N-terminal Strep-Tag II. As a control, a mutated UBAN domain (D311N-UBAN) was used. After overnight incubation at 4°C, Strep-Tactin beads (IBA) were added and incubated for 4 hr at 4°C. Beads were collected via centrifugation and washed. Laemmli sample buffer was added, and the samples were boiled for 10 min. Proteins binding to the UBAN domain were separated with SDS-PAGE and analyzed via western blotting using a ubiquitin antibody. Details of the linear and conventional ubiquitination assays can be found in the Supplemental Experimental Procedures.

Luciferase Reporter Assays

Transfected HEK 293T cells or MEFs were lysed in reporter lysis buffer (Promega) 16 hr after TNF-α treatment (10 ng/ml) or 1 day after cotransfection. After centrifugation, luciferase activity was determined luminometrically (LB96V, Berthold Technologies) by the luciferase assay system (Promega).

(C) Pathogenic parkin mutants impaired in linear ubiquitination cannot prevent stress-induced cell death. SH-SY5Y cells transiently expressing WT parkin or ΔUBL, W453X, or G430D parkin were subjected to STS and apoptotic cell death was analyzed. Quantification is based on triplicates of three independent experiments. $n \geq 2{,}500$ transfected cells.
(D) Linear ubiquitination is reduced in parkin knockdown cells. HEK 293T cells transfected with control or parkin siRNA and HOIP and HOIL-1L were treated with TNF-α (25 ng/ml, 15 min). Linear ubiquitination was determined as described in (A).
(E) Quantification of M1-linked ubiquitin chains in parkin knockdown HEK 293T cells by SRM-based mass spectrometry, as described in (B).
(F) Linear ubiquitination is reduced in parkin KO MEFs. MEFs from WT or parkin KO mice were treated with TNF-α, and linear ubiquitination was determined as described in (A).
(G) Endogenous parkin interacts with endogenous HOIP in HEK 293T cells. Immunoprecipitation (IP) using a HOIP antibody was followed by western blotting with a parkin antibody (upper panel) and vice versa (lower panel). Anti-myc was used as an unrelated antibody control.
(H) The interaction of parkin and HOIP is enhanced under cellular stress. HEK 293T cells were treated with CCCP, TG, or STS for 1 hr. Cell lysates were subjected to immunoprecipitation using a parkin antibody, and western blotting was performed with a HOIP antibody.
(I) Pathogenic parkin mutants can interact with HOIP. Lysates of HEK2 93T cells transiently coexpressing HOIP, HOIL-1L, and WT parkin or the parkin mutants ΔUBL, W453X, or G430D were subjected to immunoprecipitation using a parkin antibody or a myc antibody as a control. Immunoprecipitated proteins were analyzed by western blotting using a HOIP antibody.
(J) Endogenous parkin interacts with NEMO, and the interaction increases under cellular stress. HEK 293T cells were treated with TG, CCCP, or STS for 1 hr. Cell lysates were subjected to immunoprecipitation using a parkin antibody, and western blotting was performed with a NEMO antibody.
(K) Parkin increases ubiquitination of WT NEMO, but not of mutant NEMO defective in linear ubiquitination. Parkin-induced ubiquitination of WT NEMO was compared to that of K285R/K309R NEMO (K/R). HEK 293T cells were transiently cotransfected with WT or K/R NEMO-FLAG and parkin. NEMO was immunoprecipitated under denaturing conditions, followed by western blotting using a ubiquitin antibody.
Data represent the mean ± SEM. *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$. See also Figures S5 and S6.

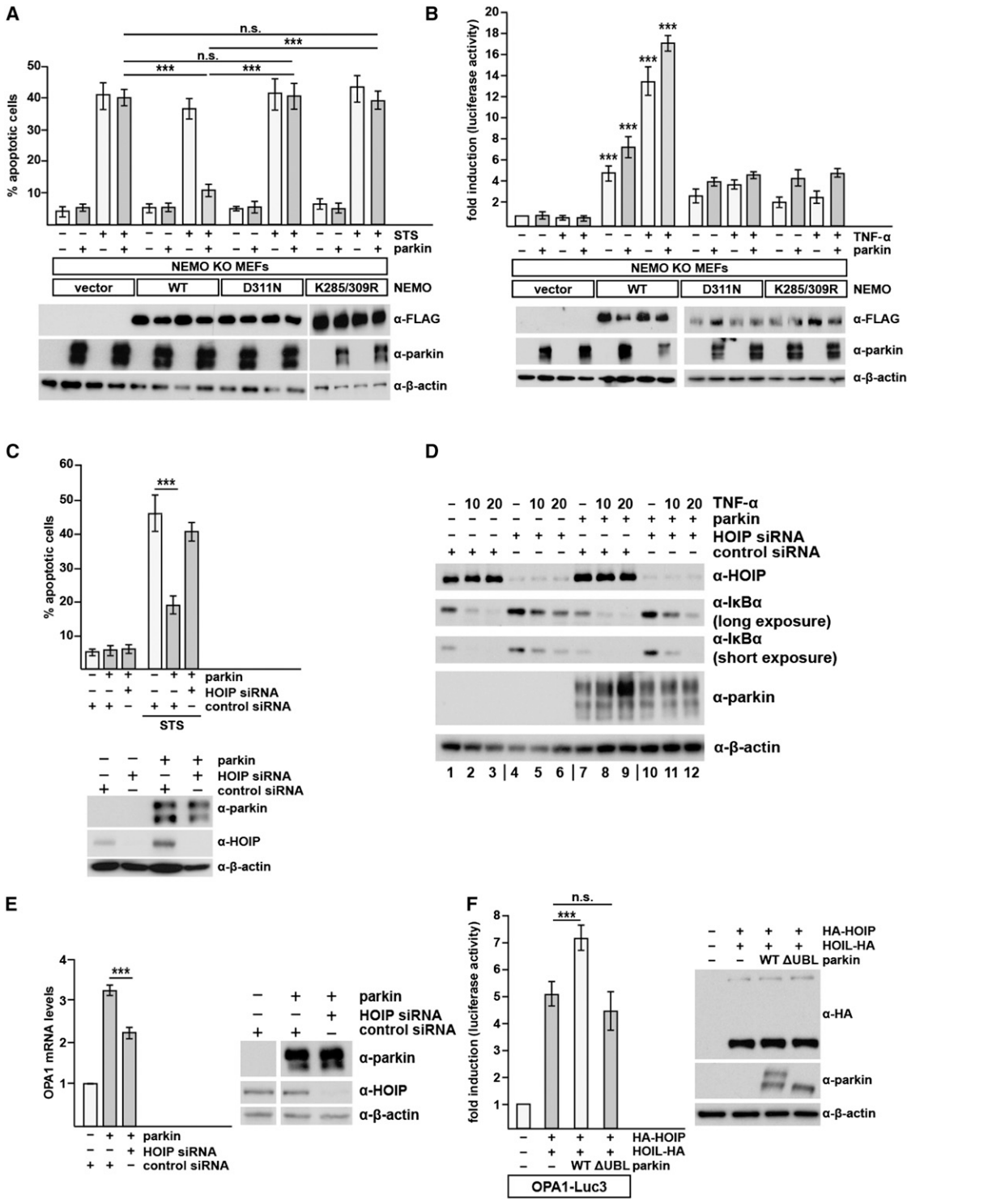

Figure 6. Linear Ubiquitination Is Essential for the Prosurvival Activity of Parkin

(A) NEMO mutants defective in either sensing linear ubiquitin chains (D311N NEMO) or being modified with linear ubiquitin chains (K285R/K309R NEMO) are not able to restore the prosurvival activity of parkin. NEMO KO MEFs were transiently cotransfected with parkin and WT NEMO or the mutants indicated. Cells were exposed to STS, and apoptosis was quantified. n ≥ 2,500 transfected cells per condition.

(B) NEMO mutants defective in linear ubiquitination cannot restore TNF-α- and parkin-induced activation of NF-κB. NEMO KO MEFs transfected as described in (A) together with an NF-κB luciferase reporter construct were treated with TNF-α (10 ng/ml), and luciferase activity was determined 16 hr after TNF-α treatment or 1 day after cotransfection. Quantifications are based on four independent experiments performed in duplicate.

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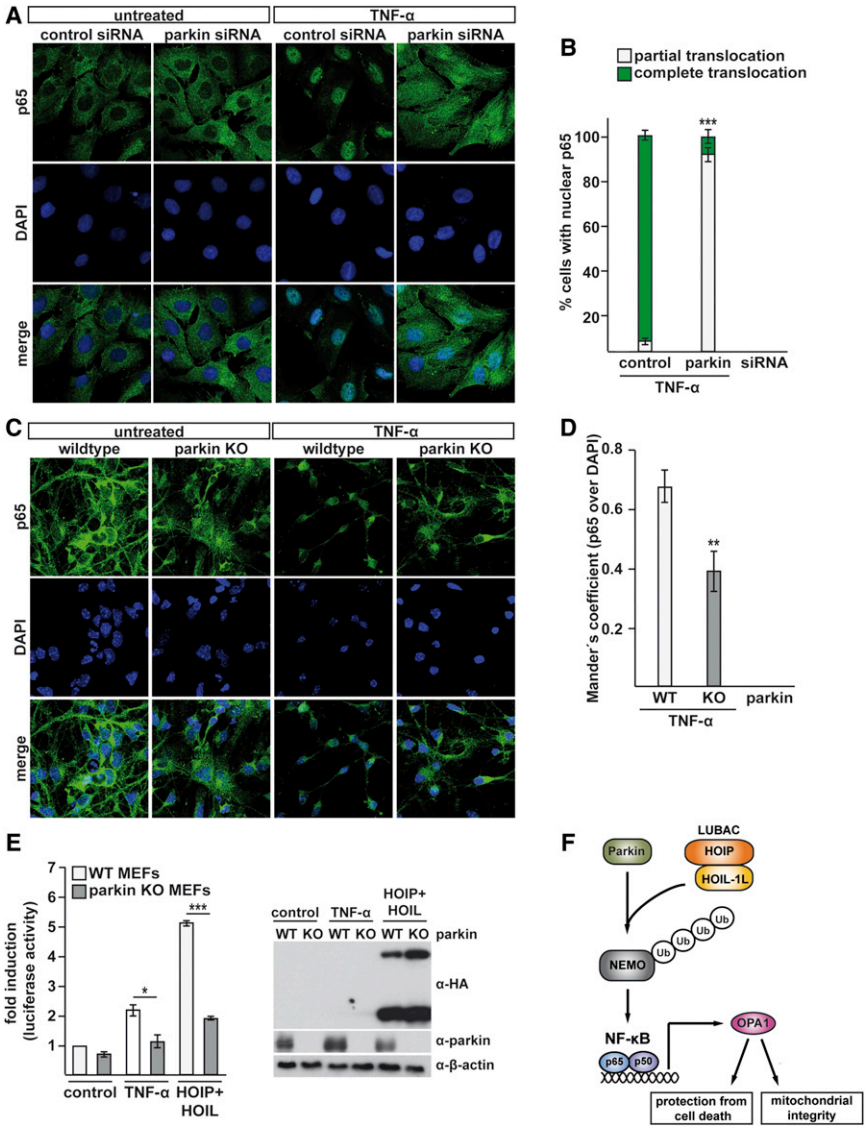

Figure 7. TNF-α-Induced Activation of the NK-κB Pathway Is Impaired in Parkin-Deficient Cells

(A–D) Nuclear translocation of p65 is decreased in parkin-deficient cells. Nuclear translocation of p65 was analyzed by indirect immunofluorescence in SH-SY5Y cells transfected with control or parkin siRNA (A and B) and in mixed mesencephalic neurons from WT or parkin KO mice (C and D) treated with TNF-α (20 ng/ml, 15 min). Colocalization of p65 and DAPI was quantified in four independent experiments performed in duplicate ($n \geq 2{,}500$ cells per condition) (B) or by Mander's coefficient ($n = 10$) (D).

(E) NF-κB reporter activity is reduced in parkin KO MEFs. MEFs from WT or parkin KO mice transfected with an NF-κB luciferase reporter construct were either cotransfected with HOIP-HA and HA-HOIL-1L or treated with TNF-α (10 ng/ml). Luciferase activity in cell lysates was determined 16 hr after TNF-α treatment or 1 day after cotransfection. Quantifications are based on at least four independent experiments performed in duplicate. Expression controls are shown in the right panel.

(F) Parkin confers stress protection via NEMO, NF-κB, and OPA1. In response to cellular stress, parkin can increase the activity of LUBAC to mediate linear ubiquitination of NEMO, which activates the IκB kinase complex. As a result, NF-κB is activated and regulates transcription of NF-κB-responsive genes. OPA1 as an NF-κB target links parkin, linear ubiquitination, and NF-κB signaling to mitochondrial integrity.

Data represent mean ± SEM. *$p \leq 0.05$; ***$p \leq 0.001$. See also Figure S7.

Measured values were analyzed with WinGlow Software (Berthold Technologies) and normalized to protein levels.

Mass Spectrometry

Following affinity purification on Strep-Tactin resin, the eluate was run on a SDS-PAGE gel and subjected to an in-gel digestion with trypsin. After Stage-Tip extraction, the eluted peptides were lyophilized and resuspended in 3% trifluoroacetic acid/5% acetonitrile buffer containing 50 fmol/μl of the heavy polyubiquitin marker peptide standards. Peptides were separated on a reversed-phase column (15 cm in length, 75 μm ID [inner diameter of the fused silica capillary tubing used to make the column], 3 μm Dr. Maisch GmbH C18) by a gradient from 4 to 42% B in 38 min, and SRM measurements were performed using a QTRAP 5500 (AB Sciex). The top two most intense transitions were selected and their peaks integrated with MultiQuant 1.2 software (AB Sciex). The mass-spectrometry-based interactor screen is described in the Supplemental Experimental Procedures.

(C) The prosurvival activity of parkin is compromised in HOIP-deficient cells. SH-SY5Y cells stably expressing parkin or control SH-SY5Y cells were transfected with HOIP-specific siRNA or control siRNA. Cells were treated with STS and apoptosis was quantified as described in Figure 1. $n \geq 2{,}500$ transfected cells per condition.

(D) The activity of parkin to activate NF-κB is reduced in HOIP-deficient cells. HEK 293T cells silenced for HOIP expression were treated with TNF-α (25 ng/ml, 10 or 20 min), and degradation of IκBα was monitored by western blotting.

(E) The activity of parkin to increase OPA1 levels is impaired in HOIP-deficient cells. WT MEFs stably expressing parkin were transfected with HOIP-specific siRNA or control siRNA. OPA1 mRNA levels were analyzed on day 4 after transfection by RT-PCR (three independent experiments with triplicate samples).

(F) Parkin increases HOIP and HOIL-1L-mediated OPA1 expression. HEK 293T cells were transfected with the OPA1-Luc3 reporter construct and HOIP and HOIL-1L together with WT or ΔUBL parkin. Luciferase activity in cell lysates was determined 1 day after cotransfection. Quantifications are based on four independent experiments performed in duplicate.

Data represent mean ± SEM. *$p \leq 0.05$; ***$p \leq 0.001$. See also Figure S7.

Statistical Analysis

Data represent the mean ± SEM. Statistical analysis was carried out using ANOVA; *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.molcel.2013.01.036.

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